PHOTOSYNTHETIC PIGMENTS: chemical structure, biological function and ecology

Edited by
Tamara K. Golovko (Russia)
Wieslaw I. Gruszecki (Poland)
M.N.V. Prasad (India)
Kazimierz Strzalka (Poland)

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Photosynthetic pigments: chemical structure, biological function and ecology. Syktyvkar, 2014. 440 p. **(**Komi Scientific Centre of the Ural Branch of the Russian Academy of Sciences**).**

This book provides an overview of photosynthetic pigments. The chapters cover a wide scope of pigment researches – from chemical structure and biological function to ecology. The basic data on structure of chloroplasts and photosynthetic apparatus in plants, metabolism of photosynthetic pigments and its regulation are reviewed. Information on physiology of pigment–protein complexes is discussed. Original materials on photosynthetic pigment characteristics of plants from various ecosystems and botanical-geographical areas are presented. Effects of environmental stresses on photosynthetic pigment-protein complexes are considered.

The book is directed at researches and professionals in plant physiology and biochemistry. It will be useful to plant biologists, ecologists and students.

Фотосинтетические пигменты – химическая структура, биологические функции и экология. Сыктывкар, 2014. 440 с. (Коми научный центр УрО РАН).

В монографии представлен обзор результатов изучения фотосинтетических пигментов. Приведены данные о структуре хлоропластов и фотосинтетического аппарата растений, метаболизме фотосинтетических пигментов и его регуляции. Рассмотрена физиология пигмент-белковых комплексов. Представлен оригинальный материал о содержании пигментов в растениях разных функциональных групп и ботанико-географических зон, обсуждены реакции фотосинтетических пигментов на стрессы.

Книга предназначена для специалистов в области физиологии и биохимии растений, будет полезна для биологов, экологов и студентов.

Reviewers

Dr. Vladimir D. Kreslavskii (Russia), Prof. Evgenia F. Markovskaya (Russia), Prof. Andrzej M. Skoczowski (Poland), Dr. Tatyana V. Zhigalova (Russia)

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CONTRIBUTORS

Olga Chivkunova Biological Faculty, M.V. Lomonosov Moscow State University, Moscow, Russia; e-mail: olga.chivkunova@mail.ru

Igor Dalke Institute of Biology, Komi Scientific Centre, Russian Academy of Sciences, Syktyvkar, Komi Republic, Russia, e-mail: dalke@ib.komisc.ru

Olga Dymova Institute of Biology, Komi Scientific Centre, Russian Academy of Sciences, Syktyvkar, Komi Republic, Russia, e-mail: dymovao@ib.komisc.ru

Vladimir Elsakov Institute of Biology, Russian Academy of Sciences, Syktyvkar, Komi Republic, Russia; e-mail: elsakov@ib.komisc.ru

Leszek Fiedor Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; e-mail: leszek.fiedor@uj.edu.pl

Masayuki Fujita Laboratory of Plant Stress Responses, Department of Applied Biological Science, Kagawa University, Kagawa, Japan, e-mail: fujita@ag.kagawa-u.ac.jp

Elena Garmash Institute of Biology, Komi Scientific Centre, Russian Academy of Sciences, Syktyvkar, Komi Republic, Russia, e-mail: garmash@ib.komisc.ru

Tamara Golovko Institute of Biology, Komi Scientific Centre, Russian Academy of Sciences, Syktyvkar, Komi Republic, Russia, e-mail: t_golovko@ib.komisc.ru

Wieslaw I. Gruszecki Department of Biophysics, Institute of Physics, Maria Curie-Sklodowska University, Lublin, Poland; e-mail: wieslaw.gruszecki@umcs.pl

Mirza Hasanuzzaman Laboratory of Plant Stress Responses, Department of Applied Biological Science, Kagawa University, Kagawa, Japan; Department of Agronomy, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh; e-mail: mhzsauag@yahoo.com

Pawel Jedynak Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; e-mail: pawel.jedynak@uj.edu.pl; p.a.jedynak@gmail.com

Malgorzata Jemiola-Rzeminska Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; e-mail: malgorzata.jemiola@gmail.com

Mikhail Khristin Institute of Basic Biological Problems, Russian Academy of Sciences, Pushchino, Moscow, Russia; e-mail: khristin_@rambler.ru

Kinga Klodawska Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow; e-mail: kingaklodawska@gmail.com

Ekaterina Kokovkina Institute of Biology, Komi Scientific Centre, Russian Academy of Sciences, Syktyvkar, Komi Republic, Russia, e-mail: kokovkina@ib.komisc.ru

Andrzej Kornas Institute of Biology, Pedagogical University, Krakow, Poland; e-mail: anko@up.krakow.pl

Bernhard Krautler Institute of Organic Chemistry &Centre of Molecular Biosciences, University of Innsbruck, Innsbruck, Austria; e-mail: bernhard.kraeutler@uibk.ac.at

Abhay Kumar Department of Plant Sciences, University of Hyderabad, Hyderabad, India; e-mail: kkumar.abhay@gmail.com

Rima Kumari Department of Plant Sciences, University of Hyderabad, Hyderabad, India; e-mail: rimabotany@gmail.com

Elzbieta Kuzniak Department of Plant Physiology and Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland; e-mail: elkuz@biol.uni.lodz.pl

Ekaterina Lashmanova Syktyvkar State University, Syktyvkar, Komi Republic, Russia; e-mail: ekaterinalashmanova@yandex.ru

Dariusz Latowski Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; e-mail: dariuszlatowski@gmail.com

Przemyslaw Malec Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; e-mail: przemyslaw.malec@uj.edu.pl

Maria Maleva Department of Plant Physiology and Biochemistry, M. Gorky Ural State University, Ekaterinburg, Russia; e-mail: maria.maleva@mail.ru

Tamara Maslova Komarov Botanical Institute, Russian Academy of Sciences, Saint-Petersburg, Russia; e-mail: zubkovaelena44@mail.ru

Maibam Dhanaraj Meitei Department of Plant Sciences, University of Hyderabad, Hyderabad, India; e-mail: rajjhcu@yahoo.in

Zbigniew Miszalski Institute of Plant Physiology, Polish Academy of Sciences, Krakow, Poland and Institute of Biology, Pedagogical University, Krakow, Poland; e-mail: miszalski@ifr-pan.krakow.pl

Kamrun Nahar Laboratory of Plant Stress Responses, Department of Applied Biological Science, Kagawa University, Kagawa, Japan, Department of Agricultural Botany, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh; e-mail: knahar84@yahoo.com

Robert J. Porra Divisionof Plant Industry, Commonwealth Scientific and Industrial Organization, Canberra, Australia; e-mail: robert.porra@gmail.com

Majeti Narasimha Vara Prasad Department of Plant Sciences, University of Hyderabad, Hyderabad, India; e-mail: prasad_mnv@yahoo.com

Hugo Scheer Department of Biologie 1 – Botanik, University of Munich, Munchen, Germany; e-mail: hugo.scheer@lmu.de

Abin Sebastian Department of Plant Sciences, University of Hyderabad, Hyderabad, India; e-mail: abinhcu@gmail.com

Alexei Solovchenko Biological Faculty, M.V. Lomonosov Moscow State University, Moscow, Russia; e-mail: solovchenko@mail.bio.msu.ru

Kazimierz Strzalka Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; e-mail: kazimierzstrzalka@gmail.com, kazimierz.strzalka@uj.edu.pl

Renata Szymanska Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; e-mail: renata.szymanska@fis.agh.edu.pl

Galina Tabalenkova Institute of Biology, Russian Academy of Sciences, Syktyvkar, Komi Republic, Russia, e-mail: tabalenkova@ib.komisc.ru

Andrzej Waloszek Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; e-mail: andrzej.waloszek@uj.edu.pl

Iljia Zakhozhiy Institute of Biology, Komi Scientific Centre, Russian Academy of Sciences, Syktyvkar, Komi Republic, Russia, e-mail: zakhozhiy@ib.komisc.ru

CONTENTS

PREFACE

Life on Earth depends on photosynthesis that is the overall process whereby plants, algae and some prokaryotes use light energy to synthesize organic compounds. In higher plants the energy of sunlight is absorbed by photosynthetic pigments, chlorophylls and carotenoids. They form assemblies with specific membrane-bound proteins in the thylakoid membranes of the chloroplasts for optimization of absorption, transfer and conversion of light energy.

Today a large amount of new information is appeared in dynamic and expanding the field of knowledge on photosynthetic pigments. The overviews of chemical structure, biological function and ecology of pigments are provided in this book, which written by 38 contributors from 8 countries. Together they cover and integrate a great part from scientific spectrum of the pigment researches.

Part 1 includes Chapters 1 to 4, presenting information on the structure of the chloroplasts and photosynthetic apparatus in higher plants. The emphasis is given to molecular biophysics of photosynthetic process. Part 2 observes the data on metabolism of photosynthetic pigments and its regulation. The information on chlorophylls and carotenoids biosynthesis and their degradation are presented in Chapters 5 to 7. The new data on chloroplasts' chlorophyll-protein complexes and chlorophyll fluorescence in greening wheat seedling are discussed in Chapter 8. Part 3 focuses the attention on physiological aspects of functioning of the pigment-protein complexes and its role in photosynthesis (Chapters 9 to 13). Part 4 provides comprehensive information on ecology of photosynthetic pigments and pigment-protein complexes (Chapters 14-21). Several examples of investigations of stress effects on the plant are presented. Some methodical aspects of pigment studies are considered in Part 5. Chapters 22 and 23 present information on non-destructive assay of leaf pigments and remote sensing approaches to the spatial-temporal estimation of a vegetation chlorophyll index. Pigments play an important role in plant biology. Plant pigments are also known to possess biological activity. They are able to contribute into human health maintenance. These aspects are considered in Chapter 24.

We should like to believe that this book will stimulate future researches of photosynthetic pigments, leading to progress in our understanding of the mechanisms of photosynthesis and in practical use of plant pigments in biotechnology and human life. We hope that this book will be helpful to researches, students and professionals.

We express our gratitude to all the contributors of this book. We are extremely grateful to Olga Dymova for her hard work and time she spend to finish this job. We express thanks to the Directorate of the Institute of Biology, Komi Science Center, Ural Branch of the Russian Academy of Sciences, for support of the project.

Tamara Golovko

Part I. STRUCTURE OF CHLOROPLASTS AND PHOTOSYNTHETIC APPARATUS IN HIGHER PLANTS

Chapter 1. STRUCTURE AND PROPERTIES OF CHLOROPLAST MEMBRANES

Renata Szymanska
Jagiellonian University, Krakow, Poland

Contents

1.1. Introduction

Life on Earth depends on photosynthesis, the conversion of light energy to chemical energy. In higher plants, photosynthesis takes place in the thylakoid membranes of chloroplasts. Among different plastids occurring in plant cells, chloroplasts are the most crucial subcellular organelles because they perform vital and exclusive processes not only life-giving photosynthesis but also others: vitamin, fatty acid and amino acid biosynthesis (Kota and Goshe, 2011). In higher plants chloroplasts are large, 5–10 μm diameter organelles surrounded by a bilayer membrane, which efficiently separates the chloroplast stroma from the cytosol. Three major structural regions can be distinguished in chloroplasts: 1) a highly organized internal membrane network formed of

flat compressed vesicles, the thylakoids; 2) an amorphous medium rich in ribosomes and soluble proteins, the stroma; 3) the double-layer chloroplast envelope (Block et al., 2007) (Fig. 1). Chloroplasts are semi-autonomous organelles – and although they contain their own DNA, which encodes approx. 80–100 proteins, more than 3000 nuclear encoded proteins are predicted to be targeted to chloroplasts (Block et al., 2007). That means that the coordination and communication between chloroplasts and nucleus must be highly specialized. The chloroplast envelope is the border between plastid and cytosol and play a crucial role in interacting with the plastid translation apparatus and in the import of nuclear-encoded proteins (Block et al., 2007).

1.2. Chloroplast envelope structure and function

The chloroplast envelope is a complex and unique biochemical machinery. It is a key structure for the integration of chloroplasts metabolism within the cell.

For preparation of the envelope membrane, chloroplasts are lysed in a hypotonic medium followed by centrifugation on a sucrose gradient (Douce and Joyard, 1982). This method is applied to obtain both inner and outer membranes and as Farro et al. (2003) calculated the cross-contamination of the envelope fraction by thylakoid proteins is about 1–3% between several independent preparation. The obtainment of a highly purified envelope membrane allowed to perform the study on metabolite transport into the intact chloroplasts (Heber, 1974; Walker, 1976) and to the recognition of the enzymatic and proteomic pattern of the membrane (Douce and Joyard, 1979, 1990; Rolland et al., 2003). Furthermore genetic and molecular approaches on the envelope membrane were applied in the context of whole plant functional studies (Weber et al., 2004).

Chloroplast membrane biogenesis requires astonishing types of lipids or lipid derivatives including: polar glycerolipids (i.e. galactolipids, phosholipids and sulfolipids), pigments (chlorophylls, carotenoids) and prenyloquinones (i.e. plastoquinones, tocochromanols) (Block et al., 2007). All these compounds are essential for the functioning of the chloroplast membrane and photosynthetic apparatus. Activation, regulation and coordination of the metabolic pathways leading to biosynthesis of membrane "bricks" are of striking importance.

1.3. Lipids

The chloroplast envelope is a double-layer, lipid-rich structure. The outer membrane layer has the highest lipid-protein ratio among all plant cell membranes – 2,5–3,0 mg lipids/mg proteins which is reflected

by the low density of these membrane – only 1,08 g/cm^3 (Block et al., 2007). In comparison, the lipid-protein ratio of the inner membrane is lower – 1,0–1,2 mg lipid/mg protein and the corresponding density is 1,13 g/cm^3 (Block et al., 2007). All plastid membranes are characterized by a low phospholipid content but instead they have a higher level of polar neutral lipids – galactolipids (Benson, 1964; Block et al., 2007). In outer envelope membrane the most abundant lipid is digalactosyldiacylglycerol (DGDG) and phosphatidylcholine (PC), whereas in the inner membrane monogalactosyldiacylgycerol (MGDG) (Block et al., 2007) (Table 1). It is worth to mention that to date, the MGDG synthesis is the best enzymatic marker for the envelope membrane (Douce and Joyard, 1990). The comparison of glycerolipids pattern of the envelope membrane isolated from spinach chloroplasts, cauliflower proplastids, pea etiplasts has shown that these patterns are almost identical (Douce and Joyard, 1990). Is has to be mentioned that glycerolipids existing in the plant membrane differ in their fatty acid composition which suggests that they originate from complex biosynthetic pathways (Mongrand et al., 1998; Block et al., 2007).

MGDG synthesis in the chloroplast envelope has been established in 1974 (Douce, 1974). Since that time it has been demonstrated that chloroplast envelope is also a place of sulfolipid and phosphatydylglycerol synthesis (Essigmann et al., 1999) as well as fatty acid metabolism (Ferro et al., 2002, 2003).

1.4. Lipid-soluble compounds

Plant membranes contain a high level of lipid-soluble compounds, which perform various biochemical and physiological roles and originate from the isoprenoid pathway: carotenoids, chlorophylls, and prenyloquinones: plastoquinone, ubiquinone, tocochromanols, phylloquinone and others (Lange and Ghassemian, 2003) (Table 1). The envelope membrane isolated from chloroplasts is yellow due to the high content of carotenoids and the absence of chlorophyll (Block et al., 2007). The dominant carotenoid in the envelope membrane is violaxanthin (Jeffrey et al., 1974). The envelope membrane is considering as a storage site of violaxanthin (serving the violaxanthin cycle which occurs in the thylakoid membrane) and it is a place of its biosynthesis (Yamamoto, 2006).

The plant chloroplast envelope is devoid of chlorophylls, but contains small amounts of their precursors: chlorophyllide and protochlorophyllide (Pineau et al., 1993) (Table 1), thus is an evidence that some chlorophyll biosynthesis steps take place in the envelope membrane. Chlorophyllide is generated by the enzyme protochlorophyllide oxidoreductase (POR) localized in the chloroplast envelope. Some studies

Table 1

Distribution of lipids and lipid derivatives in chloroplast (according to Block et al., 2007).

	Outer envelope membrane	Inner envelope membrane	Total envelope membranes	Thylakoids
Total polar lipids (mg/mg proteins)	2,5–3	1	1,2–1,5	0,6–0,8
Polar lipids (% of total)				
MGDG	17	55	32	57
DGDG	29	29	30	27
Sulfolipid	6	5	6	7
Phosphatidylcholine	32	0	20	0
Phosphatidylglycerol	10	9	9	7
Phosphatidylinositol	5	1	4	1
Phosphatidylethanolamine	0	0	0	0
Total Chlorophylls (μg/mg protein)	nd.	nd.	0,1–0,3	160
Chlorophylls (% of total in the fraction)				
Chlorophyll *a*	nd.	nd.	86	72
Chlorophyll *b*	nd.	nd.	14	28
Chlorophyll precursors (protochlorophyllide + chlorophyllide; μg/mg protein)	nd.	nd.	0,41	0–0,35
Total carotenoids (μg/mg proteins)	2,9	7,2	6–12	20
Carotenoids (% of total)				
β-carotene	9	12	11	25
Violxanthin	49	47	48	22
Lutein + Zeaxanthin	16	23	21	37
Antheraxanthin	–	5	6	-
Neoxanthin	26	13	13	16
Total prenylquinones (μg/mg protein)	4–12	4–11	4–11	4–7
Prenylquinones (% of total)				
α-tocopherol + α-tocopherylquinone	81	67	69	24
Plastoquinone-9	18	32	28	70
Phylloquinone K1	1	1	3	6

also consider their role in chloroplast development and differentiation as a signal molecules (Block et al., 2007).

The envelope membrane is also composed of several prenyloquinones of basic importance: plastophuinone-9, phylloquinone (vitamin K_1), α-tocopherol (vitamin E) (Soll et al., 1985; Lichtenthaler, 2007) (Table 1). Among them α-tocopherol is present in a higher proportion in both the outer and inner layer, but its level in the outer membrane is more pronounced. In contrast, the level of PQ-9 is higher in the inner membrane (Soll et al., 1985). Soll et. al (1985) showed that key enzymes for tocopherol and plastoquinone biosynthesis are localized in the inner envelope membrane. Recent studies showed that with exception of cytosolic enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) (Garcia et al., 1997) and tocopherol cyclase (VTE1), which is localized in the plastoglobules (Vidi et al., 2006; Ytterberg et al., 2006) other enzymes of the tocopherol biosynthetic pathway are localized in the chloroplast envelope (Vidi et al., 2006; Szymanska and Kruk, 2010). The different localization of tocopherols requires a sophisticated distribution of these compounds within chloroplasts (between envelope and thylakoids) and shed a new light into its function – a non-antioxidant role in plants (i.e. as a signaling molecules) (Szymanska and Kruk, 2010).

The role of the chloroplast membrane envelope as a place of prenyloquinones biosynthesis has been strongly supported by the findings of its proteomic analysis (*see below*). Furthermore the most abundant membrane enzyme is a S-adenosylomethionine(SAM)-dependent methyltransferease (Teyssier et al., 1995), which is involved in a plastid prenylquionones biosynthesis.

1.5. Sterols

Plastid membranes contain several sterols with the stigmat-7-enol as the main compound. The level of sterols in the plastid envelope in comparison to other cell membranes is rather low (only 7 μg/mg protein) (Block et al., 2007).

1.6. Proteins

To date, there have been various proteomic studies of the chloroplast envelope membrane (Ferro et al., 2003, 2002; Rolland et al., 2003; Armbruster et al., 2011) as well as the thylakoid membrane and lumen (Whitelegge, 2003; Friso et al., 2004). The best informative source of the plastid membrane proteome is *Plant Proteome Databases* (PPDB) (http://ppdb.tc.cornell.edu/). Isolation, purification and identification of membrane proteins with biochemical approaches is extremely difficult because it requires large amounts of detergents. Owing to that, recently

other strategies were applied to the identification of envelope proteins: genomic studies, SDS-PAGE combined with trypsin digestion and LC-MS/MS analysis (Ferro et al., 2002; Block et al., 2007), off-line multidimensional protein identification technology (MUDPIT) (Froehlich et al., 2003), bioinformatics analysis (Ferro et al., 2002, 2003; Rolland et al., 2003; Sun et al., 2004) and others.

Apart from many enzymes found in the chloroplast envelope, taking into consideration its function as a barrier between plastids and the cytosol, the envelope contains many transport systems (Ferro et al., 2002; Weber et al., 2005). Proteomic analysis allowed the identification of several transport proteins family: phosphate transporters (Weber et al., 2005), oxoglutarate/malate transporters (Ferro et al., 2002); amino acid translocators (Ortiz-Lopez et al., 2001), folate transporters (Ferro et al., 2002), S-adenosylmethionine transporter (Ravanel et al., 2004) and others.

The second very important class of protein localized in the chloroplasts envelope is a translocons family. Due to the large number of proteins encoded in the nucleus the chloroplast envelope has acquired a range of sophisticated transport systems. Those proteins, which are nuclear-encoded are first specifically recognized by outer membrane receptors, and after that are translocated across the two envelope layers (Block et al., 2007). Proteins coming into the chloroplast are bearing the N-terminal transit, removable peptides, which directs them to the right chloroplast sub-compartments (Block at al., 2007). Chloroplast protein import requires the parallel cooperation of the membrane lipid surface, integration of complexes TOC (*translocon of the outer envelope*), TIC (*translocon of the inner envelope*) and ATP (Gutensohn et al., 2006). Probably, several importing complexes exist in chloroplast envelope allowing for efficient protein transport.

1.7. Thylakoid membrane

The thylakoid membranes in higher plants have the most complex structure and organization of all biological membranes (Figure 1). The main function is to capture light quanta and drive a series of redox reactions where oxygen and ATP are produced and ferredoxin is reduced (Albertsson, 1995). In higher plants and green algae, thylakoids are differentiated into two distinct morphological domains: cylindrical, stacked grana with interconnecting regions and the unstacked membrane, called stroma lamellae (Shimoni et al., 2005; Mustardy and Garab, 2003) (Fig. 1). The grana stacks can be further divide into grana cores, grana margins and grana end membranes (Allen and Forsberg, 2011). Grana are cylindrical membrane stacks, containing ~10-20 layers of thylakoid membrane comprising 80% of the total membrane

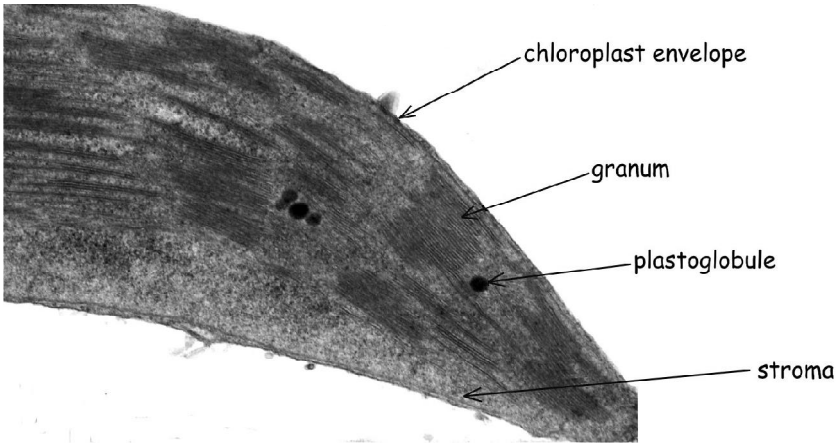

Fig. 1. Chloroplast ultrastructure (R. Szymanska).

(Kirchhoff, 2008). Electron tomography with cryo-immobilization showed that thylakoid grana membranes are built of repeating units that contain paired layers formed by bifurcation of stroma lamellar sheets, which fuse within the granum body. Each granum unit is connected to it neighbors and to the stroma lamellae (Shimoni et al., 2005). The thylakoid domains differ in protein composition and biochemical properties (Allen and Forsberg, 2001), but altogether are switched in a 3-D lamellar network that enclosed inside the thylakoid lumen (Shimoni et al., 2005). The continuum of the membrane system is crucial for the diffusion of mobile components within the network (Pali et al., 2003).

In general, thylakoids from plants grown under normal conditions are circular in shape with a diameter between 0,4 and 0,5 nm and the chlorophyll *a*/*b* ratio 3,0±0,5 (Albertsson, 1995). Biochemical and ultrastructural data showed that proteins occupy approx. 70% of the area in thylakoid membranes and 80% of the area in grana thylakoids (Kirchhoff, 2008). In higher plants, the lipid-protein ratio for thylakoid membranes is very high and reaches about 0,34 (w/w) (Pali et al., 2003). As shown in Table 1, in thylakoid membranes of higher plants MGDG accounts for more than 50% of the total lipids. The membrane also contains DGDG (~30%), SQDG (~7%) and phosphatidylglicerol (~7%). Because of its high concentration in the thylakoid membrane MGDG is the most abundant polar lipid species in nature (Pali at al., 2003). High lipid-protein ratio is probably required for the large number of cooperative interactions, and it can be a self-regulated mechanism – by non-bilayer lipids (the high non-bilayer lipids ratio in the thylakoid lipid mixture prevents membrane "dilution") (Garab et al., 2000). MGDG

is an example of a non-bilayer lipid which can form non-bilayer structures such as the reversed hexagonal phase (Pali et al., 2003). It is suggested that non-bilayer lipid structures can have multiple structural and functional roles in thylakoids, such as regulation of membrane fluidity (Latowski and Strzalka, 2007). Membrane fluidity can be also predicted from the saturated fatty acid content or phase transition temperatures of the lipids, but it is also strongly dependent on lipid compositions (Orr and Raison, 1987; Harwood, 1998). Changes in MGDG/DGDG ratio depends on environmental factors and can modify the phase-transition temperatures but more likely is involved in incorporation of more lipids into the membrane due to decreasing the nonbilayer propensity of the membrane (Garab et al., 2000).

The high protein content of the thylakoid membrane facilitates protein-protein interactions. Furthermore, protein complexes are found in large assemblies, which are spaced by lipids and these findings are in agreement with the micro-domain organization of the grana membrane (Boekema et al., 2000; Pali et al., 2003). Furthermore it was found that membrane lipids and integral membrane proteins possess different rotational and translational mobility which is extremely important to maintain molecular packing (Pali et al., 2003). For instance, EPR spectra of different spin labels in PSII-enriched membranes has shown that the majority of the lipids in these areas are less-mobile (Li et al., 1987), whereas more than 60% of total thylakoid lipids were in amorphic phase at room temperature (Li et al., 1989). It suggest that the concentration of these mobile-restricted lipids in a certain area is one of the most important structural parameters of the thylakoid membrane (Pali et al., 2003).

The protein composition of stacked grana and unstacked stroma lamellae are completely different (Dekker and Boekema, 2005). Photosystem II and its light-harvesting antenna complex are located overwhelmingly in the grana, whereas photosystem I (PSI) and ATP synthase are situated at the stroma lamellae and margins of the grana (Dekker and Boekema, 2005; Staehelin, 2003). On the stromal side PSI protrudes above the membrane (~3.5 nm), that is why it can not be situated between appressed membranes in the grana (Mullineaux, 2005). Distribution of cytochrome b_6f complex in the thylakoid membrane seems to be unrestricted (Kouril et al., 2011). PSII is built of two core dimers and a perypherial antenna system composed of six different units of the light harvesting protein family (Kouril et al., 2011).

Different location of PSI and PSII causes “lateral heterogeneity” in the thylakoid membrane. The resulting asymmetry in the structure and protein compositions has been proposed to serve several functions, including minimization of spillover of excitation energy from PSII to PSI, regulation of light energy distribution between the two photo-

systems, maximization of light trapping by PSII and protection of PSII complexes containing inactivated D1 proteins against degradation under high light intensity (Albertsson, 1995; Anderson, 1999; Shimoni et al., 2005). Thus, in addition provides a background for light harvesting and energy transfer and allows the photosynthetic machinery to adjust to fluctuating environmental conditions and provide protection against light-induced damage.

1.8. Conclusions

Chloroplasts with its membrane systems (chloroplasts envelope, grana and stroma lamellae) represent the most topologically complex organelle in the cell. The sophisticated structure of these membranes reflects the large amount of processes which take place in the chloroplasts. On the one hand chloroplasts contain highly organized thylakoid membranes which are crucial for photosynthesis. On the other hand the chloroplast envelope must have the sophisticated transporting systems. Therefore all chloroplast membranes are integrated and efficiently coordinated.

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Chapter 2. PHOTOSYNTHETIC CHLOROPHYLL-PROTEIN COMPLEXES

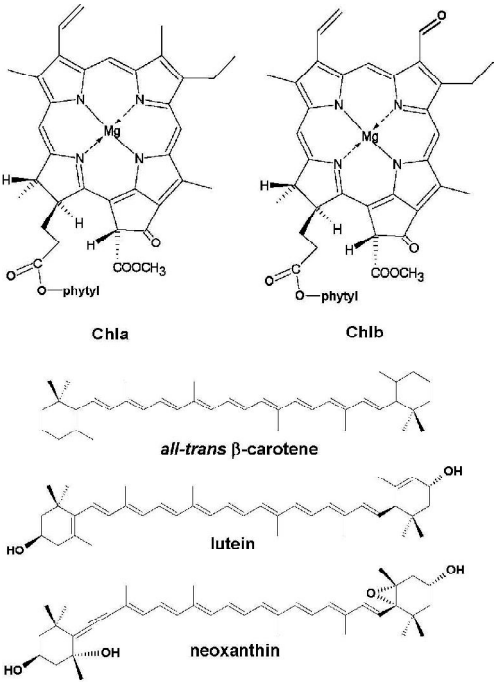
Leszek Fiedor
Jagiellonian University, Krakow, Poland

Contents

2.1. Historical perspective

The organization of the photosynthetic apparatus into specialized pigment-complexes for quite some time has not been obvious during the history of photosynthesis research. Towards the end of the 19th century, the essential role of chlorophylls (Chls) was discovered (Willstatter and Stoll, 1913) and various photophysical and photochemical mechanisms of their direct involvement in primary photosynthetic reactions had been suggested. The structures of major photosynthetic pigments in plants are shown in Fig. 1. The concept that a cooperation of two photosystems (PSs) and photons is required to drive photosynthetic CO_2 fixation and O_2 evolution emerged based on early estimations of quantum efficiency of photosynthesis and the effectiveness of photosynthetic reactions in various spectral regions, dating back to the works by Warburg and Emerson in the 1930s and 1940s (Emerson and Arnold, 1932). Other curious observations, such as the «red drop» effect and the synergetic effects of red light (>680 nm) and light of shorter wavelengths (green), confirmed the requirement of a joint action of two PSI and PSII in oxygenic photosynthesis. These and other findings could be accommodated by a model in which two distinct photochemical acts, mediated by two separate assemblies of Chls, are required to complete the photosynthetic reactions (Clayton, 1980). The schematic presentation of mutual spatial and functional relationships between

Fig. 1. Chemical structures of major pigments of plant photosystems.



the two photosystems and other components of the photosynthetic non-cyclic electron transfer chain in plants is shown in Fig. 2. A more detailed insight into the photosynthetic machinery came with the developments in flash techniques and time-resolved spectroscopies (Emerson and Arnold, 1932; Kaufmann et al., 1975; Shuvalov and Krasnovskii, 1981; Breton et al., 1986; Wasielewski et al., 1987; Durrant et al., 1992). More recently, the most intimate view has emerged with the advancements in crystallographic studies of photo-

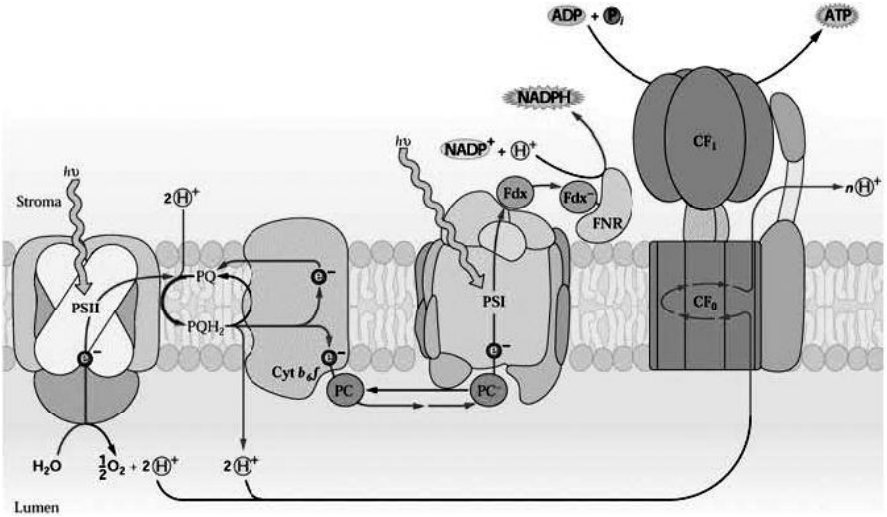


Fig. 2. Functional organization of photosystem I and photosystem II in thylakoid membrane from higher plants.

synthetic pigment-proteins, initiated by the pioneering achievements in X-ray crystallography of the bacterial photosynthetic reaction center (RC) (Deisenhofer et al., 1985; Allen et al., 1987) and in parallel in electron crystallography of plant LHCII antenna (Kuhlbrandt, 1984). Now the structurally characterized pigment-proteins extend from single complexes to large supercomplexes of nearly entire PSs.

2.2. Multifunctionality and versatility of chlorophylls in photosynthesis

Chlorophylls are the all-important cofactors in photosynthetic pigment-protein complexes in which they not only fulfill several quite diverse functions but also are indispensable for their assembly and stability. At the functional level, majority of Chls are found in LHCs, where they play an obvious role of light-harvesting chromophores. At the same time, Chls are responsible for excitation storage in LHC and its transfer towards RCs. In the latter complexes, Chls act as acceptors of excitation from LHC and then function as primary electron donors and acceptors. In addition, Chls in photosystems play a photoprotective role and constitute sites of excess energy dissipation. Clearly, this exceptional multifunctionality of Chls originates from an interplay of pigment-pigment and pigment-protein interactions, controlled and tuned in a strict manner by the protein matrix. This multifunctionality of Chls is achievable due to the presence of a highly tunable π-electron system, prone to modulation via a number of intermolecular interactions (Fiedor et al., 2003; Kania and Fiedor, 2006; Fiedor et al., 2008). At the structural level, Chls are also key components of photosystems; they are required for the assembly of individual complexes and entire photosystems. Furthermore, the non-covalent interactions of amino acid side groups with Chls provide stabilization forces, among which the coordinative bond to the central Mg^{2+} ion (Kania and Fiedor, 2006) and hydrogen bonding to the carbonyls have the largest contribution (Scheer, 1991; Grimm et al., 2006; Fromme, 2008). As the crystal structures show, in most cases, the Chls in photosynthetic complexes accept a single axial ligand (Liu et al., 2004; Amunts et al., 2007; Fromme, 2008) but a double axial ligation seems to be possible too (Fiedor, 2006). The phytyl chain is another highly conservative structural motif of Chls. Its presence only slightly affects spectral features of the pigments (Fiedor et al., 2003; Fiedor et al., 2008) but in vivo this moiety is important for hydrophobic contacts with amino acid residues and carotenoids (Crts) (Liu et al., 2004; Standfuss et al., 2005; Amunts et al., 2007). The present chapter gives an overview of the current understanding of the roles and interactions of Chls in plant photosynthetic pigment-protein complexes.

2.3. Organizations and general features of photosystems

Both PSI and PSII are supercomplexes of reaction centers and several types of LHCs (Fig. 2). The total molecular masses of PSs are approximately 600 kDa and 320 kDa, respectively. The crystallographic studies show that 17 protein subunits of PSI host in total almost 170 molecules of Chl *a*, five Crt molecules (Fig. 1), two phylloquinones (vitamin K_1) and three Fe_4S_4 centers (Fromme et al., 2001; Amunts et al., 2007). The core of plant PSI contains approximately 100 molecules of Chls, whose positions are largely identical to those in cyanobacterial PSI. The monomeric form of PSII consists of more than 14 membrane protein subunits and over 40 cofactors, including Chls, Crts, two transition metal ions, Fe and Mn, and plastoquinones (PQ). The central part of the complex, which is formed by two clusters of five transmembrane α-helices (TMHs) assigned to the D1 and D2 subunits that bind the cofactors, closely resembles the arrangement of L and M subunits and their cofactors of the purple bacterial RCs (Goldbeck, 2006; Fromme, 2008).

The organization of photosystems in the thylakoid membrane is remarkable. PSI is localized mostly in the stroma lamellae and at the edges of the grana stacks, whereas PSII is found almost exclusively in the stacked regions of the membranes. The cytochrome b_6f complex is almost evenly distributed between the two types of membranes and the ATP synthase complex is entirely localized in the stroma lamellae (Blankenship, 2002). In the oxygen-producing photosynthetic organisms, the cooperation of the two PSs is necessary to utilize the combined energy of photons to drive a series of reactions which yield reducing power, strong enough to produce NADPH, and a proton gradient, high enough to generate ATP. The combined energy of two photons captured in two consecutive steps is thus sufficient to abstract electrons from water molecules, i.e. to oxidize it, in PSII, and to produce a weak reductant plastoquinol (PGH_2) from PQ. The energy of electrons delivered from PSII is further elevated in PSI, which generates the reducing power. All primary reactions in the PSs are carried out by specialized pigment-protein complexes in which Chls play the crucial roles. Most Chls are contained in the system of antenna complexes which serve to increase the probability of capturing photons in a wide range of wavelengths, thus covering the visible spectrum. The intensive coloration of LHCs stems from a high content of Chls *a* and *b*, whose ratio in each complex varies only to a minimal degree. Besides Chls, LHCs bind yellow or orange pigments, Crts. The performance of both antenna system and photochemical RCs is very high; the quantum efficiency of excitation transfer from antenna to RC almost reaches 1, meaning that each quantum of appropriate energy absorbed by LHCs

triggers the photochemical act of charge separation in RC. Major antenna complexes LHCI and LHCII are associated with the respective photosystems. However, in particular the LHCII complex under specific circumstances changes its location and moves from PSII to PSI (Blankenship, 2002).

2.4. Photosystem II

The essential processes of photosynthetic water decomposition take place in PSII, which in this context can be regarded as a light-driven water:plastoquinone oxidoreductase. PSII generates a redox potential between +1.1 and +1.2 V, high enough to drive the oxidation of water. PSII, a supercomplex composed of more than 20 subunits embedded in the thylakoid membrane (Barber, 2003; Umena et al., 2011), is the only biological system capable of oxidizing H_2O to O_2 (Wydrzyski et al., 2005). Long before the PSII crystallization, the cofactors of charge separation and electron transfer were identified by application of a variety of biochemical and spectroscopic techniques, including EPR and time-resolved absorption and emission spectroscopies (Blankenship, 2002; Wydrzyski et al., 2005; Aartsma and Matysik, 2008). The protein and pigment composition of eukaryotic PSII is similar to that found in cyanobacteria. The functional role of some proteins associated with PSII is not yet clear. It is possible to remove (or mutate) most of these proteins and PSII still retains its photochemical activity (Blankenship, 2002).

PSII reaction center. The D1 and D2 subunits of PSII bind six Chls: P_{D1}-P_{D2} (P860), Chl_{D1} and Chl_{D2}, $ChlZ_{D1}$ and $ChlZ_{D2}$, two pheophytins, two plastoquinones and non-heme Fe (see Fig. 3A). The crystallographic studies show that the P_{D1}-P_{D2} Chls are ligated by D1-His-198 and D2-His-197, and $ChlZ_{D1}$ and $ChlZ_{D2}$ by D1-His-118 and D2-His-117, respectively. The values of 10-11 Å of the center-to-center distances between Chls P_{D1} and P_{D2}, and from these two to their «accessory» Chls Chl_{D1} and Chl_{D2} confirm the validity of the multimer model of Chl arrangement in PSII RC (Zouni et al., 2001; Umena et al., 2011). Two Crts, most likely β-carotenes, are located in the proximity of Chls in RC, one in the *cis*- and another in the *all-trans* conformation. The distances between the *cis* Crt and $ChlZ_{D2}$ (12 E) and Chl_{D2} (21 E) suggest that this pigment may be involved in the secondary electron transfer from RC to cytochrome b_{559}. Also, the close location of the two Crts to D1/D2 cluster points to their participation in the dissipation of excess energy under strong illumination. The D1/D2 heterodimer is surrounded by a cluster of six TMHs of the CP43 and CP47 inner antennae. These inner antenna arose in cyanobacteria 3 bln years ago. Chloroplasts show no obvious sequence or structural similarity to LHC. CP43, located

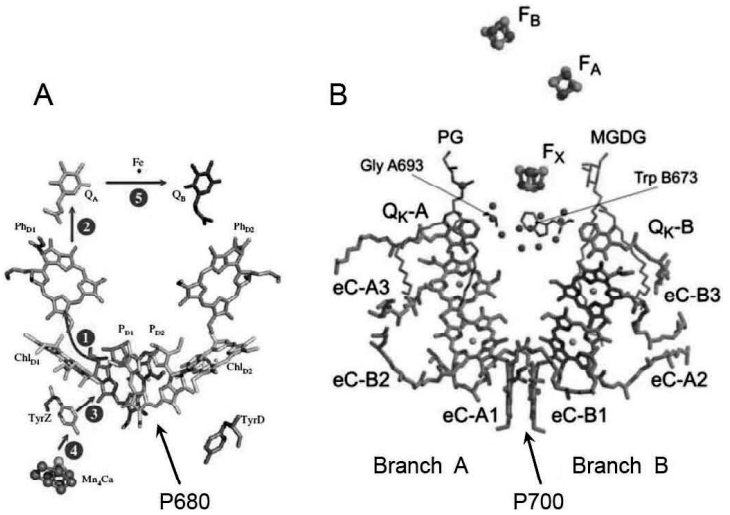


Fig. 3. Schematic organization of cofactors in the reaction centers of plant photosystem I (A), adopted after Fromme et al., and photosystem II (B), adopted from Cardona et al., 2012.

in the vicinity of the D1 protein which binds 13 Chls while CP47, associated with D2, coordinates 16 Chls. The PSII core binds other monomeric antenna complexes, CP29, CP26 and CP24. Similarly to LHCII, they bind Chla, Chlb and xanthophylls as antenna pigments.

In general, the overall structure and charge separation (see below) in PSII reaction center resemble that in purple bacterial RCs. The D1-D2-cytochrome b559 complex is biochemically sufficiently stable to survive isolation procedures as a functionally competent entity, often referred to as PSII RC (Fromme 2008), but the two types of RCs differ in some important detail and the PSII RC is more complex. Firstly, the electronic coupling between the special pair Chls in PSII is weak, of the order of 85-150 cm^{-1}, and the geometry of the special pair is different, resulting in a smaller orbital overlap. Due to rotation, there is almost no overlap of rings I in the P_{D1}-P_{D2} dimer (Fig. 3A). In effect, the P_{D1}-P_{D2} coupling is not much greater than among the remaining four RC pigments, and if compared to the couplings between the components of bacterial RC, P_{D1} and P_{D2} may be regarded as monomers. The electronic couplings between the remaining Chls and Pheos are even weaker (~50 cm^{-1}) but functionally the entire ensemble of six weakly coupled pigments can be regarded as a multimer. From a different point of view, only the interactions among the special pair Chls seem to be unique and other pigments are seen as monomeric (Cardona

et al., 2012). In either case, the special pair Chls do not create a low energy trap but should be viewed as a weakly coupled dimer whose geometry brings about a coupling with a strength and character being exceptional among the interactions between the chromophores of PSII RC. The weak interactions do not perturb the energy levels of the RC components and their absorption profiles largely overlap. In effect, the initial excitation in PSII RC does not localize on any specific cofactor; at ambient temperatures it may reside on each of four Chls and two Pheos (Groot et al., 2005).

Major antenna of photosystem II. LHCII is perhaps the most abundant membrane protein on Earth. It is richly pigmented, binding up to 50% of Chls present in chloroplasts and hence it is responsible for collection of most solar energy in the biosphere. LHCII is a prototype of a large family of membrane pigment-proteins which constitutes as much as one third of proteins in thylakoid membranes. As the other members of LHC family, LHCII is nuclear-encoded. The LHC apoproteins are the product of the *Lhc* gene superfamily which in *Arabidopsis* comprises over 30 homologous genes. In addition to the three apoproteins of the major LHCII, Lhcb2, Lhcb1 and Lhcb3, it also contains minor antenna complexes of PSII, CP24, CP26 and CP29 (Barros and Kuhlbrandt, 2009). LHCII and other members of its family occur only in plants and seem to join the basic set of photosynthetic pigment-proteins later than the PSII core antennae CP43 and CP47. The apoproteins of both core antennae are chloroplast encoded and these type of antenna complexes are more ancient then LHCII as they evolved in cyanobacteria some 3 billion year ago. The basic structural and functional form of LHCII is a trimer, whose structure was first solved

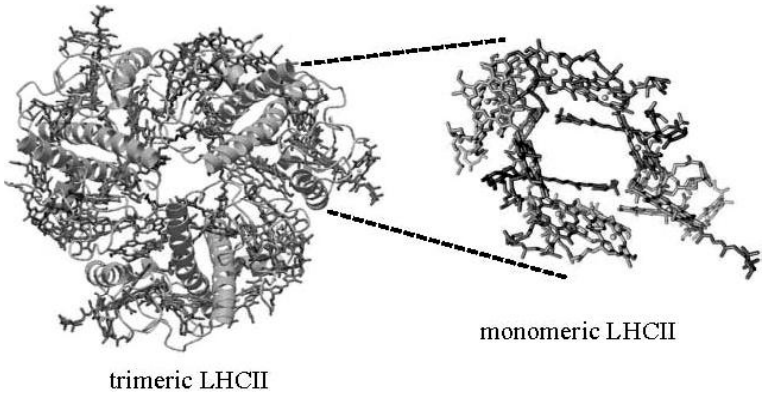


Fig. 4. Top view of the stromal side of the trimeric form and pigment localization in the monomeric form of LHCII from pea. Adopted from Standfuss et al., 2005.

by electron microscopy of 2D crystals (Kuhlbrandt, 1984) and then by X-ray crystallography on 3D crystals (Kuhlbrandt, 1987). More recently, the structure of trimeric LHCII has been obtained with 2.5-2.72 Å resolution (Liu et al., 2004; Standfuss et al., 2005). Remarkably, the trimeric form of LHCII (the structure shown in Fig. 4) hosts 54 pigment molecules of five different types and holds the chromophores in a precise orientation, which is crucial for a high efficiency of its functioning. The LHCII polypeptide is made of 232 amino acid residues and on average, 13 amino acid residues bind one pigment molecule. Each monomeric LHCII is composed of three transmembrane helices and two amphipathic helices which bind 8 Chl *a* and 6 Chl *b* molecules and 4 xanthophylls (one neoxanthin, two luteins and one violaxanthin, see Fig. 4). Besides the chromophores, LHCII tightly binds two types of lipids, phosphatidyl glycerol (DG) and digalactosyl diacyl glycerol (DGDG). The former lipid is bound at monomer interface and is required for the trimer formation while the latter one is located more peripherally. The symmetrical arrangement of the two longer transmembrane helices, A and B, defines a region of local near-twofold symmetry, and reflects an internal repeat of the apoprotein, indicating a gene duplication step in the evolution of LHCII (Barros and Kuhlbrandt, 2009).

In addition to the main function of light harvesting, LHC complexes play a key role in dissipation of excess excitation energy under strong illumination (Pascal et al., 2005; Standfuss et al., 2005), as discussed below. The overexcitation may lead to the formation of a too high proton gradient across the thylakoid membrane. In addition, Chls are very efficient photosensitizers of singlet oxygen and other reactive oxygen species from molecular oxygen (Fiedor et al., 1993; Fiedor et al., 2001) that is in situ generated by PSII at the very location of the photosensitizers. The damage to PSs caused by this photodynamic effect may thus be even more extensive. The photodamage of LHCII is prevented thanks to an efficient triplet quenching by Crts bound in the complex, accommodated in hydrophobic pockets formed either by hydrophobic amino acid residues on the surface of the A and B helices or at the monomer-monomer interface formed by Chls, hydrophobic residues and PG molecules (Liu et al., 2004). In addition to the structure-stabilizing and photoprotective roles as Chl triplet state quenchers, lutein and neoxanthin function as efficient light-harvesting pigments, complementing the light absorption in the blue-green spectral region. The rate of singlet ET between Crts and Chls depends on their mutual orientations and distances. In LHCII six Chls are located in favorable orientations and distances to two luteins to facilitate an efficient ET from lutein to Chl *a*. Also, efficient ET from neoxanthin to Chl *b* 606 and Chl *b* 608 is very likely to take place. In general, luteins transfer excitation exclusively to Chl *a* molecules while neoxanthin transfers the energy mainly to Chl *b* molecules.

2.5. Photosystem I

PSI functions as a plastocyanin:ferredoxin photo-oxidoreductase that drives electron transfer between two water-soluble redox active enzymes, plastocyanin and ferredoxin. In order to fulfill this function, PSI has to generate a high redox potential. Indeed, the redox potentials of the early electron acceptors in PSI are approximately −1V, while the excited state of P700, the special pair of Chls, is estimated to reach −1.26 V, which is the highest value found in natural systems (Goldbeck, 2006). The structure of (cyanobacterial) PSI has been determined by X-ray crystallography (Krauβ et al., 1996; Jordan et al., 2001; Amunts et al., 2007). The core of plant PSI consists of at least 17 subunits, termed PsaA to PsaL, and PsaN to PsaP. Three of them, PsaA, PsaB and PsaC, directly host the electron transfer cofactors: P700 – the heterodimer of Chl *a* and Chl *a*' with the Q_Y absorption maximum at 700 nm, two monomeric Chl *a* molecules (A_0) or eC-A2/B2, see Fig. 3B), two «accessory» Chls (eC-A3 and eC-B3), two phylloquinone molecules (A_1), and three Fe_4S_4 clusters (F_X, F_A and F_B). As shown in Fig. 3B, P700 is located at the heart of the RC while the Chls and phylloquinones are arranged pairwise in two branches A and B, related to each other by a pseudo-C_2 symmetry, which evolved from an ancient homodimeric assembly. The S diastereoisomer (non-prime) of Chl *a*, bound to the B-branch, coordinates axially His B660 and seems not to form H-bonds to the apoprotein. The R form (epimeric Chl *a*') rests on the A-branch and is axially ligated by His A680, and forms three H-bonds to the amino acid residues of TMHs A-i and A-k and a molecule of water (Fig. 3B). The significance of the intrinsic asymmetry for the functioning of PSI remains to be confirmed. However, Chl *a*' is assumed to be a conservative component of P700 in cyanobacterial, algal and plant PSI, and seems to be essential to its functioning. The ultrafast spectroscopic measurements show that both branches are active in electron transfer through PSI (see below). Chls eC-A3 and eC-B3 are hydrogen bonded to tyrosines A696 and B676, respectively (Fig. 3B). The axial ligands to these pigments are quite unusual as each is ligated by a sulphur atom of methionine residues (A688 and B668). The replacement of AMet688 by histidine which is a much stronger ligand of the central Mg^{2+} leads to the blockage of electron transfer along the A branch; which, however, does not affect the autotrophic growth of the green algae mutant. In contrast, an analogous mutation on the B branch strongly inhibits the photosynthetic competence of the mutant. This observation points to the importance of the B branch in the electron transfer in PSI. The engagement of the two branches in the electron transfer is still a matter of controversy and it seems likely that the balance between the two pathways is species-

dependent. The analysis of amino acid sequences of proteins of the PSI complexes from various types of photosynthetic organisms (plants, green algae and cyanobacteria) shows all residues that act as ligands or hydrogen bond donors to the 2nd and 3rd pairs of Chls are highly conserved. The evolutionary preservation of the same pattern of intermolecular interactions speaks for the key role of these interactions in tuning the physico-chemical properties of the cofactors of the charge separation in PSI. Yet, the main reason for the very low redox potential of the primary electron acceptor A_0 is unclear (Blankenship, 2002).

The rest of the PSI subunits have various other functions; PsaF and PsaN comprise a docking site for ferredoxin on the stromal side of the thylakoid membrane while PsaF and PsaN interact with plastocyanin, the lumenal electron donor. Additionally, the PsaF subunit is involved in close interactions with the Lhca1/Lhca4 dimer. The core antenna consists of nearly 100 Chl *a* and 20 β-carotene molecules, most of which are bound to the PsaA and PsaB subunits. The smaller subunits also bind a few pigment molecules. The Q_Y transitions of majority of Chls are located near 680 nm but a fraction exists with the Q_Y maximum shifted to 730 nm, termed «red chlorophylls». They contribute only up to 5% of the total absorption and thus cannot be easily seen in the absorption spectra of PSI but their signature is well seen in emission recorded at 77 K as a peak at 735 nm. This form is found in all PSIs studied so far and it was attributed mainly to Lhca1-4 antennae. It was also found in monomeric Chl-protein complexes reconstituted in vitro, suggesting the mechanism of their formation, namely, due to excitonic interactions between Chls bound at specific sites in Lhca. The long-absorbing Chl forms may be involved in photoprotection (dissipation excess energy without RC excitation) and/or in the absorption of light in the spectral region above 700 nm, e.g. in leaves under a canopy (Jensen et al., 2007).

The peripheral antenna of PSI, LHCI, is composed of four polypeptides, Lhca1-Lhca4, of molecular masses between 20 and 24 kDa (~160 kDa in total, including pigments), which bind Chl *a*, Chl *b* and luteins. The conservative arrangement of luteins, being in close contact with three Chls is found in both LHCII and Lhca complexes. The LHCI complex shares considerable sequence and structure homologies with LHCII, and the tertiary structure of all Lhca proteins is the same as in LHCII whereas their common motif of two central TMHs superpose almost perfectly with those of LHCII. However, they aggregate in different ways as LHCI assembles into dimers while LHCII forms trimers (Jensen et al., 2007).

2.6. LHC assembly

The apoproteins of the majority of photosynthetic pigment-protein complexes are products of nucleus-located genes and they require post-translational modifications and relocation to thylakoid membrane via the plastid membrane. The apoproteins are synthesized as precursor proteins with cleavable N-terminal presequences (i.e. transit peptides) and then imported by protein translocation system in the outer and inner envelope membranes. After the import into the stroma, the apoproteins undergo further targeting to the thylakoid membrane. For instance, the LHCII apoprotein is synthesized in the cytoplasm, transported through the outer and inner envelopes of chloroplast, and then inserted into the thylakoid membrane, which requires the engagement of the chloroplast signal recognition particle (CSRP) (Jensen et al., 2007; Barros and Kuhlbrandt, 2009). A detailed mechanism of the in vivo assembly of LHCs is not well established but a substantial piece of useful information about their folding, assembly and pigment composition and pigment binding comes from reconstitution studies. which indicate that the presence of Chls is strictly required for the folding of mature pigment-proteins and the assembly of larger functional ensembles. These in vitro studies show that the apoproteins of LHCII and other related antenna complexes are able to bind externally added pigments (Chls and Crts) and then self-assemble, forming complexes spectroscopically and biochemically very similar to the native complexes isolated from photosynthetic membranes (Plumley and Schmidt, 1987; Paulsen et al., 1990). A similar reconstitution approach was successfully applied to study minor LHCs. The LHCII apoprotein, now available via expression in bacteria, unfolded in SDS, spontaneously folds into a functional pigment-protein complex, when mixed with Chls and Crts in detergent (SDS). The pigment-triggered folding occurs in two phases, a fast one (tens of seconds) and a slower one, which takes several minutes. Binding of Chl *a* occurs in the first step while Chl *b* is bound in the second step, during which the complex reaches its mature and stable conformation (Docker et al., 2009).

2.7. Functioning of chlorophyll-protein complexes

2.7.1. Charge separation in reaction centers

Although the structures of PSI and PSII reaction centers follow a common pattern of two quasi-symmetrical branches of electron transfer cofactors, the actual pathways of charge separation in them differ considerably. The kinetics of ET in PSI is biphasic, i.e. both branches are active, while in PSII only one branch is active, similarly to the RC of purple photosynthetic bacteria.

Photosystem II reaction center

As shown in Fig. 3A, the overall scheme of charge separation in PSII RC is similar to that in photosynthetic purple bacterial RC but in addition to the above discussed structural differences and obvious difference in the energetics several other relevant differences exist at the level of primary events. Excitation energy is transferred from the antenna system to the RC, where the charge separation takes place. P680*, the excited primary electron donor, ejects an electron to the plastoquinone Q_A, via pheophytin a Ph_{D1}, forming a stabilized pair $P680^{+\bullet}Q_A-$. An electron is then transferred from Q_A to Q_B, the weakly-bound secondary plastoquinone. After the second reduction and its double protonation, Q_B leaves the RC complex and in its place another plastoquinon is accepted from the membrane «plastoquinone pool». $P680^{+\bullet}$ formed as a result of primary charge separation is one of the strongest oxidants in biological systems. The mechanism by which such a strong potential (+1.1 V) can be generated remains unclear (Blankenship, 2002). Obviously, the redox properties of electron transfer cofactors and the kinetics of electron transfer depend on the specific spatial arrangements of the components within the protein matrix, which in this way tunes their photophysical properties. There two principal factors which control the spectral and functional features of the cofactors (pigments) bound to the protein: pigment-pigment interactions (coupling) and pigment-protein interactions (Renger, 2009). The electrons required to re-reduce $P680^{+\bullet}$ to P680 are delivered, via a redox-active Tyr residue (Tyr_Z), by the Mn_4Ca cluster, which directly abstracts electrons from water molecules. At each step, the water oxidizing complex is oxidized to a higher oxidation state, and finally, after four steps of electron abstraction from water, an O_2 molecule is released.

Initially, the excitation is thought to localize on P_{D1}, which seems to be the longest wavelength pigment on the RC. Following excitation, the primary charge separation does not occur in a single step but rather several charge separated states are formed during the first few ps. After next several tens of ps, an electron transfer occurs and a more stable secondary radical pair $P_{D1}{}^{+}Ph_{D1}{}^{-}$ is created, in which the positive charge is mostly localized on P_{D1}, somewhat in contrast to the bacterial RC, where it remains delocalized on the special pair. In the following 400 ps, an electron is transferred from $P_{D1}{}^{+}Ph_{D1}{}^{-}$ to Q_A with some energy loss, which provides stabilization to this long-lived $P_{D1}{}^{+}Q_A{}^{-}$ pair. In the next step, within 0.2-0.4 ms, an electron is transferred to Q_B. In the meantime (~50 ns), the highly oxidizing cation radical $P_{D1}{}^{+}$ receives an electron from Tyr_Z (Tyr 160 of D1), forming Tyr_Z radical which in turn oxidizes the Mn_4Ca cluster and thus provides oxidative power for water splitting. After a second reduction and a double

protonation of the labile Q_B, the reduced form of plastoquinone, QH_2, is released from the RC. Its vacant site is at the end replenished from the plastoquinone pool in the thylakoid membrane, and the entire PSII RC nanodevice is ready to perform the next sequence of charge separation and electron transfer steps (Cardona et al., 2012).

Photosystem I reaction center

Theoretical analysis of electronic structure of P700 based on DFT calculations on the crystal structure of PSI showed that the Chls are strongly coupled and form a supermolecule. The asymmetry stems from a combination of asymmetric hydrogen bonding and differences in protein environment and chemical nature of the two Chls. This is well reflected in functional features of P700* as the ENDOR studies showed that the spin density is distributed asymmetrically in P700, with over 85% of the spin density residing on Chla and the remaining 15% on Chl *a*'. P700* donates an electron to the first stable electron acceptor A_0 via one of the second pair of Chls (eC-A3, Fig. 3B). This initial charge separation occurs within less than 3 ps. The crystal structure of PSI resolved at 2.5 Å supports the notion of a direct involvement of Chls on the A branch in the electron transfer to A_0. Some studies even indicate that one of these «accessory» Chls acts as the primary electron donor instead of P700. However, as mentioned already, due to a strong overlap of spectral features of numerous Chls in PSI, the spectroscopic identification of individual charge transfer steps still remains a challenge (Blankenship, 2002).

The early electron acceptors are a unique set of three iron-sulfur clusters. The three Fe_4S_4 centers have distinct EPR spectra, which have been characterized in detail (Blankenship, 2002). The electron transfer processes in photosystem I begin with excitation of P700, usually by excitation transfer from the antenna pigments. The redox potential of P700 in the excited state is lowered to as much as −1.26 V, generating the largest reducing power among the biological systems (Goldbeck, 2006). The intrinsic time for electron transfer to the primary acceptor is ~2 ps. However, the observed excited state lifetime in PSI is significantly longer, 30 ps, because most of the time the excitation does not reside on P700, but is instead on one of the many antenna pigments. The primary electron transfer produces a state in which P700 is oxidized, and the A_0 Chl *a* is reduced. The electron then moves in 25 ps to one of the phylloquinones, and in the next 200 ns from there to F_X. An electron is transferred from the PSI complex to the soluble protein ferredoxin, and ultimately $NADP^+$ is reduced. There are evidences that both branches of electron transfer cofactors are active in the charge separation in PSI RC as the electron transfer process seems to have two kinetic components. Two point mutations

of amino acid residue near phylloquinones in either of the branches affect the electron transfer kinetics in different ways (Guergova-Kuras et al., 2001). A more recent study shows that the primary electron transfer in each branch can be initiated independently and that the initial step of charge separation occurs on the eC-2/3 Chls and only then an electron moves to P700 (Muller et al., 2010).

$P700^+$ is rereduced in most organisms by electron transfer from the blue copper protein plastocyanin, although in some organisms a c-type cytochrome (cytochrome c_6) serves this role. The PsaF protein is implicated in plastocyanin binding, although the core heterodimer PsaA and PsaB complex is also involved in this process. Plastocyanin oxidation kinetics are complex and depend on whether or not the plastocyanin is bound to the PSI complex prior to P700 oxidation (Blankenship, 2002).

2.7.2. Energy transfer from antennae to the reaction centers

The mechanisms of light harvesting and excitation energy transfer within LHCII have been intensively investigated using a wide variety of spectroscopic techniques (van Amerongen and van Grondelle, 2001; Green and Parson, 2003; Fromme, 2008). Crts in LHCII, in addition to their photoprotective and structural functions, act as light absorbers complementing the light harvesting function of Chls by increasing absorption in the blue-green spectral region. The overall efficiency of Crt-to-Chl energy transfer reaches 70-90%, with the time constants ranging from 50 to 200 ps. The high resolution structural data enabled for the spectroscopic data to be interpreted and a model for the excitation energy dynamics has been proposed. The central point in this model is the rapid and efficient ET from Chl *b* to Chl *a*, which occurs in sub-ps time scale. The ET between Chl *a* molecules within the same and among the monomeric subunits takes several ps. In effect, on ps timescale the spectral equilibration takes place that leads to a rapid localization of excitations on the stromal side of the trimeric complex, mostly at its periphery (Chls 610-612). The cluster of these Chls is located at the periphery of the LHCII trimer, which seems necessary for efficient energy transfer to PS. From these sites, excitations are further conveyed to other pigment-protein complexes, either directly or via the minor LHCs. Spatially, the equilibration within the trimeric form of LHCII seems to proceed with slower rates, i.e. tens of ps. Excitation energy transfer in the minor antenna complexes follows internal pathways similar to those in LHCII but some differences have been found on the fast time scale. Additionally, the equilibration in these complexes occurs within a couple of ps, resulting in the population of the lower energy forms, which usually are located in the stromal domain (Chls 611 and 612). The ET between Chls in the

core and peripheral antennae can be well understood within the Forster model, which is valid only when weak interactions between the chromophores occur. This approach is no longer appropriate when stronger interactions between Chls take place, e.g. among the «red» Chls. The equilibration of excitation energy among Chls occurs in several hundred ps, both in the PSI core and in LHCI while among the red forms it occurs in 2-6 ps (Croce and van Amerongen, 2011).

An estimation based on the crystal structure of plant PSI suggests ET from LHCI to PSI also to take place within a few ps. Such a fast rate of ET is most likely related to the presence of linker Chls between the core and peripheral antennae. So far, no direct data are available for the ET rates in the PSI-LHCII supercomplex, however, in native thylakoid membranes it was estimated to occur in 25 ps. The main step of excitation trapping in isolated PSI by charge separation in the RC takes 20-50 ps. Its rate depends on the number of red chlorophylls present; the smaller their number, the faster the excitation trapping. The charge separation kinetics is complex and consists of at least two trapping steps with distinct spectral characteristics. The main trapping phase occurs in 20-25 ps, with a decay-associated spectrum with a maximum at 685 nm and a shoulder near 720 nm. This fast phase originates from excitations absorbed and trapped in the PSI core complex. The second, slower trapping phase, occurs in 60-120 ps and is related to a decay-associated spectrum with two maxima, one at 685 nm and another at 725 nm. This phase is attributed to excitations that are absorbed in the peripheral antennae and at slower rate transferred to the core complex. However, it is not clear what makes this phase slower as numerous linker Chls present between peripheral and core antennae should facilitate an efficient ET between these complexes. The charge separation in the PSI core complex is either trap-limited or transfer-to-the trap limited or both. In the PSI-LHCI complex the presence of linker Chls between core and peripheral antenna implies rapid ET between them and hence the trap-limited or transfer-to-the trap limited ET kinetics (Blankenship, 2002; Byrdin et al., 2002; Melkozernov et al., 2004).

2.7.3. Photoprotection and energy dissipation

There are several mechanisms involving LHCII that prevent the damages to the photosystems due to overexcitation under strong light. On the immediate level, harmful triplet states of Chls are efficiently quenched by Crts bound in the complex. Another process, referred to as nonphotochemical quenching (NPQ), operates to dissipate excess energy as heat (Blankenship, 2002). The analysis of the properties of LHCII crystals provided basis to explain the molecular mechanisms underlying NPQ (Standfuss et al., 2005; Barros and Kuhlbrandt, 2009).

And yet, short-term adaptations of photosynthetic apparatus to variations in light intensity include relocations of some antenna complexes, giving rise to the so called state transitions, due to which a balance of excitation distribution between PSI and PSII is maintained. When the overexcitation of PSI occurs under light of high intensity, LHCII undergoes phosphorylation at its N-terminus and migrates from PSII (in state I) to PSI (state II). In green algae, as much as 80% of LHCII complexes may participate in state transitions. This mechanism is of lesser significance in plants, where in state II, the efficiency of light harvesting due to LHCII attachment to PSI increases by 15-20%, corresponding to one LHCII trimer per PSI. Thus, it belongs to the system of how plants adapt to changes in light intensity by redistribution of excitation energy between the two PSs to protect them and elevate the overall efficiency of photosynthesis. The PSI supercomplex is able to interact with other photosynthetic proteins in a controlled manner. The most important of these interactions is the one with LHCII during state transition. Indeed, many studies indicate that LHCII antenna and PSI are forming a supercomplex RC-LHCI-LHCII. Structural modeling confirms such an arrangement and shows that only one of the monomeric subunits of LHCII is involved in the interaction with PSI core, likely at the PsaK site. Under low intensity light and when PSII excitation is predominant, phosphorylated LHCII relocates and binds to PSI. Another photoprotective mechanism is referred to as the xanthophyll cycle (Demmig-Adams and Adams III, 1993). It involves a series of chemical reactions, first the conversion of violaxanthin into antheraxanthin and then to zeaxanthin. The latter xanthophyll can act as direct quencher of excess excitation by accepting energy from singlet excited state of Chls (Jahns et al., 2009; Gruszecki et al., 2010). There are at least three Chl molecules in LHCII which assume close enough and favorable positions to act as efficient donors of singlet excitation to xanthophylls (see the Chapter 10). In addition, the distance smaller than 10 Å and mutual orientation of two Chls, Chl 613 and Chl 614, make them a good candidate to function as statistical pair energy trap. A structure-based mechanism of non-photochemical quenching in LHCII was proposed which involves formation of efficient ET pathways upon the aggregation of the antenna trimers in the presence of DGDG. This enables the excess excitation to migrate between neighboring trimers until it reaches one of the quenching sites, i.e. xanthophylls or Chl pair energy trap, and where its dissipation takes place in a non-radiative manner (Liu et al., 2004).

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Chapter 3. XANTHOPHYLLS OF PHOTOSYSTEM II

Wieslaw I. Gruszecki
Institute of Physics, Maria Curie-Sklodowska University, Lublin, Poland

Contents

3.1. Introduction

Carotenoids belong to a group of yellow, orange and red pigments which are ubiquitous both in the animal and plant kingdoms (Britton, 2008). In plants, they are responsible, among others, for the coloration of flowers and fruits but they are also present in leaves, in which carotenoid pigments are masked by the green color resulting from the presence of chlorophylls. It is very likely that carotenoid pigments present in the photosynthetic apparatus of plants play key physiological functions. Those functions are associated with collection of light quanta, transfer of excitation energy to chlorophylls, as well as protection of both chlorophyll molecules and entire photosynthetic apparatus against oxidative damage associated with overexcitation. Those functions of carotenoids are described, in detail, in other chapters of this book. This chapter provides introductory information regarding structural and photo-physical properties of xanthophyll pigments present in the photosynthetic apparatus of plants, and in particular associated with the pigment-protein complexes of Photosystem II.

3.2. Structure

Fig. 1 presents the chemical structure of β-carotene, the basic non-polar photosynthetic carotenoid, and the structures of the main photosynthetic xanthophylls (carotenoids substituted with polar oxygen functions). The main polar groups,which appear in the xanthophyll pigments of the photosynthetic apparatus of plants, are hydroxyl groups, located in the C3 and C3' positions (e.g. in lutein, violaxanthin and zeaxanthin) and epoxy groups present in the C5-C6 and C5'-C6' positions (e.g. violaxanthin). As can be seen from the chemical struc-

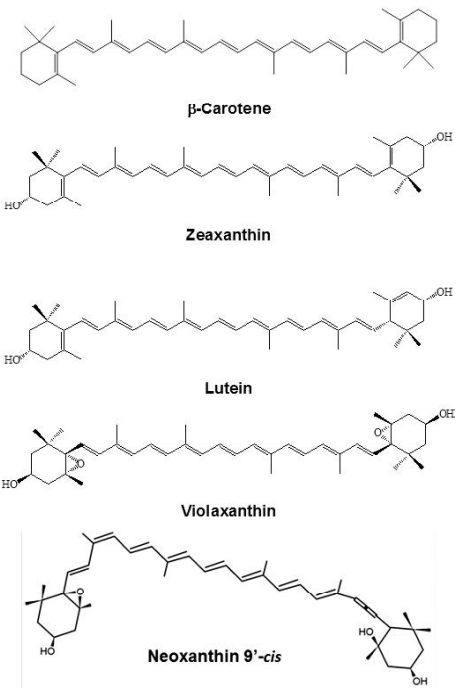

Fig. 1. The chemical structure of basic carotenoids of the photosynthetic apparatus of plants.

tures presented in Fig. 1, some xanthophylls are simply derivatives of β-carotene, such as zeaxanthin ((3R,3'R)-β,β-carotene-3,3'-diol) and violaxanthin ((3S, 5R,6S,3'S, 5'R,6'S)-5,6:5'6'-diepoxy-5,6, 5',6'-tetrahydro-β,β-carotene-3,3'-diol). Lutein has one β-ionone ring, as in the case of zeaxanthin but the second terminal ring is an ε-ring, with its double bond placed in the position 4',5' instead of 5',6' (93R,3'R,6'R)-β,ε-carotene-3,3'-diol). This means that the double bond of the ring is not conjugated with the entire double bond system of the polyene chain. Such a difference has both structural and photo-physical consequences which will be discussed below. Neoxanthin ((3S,5R,6R,3'S,5' R,6'S)-5',6'-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro-β,β-carotene-3,5,3'-triol) is a xanthophyll which has one terminal ring the same as in the case of violaxanthin but the second ring is essentially different: substituted with two hydroxyl groups located in the positions C3 and C5. Interestingly, two double bonds in neoxanthin are not conjugated but located in adjacent positions (C6=C7 and C7=C8), forming the allenic bond. In contrast to other photosynthetic xanthophylls which appear in the photosynthetic apparatus in the all-*trans* molecular configuration, neoxanthin appears in the 9'-*cis* form. It has to be mentioned that light-induced isomerization of xanthophylls, in the photosynthetic apparatus of plants, has been reported and interpreted as associated with the regulatory activity. This point will be further addressed below.

Terminal rings and polar groups are principal determinants of chemical properties of xanthophylls. On the other hand, the conjugated double bond system, common to all carotenoids, is a basic structural feature, responsible for rigid structure of a polyene chain. Conjugated double bond system is also responsible for the photo-physical properties of carotenoids, in principal for absorption of electromagnetic radiation

from the blue-green part of the spectrum, so that the remaining part of the visible light spectrum (yellow-orange-red) can reach a photodetector or our eyes, giving rise to color vision.

3.3. Spectroscopic properties

Figure 2 shows the electronic absorption spectra of selected xanthophylls isolated from the photosynthetic apparatus of plants. As can be seen, the pronounced absorption band appears in the spectral region between 350-500 nm. Very clear vibrational sub-structure of the principal absorption band, which is characteristic for each pigment, can be resolved in the absorption spectra (Fig. 2). The molecular vibrations of xanthophylls are mainly associated with the C=C stretching modes. Position of absorption maxima, on the wavelength scale, of different xanthophylls, recorded in the same solvent system, depend on the length of the conjugated double bond system (N) (Christensen et al., 2004). As can be seen, the absorption spectrum of violaxanthin (N = 9) is shifted towards the short-wavelength region with respect to lutein (N = 10) and zeaxanthin (N = 11). This is due to the fact that the longer the conjugated double bond system the lower the energy level associated with the same electronic transition. The main xanthophyll absorption band can be attributed to the single, strongly allowed electronic transition, referred to as the $S_0 \rightarrow S_2$ transition ($1A^-_g \rightarrow 1B^+_u$). The energy level diagram of carotenoids, with indicated allowed electronic transitions, is presented in Fig. 3. This electronic transition is associated with strong light absorption which can be characterized by relatively high molar extinction coefficient (~1.3×10^5 $M^{-1}cm^{-1}$). On the contrary, the $S_0 \rightarrow S_1$ electronic transition is not associated with pronounced transition dipole, due to the same point group symmetry (A), the same parity ($_g$, gerade) andthe same pseudo-parity elements ($^-$) of the ground and excited energy states: $1A^-_g$ and $2A^-_g$. Owing

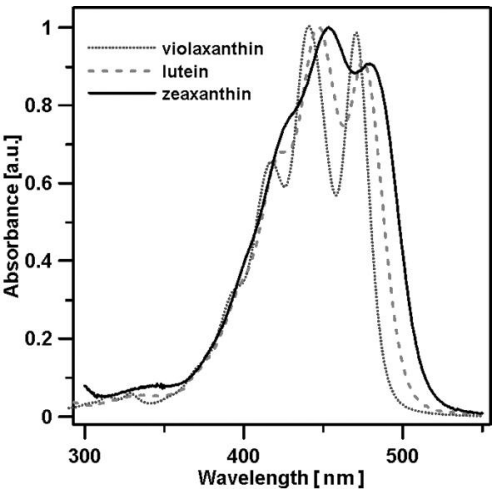

Fig. 2. Absorption spectra of violaxanthin (N = 9), lutein (N = 10) and zeaxanthin (N = 11) in the acetonitrile : methanol : water (75:20:5, v:v:v) solvent system.

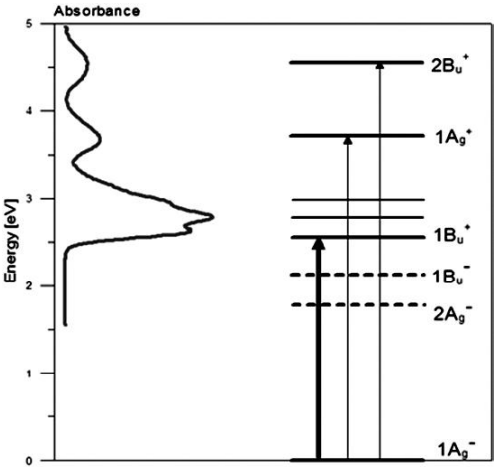

Fig. 3. The energy level diagram of a carotenoid with indicated allowed electronic transitions. The thick arrow represents the strongly allowed transition responsible for light absorption in the blue-green spectral region. The typical Uv-vis absorption spectrum of a carotenoid is also shown showing spectral bands corresponding to individual energy levels. See the text for more explanations.

to this fact the $S_0 \rightarrow S_1$ transition cannot be detected by means of conventional, single-photon absorption spectroscopy. On the other hand, this energy level can be involved in excitation energy transfer between carotenoids and chlorophylls. This point is in more detail discussed in another chapter of this book. The energy diagram presented in Fig. 3 shows also relatively weak electronic transition $1A^-_g \rightarrow 1A^+_g$. Interestingly, this transition becomes more pronounced in the UV-Vis absorption spectra after the change of the molecular configuration of a xanthophyll from the all-*trans* to a *cis* form. Owing to such a fact this electronic absorption band is often referred to in the literature as a "cis band". As can be seen from Fig. 4, the "cis band" is particularly intensive in the case of central *cis* isomers of carotenoids (e.g. 15-15'-di-*cis* violaxanthin).

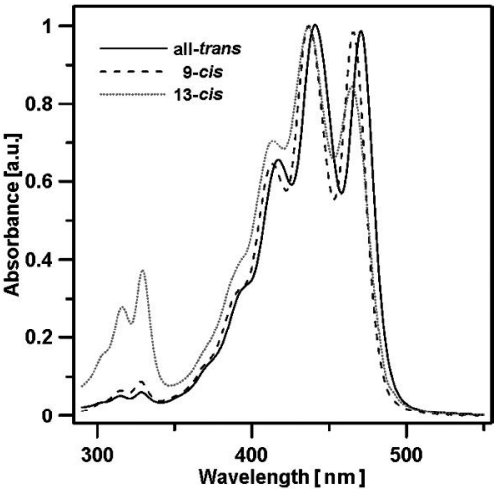

Fig. 4. Absorption spectra of three molecular configuration form of violaxanthin, all-*trans*, 9-*cis* and 13-*cis*, in acetonitrile : methanol : water (75:20:5, v:v:v) solvent system. The spectra were normalized at the maximum.

3.4. Xanthophylls in Photosystem II

According to a general conviction all photosynthetically-active xanthophylls in Photosystem II are associated with pigment-protein antenna complexes: CP 24, CP 26, CP 29 and in particular with LHCII, the most abundant membrane protein in the biosphere (Barros and Kuhlbrandt, 2009). The structure of LHCII, based on crystallographic data (Liu et al., 2004; Standfuss et al., 2005) is presented in Fig. 5, with indicated localization of lutein, neoxanthin and violaxanthin. As can be seen, two molecules of lutein are located in the core of the complex, in contrast to neoxanthin and violaxanthin, located more peripherally. Interestingly, violaxanthin is localized at the border of the trimer-forming LHCII monomers. Very often, violaxanthin is found in the LHCII preparations in the sub-stoichiometric concentrations, which reflects relatively weak binding of this xanthophyll. On the other hand, relatively weak binding of violaxanthin seems to possess a physiological meaning associated with the operation of the xanthophyll cycle (see other chapters of the book for more detailed information on the xanthophyll cycle). Enzymatic de-epoxidation of violaxanthin requires this pigment to be detached from the protein environmentand to be directly present in the lipid phase of the thylakoid membrane, in where is converted to antheraxanthin and zeaxanthin (Latowski et al., 2004). It has been proposed, based on the results of the Resonance

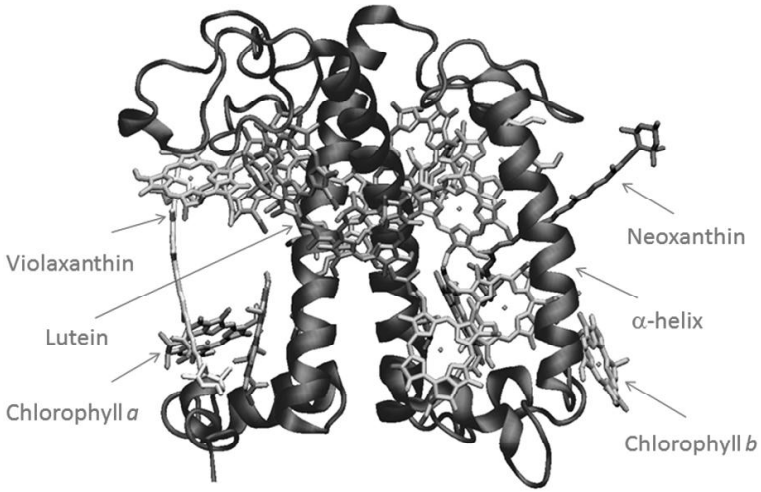

Fig. 5. Molecular structure of the LHCII pigment-protein complex from spinach, based on the crystallographic data (PDB code 1RWT). Main components are marked.

Raman studies, that the process of making violaxanthin available for enzymatic de-epoxidation, understood as detachment from its binding site in the protein, is based upon the reversible pigment photo-isomerization (Gruszecki et al., 2009). According to the mechanism proposed, the light-induced change of the all-*trans* molecular configuration decreases a stereo-chemical affinity of violaxanthin to the pigment binding pocket, which facilitates its transfer to the thylakoid lipid phase. The light-driven reaction is spontaneously reversible due to the fact that the all-*trans* molecular configuration of violaxanthin has lower steric energy. Interestingly, the LHCII-bound neoxanthin appears in the 9'-*cis* form. Neoxanthin is the only xanthophyll which protrudes out of the protein complex therefore is partially present in the lipid phase of the membrane. Such a pigment localization has pronounced physiological consequences. First of all, neoxanthin has been reported to be particularly effective in the protection of the thylakoid lipids against free-radical-induced oxidation. It has been reported that illumination of LHCII with strong light, both in isolated pigment-protein complex and in chloroplasts, induces light-driven molecular configuration change, interpreted as twisting (Ruban et al., 2007). This mechanisms has been postulated to initiate a cascade of conformational events in LHCII, resulting in singlet excitation quenching of chlorophylls via energy transfer to the S_1 energy level of one of two protein-bound luteins. Interestingly, it has been demonstrated that neoxanthin bound to LHCII can also undergo light-driven isomerization from the molecular configuration 9'-*cis* to 9',13'- and 9',13'-*dicis* forms (Zubik et al., 2011). It has been postulated that such photo-isomerization can act as a triggering mechanism which eliminates the steric hindrance and therefore enables two adjacent LHCII trimers to form a supramolecular structure characterized by efficient chlorophyll singlet excitation quenching; therefore possessing a photo-protective meaning under light stress conditions (Zubik et al., 2013). In this context it has to be emphasized that singlet excitation quenching of chlorophyll prevents formation of the chlorophyll triplet state which can be involved in photo-sensitized formation of singlet oxygen and lead to oxidative damage of the photosynthetic apparatus. Xanthophylls present in the pigment-protein complexes, in particular lutein, are also involved in photo-protection via quenching the chlorophyll triplet states.

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Chapter 4. EXCITATION ENERGY TRANSFER BETWEEN PHOTOSYNTHETIC PIGMENTS

Wieslaw I. Gruszecki
Institute of Physics, Maria Curie-Sklodowska University, Lublin, Poland

Contents

4.1. Introduction

Absorption of light quanta by a photosynthetic pigment molecule is associated with transition to one of the excited electronic states. The ground state of both groups of antenna pigments chlorophylls and carotenoids is a singlet state (the state multiplicity equals 1) therefore the allowed, light absorption-induced transition is also to one of the singlet excited states, due to the multiplicity conservation rule (see Fig. 1). There are several ways of de-excitation of singlet excited molecules. Energy of the excited singlet states can be thermally dissipated or emitted as a fluorescence. In the case of chlorophylls, the singlet excited states can be also efficiently converted to the triplet excitation via the intersystem crossing process. In terms of the photosynthetic activity of pigments all the de-excitation pathways have to be considered as an energy loose, owing to the fact that this part of energy may not be utilized to drive charge separation processes in the photosynthetic reaction centers (van Grondelle and Novoderezhkin, 2010). Fortunately, the electronic excitation energy can be transferred from one molecule to another by non-radiative processes. Owing to this fact, hundreds of the photosynthetic antenna

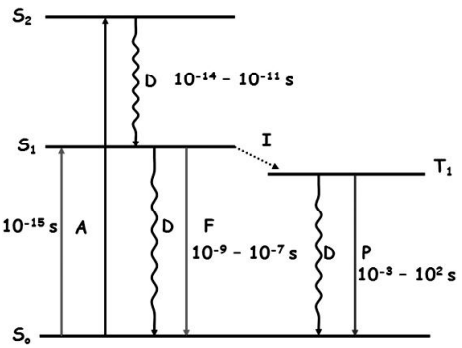

Fig. 1. General Jablonski diagram of energy levels of a molecule. The basic photo-physical processes are shown with typical characteristic times: A – light absorption, D – thermal relaxation, F – fluorescence, I – intersystem crossing, P – phosphorescence. S stands for the singlet states and T for the triplet state.

pigments, covering a broad area and different regions of the sunlight spectrum, can capture light quanta and transfer their excitations towards the reaction centers to drive photosynthesis (Croce and van Amerongen, 2011).

4.2. Theory of excitation energy transfer

Non-radiative excitation energy transfer is an amazing process. Let us imagine two molecules. One of them excited and the other one not. Suddenly, we realize that the molecule which was in the ground state is now excited and the molecule which was originally excited is currently in its ground state. Moreover, one has to state that there was no energy exchange via the fluorescence emission from the excited molecule combined with light absorption by the non-excited molecule. It means that the non-radiative excitation energy transfer between those molecules took place. It sounds like a description of a magic trick but in fact this process of non-radiative excitation energy transfer can be described in a very precise way within the framework of quantum physics. To be more precise, the process of non-radiative excitation energy transfer between two molecules can be described by two formalisms, dependent on whether the molecules are separated or remain in a close, van der Waals contact. The first one, long-range energy transfer is referred to as a Forster process (Forster, 1959) and the second one as a Dexter type energy transfer (Dexter, 1953). The processes have been named in honor of Theodor Forster and Benjamin Dexter, the physicists who contributed with their pioneer analyses to understand and describe formally the excitation energy transfer between molecules. Both energy transfers are the resonance processes. It means that the maximum performance in the transfer is achieved when the excited state of both the donor and the acceptor are isoenergetic. The principal difference between those two processes is that the Dexter mechanism is based upon both the energy and electron exchange since the Forster mechanism is a pure long-distance energy transfer between the interacting molecules. The equation 1 describes the rate of the long-distance non-radiative excitation energy transfer k_{ET}:

$$k_{ET} = A\kappa^2 \frac{\varphi_D}{\tau_D R^6} \int F_D(\nu)\varepsilon_A(\nu)\nu^{-4} d\nu, \quad (1)$$

where: A is a constant, φ_D is a fluorescence quantum yield and τ_D is a lifetime of the excited state of the donor, R is a donor-acceptor distance and the integral of the product of the normalized fluorescence emission spectrum of the donor and the molar extinction coefficient spectrum of the acceptor (both on the wavenumber scale, ν), multiplied additionally by the ν^4 is called "the overlap integral". The factor κ is called the "orientational factor" and is defined by the following dependency:

$$\kappa = (\cos\theta_{DA} - 3\cos\theta_D\cos\theta_A). \quad (2)$$

In eq. 2, θ_{DA} represents the angle between the transition dipoles of the donor and acceptor and θ_A and θ_B stand for the angles between the vector connecting the centers of the transition dipoles of the donor and acceptor molecules and the transition dipoles of the donor and the transition dipole of the acceptor respectively. The schematic visualization of the donor and acceptor transition dipoles, shown in Fig. 2, will make it more clear to imagine relative orientation of those vectors and an effect on efficiency of the energy transfer. It can be very easily calculated that the maximum excitation energy transfer takes place when both the transition dipoles, of the donor and the acceptor, are parallel to each other and, at the same time they are parallel to R. In such a case the κ^2 equals 4 and is double as high as in the case of the parallel transition dipoles but perpendicular to R ($\kappa^2 = 2$). In the case of the transition dipoles perpendicular to each other and both perpendicular to R, the factor $\kappa = 0$ which means that excitation energy transfer will not be possible. Fig. 3 presents schematically the localization of the absorption and fluorescence spectra on the wavenumber scale, of the donor and the acceptor. The marked area corresponds to the overlap integral and helps understand why excitation energy transfer is more efficient between molecules with isoenergetic energy levels. The rate of non-radiative excitation energy transfer is highly distance-dependent, as it is proportional to R^{-6}. It means that by increasing the donor-acceptor distance by a factor of 2 we decrease a rate of the energy transfer by a factor of 64. Interestingly, the rate of the non-radiative energy transfer depends also on fluorescence quantum yield of the donor. It means that the transfer would not by practically possible in the case of donor molecules with relatively low fluorescence quantum yields, despite the fact that fluorescence emission by the donor and light absorption by the acceptor do not take place. Historically, the dependency of the rate of non-radiative energy transfer on the fluorescence quantum yield of a donor created severe problems in understanding the process of efficient excitation transfer in the photosynthetic apparatus, from carotenoids to chlorophylls. This is owing to the fact that carotenoid pigments are known to be characterized by a relatively low fluorescence quantum yield. On the other hand, it appears that the short-range excitation energy transfer

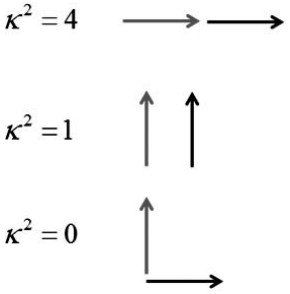

Fig. 2. A scheme to discuss the orientation factor κ. Arrows represent transition dipoles of the donor and acceptor molecules. See the text for more explanations.

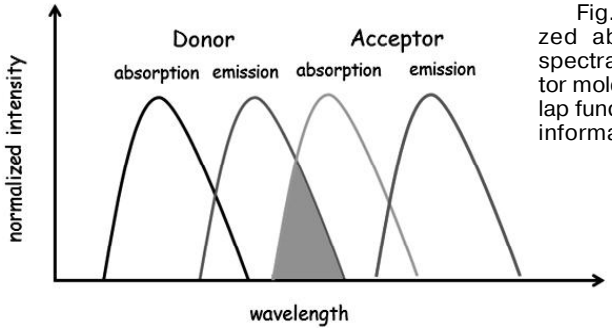

Fig. 3. A scheme of normalized absorption and emission spectra of the donor and acceptor molecules to discuss the overlap function. See the text for more information.

between molecules is not dependent on the fluorescence quantum yield of the donor. This can be seen from the formula which describes the rate of the energy transfer, elaborated by Dexter (eq. 3):

$$k_{ET} = \frac{4\pi}{h} e^{-\frac{2R}{L}} \int F_D(\nu)\varepsilon_A(\nu)d\nu, \qquad (3)$$

where L stands for the mean effective radius of the ground state wavefunction of a donor and excited state wavefunction of an acceptor.

As it can be seen, the Dexter-type energy transfer depends also on the distance between the donor and the acceptor levels on energy scale, expressed by the overlap integral, and depends on the intermolecular distance R but does not depend on a donor fluorescence quantum yield and kinetic parameters. It means that carotenoids can effectively transfer their excitation energy to chlorophylls exclusively when they are closely located, via the Dexter-type process. In fact, the short-range excitation energy transfer is only effective when the donor and acceptor molecules are in a close, van der Waals contact. This will be more easy to understand when one realizes that this is not only the excitation energy exchange but it is also associated with electron transfer: the excited state electron of the donor molecule is transferred to the acceptor and, at the same time, the ground state electron of the acceptor molecule is transferred to the donor (see Fig. 4). Owing to this simultaneous electron and energy transfer the Dexter mechanism is very often referred to as the "energy-electron exchange process".

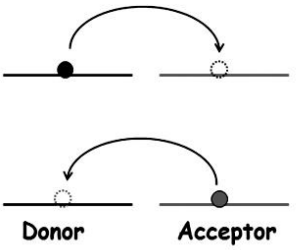

Fig. 4. Model of the Dexter-type excitation energy transfer. See the text for more explanations.

$Donor^* + Acceptor \rightarrow Donor + Acceptor^*$

4.3. Excitation energy transfer in photosynthetic apparatus

As it was pointed out above excitation energy transfer between chlorophyll molecules can be effective both in close and long distances. This is why the photosynthetic energy transfer between the individual antenna pigment-protein complexes and between the antenna complexes and the reaction centers is primarily considered as a chlorophyll-chlorophyll excitation energy transfer. In most cases, the excitation energy is transferred from the chlorophyll short-wavelength spectral forms to the long-wavelength spectral forms, in agreement to the requirement of effectively high overlap integral. Even in the closely packed antenna complexes, such as plant LHCII trimers, the inter-complex energy transfer between individual monomers takes place between the chlorophyll molecules via the Forster-type energy transfer process (Liu et al., 2004; Standfuss et al., 2005). On the other hand, the pigments bound to the single LHCII monomers are so densely packed that excitation energy transfer between the neighboring pigment molecules, both chlorophylls and carotenoids, can be efficiently realized via the Dexter-type mechanism. The binding of the pigments to the protein in the LHCII is a very interesting phenomenon. The local chlorophyll concentration in the monomer of LHCII is extremely high and corresponds to 0.3 M (Barros and Kuhlbrandt, 2009). A chlorophyll solution of such a high concentration would hardly emit any fluorescence due to the fact that most singlet excitations would be dissipated via the collisions and so-called concentrational quenching. The precise localization, orientation and spacing of both chlorophylls and xanthophylls in LHCII assures not only efficient absorption of light quanta but also intermolecular excitation energy transfer. Owing to such properties the complex can act as an efficient photosynthetic antenna, able to collect light energy which may be eventually provided to the reaction centers and to drive photosynthesis.

The most intriguing is the process of the excitation energy transfer from carotenoids to chlorophylls. The fact that the clear carotenoid contribution can be distinguished in the fluorescence excitation spectrum of chlorophyll *a* in the photosynthetic apparatus of plants, without any doubts, indicates that excitation energy is transferred from carotenoids to chlorophylls. On the other hand, there are several possible pathways of such a transfer. Fig. 5 presents the Jablonski diagram of energy levels of a chlorophyll and carotenoid molecule. As can be seen from the analysis and comparison of the energy levels shown, there are, in principle, two pathways, via which the energy of the carotenoid molecule excited to the $1B_u$ energy level via absorption of the quantum of blue light, can be realized. One pathway leads directly between the $1B_u$ and the energy levels from the Q band of the chlorophyll. The second pathway requires non-radiative relaxation of the

carotenoid excitation ($1B_u \rightarrow 2A_g$) followed by the $2A_g \rightarrow Q$ energy transfer. As can be seen from the carotenoid absorption spectrum (see the ordinate axis in Fig. 5) there is no absorption band corresponding to the $2A_g$ energy level. This makes it difficult to localize this state on an energy scale, in a direct light absorption measurement. A reason of such a situation is a fact that both the ground and the first excited singlet energy state of carotenoids are characterized by the same symmetry group (A) and the same parity (g, "gerade") and therefore the $S_0 \rightarrow S_1$ transition in carotenoids is forbidden. Localization of the $2A_g$ energy level is in principle possible by analysis of the fluorescence emission from this state, after excitation of the $1B_u$ state (which is strongly allowed) (Polivka and Sundstrom, 2004). Such a fluorescence emission can be well observed in carotenoids with relatively short conjugated double bonds (7 and shorter). Unfortunately, the carotenoid pigments present in the photosynthetic apparatus are characterized by a relatively long conjugated double bond system (eg. violaxanthin N = 9, lutein N = 10, β-carotene N = 11) and therefore fluorescence is almost exclusively emitted from the $1B_u$ energy level, which makes it not possible to assess the energy of the $2A_g$ state. Despite such severe

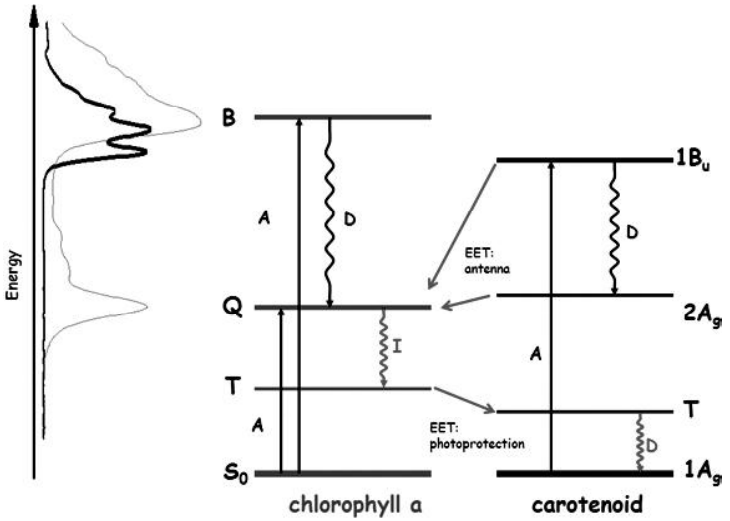

Fig. 5. Comparative Jablonski diagram of chlorophyll and carotenoid molecules. The energy levels which can be detected by single-photon absorption spectroscopy correspond to the maxima in the absorption spectra presented on the left hand side. The energy levels Q and B represent the Q and B absorption bands of chlorophyll, T denotes the triplet states. The singlet-singlet excitation energy transfer (EET) from carotenoid to chlorophyll is attributed to the antenna function and the triplet-triplet EET from chlorophyll to carotenoid is attributed to the photoprotection function (marked by arrows). See caption to Fig. 1 and the text for more explanations.

obstacles, there were several very elegant approaches, both direct and indirect, aimed to localize the $2A_g$ state of carotenoids present in the photosynthetic apparatus on an energy scale (Polivka and Sundstrom, 2004; Gruszecki et al., 2005; Christensen et al., 2004). It appears that the energies of the S_1 state of those pigments are very close to the energies of the Q_y energy level of chlorophyll *a*, which makes it very efficient to transfer energy from carotenoids to chlorophyll both via the long-distance Forster and the short-distance Dexter mechanisms.

Acknowledgements

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Part 2. METABOLISM OF PHOTOSYNTHETIC PIGMENTS AND ITS REGULATION

Chapter 5. BIOSYNTHESIS AND BREAKDOWN OF CHLOROPHYLLS

Robert J. Porra[1], Hugo Scheer[2], and Bernhard Krautler[3]
[1] Commonwealth Scientific and Industrial Organization, Canberra, Australia
[2] University of Munich, Munchen, Germany
[3] Institute of Organic Chemistry & Centre of Molecular Biosciences, University of Innsbruck, Innsbruck, Austria

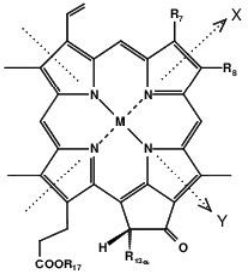
Contents

5.1. Introduction

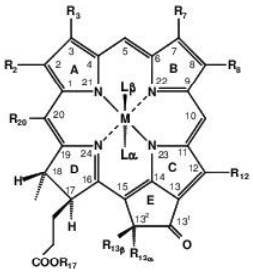
Chlorophylls are magnesium-containing tetrapyrrolic pigments with essential roles in both the harvesting and transduction of solar energy into useful biochemical energy (ATP) and reducing power (NADPH or NADH) for the photosynthetic fixation of CO_2 in higher plants, marine and freshwater algae, and in photosynthetic bacteria. This chapter describes the structures of three chemical classes of chlorophylls found in these widely-differing types of photosynthetic organisms, outlines the biosynthetic pathways leading to their formation and summarizes the recent progress in chlorophyll biodegradation.

5.2. Chemical structures, nomenclature and distribution

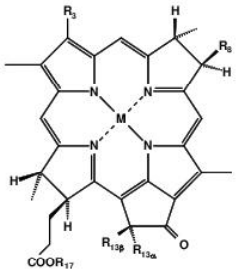
More than 100 chlorophylls (Chls) and bacteriochlorophylls (BChls) are known: a representative few are listed (Fig. 1) and their structures have been reviewed (Scheer, 2006). Chls are mostly Mg-coordination complexes of cyclic tetrapyrroles containing a fifth cyclopentenone (or *iso*cyclic) ring E, which is formed enzymically from the 13-methyl-propionate side chain of pyrrole ring C of Mg-protoporphyrin IX monomethyl ester forming, concomitantly, the 13^1-oxo and 13^2-carboxymethyl substituents (Fig. 2; see *Section* 5.5.3). Fig. 1 shows the IUPAC-IUB tetrapyrrole nomenclature (Moss, 1988), atom numbering and ring



Phytoporphyrin (PP)

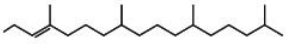


Phytochlorin (PC)



Phytobacteriochlorin (PB)

Phy =



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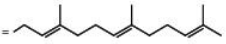

Pigment	R_2	R_3	R_7	R_8	R_{12}	$R_{13\beta}$	$R_{13\alpha}$	R_{17}	R_{20}	M	Macrocycle
Chl(ide) c_1			CH_3	C_2H_5			$COOCH_3$	H[a,h]		Mg	PP
Chl(ide) c_2			CH_3	C_2H_3			$COOCH_3$	H[a,b]		Mg	PP
Chl(ide) c_3			$COOCH_3$	C_2H_3			$COOCH_3$	H[a,b]		Mg	PP
[8-Vinyl]-PChlide a			CH_3	C_2H_3			$COOCH_3$	H		Mg	PP
Chl a	CH_3	C_2H_3	CH_3	C_2H_5	CH_3	H	$COOCH_3$	Phy[c]	H	Mg	PC
Chl a'	CH_3	C_2H_3	CH_3	C_2H_5	CH_3	$COOCH_3$	H	Phy	H	Mg	PC
[8-Vinyl]-Chl a	CH_3	C_2H_3	CH_3	C_2H_3	CH_3	H	$COOCH_3$	Phy	H	Mg	PC
8^1-OH-Chl a	CH_3	C_2H_3	CH_3	$CHOH-CH_3$	CH_3	H	$COOCH_3$	Phy	H	Mg	PC
Chl b	CH_3	C_2H_3	CHO	C_2H_5	CH_3	H	$COOCH_3$	Phy	H	Mg	PC
[8-Vinyl]-Chl b	CH_3	C_2H_3	CHO	C_2H_3	CH_3	H	$COOCH_3$	Phy	H	Mg	PC
Chl d	CH_3	CHO	CH_3	C_2H_5	CH_3	H	$COOCH_3$	Phy	H	Mg	PC
Chl d'	CH_3	CHO	CH_3	C_2H_5	CH_3	$COOCH_3$	H	Phy	H	Mg	PC
Chl f	CHO	C_2H_5	CH_3	C_2H_5	CH_3	H	$COOCH_3$	Phy	H	Mg	PC
Phe a	CH_3	C_2H_5	CH_3	C_2H_5	CH_3	H	$COOCH_3$	Phy	H	H_2	PC
BChl c	CH_3	$CHOH-CH_3$	CH_3	$C_2H_{5-n}(CH_3)_n$	CH_3/C_2H_5	H	H	Far + others	CH_3	Mg	PC
BChl d	CH_3	$CHOH-CH_3$	CH_3	$C_2H_{5-n}(CH_3)_n$	CH_3/C_2H_5	H	H	Far + others	H	Mg	PC
BChl e	CH_3	$CHOH-CH_3$	CHO	$C_2H_{5-n}(CH_3)_n$	CH_3/C_2H_5	H	H	Far + others	CH_3	Mg	PC
BChl a		$CO-CH_3$		C_2H_5		H	$COOCH_3$	Phy + others		Mg	PB
[Zn]-BChl a		$CO-CH_3$		C_2H_5		H	$COOCH_3$	Phy + others		Zn	PB
BChl b		$CO-CH_3$		$=CH-CH_3$[d]		H	$COOCH_3$	Phy + others		Mg	PB
BChl g		C_2H_3		$=CH-CH_3$[d]		H	$COOCH_3$	Far		Mg	PB
BChl g'		C_2H_3		$=CH-CH_3$[d]		$COOCH_3$	H	Far		Mg	PB
BPhe a		$CO-CH_3$		C_2H_5		H	$COOCH_3$	Phy		H_2	PB
BPhe b		$CO-CH_3$		$=CH-CH_3$[d]		H	$COOCH_3$	Phy		H_2	PB

Fig. 1. Structures of chlorophylls of the phytoporphyrin (PP), phytochlorin (PC) and phytobacteriochlorin (PB) types. X- and Y- denote the approximate directions of the spectroscopic axes. Carbon and nitrogen numbering, ring labelling and macrocycle nomenclature, according to IUPAC-IUB (Moss, 1988) is shown on the phytochlorin macrocycle and used to denote 'R' substituents in the associated Table. The carbon skeletons of the two most common esterifying alcohols, phytol (Phy) and farnesol (Far) are shown below. For each Chl macrocycle, only those substituents (R_x) that change within that class are identified and tabulated. a) Sometimes esterified; b) acrylic side chain at C-17 (17^1-17^2 double bond); c) 2,6-phytadienol in reaction centers of green bacteria; d) no H at C-8.

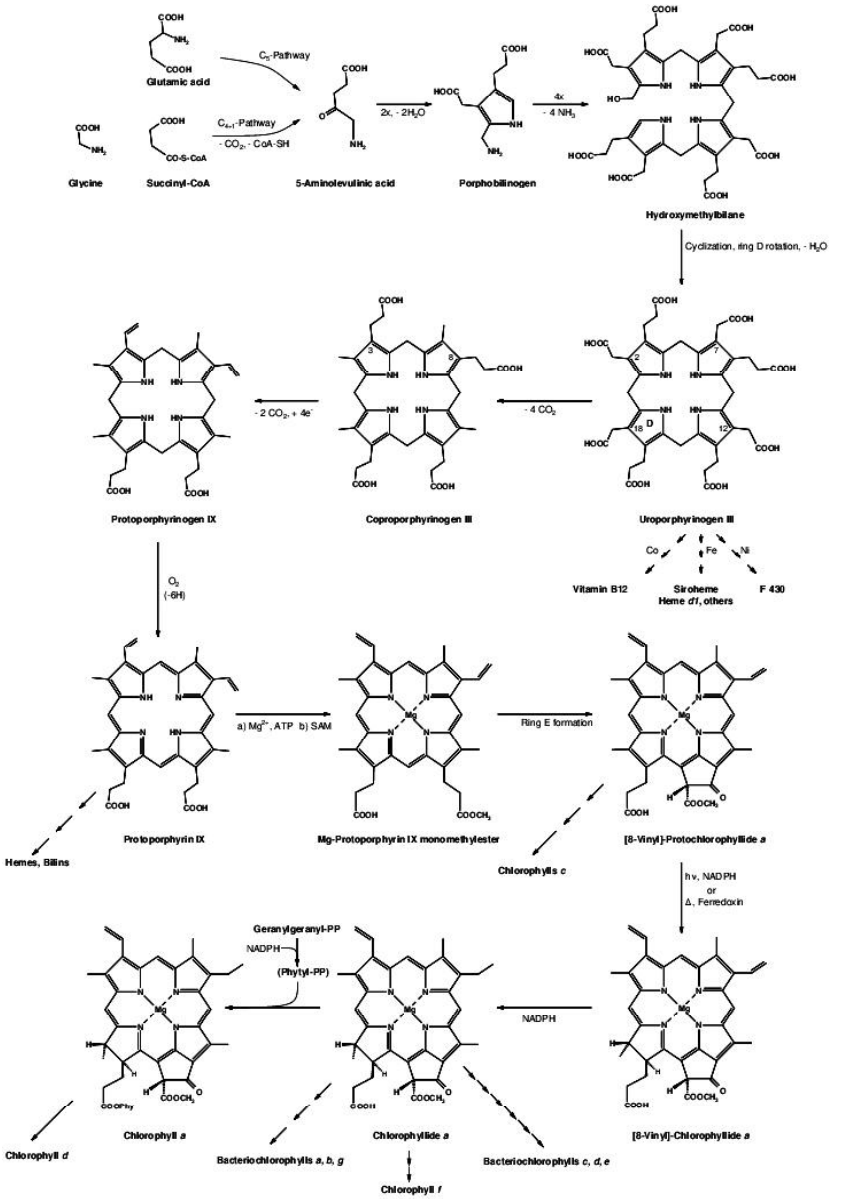

Fig. 2. The pathway of chlorophyll *a* biosynthesis showing the bifurcation points for the formation of other chlorophyll classes and other naturally-occurring tetrapyrrolic pigments.

labelling systems: the 5-ring macrocycles of the three Chl classes are distinguished from those of the 4-ring porphyrins, chlorins and bacteriochlorins by addition of the prefix, 'phyto'. Note that the 'phytoporphyrin (PP)', 'phytochlorin (PC)' or 'phytobacteriochlorin (PB)' macrocyles, as defined by IUPAC-IUB, include the 13^1-oxo group but lack a 13^2-carboxymethyl group (Fig. 1). The 17-propionic acid side chain is mostly, but not always, esterified, predominantly with a long-chain isoprenoid alcohol such as phytol, geranyl-geraniol or farnesol (Fig. 1). Derivatives lacking the esterifying alcohol are called chlorophyllides (Chlides). In one known Chl, the central Mg is replaced by Zn (Kobayashi et al., 2006). Reaction centres of photosystem II contain a demetalated Chl, pheophytin (Phe) *a* or, in a few cases, Phe *d*.

The **Mg-phytoporphyrin** type Chls comprise the Chl *c* family. Chemically they are closely related to protochlorophyllides (PChlides) but biologically they are competent light-harvesting Chls or, as they are mostly unesterified, chlorophyllides (Chlides) (Fig. 1). The macrocycle of these Mg-phytoporphyrins, unlike the Mg-phytochlorins and Mg-bacteriophytochlorins, is fully unsaturated and these c-type Chls absorb moderately at ~620 nm and about 10-fold more intensely in the Soret region (400-450 nm). Porra et al. (1997) proposed that the 7-carboxymethyl group of Chl c_3 may arise from the 7-methyl group of Chl c_2 via a 7-*gem*-diol formed by an oxygenase activity followed by dehydrogenation to a carboxyl group which is then methylated by S-adenosylmethionine. The 17-propionic acid side chain of these Chl(ide)s is often dehydrogenated to acrylic acid and, although generally unesterified, the 17-acrylate of Chl c_2 can be esterified, possibly during transport (not shown in Fig. 1). For a review of the Chl *c* family see Zapata et al. (2006).

The **Mg-phytochlorin** type Chls, possess a ring D which is *trans*-reduced at the Δ17,18 double-bond. These Chls (Fig. 1) include Chls *a* and *b* of higher plants and green algae, and Chl *d* (Kobayashi et al., 2006; Larkum et al., 2012; Manning and Strain, 1943) and Chl *f* (Akutsu et al., 2011; Chen et al., 2010) from some free-living or epiphytic prokaryotes. Bacteriochlorophylls (BChls) *c, d* and *e* are also phytochlorins: the "bacterio" prefix does not indicate possession of a phytobacteriochlorin macrocycle but rather their origin from the green photosynthetic bacteria. All these Chls are antenna Chls, but Chls *a* and *d* can also be reaction-centre Chls. Mostly, they absorb strongly and almost equally in the 400-470 nm and in the 640-700 nm regions. Chls *d* and *f* possess a formyl group on ring A at C-3 and C-2, respectively, which extends light capture for oxygenic photosynthesis from ~700 nm in Chl *a*-containing organisms to ~710 nm and to ~750nm for Chl *d*- and Chl *f*-containing organisms, respectively (Chen and Blankenship, 2011; Chen and Scheer, 2013). A Chl *e* has been reported

(Holt, 1966), but is not listed in Fig. 1 as it was not chemically characterized and its existence has never been confirmed. In addition, [8-vinyl]-Chls (also termed divinyl-Chls) containing a vinyl instead of an ethyl group at C-8 are important: they occur in marine cyanobacteria where it is estimated that they account for a considerable fraction of global photosynthesis (Goericke and Repeta, 1992; Nagata et al., 2005).

The **Mg-phytobacteriochlorin** type Chls, include BChls *a, b* and *g*, in which rings B and D are *trans*-reduced at the Δ7,8 and Δ17,18 double bonds, respectively (Fig. 1). BChls of this type possess two strong absorption bands in the near-UV (360-400nm) and the near-IR (770-795). BChls *a, b* and *g* participate in anoxygenic photosynthesis in the purple photosynthetic bacteria and heliobacteria. A functional BChl *a* in which the central Mg has been replaced by Zn was found in *Acidiphilium rubrum* (Kobayashi et al., 2006). BChl *b* possesses a labile ethylidene (=CH-CH_3) group at C-8 (Scheer et al., 1974) as does BChl *g* (Brockmann and Lipinsky, 1983).

The aforementioned pigments are the major Chls found in photosynthetic organisms. A number of additional minor pigments are known that are functional mainly in reaction centers (Kobayashi et al., 2006).

5.3. Biosynthesis of 5-aminolevulinate

The biosynthesis of chlorophylls and most hemes (Fig. 2) involves the same pathway for the conversion of 5-aminolevulinate (ALA), the first common intermediate in their biosynthesis, to protoporphyrin IX, the last common intermediate: all these common enzymes have been structurally resolved (Layer et al., 2010). ALA, however can be formed either from glycine and succinate in the Shemin or ALA synthase (ALAS) pathway (Section 5.3.1 and Fig. 3A) or from glutamate in the so-called C_5 or glutamate pathway (Section 5.3.2 and Fig. 3B). Many biosynthetic sequences have originally been discovered by isotopic labelling. Wittenberg and Shemin (1950) developed a degradation process whereby each of the 34 carbon atoms of [^{14}C]-labelled protoheme, synthesized from *either* [2-^{14}C]-glycine, [1-^{14}C]- or [2-^{14}C]-acetate *or* [1,4-^{14}C]-succinate by duck erythrocytes, could be unequivocally isolated as $^{14}CO_2$. The formation of hemes and chlorophylls by Shemin's ALAS pathway or the C_5 glutamate pathway can now be more readily determined by ^{13}C-NMR spectroscopic examination of the final tetrapyrrole product, using appropriate ^{13}C-labelled substrates (reviewed by Akhtar, 1994; Beale, 2006; Porra, 1997; Shemin, 1956).

5.3.1. 5-aminolevulinate formation by 5-aminolevulinate synthase (ALAS) pathway

Figure 3A shows the Shemin (or ALAS) pathway for the conversion of succinyl-CoA and [2^{13}C]-glycine in the presence of pyridoxal phosphate

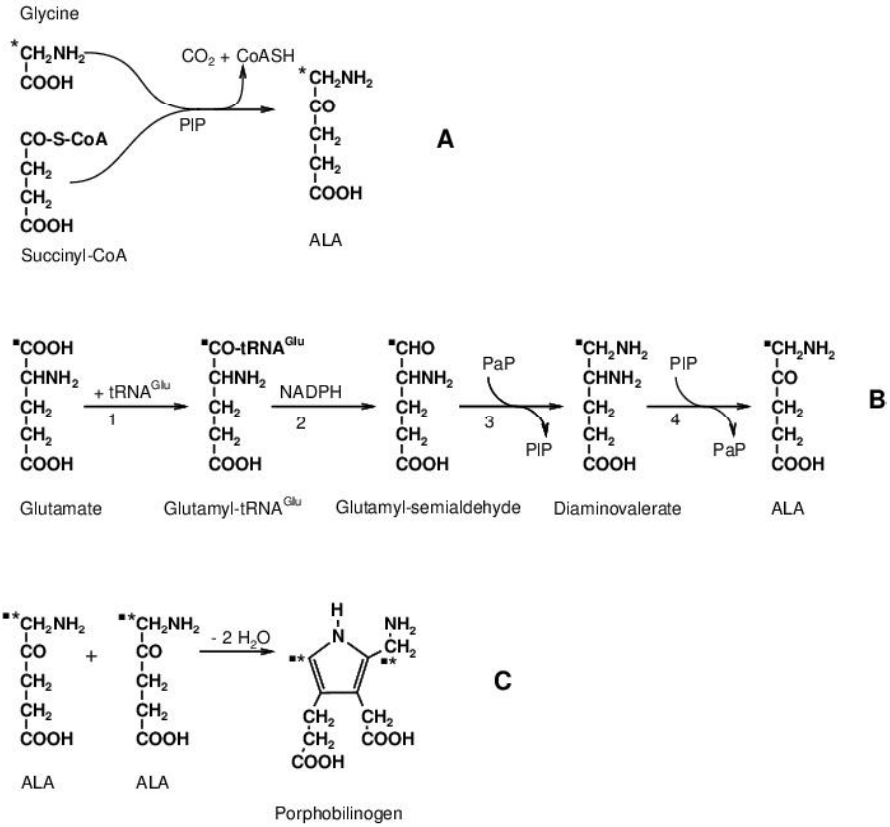

Fig. 3. Formation of 5-aminolevulinate (ALA) and monopyrrolic porphobilinogen (PBG). (A) Formation of ALA from succinyl-CoA and glycine by the ALA synthase (ALAS) route, and (B) from glutamate by the C_5 pathway. For both pathways the origin of the methylamino and α-pyrrole carbons of PBG is shown. (C) Conversion of ALA to PBG by ALA dehydratase. *Abbreviations:* PaP, pyridoxal phosphate; PIP, pyridoxamine phosphate. From Porra and Scheer (2000) with kind permission from Springer Science+Business Media.

(PaP) to [5-^{13}C]-ALA, CO_2, and CoA. ALA is the first intermediate specifically committed to tetrapyrrole formation and ALAS is a regulatory enzyme in tetrapyrrole biosynthesis. ALAS was first found in the mitochondria of mammalian, avian and fungal (yeast) cells (Jordan, 1991) where it is involved in heme formation and later found in some purple photosynthetic bacteria and other members of the α-proteobacteria; however, it could not be found in higher plants or green algae.

5.3.2. 5-aminolevulinate formation by the C_5 (glutamate) pathway

Nearly two decades passed after the discovery of the ALAS pathway, before enzymes of higher-plant and algal chloroplasts, and of many prokaryotes, were found to incorporate the intact five carbon atom skeleton of glutamate into ALA (Beale, 2006; Beale and Castelfranco, 1973) (Fig. 3B). This C_5 chloroplast-located pathway produced ALA for both heme and Chl biosynthesis in higher plants. Fig. 3B shows that the C-1 of glutamate in the C_5 pathway and the C-2 of glycine in the ALAS pathway both label the C-5 atom of ALA and, therefore, label the same carbon atoms of the monopyrrole, porphobilinogen (PBG), formed by the enzymic condensation of two ALA molecules by ALA dehydratase (Fig. 3C) and, thus, the same carbon atoms of the final tetrapyrrole. It is noteworthy that glutamyl-tRNA is formed by a glutamate ligase as an intermediate in the reduction of the C-1 carboxyl group of glutamate to the aldehyde substituent of glutamate-1-semialdehyde (GSA) catalysed by glutamyl-tRNA reductase (Fig. 3B). The preferred C_5 pathway of ALA formation from GSA via diaminovalerate (Fig. 3B) is catalysed by GSA-transaminase, in the presence of pyridoxamine phosphate (PIP) (Kannangara et al., 1994), but a lesser pathway (not shown in Fig. 3) has been demonstrated via dioxovalerate (Schulze et al., 2006). The ALAS and C_5 pathways can be distinguished by isotopic labelling (above) or by gabaculine inhibition of GSA-transaminase: ALAS is unaffected by gabaculine.

5.3.3. Distribution of the 5-aminolevulinate synthase and the C_5 pathways

Although discovered later, the C_5 pathway, from an evolutionary perspective, appears older and it is more ubiquitous than the more modern ALAS route (Avissar and Moberg, 1995; Beale, 2006; Koreny et al., 2011; Porra, 1997). Putatively it arose, like chloroplasts, from a primitive eubacterial *Cyanobacter* cell that existed in primordial O_2-sparse atmospheres where an incomplete tricarboxylic acid cycle was unable to supply succinyl-CoA for the ALAS route. The C_5 pathway is chloroplast-located in all higher plants and most green algae for the formation of Chl and for all the heme required for both chloroplasts and mitochondria; it has also been found in Rhodophyta and Bryophyta.

The C_5 pathway is found in all bacterial cells including β-, γ- and δ-proteobacteria but not in the α-Proteobacteria which contain ALAS. The α-proteobacteria include the purple photosynthetic bacteria like *Rhodobacter* (*R.*) *sphaeroides*, as well as *Methylobacterium* spp., plant symbionts (such as *Rhizobium* spp.) and obligate intracellular parasitic *Rickettsia* spp.: the latter are thought to be the progenitors of animal and yeast mitochondria which is consistent with the presence of ALAS in these mitochondria (Gray, 1998). No ALAS has been found, however,

in plant and algal mitochondria, except for two green algae: in *Euglena gracilis* and *Scenedesmus obliquus* (mutant strain C-2A'), it is involved in mitochondrial heme formation, but not in chloroplast heme or Chl biosynthesis (Kurland and Andersson, 2000).

5.3.4. Regulation of 5-aminolevulinate synthase formation

Both the ALAS and C_5 pathways of ALA formation are the initial and rate-limiting steps in heme and Chl synthesis. Strong regulation of Chl biosynthesis is essential since these free pigment as well as many porphyrin and chlorin intermediates are phototoxic: in the presence of oxygen they generate reactive oxygen species (ROS) which cause membrane destruction and cell death. The rate-limiting enzymes in the Shemin and C_5 pathways are ALAS and glutamyl-tRNA^{Glu} reductase (GluTR), respectively. ALAS (Burnham and Lascelles, 1963) and GluTR (Gough and Kannangara, 1979) both experience strong feed-back inhibition by heme. GluTR, to a lesser extent is inhibited by protoporphyrin, Mg-protoporphyrin, protochlorophyllide and chlorophyllide; there are multiple genes encoding GluTR some of which are induced by light while others are developmentally controlled (Beale, 2006). ALAS from *R. sphaeroides* is also activated by cystine- and glutathione-trisulphides but O_2 reduced activity, possibly by reducing the trisulphide concentration (Sandy et al., 1975). Both enzymes are transcriptionally regulated by environmental conditions such as light/dark conditions and oxygen tension. For example, ALAS is induced and BChl *a* formation in the facultative photosynthetic bacterium, *R. sphaeroides*, when respiring, dark-grown cells become photosynthetically active on transfer to light and anaerobic conditions; however, introducing O_2 completely inhibits BChl *a* formation and diminishes ALAS significantly, but not completely, so that heme biosynthesis can continue for respiratory processes (Lascelles, 1960). Regulation of ALAS has been extensively reviewed (Beale, 2006). Interestingly, ALAS can be induced by some chemicals including polyhalogenated aromatic compounds leading to dramatically excessive phototoxic porphyrin formation that can be used to control weeds (Rebeiz et al., 1995).

5.4. Conversion of 5-aminolevulinate to protoporphyrin IX

5.4.1. Formation of monopyrrolic porphobilinogen

Monopyrrolic porphobilinogen (PBG), with propionate and acetate side chains, is formed by a Knorr type condensation of two molecules of ALA (Fig. 3C) catalysed by ALA dehydratase (ALAD) (Jordan, 1991; Porra, 1997). This intermediate is common to the biosynthesis of all tetrapyrroles (Fig. 2). ALAD is octameric in animal, and yeast, and some bacterial (*Escherichia coli*) cells and binds two Zn^{2+} ions per

monomer; however, ALAD in *R. sphaeroides* and in chloroplasts is a hexamer and requires Mg^{2+} ions. ALAD is a soluble enzyme and, like all the enzymes of Chl biosynthesis, is located within the chloroplast of higher plants and green algae either in the stroma or attached to thylakoid membranes.

5.4.2. Conversion of porphobilinogen to uroporphyrinogen III

The mechanism of formation of the asymmetric isomer III of uroporphyrinogen, a common intermediate in the biosynthesis of all naturally-occurring tetrapyrrole pigments, has long fascinated biochemists (Battersby and Leeper, 1990; Jordan, 1991). It is catalysed by two enzymes, PBG deaminase (PBGD) and uroporphyrinogen (urogen) III cosynthetase. The PBGD synthesizes from PBG its own enzyme-bound dipyrrole coenzyme releasing two molecules of NH_3. Further sequential deamination of another four molecules of PBG forms an enzyme-bound hexapyrrole which then splits leaving the enzyme-bound dipyrrole complex and releasing a linear octacarboxylic tetrapyrrole, 1-hydroxymethylbilane (HMB I) which belongs to the isomer I series with an alternating sequence of propionate and acetate side chains (Fig. 4). Urogen III cosynthetase, which is closely associated with PBGD, binds the linear HMB I and converts it to cyclic urogen III *via* an

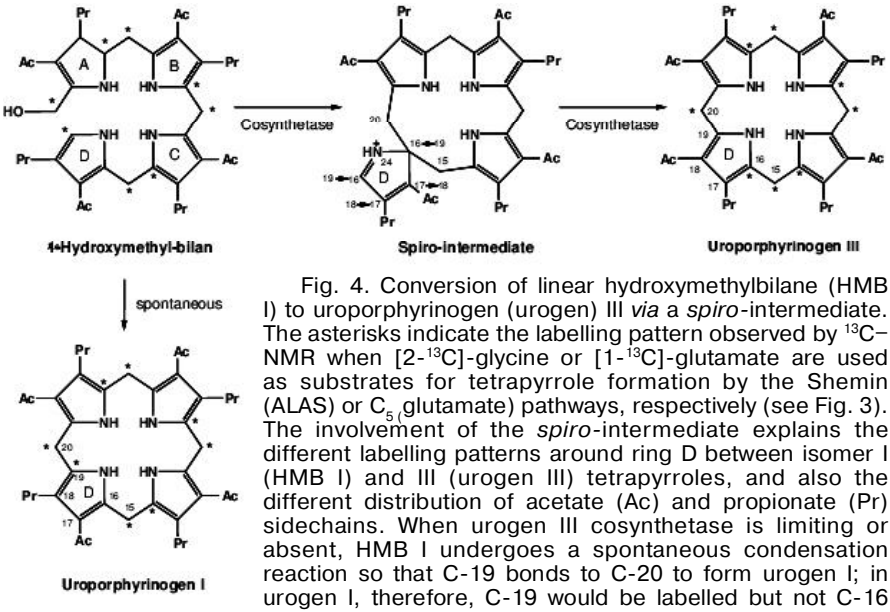

Fig. 4. Conversion of linear hydroxymethylbilane (HMB I) to uroporphyrinogen (urogen) III *via* a *spiro*-intermediate. The asterisks indicate the labelling pattern observed by ^{13}C–NMR when [2-^{13}C]-glycine or [1-^{13}C]-glutamate are used as substrates for tetrapyrrole formation by the Shemin (ALAS) or C_5 (glutamate) pathways, respectively (see Fig. 3). The involvement of the *spiro*-intermediate explains the different labelling patterns around ring D between isomer I (HMB I) and III (urogen III) tetrapyrroles, and also the different distribution of acetate (Ac) and propionate (Pr) sidechains. When urogen III cosynthetase is limiting or absent, HMB I undergoes a spontaneous condensation reaction so that C-19 bonds to C-20 to form urogen I; in urogen I, therefore, C-19 would be labelled but not C-16 this situation, however, is reversed in urogen III.

intermediate with a *spiro* C-16 atom. Later, following a 180° rotation of ring D of the *spiro*-intermediate, the original C-16 atom condenses with CH_2OH-group of HMB I to become the C-19 of urogen III (Fig. 4); thus, the acetate and propionate groups at C-17 and C-18 of HMB I are reversed in urogen III. This rationalizes the different patterns of asterisks between isomer I and III tetrapyrroles (Fig. 4) where the asterisks denote the carbon atoms labelled by either [2-^{13}C]-glycine or [1-^{13}C]-glutamate in ALAS- or C_5-pathway containing organisms, respectively, as obtained in the ^{13}C-NMR studies of chlorophyll formation (Oh-Hama et al., 1982, 1985, 1986; Porra et al., 1983). When urogen III cosynthetase is lacking or limiting, HMB I is spontaneously converted to urogen I and then decarboxylated by uroporphyrinogen III-decarboxylase (UrogenD) to coproporphyrinogen (coprogen) I which accumulates because it is not a substrate for coprogen III oxidative decarboxylase. Urogen I and coprogen I, like all porphyrinogens, are hexahydroporphyrins that can be readily oxidized in air to the phototoxic porphyrins, uroporphyrin (uro) I and copro I, respectively.

Urogen III is the bifurcation point for the formation of natural cobalt (cobalamines), nickel (coenzyme F_{430}), some iron-containing tetrapyrroles (sirohemes), and an anoxic pathway to protoporphyrin (Bali et al., 2011) (Fig. 2)

5.4.3. Decarboxylation of uroporphyrinogen III to coproporphyrinogen III

Urogen III decarboxylase (UrogenD) has low substrate specificity and can decarboxylate both urogens I and III (see Section 5.4). Urogen D has been extensively reviewed (Akhtar, 1994; Castelfranco and Beale, 1981; Porra, 1997; Porra et al., 2011). The structures of hepta-, hexa- and penta-carboxylic porphyrins found in the urine of porphyric patients, and formed by aerobic oxidation of the corresponding porphyrinogens, indicates that the acetate groups on urogen III at C-18, -2, -7 and -12 on rings D, A, B and C, respectively, were decarboxylated in that clockwise order. This decarboxylation order was maintained when ALA or PBG were offered to UrogenD as substrates but a random order resulted with urogen III as substrate thus suggesting that PBGD, urogen III cosynthetase and UrogenD are closely associated and offer PBG at or below the limiting rate so that the intermediates are formed consistent with their affinity constants.

5.4.4. Oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX

Coprogen III-oxidase is an isomer III specific oxidative decarboxylase of coprogen III. It decarboxylated the propionate side chains at C-3

and -8, on rings A and B, respectively, and in that order to form the vinyl groups of protoporphyrinogen (protogen) IX (Akhtar, 1994): protogen IX is the series III derived isomer form of cyclic tetrapyrroles with three (rather than two) different substituent side chains. This reaction occurs aerobically in mammalian, avian, higher-plant and some bacterial cells; however, the reaction also occurs in anaerobic organisms containing tetrapyrrole pigments indicating the operation of alternative electron acceptors to O_2. The enzyme has no known cofactors.

5.4.5. Oxidation of protoporphyrinogen IX to protoporphyrin IX

All porphyrinogens in tetrapyrrole biosynthesis are readily oxidized to porphyrins in air; nonetheless, there is a flavoprotein enzyme, protogen IX oxidase (PPOX) which accelerates this oxidation of protogen IX to protoporphyrin (proto) IX. PPOX activity, in higher-plant tissues, is found in plastids and mitochondria. PPOX is encoded by two genes forming two enzymes, PPOX I and II. The *PPOX II* gene encodes for two enzymes PPOX IIL (57 kDa) and PPOX IIS (55 kDa). PPOX I is mainly located on the outer, stromal side of thylakoid membranes but some also on the inner plastid envelope where PPOX IIL is also located. PPOX IIS is located on the inner mitochondrial membrane (Yaronskaya and Grimm, 2006). PPOX is inhibited by the diphenylether-type herbicides causing accumulation of excessive amounts of protogen IX which are oxidized to the phototoxic proto IX by non-specific herbicide-resistant peroxidases causing cell death.

5.5. Formation of chlorophylls from protoporphyrin IX

5.5.1. Magnesium chelatase

While ferrochelatase is a single subunit enzyme inserting Fe^{2+} ions into proto IX to form protoheme IX and requires no cofactors, the enzymic insertion of a Mg^{2+} ion into proto IX is a far more complex reaction. It is a highly ATP-dependent reaction requiring the difficult removal of H_2O from the coordination sphere of the Mg^{2+} ion, the deprotonation of the pyrrole nitrogen atoms, coordination of the Mg^{2+} ion to the pyrrole nitrogens and finally the completion of the penta- (or hexa)-coordination sphere of the Mg atom with axial ligands. Mg-chelatase is a soluble enzyme system encoded by three genes *BchI, BchD* and *BchH* in purple bacteria and *ChI, ChD* and *ChH* in higher plants and green algae which produce I, D and H protein subunits which comprise the chelatase. It has been proposed that a D subunit aggregate is dissociated in the presence of Mg^{2+} and ATP, and a single D subunit, after phosphorylation, then combines with two I subunits (Reid and Hunter, 2004; Walker and Willows, 1997). An H subunit combines with the two substrates, proto IX and Mg^{2+} which then combines with the I_2-D complex. The newly formed H-I_2-D complex

catalyses both Mg^{2+} ion insertion and ATP hydrolysis and it is suggested that the H_2O molecule required for that hydrolysis comes from the coordination sphere of the Mg^{2+} ion thus facilitating chelation to form Mg-protoporphyrin IX (Mg-proto).

Being the first enzyme in the Mg branch of metallo-tetrapyrrole pigment formation, Mg chelatase is a likely candidate for regulation of the Mg-branch of Chl formation. The high ATP dependency, the possible phosphorylation of the D subunit and the organization of the active multi-subunit chelatase all offer possibilities for regulation mechanisms.

5.5.2. Formation of Mg-protoporphyrin IX-monomethylester

Enzymically active preparations from higher plants, *Euglena gracilis* and *R. sphaeroides* have catalyzed the methylation of the 13-propionate side chain of Mg-proto in the presence of ATP and S-adenosylmethionine forming Mg-protoporphyrin IX-monomethylester (Mg-proto-MME). ^{13}C-NMR spectroscopy of Chls and BChls synthesized in the presence of [2-^{13}C]-glycine by green algae (Oh-Hama et al., 1982), by *R. sphaeroides* adapting from respiratory to photosynthetic conditions (Oh-Hama et al., 1985) and by etiolated maize leaves during greening (Porra et al., 1983) revealed that the 13^4 methyl ester carbon of the newly-formed Chls and BChls derived from the C-2 of glycine *via* S-adenosyl-methionine.

5.5.3. Mg-protoporphyrin IX-monomethylester cyclase

This enzyme converts the C-13 methylpropionate side chain of Mg-proto-MME to the cyclopentenone (or isocyclic) ring E of [8-vinyl]-protochlorophyllide *a* (also known as DV-PChlide since all Chl a related derivatives possess a 3-vinyl group) with concomitant formation of the 13^1-oxo group which is common to all Chls and BChls (Fig. 1). [8-vinyl]-PChlide is the last common biosynthetic intermediate to a vast array of Chls and BChls, and is also the bifurcation point for formation of the Chl *c* family (cf. Fig. 1) of which it is also a member (Helfrich et al., 1999) (Fig. 2).

Two pathways of ring E formation exist which involve either an oxygenase or a hydratase, and mechanisms have been proposed (Porra and Scheer, 2000). ^{18}O-mass spectrometry studies with greening maize leaves (Porra et al., 1994) and with the green algae, *Chlorella vulgaris* (Schneegurt and Beale, 1992), confirmed that the 13^1-oxo groups of Chls *a* and *b* derived from O_2, not H_2O, and thus involved the aerobic oxidase mechanism. This oxidase mechanism was also found in two purple bacteria *Roseobacter denitrificans* and *Rhodovulum sulfidophilum* although the latter also contained the alternative hydratase pathway: a third member of the purple bacteria, *R. sphaeroides,* employs only

the hydratase mechanism (for a review see Porra and Scheer, 2000. To our knowledge, little is known on the cyclase reaction *in vitro* (Bollivar, 2003), but much indirect biochemical and genetic information has been accumulated over the past decade. Knockout studies with the purple bacterium, *Rubrivivax gelatinosus*, revealed two genes, *acsF* and *bchE* that are required for ring E formation under aerobic and oxygen-depleted conditions, respectively (reviewed by Liu and Zheng (2008)). AcsF is iron dependent, while BchE is an enzyme of the SAM-radical class (Booker, 2009) that is also involved in bilin synthesis in photosynthetic organisms (Dammeyer and Frankenberg-Dinkel, 2008). Products of two genes are also involved in cyclization reaction under aerobic and oxygen-depleted conditions in the cyanobacterium, *Synechocystis sp.* PCC 6803 (Peter et al., 2009). In higher plants, CHL27 is (a component of) the cyclase, its concentration affects early and late steps of Chl synthesis (Peter et al., 2011).

5.5.4. 8-vinyl reductase activity

This enzyme is thought to be an NADPH-dependent enzyme normally reducing the 8-vinyl group of [8-vinyl]-PChlide *a*; however, some Chl derivatives (as well as PChlides) retain the 8-vinyl group; they are sometimes referred to as divinyl- (DV-) pigments, and the chlorophylls containing only the C-3 vinyl group and an ethyl group at C-8 as monovinyl- (MV-) pigments. This led Rebeiz et al. (1983) to propose that 8-vinyl reductase has a wide substrate specificity, possibly reducing any intermediate between protogen IX to chlorophyllide (Chlide) *a* containing the 8-vinyl group; thus, they suggested that there could be two parallel linear pathways rather than a single sequence in which [8-vinyl]-PChlide *a* was the only substrate of the reductase. With a wide specificity for the 8-vinyl reductase, the Chl c_1 (monovinyl, Fig. 1), could arise either from [8-vinyl]-PChlide *a* or from Chl c_2 (divinyl) by 8-vinyl reduction: the 17-propionate side chain of PChlide would also require dehydrogenation to an acrylate. A gene for the reductase has been identified in the higher plant, *Arabidopsis thaliana*, and in the cyanobacterium, *Synechocystis sp.* PCC6803, but is missing in *Prochlorococcus marinus* thus explaining the exclusive presence of [8-vinyl]-Chls *a* and *b* in this abundant organism (Nagata et al., 2005). Reduction of the 8-vinyl after reduction of ring D, that is, at the phytochlorin stage, is suggested to be the major pathway in *Arabidopsis thaliana* (Nagata et al., 2007). Multiple genes for the reduction have been found in green bacteria (Liu and Bryant, 2011b). The gene from *Dunaliella* has been cloned (Shang et al., 2012). Recent evidence points to a co-evolution of reduction of the 8-vinyl-group and the Chl binding proteins that is relevant for the tolerance to high-light which indicates a surpisingly profound functional effect of this seemingly minor variation (Ito and Tanaka, 2011).

5.5.5. Reduction of protochlorophyllide *a* to chlorophyllide *a* by reduction of ring D

PChlide *a* is reduced to Chlide *a* by a stereospecific *trans*-reduction of the Δ17,18 double bond of ring D catalysed either by a light-dependent PChlide *e* oxidoreductases (LPOR) present in angiosperms that etiolate in darkness or by a light-independent or dark form (DPOR) present in gymnosperms, mosses, green algae, cyanobacteria and phototrophic bacteria that can form Chls in the dark (for reviews see Porra, 1997 and Rudiger, 2006).

The angiosperm LPOR combines with its substrates to form a photoactive ternary complex LPOR:NADPH:PChlide *a* which, when illuminated, catalyses the reduction of ring D forming a LPOR:$NADP^+$:Chlide *a* complex which releases Chlide *a* to be quickly replaced by another PChlide *a* thus forming a new ternary complex. PChlide *a* and its [8-vinyl] (= divinyl) form were both substrates for LPOR from purple bacteria and cyanobacteria.

Three forms of LPOR, namely PORA, PORB (Armstrong et al., 1995) and PORC (Su et al., 2001), were found in *Arabidopsis thaliana*. PChlide *a* is present in large amounts in etiolated tissues where large amounts of light-labile PORA also exist. In a dark ATP-dependent reaction, PORA combines with PChlide *a* thus reducing the phototoxicity of the latter, but PORA is destroyed by light. PORB, a very stable LPOR, is present in both etioplasts and chloroplasts. PORC is present in etioplasts but is found in plastids only after long (6 h) illumination periods. These three forms of LPOR may allow plants to adapt to very different light regimes, but no structures are available for any of them.

Light-independent DPORs are encoded by three genes in photosynthetic bacteria and green algae which show considerable homology with the three nitrogen-fixing genes of *Azotobacter vinelandii* (Fujita and Bauer, 2002; Porra, 1997). The structures for two DPORs have been solved. They rationalize the *trans* reduction at C-17/18 by protonation of C-17 by the neighboring propionic acid side chain, which concurrently inhibits reduction of (esterified) PChl. The subunits of the DPOR complex interact only transiently for electron transfer (Brocker et al., 2010).

The products of these oxidoreductases, both Chlide *a* and [8-]-Chlide *a*, are:

(1) substrates for Chl synthase that esterifies the 17-propionate with a long chain isoprenoid alcohol to form Chl *a* and [8-vinyl]-Chl *a*;

(2) substrates for oxygenase activity leading to Chlide *b* and [8-vinyl]-Chlide *b* formation; and,

(3) possible bifurcation points for the biosynthesis of phytobacteriochlorin-type BChls *a, b* and *g* (Fig. 2).

5.5.6. Bacteriochlorophyll formation: reduction of ring B by chlorin reductase

The biosynthesis of bacteriochlorophylls in purple bacteria has been reviewed (Chew and Bryant, 2007; Willows and Kriegel, 2009). The Δ7,8 double bond of the monovinyl compound, Chlide *a*, or rather its [3-CH_3CHOH]-derivative, is reduced by chlorin reductase, COR, which is encoded by three genes, *bchX, bchY* and *bchZ*. COR is homologous to the dark PChlide *a* reductase (Watzlich et al., 2009). Two more genes *bchF* and *bchC* encode a hydratase and a dehydrogenase, respectively, to convert the 3-vinyl group to the 3-acetyl of BChlides *a*. *bchJ* encodes a 8-vinyl reductase, but it is not clear at which stage of BChl *a* formation the C-8 vinyl substituent on ring B is reduced. BChlide *a* is then phytylated by BChl-synthase, encoded by gene *bchG,* to form BChl *a* (for a review see Frigaard et al., 2006).

The biosynthesis of BChls *b* and *g* is unknown. BChl *g* is isomeric with Chl *a* and, formally, it can be generated by a double-bond migration from the endocyclic Δ7,8- to the exocyclic Δ8,8^1 position (Hunt et al., 1983) and BChl *b* can be similarly generated from [3-acetyl]-Chl *a*. Alternatively, with [8-vinyl]-Chlide *a* as substrate, a chlorin reductase plus an 8-vinyl isomerase, which effects an inward migration of the 8-vinyl (-CH=CH_2) double bond to form the 8-ethylidene (=CH-CH_3) group (Scheer et al., 1974), would produce BChl *g* which possesses a 3-vinyl group. This could then be converted to the 3-acetyl group of BChl *b* by hydratase and reductase activity. ^{18}O-labelling and mass spectrometry studies confirmed that the 3-acetyl group oxygen of BChl *a* in *R. sphaeroides, Roseobacter denitrificans* and *Rhodovulum sulfidophilum* were all derived from $H_2{}^{18}O$ by hydratase activity (Porra and Scheer, 2000).

5.5.7. Esterification of chlorophyllides to chlorophylls by chlorophyll synthase

The 17-propionic acid side chain of most Chls and BChls, but not of most Chls *c*, are esterified with a long chain (C_{20}) polyisoprenoid alcohol (phytol) catalysed by Chl-synthase which is encoded by the *chlG* or *bchG* genes (for reviews see Frigaard et al., 2006 and Rudiger, 2006). Chl-synthase preferentially esterifies Chlides *a* and *b* in the presence of geranyl-geranyl (GG) diphosphate to Chls a_{GG} and b_{GG}: phytyl diphosphate and GG plus ATP were less favoured substrates and PChlide *a* was inert showing that esterification occurs only after reduction of ring D (Rudiger, 2006). A reductase encoded by the *chlP* gene sequentially reduces the double bonds of the GG moiety at C-6, -10 and -14, leaving only the one double bond of phytol at C-2.

5.5.8. Oxidation of chlorophyllide *a* to chlorophyllide *b*

^{18}O-labelling and mass spectrometry experiments, using $^{18}O_2$ and $H_2{}^{18}O$ as substrates proved that the 7-formyl oxygen of Chl *b* derived

from molecular oxygen (Porra et al., 1994; Schneegurt and Beale, 1992), indicating an oxygenase mechanism for the conversion of the 7-methyl into a 7-formyl group. The enzyme, Chlide *a* oxygenase (CAO), encoded by the *CAO* gene, converts Chlide *a* to Chlide *b*: Chl *a* was not a substrate (Tanaka et al., 1998). The reaction involves two sequential oxidative steps catalyzed by CAO: firstly to [7-CH_2OH]-Chlide *a* and then to the *gem*-diol [7-$CH(OH)_2$]-Chlide *a* which spontaneously dehydrates to Chlide *b* (Fig. 5) (Oster et al., 2000).

Chl *d* is generated from Chl(ide) *a*, but, unlike the biosynthesis of Chl *b*, this involves the oxidation of the 3-vinyl group with the loss of the C-3^2 atom; the reaction is carried out by a single oxygenase (Schliep et al., 2010). The 2-formyl group in Chl *f* is derived from the 2-methyl group (Chen et al., 2010): the reaction is formally similar to the biosynthesis of Chlide *b* from Chlide *a*, but currently neither the precursor nor the enzymes are known (Chen and Scheer, 2013; Schliep et al., 2013).

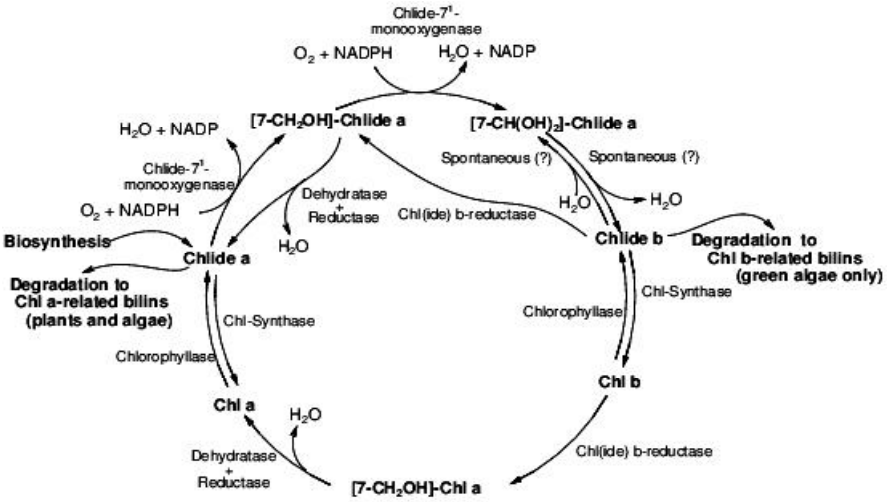

Fig. 5. The chlorophyll cycle linking chlorophyll (Chl) biosynthesis and biodegradation. This scheme, modified from Oster et al. (2000), shows the conversion of chlorophyllide (Chlide) *a* to Chlide *b* by two successive and irreversible mono-oxygenase reactions and the reverse conversion back to Chlide *a* by a reductase/dehydratase/reductase sequence. This cycle is not a continuous futile metabolic cycle but, rather, a switch linking Chl biosynthesis and biodegradation processes, and possibly a mechanism to fine tune Chl *a/b* ratios during adaptation to new light regimes. From Porra and Scheer (2000) with kind permission from Springer Science+Business Media.

5.5.9. The reverse reduction of chlorophyllide b to chlorophyllide a and the 'chlorophyll cycle'

Oxygenase reactions are strongly exothermic and irreversible (Hayaishi, 1974) but Ito et al. (1996) demonstrated that two reductases in etioplast membranes could reduce the 7-formyl of Chl *b* and Chlide *b* to the 7-methyl of Chl(ide) *a*. The reduction of Chl *b* to [7-CH_2OH]-derivatives required NADPH and the following reduction and dehydration to the 7-methyl group of Chl *a* derivatives required reduced ferredoxin (Scheumann et al., 1998) (Fig. 5): both enzymes have been identified in *Arabidopsis* (Meguro et al., 2011). The discovery of these reactions led Oster et al. (2000) to propose the 'Chl cycle' (Fig. 5) to fine tune changes in Chl *a*/*b* ratios to enable photosynthetic cells to adjust to new light regimes rather than by the more cumbersome method of degradation and/or *de novo* synthesis of Chls (Porra et al., 2011).

The initial de-esterification step in Chl degradation is shown in Fig. 5 so that Chlides *a* and *b* become the exit points for the further degradation of Chls (see following sections). Chlide *a* is the only exit point in higher plants but algae have two exit points: Chlide *a* and *b* (Fig. 5).

5.5.10. Chlorophylls from Green Bacteria

The BChls *c, d* and *e* are of bacterial origin, the green sulfur and non-sulfur bacteria, but they are, in spite of their names, phytochlorins rather than phytobacteriochlorins. They are a large and peculiar group of pigments because each member comprises a complex set of homologues that differ by methylation, esterifying alcohol, and the stereochemistry at C-3^1 by a period. The biosynthetic routes of these three BChls have been elucidated with the green sulfur bacterium, *Chlorobaculum tepidum*: the pathway to all three pigments branches off at the Chlide *a* level (see Fig. 2) and has been reviewed (Chew and Bryant, 2007). Peculiar reactions, unlike reactions found with other Chls, are the loss of the carboxymethyl group at C-13^2 and the methylations at C-8^2, C-12^2, and C-20.

The decarboxymethylation is carried out by a single enzyme, BciC, that has no homologs in the databases (Liu and Bryant, 2011a). In its absence, the respective Chls accumulated in the bacterium are methylated at C-8^2 and C-12^2 to a lesser degree than the BChls *c*/*d* contained in the wild-type. No derivatives containing a 20-methyl group were found and neither pigment was esterified with farnesol; thus, it is likely that decarboxymethylation precedes the methylations and also the esterification of the 17-propionic acid side chain. A Chl derivative lacking the carboxymethyl group has also been identified in degreening *Euglena* (Schoch et al., 1981). Methylation at C-20 is carried out by BchU, which transfers a methyl-group from SAM by an S_N2 mechanism

(Maresca et al., 2004; Wada et al., 2006). BchQ and BchR methylate C-8^2 and C-12^2, respectively, and belong to the radical SAM class (Gomez Maqueo Chew et al., 2007).

5.6. Chlorophyll breakdown

5.6.1. Introduction

As described above, the basic pathway of Chl-biosynthesis was steadily uncovered during the last century (Grimm et al., 2006). About 20 years ago, however, the equally fascinating natural disappearance of Chl was still a complete enigma (Matile, 1987). In the time since, this puzzle has been solved to a significant extent (Hortensteiner and Krautler, 2011; Krautler and Hortensteiner, 2013; Krautler and Matile, 1999; Matile et al., 1996; Moser et al., 2009b). In the late 1980s, Philippe Matile and his co-workers produced the first evidence for the existence of colourless Chl-catabolites in senescing leaves (Matile et al., 1996). The putative main catabolite **1** from senescent leaves of barley (*Hordeum vulgare*) was characterized in the Krautler laboratories as the linear tetrapyrrole 3^1,3^2,8^2-trihydroxy-1,4,5,10,15,20- (22H, 24H)-octahydro-13^2-[methoxycarbonyl]-4,5-dioxo-4,5-seco-phytoporphyrinate. This structure analysis established the role of **1** (now called *Hv*-NCC-1) as a Chl-catabolite (Fig. 7) (Krautler et al., 1991).

5.6.2. Chlorophyll Catabolites in Leaves

5.6.2.1. Non-Fluorescing Chlorophyll Catabolites

The 'non-fluorescing' Chl-catabolite (NCC) **1** was the first non-green Chl-catabolite from higher plants to be clearly identified (Krautler et al., 1991). This discovery spurred the search for NCCs in other higher plants, and NCCs were soon discovered, for example, in oil seed rape (*Brassica napus*) (Muhlecker and Krautler, 1996), in tobacco (*Nicotiana rustica*), in corn (*Zea mais*), in spinach (*Spinacia oleracea*), in the Katsura tree (*Cercidiphyllum japonicum*) and in *Arabidopsis thaliana* (Moser et al., 2009b; Muller et al., 2006; Pruzinska et al., 2005). In all of the known NCCs, the asymmetric 15-position has a common absolute configuration (according to their circular dichroism spectra). However, they exist in two categories with respect to the stereochemistry at the 1-position (Krautler and Matile, 1999; Oberhuber et al., 2008). The structures of NCCs gave the first indications for the essential transformations occurring during the degradation of Chl: accordingly, NCCs should be formed, in a formal sense, by an oxygenolytic opening of the Chl-macrocycle at its 'northern' C-5 meso-position (Krautler et al., 1991) and not, as expected, at the 'western' C-20 position (Brown et al., 1991). With one exception (Muller et al., 2006), all of these colourless tetrapyrroles not only had the same basic skeleton as **1**, but

they also carried a methyl group at their 7-position (Chl numbering), meaning that they were more closely related to Chl *a* than to Chl *b*. This observation has now been rationalized since Chl (or Chlide) *b* is converted into Chl or (Chlide) *a* in an early step of Chl-breakdown in higher plants (see Hortensteiner and Krautler (2011), and as part of the proposed 'Chl cycle' shown in Fig. 5 (above). However, in the green alga *Chlorella protothecoides*, both Chls *a* and *b* can be degraded directly as suggested by identification of red tetrapyrrolic breakdown products with a formyl group at their 7 position, analogous to Chl *b* (Iturraspe et al., 1994).

5.6.2.2. Fluorescing Chlorophyll Catabolites

The slowly accumulating knowledge on Chl-catabolites provided a solid basis to guide further considerations about Chl-breakdown in higher plants (Fig. 8): in senescent leaves, traces of blue fluorescent compounds were discovered (with luminescence maxima near 450 nm), which were tentatively named 'fluorescent' Chl-catabolites (FCCs). The rather unpolar FCC **2** was characterized structurally as a linear tetrapyrrole and a rational precursor of NCCs (Muhlecker et al., 1997). Since the FCC 2 showed the same peripheral substituents as pheophorbide (Pheide) *a*, it was named 'primary' FCC (*p*FCC, 2): this *p*FCC was assumed to be formed from Pheide *a* by a minimum of two enzymic reactions. Based on structural considerations, the so far elusive 'red Chl catabolite' of higher plants (RCC, **3**) was postulated to be directly reduced to **2** (Muhlecker et al., 1997). Indeed, when RCC (**3**) became available from a partial chemical synthesis (see e.g. Moser et al. (2009b), it was shown to be reduced to **2** by an enzyme, now called RCC-reductase (RCC-R) (Hortensteiner and Krautler, 2011) . Likewise, the key enzyme that accomplishes the critical opening of the macrocycle of Pheide *a* to RCC (**3**) was identified and was characterized as Pheide *a* monooxygenase (PaO) (Fig. 8).

RCC-R is a cofactor-independent, ferredoxin-dependent enzyme, and the mechanism of the reduction of RCC (**3**) by RCC-R to *p*FCC (**2**) was a puzzle. Non-enzymic electrochemical reduction of RCC (**3**) gave **2**, representing a biomimetic version of the biological path (Oberhuber et al., 2008). Typical FCCs are observed only fleetingly and, in weakly acidic medium as exists in vacuoles, they isomerize rapidly to the corresponding NCCs as the 'last' of the well established steps of Chl-breakdown (Oberhuber et al., 2003). Indeed, the NCCs that result from natural Chl-breakdown appear to be contained in the vacuoles (Matile et al., 1996) (Fig. 6).

5.6.2.3. Colored Chlorophyll Catabolites

For many years, the search for coloured Chl-catabolites in autumn leaves was unsuccessful (Matile, 1987) and this can now be explained

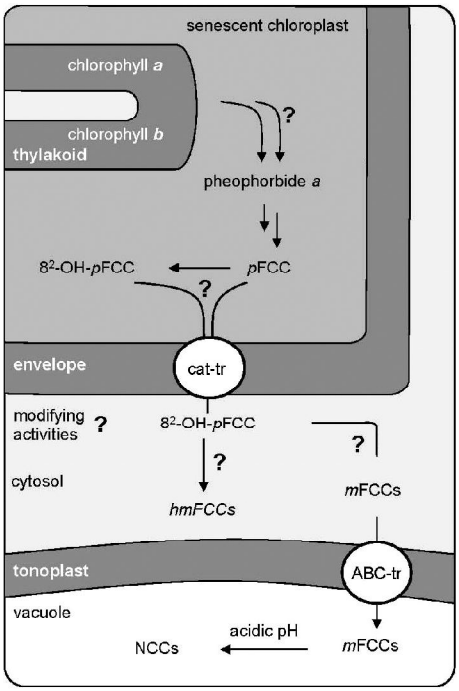

Fig. 6. Topographical model of Chl-breakdown in senescent leaves (Hortensteiner and Krautler, 2011). Chls are degraded by chloroplast enzymes to pFCC (2), or – possibly – to the hydroxylated 8^2-OH-pFCC. FCCs (pFCC or 8^2-OH-pFCC) are exported through the envelope with assistance of the 'catabolite' transporter (cat-tr). In the cytosol, 8^2-OH-pFCC is converted into modified FCCs (mFCCs) or, alternatively, to persistent, 'hypermodified' FCCs (hmFCCs, such as 6) by mostly still hypothetical enzymatic reactions. FCCs (such as 8^2-OH-pFCC and mFCCs) may then be transported by a tonoplast-bound ABC-transporter (ABC-tr) into the vacuole, where they isomerize spontaneously to the corresponding NCC (such as 4).

because it is the colourless NCCs that appear to be the typical products of Chl-breakdown in higher plants (Krautler and Hortensteiner, 2006). Likewise, the so called 'urobilino-genoidic' Chl-catabolites, discovered in senescent barley leaves, are also colourless (Losey and Engel, 2001) as are the related dioxobilanes in the autumn leaves of Norway maple (Muller et al., 2011). All of these findings are consistent with Chl-breakdown as source, basically, of colourless tetrapyrroles (Krautler and Hortensteiner, 2006). However, yellow linear tetrapyrroles were found in freshly collected, senescent leaves of *C. japonicum* (Moser et al., 2008b) (see Fig. 8). The natural yellow Chl-catabolite (YCC) **5** is identical with an oxidation product of the NCC **4** (Moser et al., 2008b). Thus, Chl-catabolites contribute directly to the colours in autumn leaves. Remarkably, the structural formula of the YCC **5** exhibits a chromophore type (Moser et al., 2008b), as is also present (two times per molecule) in the heme degradation product bilirubin (Falk, 1989).

5.6.3. Chlorophyll Catabolites in Fruits

The development of yellow and red colours is an easily observed and indirect sign of Chl breakdown during the ripening of many fruit. The first unambiguous identification of Chl catabolites in fruit was achieved in extracts of ripe 'Golden Delicious' apples (*Malus sylvestris*) and of 'Williams' pears (*Pyrus communis*); indeed, two common NCCs

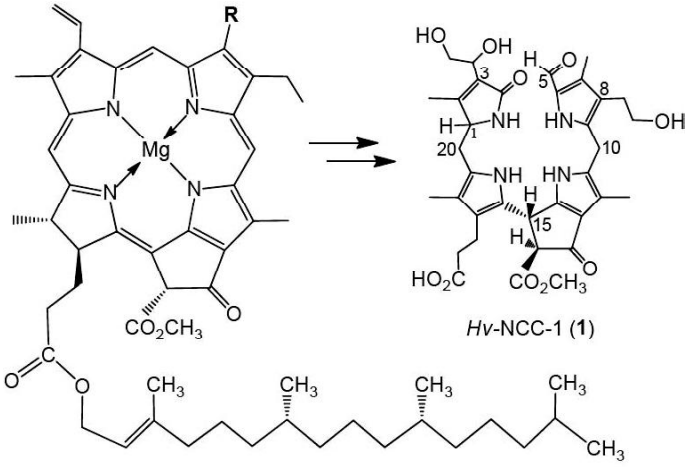

Fig. 7. A colourless chlorophyll-catabolite in senescent leaves. The 'non-fluorescent' Chl catabolite 1, named *Hv*-NCC-1, was found as the main degradation product of chlorophyll in senescent primary leaves of barley (*Hordeum vulgare*) (Krautler and Matile, 1999).

were discovered in these fruits that were also present in the senescing leaves of these two fruit trees. One of them is identical to the widely distributed NCC **4** (Muller et al., 2007) (see Fig. 8). This suggested a common path of Chl breakdown in senescent leaves and in ripening fruit (Muller et al., 2007). The frequently-found NCC **4** has been examined with respect to its properties as an antioxidant and was found to be very effective in suppressing autoxidation processes, for example, of linoleic acid (Muller et al., 2007). Thus, the discovery of NCCs as previously ignored antioxidants in fruits may now validate the old saying '*An apple a day keeps the doctor away*'.

Yellow peels of ripe bananas (*Musa acuminata*, Cavendish cultivar) provided an unexpected surprise, namely, that polar blue fluorescent Chl-catabolites (FCCs) accumulate as the apparently predominant products of Chl-breakdown (Moser et al., 2009a; Moser et al., 2012): the main FCC is the FCC-diester **6**, in which the propionate group is esterified (Fig. 8). Such esterification in natural FCCs (or NCCs) was hitherto completely unknown. The existence of a free propionic acid function in other FCCs is considered particularly relevant for catalysis of the highly stereo-selective isomerisation of typical FCCs to NCCs (Oberhuber et al., 2008). The unprecedented existence of a propionate

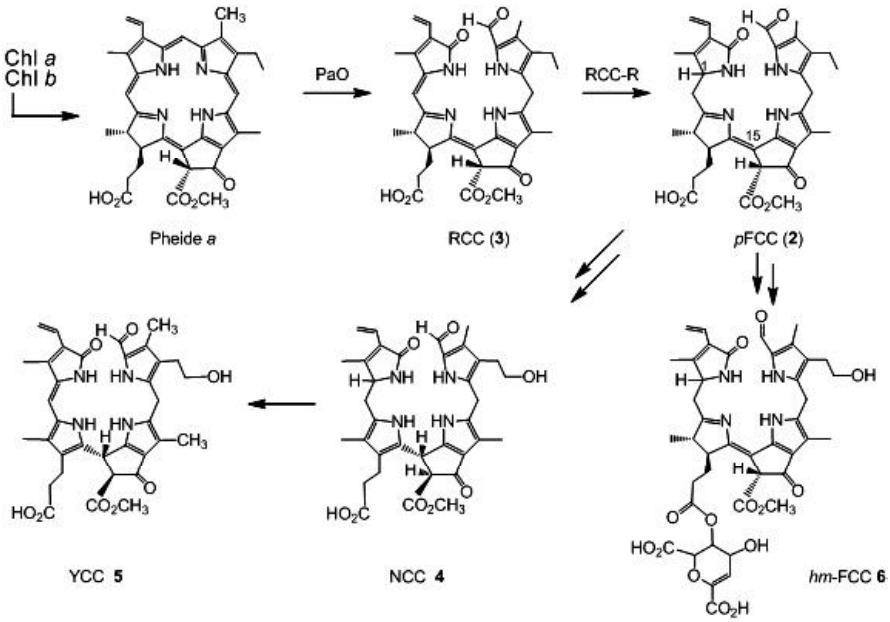

Fig. 8. Outline of chlorophyll breakdown in higher plants (Hortensteiner and Krautler, 2011; Moser *et al.*, 2009b). Chlorophylls (Chl *a* or Chl *b*) are degraded via pheophorbide *a* (Pheide *a*), which is oxygenated by Pheide *a* mono-oxygenase (PaO) to furnish the elusive «red» Chl catabolite (RCC, 3). RCC is reduced by RCC reductases (RCC-Rs) to primary 'fluorescent' Chl-catabolites (*p*FCCs, 2), which are formed as (C-1)-epimers. The *p*FCCs are transformed into 'non-fluorescent' Chl-catabolites (NCCs), such as the NCCs 1 and 4. Further oxidation of NCCs in senescent leaves furnishes yellow Chl-catabolites (YCCs), such as the YCC 5, that contribute to the colours of autumn leaves. In ripening bananas, and in some senescent leaves, 'hyper-modified' FCCs (*hm*FCCs) occur, such as 6, which make yellow bananas luminesce blue, when illuminated with UV-light.

ester group in the Mc FCC **6** provides a mechanistic explanation for the surprising persistence and accumulation of such 'hypermodified' FCCs (*hm*FCCs) in the peels of yellow bananas (Moser et al., 2008a). The *hm*FCCs explain the previously unnoticed blue luminescence of ripe(ning) bananas and represents one more striking feature of Chl-breakdown (Moser et al., 2009a)

Nowadays, many of the basic features of Chl-breakdown in higher plants have been discovered. It is accomplished by enzyme catalyzed formation of Pheide *a* from the Chls (*a* and *b*), followed by two enzyme catalyzed steps that transform Pheide *a* to the colourless *p*FCCs. From these *p*FCCs, modified FCCs (*m*FCCs) are formed and transported into

the vacuoles, where they isomerise spontaneously to colourless NCCs (Hortensteiner and Krautler, 2011; Krautler and Hortensteiner, 2006). Thus, in senescent leaves and in ripening fruit Chl breakdown furnishes natural linear tetrapyrroles that are related to heme-degradation products, such as bilirubin and other natural bilins (Krautler and Hortensteiner, 2013). Further studies on the structures and chemical reactivity of Chl-catabolites are needed to answer pertinent questions about the 'how and why' of Chl-degradation in plants and other photosynthetic organisms. The possible physiological roles of Chl-catabolites in plants remain to be elucidated. It is also of interest to learn to what extent Chl catabolites are pigments that may serve the communication between plants and animals.

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Chapter 6. LIGHT REGULATION OF CHLOROPHYLL BIOSYNTHESIS IN ANGIOSPERM PLANTS: THE ROLE OF PHOTORECEPTORS AND THE PHOTOMORPHOGENESIS REPRESSOR COP1

Pawel Jedynak, Kazimierz Strzalka and Przemyslaw Malec
Jagiellonian University, Krakow, Poland

Contents

6.1. Introduction

Chlorophylls are plant pigments that are essential for the harvesting the solar energy required for electron transport within the reaction centers of the photosynthetic apparatus (Tanaka and Tanaka, 2006). In plants, chlorophyll biosynthesis is a coordinated process that is completed by a series of subsequent reactions catalyzed by a set of enzymes and may be divided into four main phases, i.e. the synthesis of porphobilinogen (PBG), protophorphyrin IX, protochlorophyllide a and chlorophyll *a* (Chl *a*), respectively (Tanaka and Tanaka, 2007) (for more details see Chapter 5). In the first phase, glutamic acid is converted into 5-aminolevulinic acid (5-ALA), which is a tetrapyrrole precursor. Further, two 5-ALA molecules are condensed to porphobilinogen. The subsequent transformation of porphobilinogen into protophorphyrin IX includes several enzymatic reactions. Next, after the incorporation of Mg, monovinyl-photochlorophyllide *a* is formed (Tanaka and Tanaka, 2007; Tanaka et al., 2011). In angioperms, the subsequent reduction of monovinyl-protochlorophyllide *a* (Pchlide) to chlorophyllide *a* (Chlide) requires light. It is catalyzed by light-dependent photochlorophyllide oxidoreductase (LPOR) in the presence of NADPH as a reducing cofactor (Schoefs and Franck, 2003; Masuda and Takamiya, 2004). In a model plant species, *Arabidopsis thaliana*, three genetically and functionally different isoforms of LPOR were identified (Armstrong et al., 1995; Runge et al., 1996; Masuda and Takamiya, 2004). The LPOR-driven Pchlide photoreduction constitutes a crucial step during developmental

conversion to photoautotrophy and is accompanied by profound changes in plant morphology and physiology, which occur after the first exposure of emerging angiosperm seedlings to light (deetiolation) (Schoefs and Franck, 2003). Finally, chlorophyllide *a* is converted into Chl *a* after esterification (Schoefs, 2001; Masuda and Takamiya, 2004). Recently, the chlorophyll biosynthesis pathway has been the subject of intensive studies (Bollivar, 2006; Masuda, 2008). Both metabolic intermediates (Vavilin and Vermaas, 2002; Masuda and Fujita, 2008) and the vast majority of genes encoding enzymes involved in this process were identified and characterized in model organisms (Suzuki et al., 1997; Lange and Ghassemian, 2003; Bollivar, 2006). This progress facilitated the subsequent analysis of the molecular mechanisms which are involved in the regulation of chlorophyll biosynthesis.

Both chlorophyll precursors and the chlorophyll itself are potential photosensitizers. Excessive concentrations of these compounds may cause photooxidative damage to plant cells (Cheminant et al., 2011). Therefore, the regulation of the chlorophyll biosynthetic pathway as well as its adaptation to changing environmental conditions and phases of an organism's life cycle are crucial for plant growth and development. Recent progress in the regulation of chlorophyll metabolism has been extensively reviewed (Vavilin and Vermaas, 2002; Tanaka and Tanaka 2006; Tanaka et al. 2011). This chapter presents a brief overview of the light signaling mechanisms involved in the regulation of the activity of chlorophyll biosynthesis pathway in angiosperm plants.

6.2. The plant photoreceptor system

Light is the one of most important environmental factors which determines plant vegetation. Plants are able to perceive changes in the fluence rate, spectral quality, direction and rhytmicity (e.g. day/night length) of light. In order to sense the light incoming to the Earth's surface, plants evolved a complex photoreceptor system. Photoreceptors are chromoproteins, absorbing light of different wavelengths. In particular, phytochromes can absorb red/far red light (600-750 nm), while blue/UVA (320-500 nm) light is sensed by cryptochromes, phototropins and ZTL family proteins. UVB (280-320 nm) is perceived by the recently discovered UVR8 protein (Sullivan and Deng, 2003; Christie, 2007; Franklin and Quail, 2009; Inigo et al., 2012).

The most characterized phytochromes consist of a linear tetrapyrrole chromophore covalently attached to an apoprotein (120 kDa) at its amino terminal region (Franklin and Quail, 2009). In *Arabidopsis*, five phytochrome apoprotein genes (*PHYA-E*) were identified. These genes encode phytochromes with distinct stability, intracellular localization and physiological function (Smith, 1995). Upon light absorption,

phytochromes preexisting in a red light absorbing form (P_r) are rapidly converted to a far-red light absorbing form (P_{fr}). The modes of phytochrome action have been classified into the following groups: very low fluence rate responses (VLFR) activated at very low P_{fr} levels, low fluence rate responses (LFR) characterized by red/far red reversibility and high irradiance responses (HIR) induced both by red (R) and far red (FR) light (Nagy and Schafer, 2002).

Cryptochromes are plant blue/UVA photoreceptors. Two cryptochrome genes – *CRY1* and *CRY2* – were identified in *Arabidopsis* (Lin and Todo, 2005). Cryptochromes are chromoproteins containing flavin adenine dinucleotide (FAD) and pterin as chromophores (Cashmore et al., 1999). Although they show a structural similarity to prokaryotic photolyases, no photolyase activity has been attributed to plant cryptochromes (Lin and Todo, 2005).

Phototropins are blue/UVA plant photoreceptors. *PHOT1* and *PHOT2* genes encode phototropins in *Arabidopsis*. Phototropin apoproteins are able to bind flavin mononucleotide (FMN). They contain two conserved LOV (**L**ight, **O**xygen, **V**oltage-regulated) domains at the amino terminus and a serine/threonine kinase domain at the carboxy terminus (Huala et al., 1997; Christie, 2007). The second group of LOV photoreceptors, structurally and functionally different from phototropins, is comprised of several members of ZTL (Zeitlupe) family, containing a single LOV domain. They are predominantly involved in the regulation of flowering and circadian rhytmicity (Harmer, 2009).

The UVR8 photoreceptor is a dimer-forming protein, with specific tryptophan residues at the dimer interface (Kliebenstein et al., 2002; Rizzini et al., 2011). Upon the absorbance of UVB wavelengths it undergoes monomerization caused by a disruption of interactions at the interface. The released monomers were found to interact with COP1 E3 ubiquitin ligase (Christie et al., 2012).

The perception of light leads ultimately to the activation of transcription factors which recognize specific sequences within the regulatory regions of light-regulated genes (Terzaghi and Cashmore, 1995). This information transfer is provided by intracellular signaling molecules, triggered upon excitation of photoreceptors. Molecular and genetic screens have identified specific regulators of light signaling, acting downstream of photoreceptors. In particular, mutations in multiple *Arabidopsis* loci referred to as *cop/det/fus* (**CO**nstitutive **P**hotomorphogenesis/**DE**e**T**iolated/**FUS**ca) genes, result in dark-grown seedlings that phenotypically mimic light-grown wild-type ones (Chory and Peto, 1989). The products of these genes were found to form multisubunit complexes – "zomes", involved in the control of plant photomorphogenic processes via regulated proteolysis. The pleiotropic COP/DET/FUS group of proteins defines three complexes: the COP1 complex, the

COP9 signalosome (CSN) and the CDD complex (Strickland et al., 2006). COP1 (constitutive photomorphogenesis 1) is a 76 kDa protein, containing an N-terminal RING finger domain, a coiled-coil domain and a C-terminal set of WD-40 repeats. COP1 is an E3 ubiquitin-protein ligase involved in the signaling pathways which mediate gene expression in both plant and animal cells (Yi and Deng, 2005). In plants COP1 plays a significant role in the derepression of light-regulated genes, especially during deetiolation (Ma et al., 2002).

6.3. Red/far red light

In angiosperm plants, the chlorophyll pool is an effect of the *de novo* synthesis, which takes several hours after the first exposure to light. This delay is caused by the inhibition of 5-ALA synthesis in darkness and is referred to as the lag phase of chlorophyll production (Castelfranco and Beale, 1986). Early observations demonstrated that the lag phase can be overcome by the preliminary irradiation of etiolated seedlings with red light, which additionally increases the final content of chlorophyll in the plant after its transfer to standard light conditions (Masoner et al., 1975; Yoshida et al., 1987). Red light-induced breaking of the lag phase is fully reversible after a subsequent FR pulse, but only up to first the 45 minutes (in the case of *Sorghum vulgare*) after irradiation with R. The maximal time of photoreversibility corresponds with the relative ratio of activated phytochrome to total phytochrome content (P_{fr}/P_{total}). For *S. vulgare* this parameter can reach 0.15. Then, a gradual weakening of the response to FR occurs and there is no observable effect of FR after two hours (Oelze-Karow et al., 1982). The similar weakening of R-induced chlorophyll synthesis by FR in etiolated seedlings were observed in various species of angiosperm plants, although the maximal time of photoreversibility varied (Horwitz et al., 1988). Further studies of phytochrome responses with *fhy* mutants have shown that PHYA-dependent signaling is crucial for the FR-induced inhibition of greening of etiolated *Arabidopsis* seedlings (Barnes et al., 1996).

The phytochrome regulatory pathway also determines the maximal chlorophyll content in mature, light-grown plants. Again, red light increases the total chlorophyll content. Moreover, the quantitative composition of chlorophylls a and b can be altered depending on the red/far red ratio. Red light typically favors chlorophyll *b* production, which in turn leads to a decrease in the chlorophyll *a*/*b* ratio. Additionally, the final value of the chlorophyll *a*/*b* ratio under red light irradiation also depends on the age of the plant and may therefore vary within fairly wide limits (e.g. from 1,0 to 2,2 in *Chenopodium rubrum*) (Frosch et al., 1985).

Glu-tRNA reductase which catalyzes the reduction of Glu-tRNA to glutamate 1-semialdehyde with the release of intact tRNA and the glutamate 1-semialdehyde 2,1-aminomutase converting glutamate 1-semialdehyde to 5-ALA were probably the first enzymes of chlorophyll biosynthesis pathway, whose transcript levels were found to be elevated by light (Ilag et al., 1994). Later studies revealed that the *HEMA1* gene which encodes glutamyl-tRNA reductase is regulated at the transcription stage by phytochromes both in LFR and FR-HIR modes (McCormack et al., 2001).

Phytochrome activation allows its import into the nucleus where it binds to PIFs (**P**hytochrome **I**nteracting **F**actor) or PILs (**P**hytochrome **I**nteracting Factor-**L**ike), basic helix-loop-helix type class transcription factors (Bae and Choi, 2008). The light-activated phytochrome inhibits the activity of these transcription factors, which are phosphorylated and then degraded in a 26S proteasome-mediated pathway (Park et al., 2004; Al-Sady, 2006). Particularly, PIF1 and PIF3 play an important role in the regulation of chlorophyll biosynthesis in plants (Shin et al. 2009, Stephenson et al. 2009). In the *pif1* mutant, the chlorophyll accumulation was higher then in wild type *Arabidopsis* in the presence of light (Huq et al., 2004). On the other hand, *PIF1* gene overexpression significantly reduced the accumulation of chlorophyll (Moon, 2008). It was also demonstrated that PIF1 can specifically recognize G-box (CACGTG) motifs in promoters of heme oxygenase and ferrochelatase genes, encoding factors involved in the metabolism of heme (Martinez-Garcia, 2000; Moon, 2008).

Also, PIFs are involved in the expressional regulation of the NADPH/protochlorophyllide reductase C (LPOR C) gene, one of the three LPOR isoforms found in *Arabidopsis thaliana* (Moon, 2008). LPORs catalyze the key stage of chlorophyll biosynthesis – the conversion of protochlorophyllide into chlorophyllide. In angiosperms, the activity of LPOR is regulated by light, which is essential for the activation of that enzyme. Thus, in darkness, the synthesis of chlorophyllide cannot be completed (Beale et al., 1999). Moreover, the main forms of LPOR present in etioplasts (plastids of dark-grown plants) are LPOR A and B, which are not directly regulated by PIFs. Surprisingly, the LPOR C level rises in the presence of light (Masuda et al. 2003). The synthesis of protochlorophyllide must be tightly regulated and correlated with the LPOR level, because of potential protochlorophyllide phototoxicity. In the presence of light protochlorophyllide can act as a photosensitizer, generating reactive oxygen species, which can be harmful to plant cells (Egorov et al., 1990; Ma et al., 2003). That is why in etiolated plants a significant pool of protochlorophyllide is bound in the Pchlide/LPOR/NADPH complexes which are present in the structure of prolamellar bodies (Sperling et al.,

1998). After a millisecond flash of light the complexed pigment pool is rapidly converted into a much less reactive chlorophyllide (Griffiths, 1975, Schoefs et al., 2000). The inactivation of genes encoding both PIF1 and PIF3 factors results in excessive Pchlide accumulation in dark-grown seedlings, so these transcription factors are considered to be negative regulators of chlorophyll biosynthesis (Shin et al., 2009). Also, both PIF1 and PIF3 were found to negatively affect the expression of enzymes active at earlier stages of chlorophyll biosynthesis. In particular, the *Arabidopsis HEMA1* gene encoding glutamyl-tRNA reductase and the *CHLH* gene, encoding the largest subunit of Mg-chelatase have been demonstrated to be regulated by phytochrome/PIF signaling pathway(s), as both *pif1* and *pif3* mutants showed an elevated expression of these genes during etiolated growth (Stephenson et al., 2009). Interestingly, initial observations suggested that PIF3 can act as a stimulating agent of chlorophyll biosynthesis because *pif3* mutants exhibited a delayed accumulation of chlorophyll upon illumination (Monte et al., 2004). However, this phenomenon can be explained by the activity of a separate regulatory mechanism or (more probably) as the effect of the rapid degradation of accumulated Pchlide during light exposure (Duek et al., 2005, Stephenson et al., 2009).

6.4. Blue/UVA light

While red light stimulates chlorophyll synthesis, blue light usually decreases its total accumulation and elevates the chlorophyll *a*/*b* ratio to values of about 2,4-3,0. Both effects are directly proportional to the light intensity. However, the total chlorophyll content within a single plant becomes more constant under blue light and this effect is not dependent on plant age, as it is observed upon red light illumination (Frosh et al., 1985). Most of the processes initiated by blue light involve cryptochromes. In *Arabidopsis thaliana* two main isoforms are known – CRY1 and CRY2. It is believed that cryptochromes play a significant role in the coordination of chlorophyll synthesis in the presence of blue light (Liscum et al., 2003). Dimers of these photoreceptors were found to interact with COP1 protein in the dark (Wang et al., 2001, Yang et al., 2001). It was suggested that a flash of blue light induces a conformational change within cryptochrome molecules, which are transferred into COP1, leading to its rapid inactivation (Yi et al., 2005). However, CRY-COP1 interaction alone is not sufficient to disable the E3 ligase activity of COP1 (Wang et al., 2001, Yang et al., 2001).

The discovery of the formation of functional complexes by the direct interaction of CRY and SPA proteins (from SPA1 to SPA4, Suppressor of PHYA) demonstrated the existence of a COP1-dependent CRY signaling pathway. SPA binding was found to enhance COP1 activity

towards LAF1 (signaling associated with the PHYA) (Seo et al., 2003). In contrast, the interaction of COP1 with HY5 was weakened by SPA binding (Saijo et al., 2003). Moreover, it seems that SPA factors are critical for the functioning of COP1 because the *spa* quadruple mutant shows a phenotype similar to the *cop1* mutant (Laubinger et al., 2004). It was demonstrated that CRY initiates a blue light-induced dissociation of SPA from COP1 protein. Interestingly, the cellular location of CRY1 is regulated by the presence of light. In darkness both CRY1 and COP1 are located in the nucleus, while they are displaced to the cytoplasm upon illumination. It must be emphasized, that the molecular processes accompanied with the relocation of COP1-CRY1 complexes to the cytoplasm are still unclear. In contrast, CRY2 remains only in the nucleus (Lian et al., 2011).

In the presence of light cryptochromes were found to be important regulators of the expression of genes encoding subunits of antenna complexes (LHCa and LHCb) (Mazzella et al., 2001). It seems that CRY do not bind to any particular DNA sequence (Cutler et al., 2000, Lin et al., 2005). It is also believed that the main effect of CRY on chlorophyll synthesis is based on promoting the accumulation of HY5 (Liscum et al., 2003). Relatively few genes are directly activated by CRY and *SUB1* is one of them. The *SUB1* gene encode the calcium-dependent protein involved in PHYA-mediated singnaling. SUB1 inhibits the accumulation of HY5 (Guo et al., 2001). Another gene activated by CRY is *GBF1*. 6-day seedlings of *gbf1* mutants grown in continuous white light exhibit (like the *cop1* mutant) a decreased chlorophyll content. The *gbf1/cop1* double mutant exhibits an even stronger negative effect on chlorophyll accumulation. Furthermore, preliminary irradiation of etiolated seedlings of *gbf1* and *spa1* mutants with far red enhance their sensitivity to white light-induced bleaching. This effect is even stronger in seedlings of the *gbf1/spa1* double mutant. Interestingly, *CAB1* gene expression is increased in the *cop1* and *spa1* mutants, but significantly reduced in the *gbf1* and the *gbf1/cop1* mutants. Thus, COP1 and GBF1 act antagonistically. Moreover, in darkness the level of GBF1 protein is reduced – that protein is degraded by the 26S proteasome-dependent pathway, but independently of the COP1/SPA1 mediated mechanism (Mallappa et al., 2008).

The roots of plants normally (in the absence of light) do not contain any chlorophylls, but are capable of greening after exposure to light. The spectral quality of light has a significant impact on chlorophyll accumulation in greening roots. In wild-type roots, blue light causes chlorophyll accumulation up to 15-fold higher, comparing to roots irradiated with red light. In the *cop1* and *det1* mutants, the chlorophyll accumulation was comparable in roots irradiated with blue and red light. In contrast, roots of the *cry2* and *cry1/cry2* double mutants

exhibited a much stronger reduction of chlorophyll accumulation when irradiated with red light, but an astonishing decrease in chlorophyll content (up to 95% compared to wild type) was observed in the *cry1* mutant. Interestingly, the *phya/b* double mutant showed no capacity for root greening under blue light, suggesting a synergistic effect on chlorophyll synthesis between the PHYA-, PHYB- and CRY1-mediated pathways in greening roots. Moreover, a combination of blue and red light irradiation stimulates chlorophyll synthesis much more strongly than when only one light wavelength is used. This effect was not observed in the *phya* mutant. In roots, the stimulatory effect of blue light on the expression of *LHCb*1* gene is c.a. 7 times higher then that of red light (Usami et al., 2004).

Early observations also indicated that the blue light initiated the development of what are known as "sun type" chloroplasts (a plastid phenotype typical in plants grown in a high intensity of light). This effect was associated with increased levels of cytokinins (Lichtenthaler et al., 1980). It is also known that cytokinins promote chlorophyll synthesis (Canakci et al., 2009) by stimulating the synthesis of aminolevulinic acid (Ueda et al., 2006), but there is no evidence for any direct role of cryptochromes in the regulation of cytokinin levels. It turns out, however, that the cytokinin level can affect HY5 accumulation, thus contributing to an increase in the level of chlorophyll (Vandenbussche et al., 2007).

There are limited observations concerning other blue light receptors. It is known that PHOT1 is necessary to destabilize the mRNA of LHCb at high light intensities (Folta et al., 2003). Moreover, both PHOT1 and PHOT2 are involved in the regulation of intracellular Ca^{2+} levels (Harada et al., 2003) and therefore in control of calcium-dependent protein activity. These results suggest that phototropin signaling may indirectly influence chlorophyll accumulation.

6.5. UVB light

Continuous, low-dose exposure to UVA has a relatively small, negative effect on the synthesis of chlorophyll in growing plants, while UVB can cause a significant reduction in the level of chlorophyll in plants (Jayakumal et al., 2004). The effect of UVB on chlorophyll content may also be dependent on the plant species and light fluence (Larsson et al., 1998). UVB affects the reduction of the chlorophyll *a/b* ratio, probably by inhibiting the synthesis or by enhancing the degradation of chlorophyll a (Strid et al., 1990, Larsson et al., 1998, Choi et al., 2003). Interestingly, UVA radiation stimulates the synthesis of chlorophyll in etiolated seedlings. This effect was not observed in the double mutants *hy6/blu1* (Young et al., 1992). UVA stimulates the

synthesis of the ELIP proteins involved in binding chlorophyll released from the LHC under stress conditions. Both UVA and UVB are necessary to sustain the presence of ELIP in the cell (Adamska et al., 1992).

6.6. The role of COP1

Importantly, *pif1* and *pif3* mutants exhibit some features of photomorphogenic development even in the absence of light, and the double *pif1/pif3* mutant has been found to exhibit constitutively a photomorphogenic phenotype in darkness, including opened cotyledons and a reduced hypocotyl length (Stephenson et al., 2009). The same constitutive photomorphogenic phenotype is observed in the case of another group of mutants, known as *cop/det/fus* (Chory et al., 1989, von Arnim et al., 1994, Hardtke et al., 2000). Products of *cop/det/fus* genes were found to encode the regulatory proteins which participate in the 26S proteasome-mediated degradation of basic helix-loop-helix transcription factors, although differently from PIF (Kwok et al., 1999, Hardtke et al., 2000). COP/DET/FUS proteins are also required for a degradation of phytochrome A and the PHYA-mediated regulatory proteins such as LAF1 (**L**ong **A**fter **F**ar red 1) in darkness (Seo et al., 2003, 2004).

In particular, in *cop1* and some related mutants protochlorophyllide accumulation in the dark has been found to be significantly elevated (Sperling et al, 1998). This effect has been accompanied with a remarkable decrease in the expression of the *LPORA* and *LPORB* genes (Lebedev et al, 1995).

It was shown that the presence of COP1 protein, which is an E3 ligase involved in ubiquitin-dependent protein labeling degraded by 26S proteasome (Saijo et al., 2003), is required for the accumulation of PIF transcription factors in darkness (Bauer et al. 2004). This effect was initially associated with previously observed COP1-mediated PHYA degradation (Seo et al., 2004). However, PIF1 and PIF3 can also interact with PHYB, which is not degraded upon illumination (Huq et al., 2004; Stephenson et al., 2009). In addition, COP1 protein accumulation occurs in the nucleus only in the dark, whereas in these conditions PHY is present in the cytoplasm (Boccalandro et al, 2004, Osterlund et al., 1998). As the PHY-mediated export of COP1 from the nucleus in the light is very slow and can take several hours (von Arnim et al., 1997), it is possible that PIF1/PIF3 activity could be under the control of other unknown regulators degraded in the dark in a COP1-dependent manner. It was also suggested that the regulation of chlorophyll synthesis mediated by PIF1 and PIF3 factors by a mechanism involving COP1 proteins are separate phenomena (Shin et al. 2009).

Also EIN3/EIL1 (**E**thylene **In**sensitive 3/EIN3-**L**ike 1) proteins have an important role in the regulation of chlorophyll biosynthesis. They were identified as factors involved in plant responses to ethylene (Zhong et al., 2009). The accumulation of these proteins has been found to positively correlate with the presence of COP1 protein. Moreover, the promoters of *LPORA* and *LPORB* genes contain an EBS (**E**IN3 **B**inding **S**ite or F-box) conservative motif (Zhong et al., 2009). EIN3 is involved in the inhibition of PIF1-independent protochlorophyllide accumulation in the dark. The presence of EIN3/EIL1 proteins has been shown to stimulate *PIF3* gene expression (Zhong et al., 2010). *EIN3/EIL1* overexpression in the *cop1* mutant leads to a significant reduction in the Pchlide level accumulated in darkness (Zhong et al., 2009).

COP1 also controls chlorophyll biosynthesis by regulating the levels of the HY5 (elongated **Hy**pocotyl 5) transcription factor, one of the major substrates of this ligase (Hardtke et al. 2000). HY5 is directly involved in initiating most of the morphological changes during deetiolation. In particular, HY5 activates the synthesis of chlorophyll binding proteins, indispensable for the assembly of the photosynthetic apparatus (Oyama et al., 1997, Lee et al., 2007). HY5 also activates the expression of another transcription factor that promotes photomorhogenesis – LZF1 (**L**ight-regulated **Z**inc **F**inger 1), which is also a substrate for COP1. LZF1 gene expression can also be directly induced by light, so LZF1 protein is present in the nucleus even in the absence of HY5, although its level is significantly lower. Chlorophyll content is remarkably lowered in *lzf1* mutants, while the overexpression of the *LZF1* gene significantly increases chlorophyll accumulation. Similar effects are observed in a HY5 mutant (Chang et al., 2008). The reduction of chlorophyll accumulation is enhanced in the case of the double *lfz1/hy5* mutant. However, the overproduction of the LFZ1 protein in *hy5* mutants is not sufficient to restore the level of chlorophyll to a level typical of wild type *Arabidopsis* (Chang et al., 2008). This suggests that HY5 regulates the chlorophyll content through the activation of other factors, independently from LFZ1.

In darkness, the Pchlide level can also be regulated through the FLU protein (**Flu**orescent in blue light) in *Arabidopsis thaliana* (Meskauskiene et al., 2001) or TIGRINA in *Hordeum vulgare* (Gough et al., 1979, Lee et al ., 2003). When Pchlide content (unbound with LPOR) reaches a critical concentration, the FLU protein inhibits the activity of glutamyl-tRNA reductase, the enzyme synthesizing one of the first intermediates of the chlorophyll biosynthesis pathway, thus the decreasing 5-aminolevulinic acid (5-ALA) accumulation (Meskauskiene et al., 2002). Etiolated seedlings of the *flu* mutant exhibit a highly elevated Pchlide level, despite the presence of pathways associated with COP1. It is believed that COP1- and FLU-dependent

mechanisms regulating chlorophyll biosynthesis are separated (Meskauskiene et al., 2002), because the absence of COP1 ligase does not affect the FLU protein level and mutation in the *FLU* locus does not correspond with any changes in COP1 protein content. A FLU-mediated pathway therefore, seems to provide an additional mechanism for preventing excessive Pchlide accumulation in etioladed seedlings.

The role of COP1 and FLU proteins in the regulation of chlorophyll biosynthesis is not restricted to effects observed in the dark. COP1 also controls the process of chlorophyll accumulation in plants growing in the presence of light. In fact, the total chlorophyll content is increased in viable (passing full life cycle) *cop1* mutants (Sperling et al., 1998). This phenomenon is also observed in the *flu* mutant (Meskauskiene et al., 2002). Also, COP1 protein is responsible for the distribution of the pigment and the development of chloroplasts, because the lack of functional COP1 leads to the abnormal formation of Pchlide-containing plastids in root cells grown in the dark (Seyedi et al., 2001).

6.7. Perspectives

The results presented in this chapter indicate that chlorophyll biosynthesis in angiosperm plants can be regulated by light of different spectral ranges (from UVB to FR). In fact, both specific photoreceptors and cellular regulators of protein degradation are involved in the transcriptional regulation of some key enzymes of the chlorophyll biosynthesis pathway. A summary diagram for the reported interactions and possible roles of particular proteins is presented in Fig. 1. As can be seen, the apparent links with recently recognized light signaling pathways could be demonstrated for only a few enzymes including Glu-tRNA-reductase, Mg chelatase and LPOR's. Moreover, there are a number of light effects on chlorophyll synthesis/accumulation known only from physiological observations. The most important are depicted in Fig. 2. Thus, a detailed explanation of the light regulatory network in chlorophyll biosynthesis in angiosperms at the molecular level still remains a long-term goal for many laboratories. However, selected recent findings point out several areas of further research.

In particular, some chlorophyll synthesis genes may form clusters within a gene expression network (Masuda and Fujita, 2008). For example, the recently identified GOLDEN2-LIKE (GLK) 1 and GLK2 transcription factors can induce the simultaneous expression of several genes of chlorophyll biosynthesis pathway including the *HEMA1*, *CHLH*, *CHL27*, *LPORB* and *CAO* upon illumination (Waters et al., 2009). Similarly, genome-wide chromatin immunoprecipitation demonstrated that there are more than 10 genes involved in chlorophyll biosynthesis transcriptionally controlled by HY5 (Lee et al., 2007).

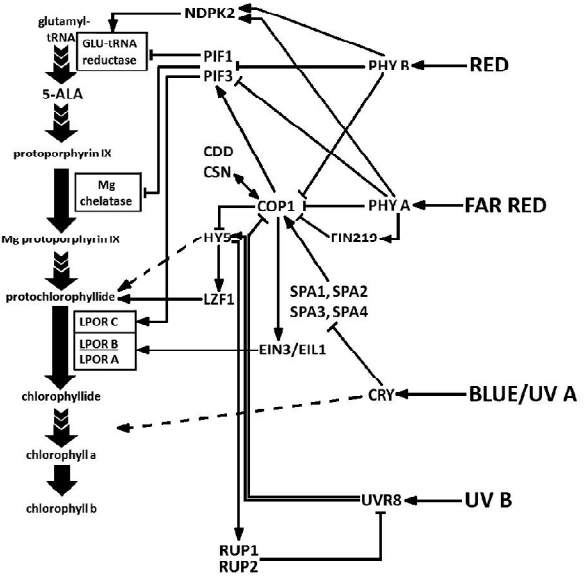


Fig. 1. A simplified diagram illustrating the role of light in the regulation of the chlorophyll synthesis pathway in angiosperms

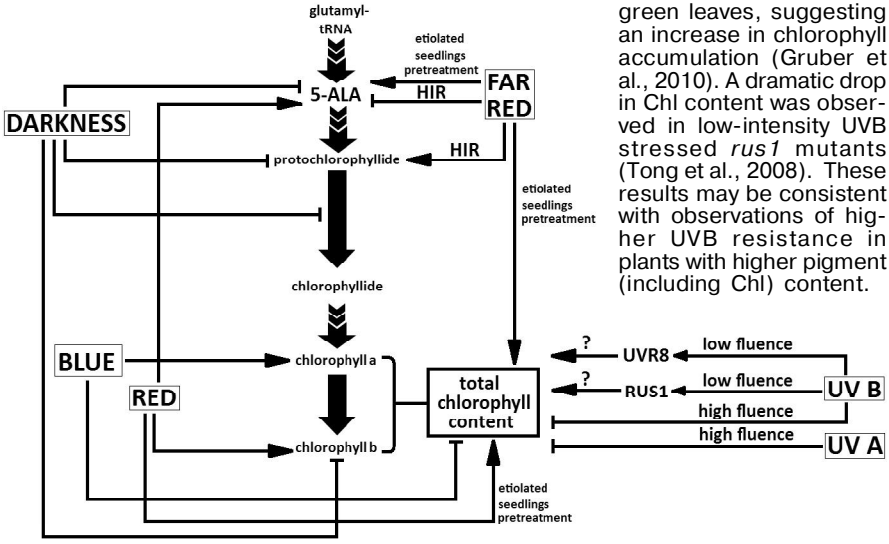
Chlorophyll biosynthesis in angiosperms can be modulated by specific light wavelengths, resulting in specific and controlled activation or down-regulation of particular genes. In darkness COP1 (E3 ubiquitin ligase) accumulation occurs and its subsequent activation by SPA proteins leads to ubiquitylation and further degradation via the 26S-proteasome of transcription factors (like HY5 or LZF1) (Chang et al., 2008). Thus, COP1 drives change in the gene expression pattern, leading to an inhibition of Chl synthesis at early stages and ensuring a rapid Pchlide to Chlide conversion of the initial Pchlide pool upon illumination, due to the activation of *POR* genes expression. In contrast, phytochromes (PHYA and PHYB) activated by red and far red light, inhibit PIF factors, allowing 5-ALA *de novo* formation. Additionally, phytochromes directly (promoting COP1 export to cytoplasm) or indirectly (i.e. via FIN219 protein) negatively affect COP1 activity (Yi et al., 2005; Wang et al., 2011). PHYA/B also trigger Chl biosynthesis directly, through the stimulatory effect of NDPK2 on Mg chelatase (McCormac and Terry, 2002; Yang et al., 2005). Blue light affects Chl synthesis through CRY (cryptochrome) photoreceptors, which deactivate COP1 E3 ligase by promoting the dissipation of SPA proteins from the complex. Thus, COP1 inhibition allows HY5 accumulation and initiates a cascade of gene expression that promotes chl synthesis (Yi et al., 2005). CRY receptors are important for Chl synthesis regulation in some tissues (i.e. in roots). However, in light-grown plants the CRY-mediated signal is widely modulated by both PHYA and PHYB (Neff et al., 1998; Usami et al., 2004). UVB radiation also inhibits COP1 activity, but, due to the HY5-mediated activation of the RUP1 and RUP2 proteins it regulates the activity of UVB sensory protein UVR8 by a negative feedback (Gruber et al., 2010).

These results suggest that enzymes of the chlorophyll biosynthesis pathway might be under transcriptional control of a limited number of "master regulators" associated with light signaling cascades.

The role of COP1 in the transcriptional regulation of plant photomorphogenesis is well established (Ma et al., 2002). The central role of COP1 in both the phytochrome- and Blue/UV – mediated regulation of

Fig.2. Notable physiological effects of light on chlorophyll biosynthesis.

In dark-grown angiosperms chlorophyll (Chl) biosynthesis is stopped at the protochlorophyllide (Pchlide) stage, due to specific, light-dependent LPOR activity. As a consequence, Pchlide accumulation results in a rapid inhibition of the formation of 5-ALA (negative feedback loop), the initial Chl precursor. Thus, Pchlide content is tightly controlled (Beale et al., 1999). Additionally, a preferential decrease in Chl *b* was observed in adult plants during a dark period (Frosch et al., 1985). Red light has a positive effect on the total Chl content and significantly decreases the Chl *a*/*b* ratio in mature plants, mainly through an increase in Chl *b* formation (Frosch et al., 1985). Additionally, preirradiation of etiolated seedlings with red light increases the maximal Chl content formed after a subsequent transfer to light conditions (Masoner et al., 1975). In contrast, blue light has a negative effect on total Chl content in mature plants and (unlike red light) increases the Chl *a*/*b* ratio (Frosch et al., 1985). Far red (FR) can have either an inhibiting or stimulating effect on chlorophyll synthesis depending on its dose and physiological context. An FR pulse reverses the stimulating upshot of red light on 5-ALA synthesis during deetiolation, thus slowing down pigment accumulation. Moreover, an FR pulse does not have an inducible effect on 5-ALA formation and notably, is not sufficient for the photoconversion of the Pchlide pool accumulated in etiolated seedlings (Klein et al., 1977). Importantly, prolonged irradiation with FR (FR-HIR) can even completely block chlorophyll synthesis via the irreversible inhibition of *HEMA1* and *POR* genes expression. Thus, etiolated seedlings may not green at all in continuous FR (McCormac et al., 2002). In a photoperiod 12FR:12dark, Chl (particularly Chl *b*) accumulation is much slower than in white light treatment (De Greef et al., 1971). FR-greening significantly blocks the increase in Pchlide level in FR-HIR irradiated seedlings (McCormac et al., 2002). The preirradiation of etiolated seedlings with FR can have stimulative effect on Chl total content after the transfer of these plants to standard growth conditions (Masoner et al., 1975). Both red and far red light pretreatment of etiolated seedlings can shorten the lag phase (initial retardation) of chlorophyll biosynthesis (Klein et al., 1977). In contrast, UV radiation, especially in high dosages, can decrease the Chl level via photooxidation, (Jayakumar et al., 2004). UVB at low intensities might stimulate Chl biosynthesis. UVBR8 overexpressing mutant lines have dark green leaves, suggesting an increase in chlorophyll accumulation (Gruber et al., 2010). A dramatic drop in Chl content was observed in low-intensity UVB stressed *rus1* mutants (Tong et al., 2008). These results may be consistent with observations of higher UVB resistance in plants with higher pigment (including Chl) content.



chlorophyll biosynthesis raises the question of its function as a main integrator of light signals perceived by the various photoreceptors which regulate chlorophyll synthesis. The role of CSN and CDD complexes directly interacting with COP1 (Yi and Deng, 2005) in chlorophyll synthesis is not known. Similarly, there is only limited data suggesting the participation of phototropins in the regulation of Pchlide accumulation (Mysliwa-Kurdziel et al., 2012a).

Also, recent data indicate that the transcription of carotenoid, chlorophyll and plastidial isoprenoid biosynthesis genes might be cross-coordinated at the cellular level (Meier et al., 2011). In *Arabidopsis*, Pchlide accumulation is accompanied by differences in xanthophyll composition, providing evidence that enzymes of the carotenoid biosynthesis pathway downstream of phytoene synthase and ξ-carotene desaturase may be involved in the regulation of greening capacity in angiosperms (Mysliwa-Kurdziel et al., 2012b). As carotenoid biosynthesis has been found to be regulated by a phytochrome/PIF cascade (Toledo-Ortiz et al., 2010), the existence of a broader light signaling network, controlling simultaneously both carotenoid and chlorophyll metabolic pathways should be considered.

It is expected that the introduction of techniques of global analysis, together with system biology tools may facilitate the rapid identification of both those chlorophyll synthesis genes whose expression is altered under particular light conditions and the regulatory proteins involved in light signal transduction.

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Chapter 7. BIOSYNTHESIS AND DEGRADATION OF CAROTENOIDS

Malgorzata Jemiola-Rzeminska and Kazimierz Strzalka
Jagiellonian University, Krakow, Poland

Contents

7.1. Introductory remarks

Within the diverse family of isoprenoids, carotenoids comprise a group of pigments that are naturally found in photosynthetic organisms and some non-photosynthetic bacteria, and also in fungi. Most of them are tetraterpenes derived from the 40-carbon isoprenoid phytoene. While the majority of carotenoids have a linear backbone or contain one or more cyclic β-ionone or ε-ionone rings, capsanthin and capsorubin are unique with their unusual cyclopentane ring. There are two major classes of carotenoids, i.e. nonoxygenated ones referred to as carotenes and their oxygenated forms – xanthophylls. The most ubiquitous representatives of carotenes are β-carotene, which is found both in chloroplasts and chromoplasts, and lycopene, which is present exclusively in the chromoplasts of some flowers and fruits. Xanthophylls are most abundant in light-harvesting complexes. Apart from the fundamental role they play in photosynthesis, which includes light harvesting and photoprotection as well as photosystem assembly, photomorphogenesis, nonphotochemical quenching and lipid peroxidation, carotenoids are precursors for plant hormones, abscisic acid (ABA)

and strigolactones. Furthermore, they are indispensable not only to the organisms where they are synthesized, but also play a critical part in human nutrition as an important source of retinoids (including vitamin A) and antioxidants. It is beyond the scope of this chapter to describe carotenoid properties and functions in detail (see Britton, 1995; Cuttriss and Pogson, 2006; Demmig-Adams et al., 1996; Frank and Cogdell, 1996; Yamamoto and Bassi, 1996). Nevertheless, one must bear in mind that it is their health promoting properties that has enhanced interest in carotenoid biosynthesis and accelerated its metabolic engineering in plants.

This chapter is aimed at presenting some fundamental aspects of carotenoid biosynthesis and catabolism. For more detailed information the reader is directed to the following excellent reviews (Cunningham, 2002; DellaPenna, 2005; Hirschberg, 2001 Sandmann, 2002; von Lintig et al., 2005).

7.2. Carotenoid biosynthesis

The carotenoid biosynthetic pathways, like some other plant compounds, are special in that their enzymes are extremely difficult to purify and analyze due to their low specific activity and poor stability. That is the main reason why much of our understanding of the genes and enzymes of carotenoid biosynthesis comes from the comparative genomics, biochemical genetics and molecular approaches.

7.2.1. Early steps in biosynthesis

The initial steps of carotenoid synthesis in higher plants have been extensively studied and are covered in detail elsewhere (Cunningham and Gantt, 1998; Lichtenthaler, 1999). The basic C5 element, isopenthenyl diphosphate (IPP), comes from the methylerythritol 4-phospate (MEP) pathway located in plastids. Organisms including archaea, some bacteria and fungi which do not contain the MEP pathway utilize the mevalonate (MVA) pathway for the synthesis of the C5 precursors for carotenoid formation. The assembly of C5 building blocks carried out by IPP isomerase (IPI) and GGPP synthase (GGPS) results in the formation GGPP (C20). Then, two molecules of GGPP condensate by the action of phytoene synthase (CrtB in bacteria, PSY in plants) which leads to 15-*cis*-phytoene, the first dedicated compound in the carotenoid pathway (see Fig. 1). The next step involves the conversion of phytoene into lycopene. In plants, apart from two desaturases, phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS), isomerases CrtISO and ZiSO are engaged at this stage, while the bacterial phytoene desaturase, CrtI, is capable of introducing four double bonds into phytoene to yield all-*trans*-lycopene (Cunningham and Gantt, 1998). Moreover,

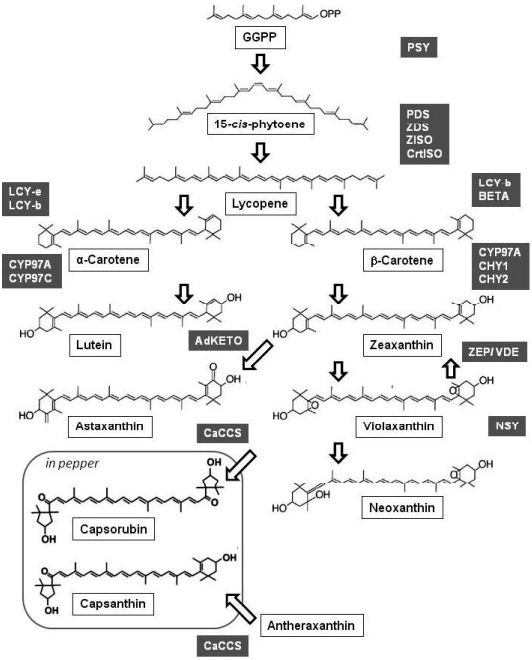

Fig. 1. The carotenoid biosynthetic pathway. Compound names are given in black, the plant enzymes are in white; AdKETO: ketolase; BETA: chromoplast-specific beta-cyclase; CaCCS: capsanthin/capsorubin synthase; CHY1, CHY2: non-heme carotene hydroxylases; CrtISO: carotene isomerase; CYP97A, CYP97C: cytochrome P450 carotene hydroxylases; GGPP: geranylgeranyl diphosphate; LCY-b: β-cyclase; LCY-e: ε-cyclase; NSY: neoxanthin synthase; PDS: phytoene desaturase; PSY: phytoene synthase; VDE: violaxanthin de-epoxidase; ZDS: z-carotene desaturase; ZEP: zeaxanthin epoxidase; ZISO: z-carotene isomerase. See text for details.

in photosynthetic tissues this step requires the presence of plastid terminal oxidase and plastoquinone (Beyer, 1989; Carol et al., 1999; Norris et al., 1995).

7.2.2. The branch-point in carotenoid biosynthesis

Following the formation of lycopene, the carotenoid biosynthesis pathway goes through two main branches (see Fig. 1) according to different cyclic end-groups. β-carotene and its derivatives zeaxanthin, violaxanthin, antheraxanthin and neoxanthin, which have two beta rings, form the β,β branch. The characteristic feature of the other branch, the β,ε branch, with α-carotene and its derivatives being representative, is the presence of one beta and one epsilon ring. The two cyclases, ε-cyclase (LCY-e) and β-cyclase (LCY-b), act either together on the two ends of the lycopene molecule, which results in α-carotene formation (Cunningham , 1998; Pogson et al., 1996) or only LCY-b alone is involved (β,β branch). The bacterial homolog of the latter is known as CrtY, while its paralog in tomato chromoplasts is referred to as BETA. Although the ε ring differs from the β ring only in the position of the double bond within the cyclohexane ring, carotenoids with two ε rings seldom occur in plant and algae (Goodwin, 1980). One

of the rare examples is lactucaxanthin, which is found in lettuce and is formed by a single ε-cyclase by adding two ε-rings to lycopene (Cunningham and Gantt, 2001).

7.2.3. Biosynthesis of xanthophylls

Xanthophylls, which comprise the majority of the carotenoid pigments in plant thylakoid membranes, are produced upon the hydroxylation of C3 atoms in each ring of α and β carotenes. Since the chirality of the hydroxyl on the β ring is opposite to that of the β ring of lutein, the involvement of enzymes of two different kinds is required. Both hydrocarbons can undergo hydroxylation which is conducted by non-heme (CHY1, CHY2) or cytochrome P450 (CYP97A, CYP97C) hydroxylases. In the case of α carotene, by the action of a β ring hydroxylase zeinoxathin is formed first, which is then converted to lutein by ε-ring hydroxylase. The hydroxylation of β-carotene is a two-step reaction with β-cryptoxanthin as an intermediate product. In green tissues β-xanthophylls are epoxidised and de-epoxidised by zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), respectively. These reactions are light-controlled and are known as the xanthophyll cycle. Under low light, violaxanthin is formed by the action of ZEP *via* the intermediate, antheraxanthin, whereas VDE is responsible for the accumulation of zeaxanthin in excessive light. Such interconversions play a key role in the regulation of the energy flow to the PSII reaction centers and therefore in photoprotection. The final step in the classical carotenoid biosynthetic pathway is the formation of neoxanthin from violaxanthin by neoxanthin synthase (NSY).

However, some plant species possess unique biosynthesis routes, which yield the production of exclusive carotenoids. Large amounts of the red ketocarotenoid astaxanthin widely found in algae but absent from most plant species, is synthesized in *Adonis aestivalis* petals (Cunningham and Gantt, 2005). The formation of astaxanthin requires the addition of carbonyl moieties at the C4 position of the β ring of zeaxanthin and is accomplished by the action of ketolase AdKETO, a CHY paralog. *A. aestivalis* is also known to produce other unusual ketocarotenoids including adonirubin, adonixanthin and 3-hydroxyechinenone (Cunningham and Gantt, 2005). A single bi-functional enzyme, capsanthin-capsorubin synthase (CCS), was shown to be responsible for converting antheraxanthin to capsanthin and violaxanthin to capsorubin – ketoxanthophylls, which accounts for the red color of pepper fruits (Bouvier et al., 1994). In tropical trees a lutein epoxidation -de-epoxidation cycle has been described (Matsubara et al., 2007).

7.3. Carotenoid cleavage

The carotenoids present in photosynthetic organisms and those taken up by animals by virtue of their diet undergo catabolic processes. One of the most important mechanisms that leads to carotenoid degradation is oxidation by non-specific enzymes, including lipoxygenases and peroxidases, or (photo)chemical oxidation. Carotenoid cleavage enzymes are referred to as carotenoid cleavage dioxygenases (CCDs), which are related to carotenoid dioxygenase or 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) (Walter and Strack, 2011). Although the particular mechanisms involved are still poorly understood (Carail and Caris-Veyrat, 2006), the CCD gene family is responsible for the formation of a number of terpenoid products with defined structures in diverse organisms, collectively known as apocarotenoids (Auldridge et al., 2006; Bouvier et al., 2005; Giuliano et al., 2003). An excellent source of more detailed information on enzymatic cleavage products and their biogenesis are reviews published recently (Ohmiya, 2009; von Lintig, 2010; Walter, 2010).

7.3.1. Apocarotenoid phytohormones

Reviewed in (Nambara and Marion-Poll, 2005) the reader can find the pathway for the formation of the plant hormone ABA. It involves NCEDs which cleave 9-*cis*-neoxanthin or 9-*cis*-violaxanthin to form xanthoxin, which is further modified to produce ABA. It is worth mentioning, that NCEDs are unique among the CCDs in that they accept only *cis*-isomers of their substrates and the isomerization step of all-*trans* to the *cis* forms of NCED substrate is one of the few steps in ABA synthesis and catabolism which are still elusive. The current focus in ABA research is on its perception and signaling (Raghavendra et al., 2010) as well as its new roles in plant disease resistance (Wasilewska et al., 2008).

In recent years, a body of evidence has been accumulated for the requirement of other carotenoid-derived signaling compounds to regulate some aspects of plant development, in particular apical dominance and branching (Beveridge et al., 2003; McSteen and Leyser, 2005). Interestingly, the inspiration for the chemical nature of the novel apocarotenoid hormone controlling plant shoot branching came from a study on the biosynthetic pathway of strigolactones. The latter, since the nineteen sixties, have been known as signals expelled from plant roots into the rhizosphere and used by parasitic weeds to detect the presence of host plants and initiate germination of their seeds (Bouwmeester et al., 2003; Humphrey et al., 2006). Strigolactones are derived from the carotenoid pathway in the same way as ABA, and are assumed to diverge at β-carotene (Matusova et al., 2005). To date, 15

different members have been structurally characterized, but even more have been tentatively identified (Xie et al., 2010). Their function, as allelochemicals in symbiosis with arbuscular mycorrhizal fungi, was discovered only recently (Akiyama et al., 2005). Even later, strigolactones were found to play a key role in shoot branching inhibition and to exert effects on different developmental processes including root development, seed germination, hypocotyl elongation, and secondary growth. Various aspects of strigolactone functions and biosynthesis can be found in (Cheng et al., 2013) and in a special issue of Plant Cell Physiology (Yamaguchi and Kyozuka, 2010).

7.3.2. Plant apocarotenoid pigments

It is well known that the pigment character of apocarotenoids is determined by a system of conjugated double bonds of sufficient length. However, with the exception of yellow-orange azafrin, which occurs abundantly in the roots of *Escobedia scabrifolia*, a plant endemic to tropical America (Eschenmoser et al., 1982), no other monocyclic C_{27} apocarotenoids have been used as food colorants on a larger scale. Instead, two diapocarotenoids, bixin (Bouvier et al., 2003; Siva et al., 2010) and crocin (Bouvier et al., 2003), which result from the symmetrical cleavage of the 5-6/5'6' or the 7-8/7'-8' double bonds, are of high commercial value.

7.3.3. Scent and aroma compounds

Apart from aromatic constituents such as esters, terpenes and pyrazines, carotenoid biodegradation products comprising 9-13 carbon atoms have been found to be important components of various scent and aroma compounds (see Walter and Strack, 2011 for more details). Among them, C_{13} apocarotenoids including β-ionone, α-ionone, β-damascenone, damascene are predominant with their extremely low odor thresholds.

7.3.4. Vitamin A

Carotenoids with an unmodified β-ionone ring, such as β-carotene, have provitamin A activity and can be utilized by animal carotene dioxygenase to produce retinaldehyde (vitamin A). This C_{20} carotenoid cleavage product is of great importance in mammalian physiology. Retinal and retinoic acid derivatives serve as visual pigments or signaling compounds in development, respectively. Although the key reaction in vitamin A and retinoid formation was already known to be in the 15-15' cleavage of β-carotene and related provitamin A compounds, rapid progress in the understanding vitamin A synthesis began in 2000 (von Lintig et al., 2005) when a β-carotene 15,15'-dioxygenase was cloned from *Drosophila melanogaster* (von Lintig and Vogt, 2000)

and chicken (Wyss et al., 2000) based on similarity to plant CCDs. However, vitamin A deficiency remains a significant global health problem (Sommer and Davidson, 2002; World Health Organization, 2003).

7.4. Regulation of carotenoid metabolism

In general, the composition and relative abundance of various carotenoids in green tissues is relatively conserved among most plant species. Conversely, carotenoid species and amounts in non-green tissues, such as flowers, fruits and seeds vary broadly. Moreover, they are influenced by many factors, including the developmental stage, environment, stress as well as their combination (Howitt and Pogson, 2006). The steady-state levels of carotenoids are determined by a complex interplay of the biosynthesis rate, cell storage capacity and the rate of catabolism and degradation. This makes the study of carotenoid regulation extremely demanding.

7.4.1. Transcriptional regulation of genes engaged in carotenoid biosynthesis

The predominant factor of carotenoid biosynthesis is the control of the transcription of genes coding the enzymes involved in this process. At this first level of regulation of carotenoid accumulation in plants, gene expression is regulated by such factors as developmental cues, light signaling, the circadian clock, ABA-mediated feedback and epigenetic mechanism.

One of the best studied systems for the regulation of carotenoid biosynthesis is that of tomato and pepper fruits and flowers where the accumulation of specific carotenoids has been shown to coincide with increased expression of upstream carotenogenic genes and reduced expression of downstream genes (Hirschberg, 2001).

As a consequence of the structural role carotenoids play in photosynthetic systems, the regulation of their biosynthesis in the green tissues of plants must be correlated with the other cellular processes that account for the assembly of the photosynthetic apparatus. However, apart from certain carotenoid biosynthetic genes, including PSY, which is regulated by light through a phytochrome-mediated process (von Lintig et al.,1997; Woitsch and Romer, 2003), the transcript levels of carotenoid genes collectively are not light dependent (Fraser and Bramley, 2004).

The accumulation of several groups of MEP-derived plastidial isoprenoids, including the hormones gibberelins and carotenoid-derived ABA, is governed by diurnal oscillation (Aharoni et al., 2003; Barta and Loreto, 2006; Dudareva et al., 2005; Hedden and Kamiya, 1997).

These data suggest that the production of carotenoids in plants is controlled by the circadian clock enabling better protection against oxidative damage. However, this has still to be proved experimentally.

In organs that are not directly dependent on light signals, e.g. roots, a positive correlation has been observed between PSY expression and carotenoid levels (Maass et al., 2009). The high proportion of β,β-xanthophylls they accumulate might serve as a reservoir for ABA precursors, although insufficient in the case of drought or saline stress conditions (Arango et al., 2010; Li et al., 2008a, 2008b; Welsch et al., 2008). Thus, upregulation mediated by ABA may confirm the feedback regulatory mechanism by which PSY expression is regulated in the root in response to abiotic stress.

Recently, it was found that the expression of the gene encoding the *Arabidopsis* CRTISO1 isoform appears to be specifically regulated by CCR1/SDG8, a chromatin-modifying histone methyltransferase enzyme (Cazzonelli et al., 2009) indicating the epigenetic mechanism that governs the transcriptional regulation of biosynthetic genes involved in carotenoid metabolism.

7.4.2. Post-transcriptional regulation of carotenoid accumulation

To explain the poor correlation between gene expression patterns and the carotenoid accumulation found in many cases, a number of post-transcriptional regulatory mechanisms have been postulated. They include the modulation of enzyme levels and activities, metabolite channeling by multi-enzyme complexes, sequestration and storage capacity and carotenoid turnover.

In functional chloroplasts light-driven processes lead to non-enzymatic isomerisation and thus were shown to substitute, at least to some extent, the activities of the Z-ISO and CRTISO isomerases (Breitenbach and Sandmann, 2005; Isaacson et al., 2002; Li et al., 2007; Park et al., 2002; Sandmann, 2009). Moreover, the activity of carotenoid biosynthetic enzymes modulated by photosynthetic redox system are influenced by light. The MEP pathway enzymes DXR, HDS and HDR are the targets of thioredixin (Balmer et al., 2003; Lemaire et al., 2004), whereas the carotenoid desaturases PDS and ZDS use plastoquinone as a hydrogen acceptor (Carol and Kunz, 2001). The latter, like CRTISO, LCYB, LCYE and ZEP, contain a conserved FAD-binding motif that suggest the involvement of the redox balance in the corresponding enzymatic reactions (Buch et al., 1995; Hugueney et al., 1992; Isaacson et al., 2004; Marin et al., 1996; Mialoundama et al., 2010; Schnurr et al., 1996; Yu et al., 2010, 2011). However, the most evident proof of light-driven regulation is observed in the case of ZEP and VDE playing a key role in the xanthophyll cycle aimed at photoprotection and discussed in detail in Chapter 12.

In non-photosynthetic tissues, the main limiting factor for carotenoid biosynthesis is PSY activity (Maass et al., 2009). This enzyme also illustrates by example the necessity of membrane association for carotenoid biosynthesis. This can be accomplished *via* membrane attachment and spanning domains, or by interactions with the protein harboring those domains that might be responsible for anchoring the whole complex. A hypothetical model for multi-enzyme complexes involved in carotenoid biosynthesis in chloroplast is illustrated in (Ruiz-Sola and Rodriguez-Concepcion, 2012).

In plants, *de novo* synthesis of carotenoids takes place in almost all types of plastids. While in chloroplasts they are located in photosynthetic membranes and integrated with chlorophyll-binding proteins to form pigment-protein complexes, in chromoplast carotenoids associate with polar lipids and proteins in carotenoid-lipoprotein sequestering substructures. It helps to retain a high level of carotenoids and also prevents negative feedback to the biosynthetic pathway by the end products. Importantly, expression of the genes encoding carotenoid-associated proteins is well associated with chromoplast development and carotenoid accumulation (Leitner-Dagan et al., 2006; Simkin et al., 2007).

The mechanism responsible for controlling carotenoid degradation and turnover is still a subject of extensive studies because some apocarotenoids such as the hormones ABA, strigolactones and others still awaiting identification, are well known to play essential regulatory functions as described in (Nambara and Marion-Poll, 2005; Van Norman and Sieburth, 2007; Xie et al., 2010).

7.5. Concluding remarks

Since the attractive colors of many fruits, vegetables and flowers are due to the presence of carotenoids, the economic value of a crop is highly dependent on these isoprenoid compounds. Moreover, they are essential as feed and food additives as well as of great importance for the cosmetic and pharmaceutical industries. However, it is mainly due to their health-related properties that the manipulation of carotenoid biosynthesis in plants has contributed to a carotenoid-enriched diet with improved health benefits. The main aspects of carotenoid biotechnology are reviewed elsewhere (Botella-Pavia and Rodriguez-Concepcion, 2006; Fraser and Bramley, 2004; Giuliano et al., 2008; Lee and Schmidt-Dannert, 2002; Sandmann, 2001; Sandmann et al., 2006).

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Chapter 8. CHLOROPLAST CHLOROPHYLL-PROTEIN COMPLEXES AND CHLOROPHYLL FLUORESCENCE IN WHEAT SEEDLINGS DURING DE-ETIOLATION

Elena Garmash[1], Mikhail Khristin[2], Olga Dymova[1], and Tamara Golovko[1]
[1] Institute of Biology, Russian Academy of Sciences, Syktyvkar, Russia,
[2] Institute of Basic Biological Problems, Russian Academy of Sciences, Pushchino, Moscow, Russia

Contents

8.1. Introduction

The greening process in a plant involves light-induced biogenesis of plastids and maturation of thylakoid membranes in chloroplasts. This process is characterized by chlorophyll formation (protochlorophyllide-chlorophyllide transformation) which is catalyzed by light-dependent protochlorophyllide reductase (LPOR; EC 1.3.1.33). In seedlings grown in the dark, proplastids develop into etioplasts and contain prolamellar bodies (PLBs) which rapidly transform into chloroplasts with thylakoids in the light (summarized in Solymosi and Schoefs, 2010). This process is called de-etiolation. The development of functional chloroplasts is accompanied by the synthesis and assembly of two photosystems – I and II – the key components of the light reactions of photosynthesis. During de-etiolation, thylakoid membranes undergo considerable elaboration and structural reorganization as grana are formed. Lipid and carotenoid composition of thylakoids also changes (Vothknecht

and Weshoff, 2001). In etioplasts and etio-chloroplasts, carotenoids have been found to be essential for stabilizing the fold structure of antennae apoproteins and reconstructing pigmented light-harvesting complex of PSII (LHCII) (Kuhlbrandt et al., 1994; Gastaldelli et al., 2003; Woitsch and Romer, 2003 etc.). Carotenoids are involved in light harvesting and protection against excessive light energy (Sieferman-Harms, 1985; Havaux, 1998; Niyogi, 1999; Latowski et al., 2004). In addition, the presence of the xanthophyll cycle (VXC) enhances plant photoprotection (Niyogi et al., 1998). In mature green tissues of higher plants, VXC pigments participate in non-photochemical quenching, a mechanism in which violaxanthin (Vx) is de-epoxidised to zeaxanthin (Zx) and excess energy is dissipated into heat (Demmig-Adams, 1990; Gastaldelli et al., 2003; Latowski et al., 2004).

There is a lot of information about the process of chloroplasts maturation and assembly of photosystems during greening. However, the rate of biogenesis of PSI and PSII as well as chlorophyll synthesis can be different among species and depends on light conditions. In addition, the role of carotenoids, in particular, those of VXC pigments in protection of developing photosynthetic apparatus against toxic effects of light is not completely understood yet.

This article briefly reviews the known information about the structure, assembly, and functions of PSII and PSI in autotrophic plant cells. Based on our own experimental data the chronology of photosystems formation during de-etiolation of the first wheat leaf is described. We have also studied developmental changes in non-photochemical quenching of chlorophyll fluorescence and its relationship with the activity of VXC of the greening leaf. We suppose that the energy dissipation processes during de-etiolation can intensify to protect the developing photosynthetic machinery against light-induced oxidative stress.

8.2. Photosystems I and II

8.2.1. Localization

In green mature tissues chlorophyll is embedded in special units called photosystems. There are two photosystems involved in the light reaction, photosystem I and II (PSI and PSII). Each photosystem is a pigment-protein complex located in the thylakoid membrane. It contains 200-400 pigment molecules, and is composed of two linked components: the antenna complex and the reaction centre (reviewed in Erickson, 1998; Raven et al., 2003). Two Chl *a* molecules of the PSI reaction centre are known as P700 (700 is the wavelength of the absorbance peak for the pigment). Two Chl *a* molecules of the PSII reaction centre form P680 with the absorbance maximum at this wavelength (Nelson

and Yocum, 2006). PSI localizes in stroma lamellae and stroma-exposed grana surface. PSII is found in the grana. PSII is the first protein complex in the light-dependent reactions and is defined as a water-plastoquinone oxidoreductase. PSI is the second photosystem (it is named 'I' since it was discovered before photosystem II) in the photosynthetic light reactions and acts as plastocyanin-ferredoxin oxidoreductase (Nelson and Ben-Shem, 2004). In plants, PSI, PSII, cytochrome (Cyt) b_6f complex and F-ATPase are the four main complexes of the light reaction. Plastocyanin (PC), Fd, and FNR are proteins that assemble with the photosystems to perform the redox reactions (Nelson and Yocum, 2006).

8.2.2. Light reactions

In the light, the exited Chl *a* enables a charge separation and electron flow in consequence. The remaining $P680^+$ is then reduced with an electron deriving from water molecules leading to their splitting after P680 has been excited four times. This step creates oxygen and hydrogen and starts the buildup of proton motive force in the thylakoid lumen (reviewed in Raven et al., 2003). Then the electron passes an electron transport chain that links PSII with PSI via the mobile carrier plastoquinone (PQ) and the Cyt b_6f complex and a plastocyanin (PC). The Cyt b_6f is the second proton pump in the electron chain and amplifying the level of protons in the thylakoid lumen relative to the stroma. Cyt b_6f contributes to the proton motive force in a mechanism known as the Q-cycle. In PSI the light is captured in the antenna proteins (LHCI) and funneled to the Chl *a* in the reaction centre (P700). Then the electron from P700 passes several carriers and finally reduces Fd on the stroma side of the PSI and then can be captured by NADP reductase which produces NADPH of the $NADP^+$ (reviewed in Nelson and Ben-Shem, 2004).

The PSI can also work independently of photosystem II in a reaction called cyclic electron flow. This reaction enables ATP synthesis; however, no NADPH is produced. Thus, the photosystems together form a unique photosynthetic chain which is able to extract electrons from water, to reduce $NADP^+$ and to generate ATP, creating oxygen as a byproduct. The ATP and the NADPH molecules made in the light reactions fuel the carbon fixation (the dark reactions). These reactions fix carbon dioxide from the air (in terrestrial plants) and enable sugar synthesis. The net product of photosynthesis is glucose ($C_6H_{12}O_6$) but the primary product of carbon fixation is either a three or a four carbon molecule in the C_3 and C_4 plants respectively.

8.2.3. The composition of PSI and PSII

At least 16 chloroplast- and 11 nucleus-encoded polypeptide subunits have been identified as the main constituents of the chloroplast PSII (Erickson, 1998). In green plants, the native form of PSII is surrounded by the light-harvesting complex (LHCII complex) and thus it is called the PSII–LHCII supercomplex (reviewed in Minagawa and Takahashi, 2004). The central position in this complex is occupied by the reaction center (RC), which is composed of a D1/D2 heterodimer and a few intrinsic low molecular weight polypeptides. RC is surrounded by other subunits to form the 'core' complex, which includes the core antenna proteins, CP43 and CP47, and several additional small polypeptides. The PSII–LHCII supercomplex contains PSII core complex and minor and major antennae.

PSI contains 14 subunits; 5 proteins are chloroplast encoded, while the rest is nuclear encoded (Wollmann et al., 1999; Schwabe and Kruip, 2000; Shen et al., 2002; Fromme and Mathis, 2004). About 100 Chl *a*, 20 carotenoids, 2 phylloquinone molecules and three 4Fe-4S iron-sulphur clusters are coordinated with the protein subunits. The functional 'heart' of the PSI is heterodimer PsaA/PsaB. Additionally, PsaC protein binds two [4Fe-4S] clusters which serve as a terminal electron acceptor to allow for stable charge separation within PSI.

Development of etioplasts into chloroplasts is accompanied by the biogenesis and organization of the thylakoid membrane, including the synthesis and assembly of photosystems components. This transformation consists of two processes: the expression of genetic information and the assembly of the gene products to form the functional structure of photochemical systems. Some components of the thylakoid electron transport system (Cyt b_{559}, b_{563}, and *f*) and PC are already present in etioplasts, other components are formed after illumination (summarized in Ohashi et al., 1989).

8.2.4. The assembly of PSI and PSII

The structure and composition of PSI and PSII have been studied extensively, but less is known about the assembly and especially about the chronology for the assembly components buildup into functional chlorophyll-binding protein complexes.

Because of the structural complexity of PSII, its assembly is likely to be a multi-step process assisted by molecular chaperones or other factors (Baena-Gonzalez and Aro, 2002; Erickson, 1998; Hippler et al., 2002). A model for the assembly of the PSII–LHCII supercomplex was proposed by Minagawa and Takahashi (2004). In short, the proposed assembly steps include formation of a precomplex consisting of Cyt b_{559} and D2, integration of D1 into the precomplex to form RC, integration of different core antenna proteins to form sub-complexes and

PSII core complex, binding extrinsic polypeptides on the lumenal side of PSII core complex, dimerization of PSII core complex, synthesis of major and minor antenna complexes independently of the assembly of PSII core complex, and association of PSII core complex dimer and outer antenna to form PSII–LHCII supercomplex.

The sequence of the subunit assembly to yield functional PSI includes folding of the core consisting of heterodimers PsaA and PsaB, on which the other subunits are assembled (Schwabe and Kruip, 2000; Shen et al., 2002). The core binds P700, Chl *a*, and phylloquinone and 4Fe-4S iron-sulphur cluster. Then PsaC protein becomes assembled into PSI. In case of higher plants, only monomeric PSI can be found.

8.2.5. The chronology for the assembly of PSI and PSII

Upon illumination of dark-grown seedlings, etioplasts differentiate into photosynthetically active chloroplasts within few hours. Parts of PSI and PSII are already visible after one hour of illumination of an etiolated plant (Dreyfuss and Thornber, 1994). The induction of PSI activity precedes the activity of PSII. However, the activity of PSI is linked to that of PSII, hence NADP production doesn't start earlier than four hours after the start of illumination when PSI is able to reduce Fd. The electron transport chain is complete after four hours (Ohashi et al., 1989). However, the full assembly of the PSII–LHCII supercomplex begins much later – after 12-18 h exposure to continuous light, when trimeric LHCIIb complex formation (from the previously made monomeric LHCIIb complexes) is nearly complete (Dreyfuss and Thornber, 1994).

8.3. The role of thermal dissipation and violaxanthin cycle (VXC) in protection of chlorophyll against photooxidative damage

At present it is clear that under non-limiting light conditions plants gain far more energy than they can utilize for photosynthetic CO_2 fixation (Wilhelm and Selmar, 2011). Moreover, light stress is thought to be a normal condition for photosynthesis in plants. Excessive light energy induces generation of reactive oxygen species (ROS). Most of the ROS produced originate from chloroplasts, especially in the light (Foyer and Noctor, 2000). ROS can act as signaling molecules, especially in response to various stress factors (Gechev et al., 2006). On the other hand, surplus of ROS is harmful because of their detrimental effect on all important macromolecules: proteins, pigments, lipids, and nucleotides. The excess of ROS may affect cell structures due to their free diffusion through the cellular compartments (Sweetlove et al., 2002) including chloroplast envelope membranes (Mubarakshina et al., 2010). Plants developed protection and repair mechanisms to

prevent photo-oxidative damage and to maintain the physiological state of cell metabolism. Among them, energy-dissipating systems in chloroplasts play a crucial role in defense against excessive light energy.

In chloroplasts, thermal dissipation of light energy prevents triplet chlorophyll formation. It is known that under excessive light, up to 90% of the absorbed light can be dissipated via the nonphotochemical quenching pathway (NPQ) within the PSII–LHCII (Horton et al., 1994, Demmig-Adams and Adams III, 1996; Ruban et al., 2001, 2007). Several processes can contribute to non-photochemical quenching, but the major fraction of NPQ is dependent on the energization of the thylakoid ΔpH formed under the influence of illumination and is called qE (Quick and Stitt, 1989; Krause and Weis, 1991; Horton et al., 1994). qE is also related to the functioning of the xanthophyll cycle (VXC) (Demmig-Adams, 1990; Horton et al., 1994; Niyogi et al., 1998; Gilmore, 1997; Latowski et al., 2004; Nowicka et al., 2009). VXC is the light-dependent interconversion of xanthophyll pigments: violaxanthin (Vx), antheraxanthin (Ax), and zeaxanthin (Zx). Two enzymes, violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZE), are engaged in the xanthophyll cycle (Latowski et al., 2004). Localized in thylakoid lumen and activated by acidic pH, VDE catalyses de-epoxidation of Vx to Zx. It was suggested that the process of making LHCII-bound Vx available for enzymatic de-epoxidation depends not only on acidification but also on light intensity (Gruszecki et al., 2009). The mechanism of qE is still under debate. Zx is thought to bind with PsbS, a subunit of PS II, accepting excitation energy from chlorophyll and yielding massive energy dissipation (Szabo et al., 2005). It is not excluded that xanthophyll dependence of qE is due to other pigment-binding proteins, probably of the Lhcb type (chlorophyll *a*/*b*-binding proteins) (Bonente et al., 2008).

8.4. Biogenesis of photosystems I and II and developmental changes in energy dissipation processes during conversion of etioplasts to chloroplasts

The developmental changes of photosynthetic machinery including chronology of photosystems formation, and thermal dissipation processes associated with the activity of developing chloroplasts in etiolated wheat (*Triticum aestivum* L., var. Irgina) seedlings during greening for 48 h under continuous light conditions at the level of illumination 190 μmol (photon) m^{-2} s^{-1} were studied (Garmash et al., 2013). All measurements were carried out on the first leaf blade, the top segment (1.5 cm) of which was removed and the next 2-3-cm-long segment was taken for the experiment. Etioplasts of 5-day-dark-grown leaves contained regular PLBs. After 1 h of illumination, plastids

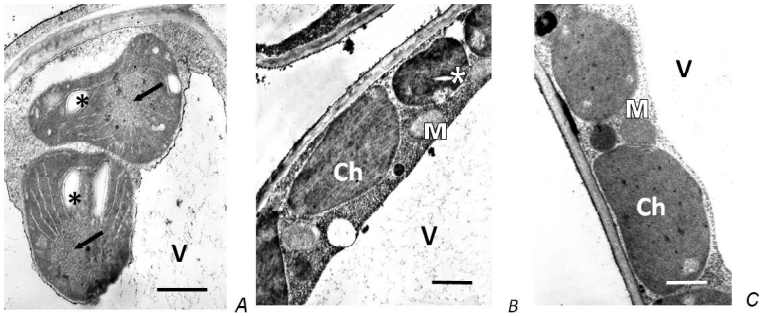

Fig. 1. Electron micrographs of mesophyll cells transverse section after illumination for 1 h (*A*), 6 h (*B*), 24 h (*C*). Ch – chloroplast, M – mitochondrion, V – vacuole; * – starch grain. The arrows indicate prolamellar body. Bars – 1 μm. *Source*: Garmash et al. 2013.

contained few thylakoid membranes which protruded from PLBs (Fig. 1*A*). After 6 h of greening PLBs were absent and plastid membranes were completely reorganized into thylakoids (Fig. 1B). Chloroplasts still contained starch grains. By the end of the 1st day of illumination, chloroplasts had well-developed grana (Fig. 1C) consisting of 6.1 ± 1.7 thylakoids.

During the greening process the amount of chlorophylls, mainly Chl *a*, increased significantly (Fig. 2). After 48 h of greening, the concentration of chlorophylls and carotenoids increased by 30 and 7 times, respectively. The relative amount of carotenoids in total pigment pool decreased during the greening process.

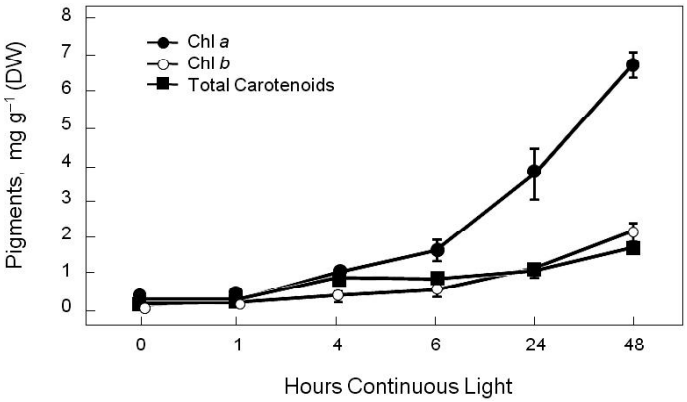

Fig. 2. Pigment content in wheat leaves during greening. Data are presented as mean values ± SE (n = 4–5) taken during three independent experiments.

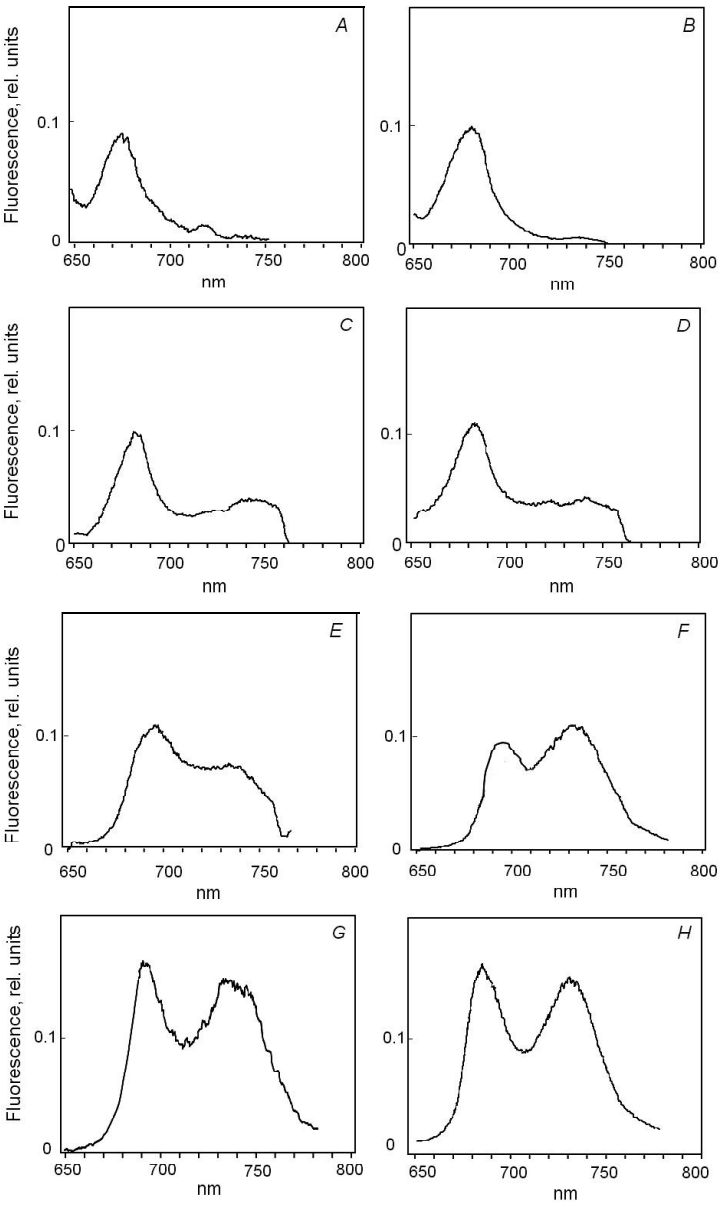

Fig. 3. Low temperature (77°K) fluorescence emission spectra of greening wheat leaf after illumination for 1 min (*A*), 5 min (*B*), 1 h (*C*), 2 h (*D*), 4 h (*E*), 6 h (*F*), 24 h (*G*), and 48 h (*H*).

Low temperature (77K) fluorescence emission spectra of the greening leaf revealed the chronology of Chl biosynthesis and Chl integration into pigment-protein complexes. During the first minutes of illumination the emission band from 655 to 710 nm with the maximum of 675 nm was detected (Fig. 3 A, B). This band characterized the presence of Chl precursors. After 1 h of greening the shift of fluorescence with the maximum of 680 nm indicated integration of Chl in developing pigment-protein complexes of PSI and PSII (Fig. 3 C, D).

The structural and functional state of LHCII complex changed during the whole period of de-etiolation. The intensity of the emission peak at 682 nm reflecting the presence of LHCII increased during de-etiolation (Fig. 3 E) and reached its maximum after 6 h of illumination (Fig. 3 F). The presence of the marked emission peaks of PSI and PSII was detected by the end of one-day period of light exposure (Fig. 3 G, H). The fluorescence maxima of 685 and 695 nm characterize developed pigment-protein complexes of PSII, while the maximum of 730 nm indicates the complete assembly of PSI. According to our data, the accumulation of Chl and biogenesis of both photosystems in wheat leaf during greening requires more time than it was earlier revealed, in particular, for etiolated barley seedlings exposed to light (Shlyk et al., 1984; Dreyfuss and Thornber, 1994). Thus, according to the data from electron microscopy and 77K fluorescence emission spectra, thylakoid membranes and light-harvesting complexes were formed by the end of the 6 h period of light exposure, but PSI and PSII were completely developed only after 24 h of de-etiolation. Wheat seedlings were exposed to a relatively mild level of illumination. This level of illumination (190 μmol (photon) m^{-2} s^{-1}) is in the curvature region (the transition from the light-limited phase to the light-saturated plateau) of the light-response curve of the net CO_2 exchange rate of the first mature wheat leaf (Garmash et al., 2013). The curvature region corresponds to the typical light intensities of plant habitat (Tooming, 1984, Garmash and Golovko, 1997). However, during the initial few hours of greening the etiolated leaves were probably forced to with-

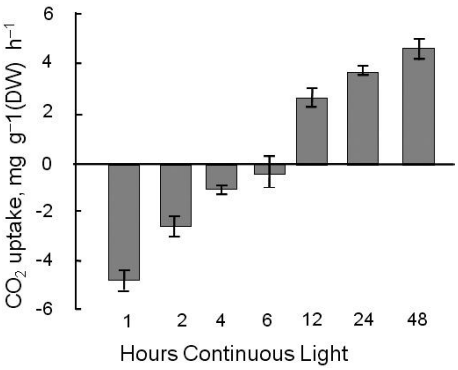

Fig. 4. Net CO_2 exchange (net photosynthetic rate) in wheat leaves during greening. Data are presented as mean values ± SE (n = 3 – 6) taken during three independent experiments. *Source*: Garmash et al. 2013.

stand an excess light energy and to defend the developing photosynthetic apparatus.

It was found that net-photosynthesis rate became positive only after 6 h of illumination (Fig. 4) when chloroplasts became more mature (Fig. 1 B) and the intensity of emission spectra of PSII and PSI reached no less than half of their maximal value (Fig. 3 F). We estimated the functionality of the photosynthetic machinery and the cell membrane state by measuring chlorophyll fluorescence and lipid peroxidation parameters (Table 1).

The first two hours of de-etiolation were characterized by low values of F_v/F_m, Φ_{PSII}, and coefficient of photochemical chlorophyll quenching (qP) in leaves. The low F_v/F_m was due to low values of F_o and F_m (data are not presented) that were the result of the incomplete light-harvesting complexes. Thereafter, a gradual increase of F_v/F_m and Φ_{PSII} was detected. By the end of the 24-h light period the F_v/F_m reached about 0.8, a normal value for a healthy mature leaf. These results agree with the common dynamics of photosynthetic apparatus development under continuous light. The light-triggered reduction of Pchlide to chlorophyllide (Chlide), as a prerequisite for chlorophyll synthesis, lasts for 4–6 h of illumination, when the concentration of LPOR as the most abundant protein associated to PLBs doesn't change dramatically (Reinbothe et al., 1999, Solymosi and Schoefs, 2010). According to our data, PLBs were already absent after 6 h of de-etiolation. But, the

Table 1

Parameters of chlorophyll fluorescence (F_v/F_m and Φ_{PSII}-maximum and actual photochemical efficiency of PSII, respectively; qN and qP-coefficients of non-photochemical and photochemical quenching, respectively), and level of lipid peroxidation presented as thiobarbituric acid-reactive-substances (TBARS) content in wheat leaves during greening

Hours continuous light	F_v/F_m	Φ_{PSII}	qP	qN	TBARS, nmol g^{-1} (FW)
1	0.45 ± 0.03	0.22 ± 0.02	0.78 ± 0.01	0.32 ± 0.01	15.0 ± 1.0
2	0.48 ± 0.02	0.33 ± 0.02	0.86 ± 0.01	0.38 ± 0.03	20.2 ± 0.9
4	0.64 ± 0.02	0.54 ± 0.01	0.90 ± 0.01	0.50 ± 0.04	19.5 ± 1.0
6	0.69 ± 0.01	0.58 ± 0.14	0.94 ± 0.01	0.56 ± 0.01	28.9 ± 2.2
12	0.74 ± 0.01	0.64 ± 0.16	0.93 ± 0.02	0.59 ± 0.01	32.4 ± 2.2
24	0.76 ± 0.01	0.67 ± 0.12	0.96 ± 0.01	0.40 ± 0.03	22.3 ± 2.0
48	0.80 ± 0.01	0.74 ± 0.01	0.94 ± 0.03	0.45 ± 0.04	23.0 ± 1.6

Data are presented as mean values ± SE (*n* for TBARS content = 3–6; *n* for F_v/F_m, Φ_{PSII}, qN, qP = 5–12) taken during three independent experiments.

Source: Garmash et al. 2013.

formation of fully functional photosynthetic machinery including build-up light-harvesting complexes and integration of PSI and PSII into the membrane requires longer illumination (more than 6 h, Fig. 3).

The changes of qN value reflecting the non-photochemical quenching of chlorophyll fluorescence and lipoperoxidation activity (LPA) during the greening process were similar (Table 1). They were low after 1 h of illumination, thereafter qN and LPA increased and were the highest after 12 h of greening, and then they decreased again. The low values of qN and LPA during the first few hours of de-etiolation confirm that etioplasts have their own effective defense against photooxidation. There is evidence that LPOR plays a vital role in avoiding ROS formation (Schoefs and Franck, 2003, Solymosi and Schoefs, 2010).

To get more insight into the characteristics of qE – the ΔpH-dependent fast inducible non-photochemical quenching, we have studied the qN induction and relaxation. Time courses for induction and relaxation of qN are presented and described in detail in our work (Garmash et al., 2013). It was shown that between the 1st and the 2nd h of greening, the qN parameter rose rather slowly and reached steady-state values in 200 s. During the next hours of greening the qN parameter increased faster. By 48 h, the leaves needed only 40 s to reach the steady state. The maximal qN steady-state level was observed between the 6th and 12th h of greening. After switching off the actinic light, qN decreased slowly during the first two hours of greening, but after 6 h of greening qN decreased faster.

The increase in qN is likely due to the activation of systems dissipating excess light energy in developed chloroplasts. We have studied the VXC activity along with the kinetics of qN induction and relaxation. It is known that the generation of qE requires a lumen pH below 6 and proceeds during 10 min of illumination at saturating light intensities (Quick and Stitt, 1989; Krause and Weis, 1991; Horton et al., 1994; Kalituho et al., 2007).

According to our experiments, etiolated leaves accumulated carotenoids (Fig. 2), the major part of which was represented by xanthophylls (Garmash et al., 2013). To examine how VXC operates during the greening process, we exposed dark-acclimated plants to high light (1200 μmol(photon) m^{-2} s^{-1}) and then again to darkness. HPLC separation of extracts from leaf samples taken at certain periods of time allowed us to observe changes in the level of VXC pigments. During the greening process of the dark-acclimated leaves, Vx increased from 60 to 94% of the total pool of xantophyll cycle pigments (VAZ); relative contents of Ax and Zx decreased from 31 to 3% and from 9 to 3% of VAZ, respectively (Fig. 5 A-C). After 1 h of greening Vx and Ax levels decreased during light exposure, and there was no epoxidation after placing the plants into darkness indicating disturbed VXC functioning.

After 2 h of illumination, a greater amount of Vx and Ax (25 and 18% respectively) was de-epoxidized, but there was no normal epoxidation – only Ax was used as a substrate for ZE. Normal operation of VXC was observed after 4 h in the presence of light. However, the amount of pools of converted xanthophylls was different. After 6 and 12 h of greening it was the highest, thereafter the pools of converted xanthophylls decreased.

Thus, on the first stage (1–2 h) of greening VXC didn't function normally (Fig. 5). Moreover, slow induction and dark relaxation of NPQ during this period of de-etiolation confirmed that energy dissipation was not connected with the operation of VXC. It is known that etiolated leaves contain a significant amount of xanthophylls and violaxanthin de-epoxidase but VXC doesn't protect Pchlide and Chlide molecules in etioplasts (Franck and Mathis, 1980; Ignatov et al., 1983; Pfundel and Strasser, 1988). The accumulation of xanthophylls has been found to

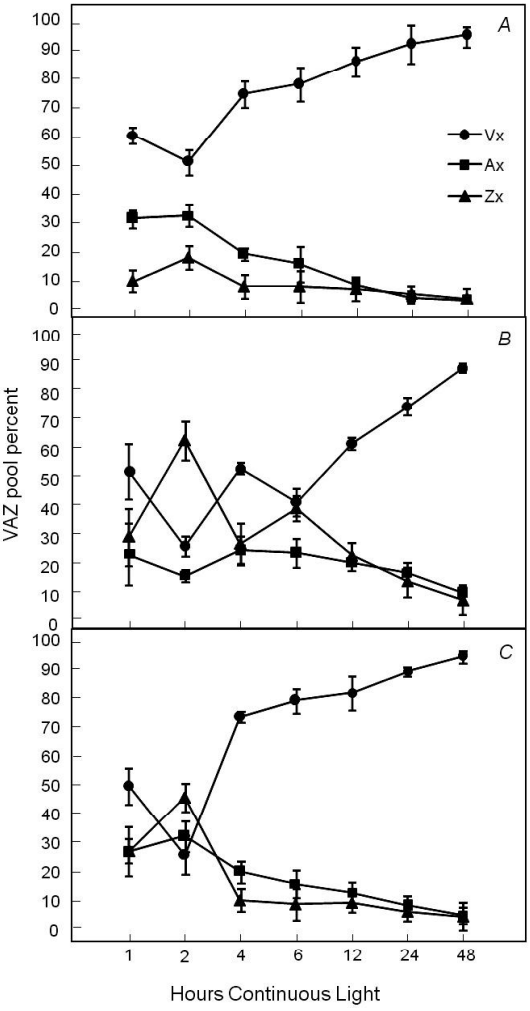

Fig. 5. Relative percentage of xanthophylls cycle pigments (VAZ) in wheat leaves during greening: *A* – control (after 1 h of darkness), *B* – after 30 min of light exposure (1200 μmol (photon) m^{-2} s^{-1}) and subsequent 1 h of darkness (*C*). Data are presented as mean values ± SE (n = 3) taken during three independent experiments. *Source*: Garmash et al. 2013.

be essential for PLBs formation (Park et al., 2002; Cuttris et al., 2007).

The highest induction and relaxation as well as maximal qN values were observed between the 6th and the 12th h of illumination when VXC functioned more effectively. During the next hours of the greening process, the induction of qN was fast but the maximal qN values were lower. It correlated with the decreasing level of Zx and Ax in dark-acclimated leaves and VXC activity in general. These results are supported by the literature data. It was shown that the contact between chlorophylls and carotenoids becames closer and, correspondingly, the efficiency of chlorophyll triplet state quenching by carotenoids increased during the later phase of cucumber cotyledons greening (after 3 h of continuous illumination) (Waloszek et al., 2002).

8.5. Conclusion

Overall, de-etiolation as light-stimulated chloroplasts development from etioplasts is a complicated and highly regulated process. We have found that thylakoid membranes and light-harvesting complexes were formed by the end of the 6-h period of continuous light exposure, but PSI and PSII were completely developed only after one day of de-etiolation. Dramatic physiological changes connected with intensification of protective cellular mechanisms against photodestruction in a greening wheat leaf occurred during the first 12 h of continuous illumination. During the first 2 h of greening, the level of lipoperoxidation activity was low, and etiolated leaves are thought to avoid ROS formation due to functioning of LPOR in PLBs as shown by Schoefs and Franck (2003). After 4 h of illumination the lipoperoxidation processes were intensified, and the developing photosynthetic machinery needed protection against irreversible damage caused by ROS. During this period the energy dissipation processes in chloroplasts increased significantly. It was found that the increase in VXC activity was accompanied by a fast rate of induction of non-photochemical quenching of chlorophyll fluorescence. The values of these parameters were at maximum during the later phase of de-etiolation, (between the 4th and 12th h of illumination), when most prolamellar bodies converted into thylakoids, and had the greatest activity until the photosystems were almost completely developed. With maturation of chloroplasts, the activity of the energy dissipation and lipoperoxidation processes decreased. Our results show that the induction of thermal dissipation processes in chloroplasts is highly regulated by the level of photosynthetic machinery development and light stress susceptibility of de-etiolating leaves.

Acknowledgments

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Part 3. PHYSIOLOGY OF PIGMENT-PROTEIN COMPLEXES

Chapter 9. CHLOROPHYLLS AND THEIR ROLE IN PHOTOSYNTHESIS

Olga Dymova[1], and Leszek Fiedor[2]
[1] Institute of Biology, Russian Academy of Sciences, Syktyvkar, Russia
[2] Jagiellonian University, Krakow, Poland

Contents

9.1. Introduction

Chlorophylls (Chls) are among the compounds most important to life on the Earth because of their crucial role in photosynthesis. It is not only the primary source of energy to almost all forms of life on the planet but also the source molecular oxygen, which also contributed to its geology on global scale by creating oxidative atmosphere. Chls are widely distributed in biosphere as the green pigments responsible for the coloration of plants, at the same time giving the men deeply aesthetic impressions. In fact, the seasonal changes in chlorophyll (Chl) content in flora are the only biological process visible from space. What is perhaps even more amazing is the versatility of (bacterio)chlorophylls (B)Chls in photosynthetic apparatus where they function as antenna pigments, active in the photon capture, excitation energy storage and transfer, as well as both the electron donors and acceptors in photosynthetic reaction centers (RCs). On top of that, in practically all photosynthetic pigment-proteins they play a key structural role. These

features render (B)Chls indispensible for photosynthesis and for all these reasons, for over a century, these fascinating pigments are a subject of intensive investigations in many laboratories all over the world (Vernon and Seely, 1966; Scheer, 1991; Grimm et al., 2006).

The term «chlorophylls» was first used by Pelletier and Caventou in 1818 to describe the green pigments that are involved in photosynthesis in higher plants. The strong absorption of light by Chl in the red part of the spectrum was first determined and the role of this pigment in photosynthesis was suggested by Timiriazeff as early as in 1877 while the chromatographic separation of Chls from plant extracts was first achieved by Tswett at the beginning of the XX century. Fundamental investigations on the role of Chls in plants was also done by Lubimenko (1916). He determined Chl content of 623 plant species from the tropics, subtropics and temperate zones, and developed the idea of shade and sun plants. Later other authors continued to study Chl content of plants from different botanic-geographical zones (Seybold and Egle, 1940; Montfort, 1950; Tieszen, 1970; Luk'yanova et al., 1986; Gerasimenko et al., 1988; Kornyushenko and Soloveova, 1992; Maslova and Popova, 1993). The mechanisms of functioning of (B)Chls in photosynthesis are intensively investigated since the very moment of the discovery of their crucial involvement in this multi-step complicated process. The chemical characterization of Chls dates back to the identification of Chls to be porphyrins and then as the Mg-complexes by Schunck and Marchlewski (Schunck and Marchlewski, 1894), and to the works of Willstatter and Stoll, who were the first to show that Chls are the major photoactive components of plant photosynthetic apparatus (Willstatter and Stoll, 1913). For this discovery and other work on Chl (bio)chemistry, the two researchers were awarded the Nobel Prize in 1915. The correct chemical formula of Chl *a* was proposed by Hans Fischer in Munich. Also the total synthesis of Chl had been attempted by chemists. It was done by Woodward and his coworkers in Manchester in 1960, who for this collective achievement received the Nobel Prize in 1964.

9.2. Major characteristics of chlorophylls

The (B)Chls belong to cyclic tetrapyrroles (Fig. 1) and hence are structurally related to the porphyrins, showing similarity to another pigment, heme, found in hemoglobin, except that in heme the central atom is iron, whereas in Chls it is Mg^{2+} ion. The common evolutionary origin of these two tetrapyrrolic pigments is supported by the fact that they share their biosynthetic pathway up to the point of insertion of the central metal ion by the respective chelatase. On further stages, an isocyclic ring V is added to the (B)Chl macrocycle by the side chain

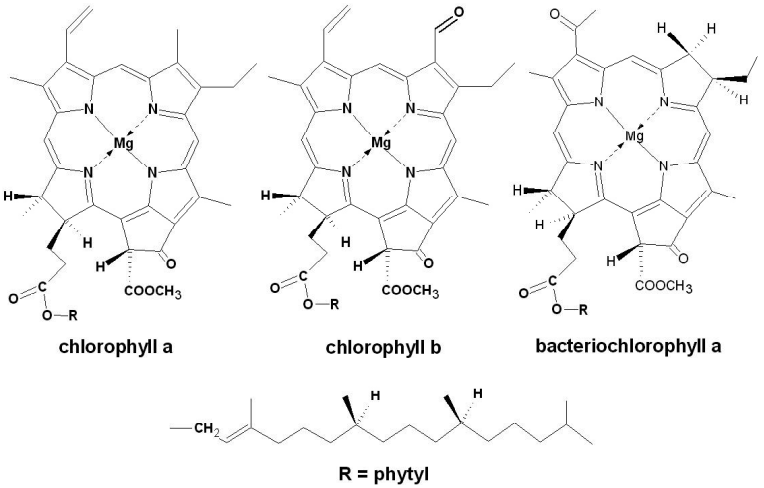

Fig. 1. Structural formulae of chlorophyll *a*, chlorophyll *b*, bacteriochlorophyll *a* and their side chain phytyl.

cyclization and one or two pyrrole rings undergo reduction, forming chlorin (in Chls) or bacteriochlorin (in BChls), respectively.

In higher plants there are two types of Chls, named Chl *a* and Chl *b*, both contain three centers of chirality (Fig. 1). Cyanobacteria contain only Chl *a*. The chemical formula of the former pigment is $C_{55}H_{72}N_4O_5Mg$ and Chl *b* differs from Chl *a* only at the C-7 position, where the formyl replaces the methyl group. Chl *b* is synthesized by enzymatic oxygenation of Chl *a* and the oxygen in the formyl group is derivated from molecular oxygen. Among the bacteriochlorin derivates, BChl *a* is the most common pigment, present as the main photosynthetic pigment in purple photosynthetic bacteria. As a result of the reduction of ring II, its molecule contains additional two chiral centers.

Electronic structure. The versatility of (B)Chls stems from the properties of their electronic structure. In principle, their electronic structure is similar to that of porphyrins whose main feature is the system of delocalized π-electrons, due to the presence of many interchanging C-C and C=C bonds. The properties of this π-electron system can be quite well explained within the so called four-orbital model developed by Gouterman (Gouterman et al., 1972). The electronic properties (electronic transitions, redox potentials, etc.) of these aromatic molecules depend on the movements of electrons between the HOMO and LUMO levels, each composed of two orbitals of almost the same energy in porphyrins (quasi-degeneration) and of differing energies in (B)Chls. In more symmetric porphyrins, the transitions between the

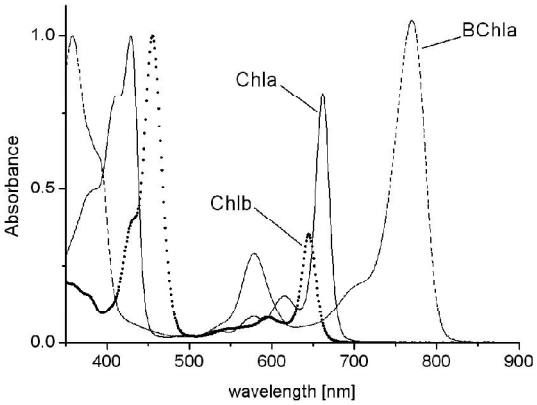

Fig. 2. Absorption spectra of isolated chlorophyll *a*, chlorophyll *b* and bacteriochlorophyll *a* in acetone. The spectra of these pigments in their native environment of proteins are different due to various intramolecular interactions.

HOMO and LUMO are either additive or cancel each other, reflected in their absorption spectra (Fig. 2) as a very intense high-energy band (Soret) and a series of weak bands at lower energies (Q bands). Due to the change of molecular symmetry in chlorins and bacteriochlorins, the electronic levels in (B)Chls become separate and give rise to characteristic shape of absorption spectra of these pigments, which result from one and two electron promotions between the split levels. What is important from the point of view of photosynthetic functions of these pigments, it is the fact that their delocalized π-electron system, and thus their electronic properties, can be modulated and fine-tuned to suit a particular task (e.g. energy or electron transfer) via several types of weak intermolecular interactions.

Spectral and redox properties. The spectral properties of Chls are essential for the function of Chl in harvesting light energy and in the transduction of that light energy for photosynthesis. (B)Chls absorb light very effectively in the range between 350 and 500 nm and between 600 and 800 nm, and that is why they appear green (or blue in the case of BChl *a*). The differences between the absorption spectra of Chl *a* and Chl *b* (Fig. 2) are caused by a coupling between the macrocyclic π-electron system and the π-electrons from the keto group in Chl *b* and these two Chls complement each other in absorbing sunlight (white light). Plants are able to derive energy from the blue and red parts of the spectrum, however, there is still a broad spectral region, between 500-600 nm, where light is absorbed less strongly and accessory pigments, e.g. carotenoids (Cars), supplement Chls. Since light in the green region is partly reflected or transmitted, the photosynthetic organs of plants appear green. Due to a high content of Chls in plant tissue they mask other less intense colors. Some of these more delicate colors (from other pigments, such as carotenoids and anthocyanins) are revealed when the Chl molecule decays in the autumn, and the woodlands turn red, orange, and golden brown.

In almost all aspects, the photophysical features of (B)Chls bound within the photosynthetic complexes differ remarkably from those of isolated pigments in organic solvents (Fig. 2), which is a manifestation of how their properties are fine-tuned in photosystems. Obviously, such a fine-tuning is needed to enable (B)Chls to perform their photosynthetic functions. On the other hand, these pigments were selected/designed by nature because their electronic structure is prone to such a modulation. In all types of (B)Chl-binding complexes, perhaps the most pronounced change concerns the singlet excited state lifetime, which is shortened from 3-6 ns to below 1 ns, due to the pigment aggregation. In result, the chances of excited triplet state formation are largely reduced. This is of extreme importance to oxygenic organisms because this way the generation of harmful reactive oxygen species (ROS) can be avoided. Another effect is the lowering of the S_1 state energy, which is important to the formation of excitation energy sinking and funneling (down-hill energy transfer). The drop in the S_1 state energy is sometimes not so obvious for Chls (e.g. P680, P700) but in the case of BChls, it is markedly lower, thus favoring absorption of light in the near IR range and enabling the photosynthetic bacteria to carry out photosynthetic processes in deeper water reservoirs and under the screen of Chl-containing species. In addition, the close contacts of the cofactor π-electron systems is suitable for loss-free energy transfer/storage within the antenna complexes, whereas in RCs it facilitates rapid and directional electron transfer (Blankenship, 2002).

Aggregation properties of chlorophylls. The functioning of photosynthetic apparatus relies on the aggregated forms of the pigments, whose type and size is strictly controlled by the protein matrix. In contrast, the easily observed aggregation of isolated (B)Chls in organic solvents or aqueous media, due to their insolubility, leads to the formation of very undefined types of aggregates or oligomers.

In the photosynthetic ensembles, the pigment molecules remain in close contact with each other and their photophysical properties undergo specific changes. These weak intermolecular interactions contribute to fine-tuning of the cofactors to their function in energy or electron transfer. The largest contribution to this fine-tuning comes from the so called excitonic interactions, which lead to significant shifts in the electronic properties of the cofactors (Scherz and Parson, 1984; Scherz et al., 1990). As mentioned above, these effects are most clearly seen in the case of BChls which are found in bacterial photosystems, resulting in a red shift of their Q_Y transitions even exceeding 100 nm. The π–π interactions between the macrocycles provide the major forces that bind pigment molecules within their assemblies in photosynthetic complexes. In the absence of proteins, also the coordination bond between the central Mg ion and the side carbonyl groups is involved in aggregate formation (Sauer et al., 1966).

Involvement of chlorophylls in antenna processes and charge separation in RC. Intriguingly, in spite of tremendous effort invested by scientists all over the world to reveal the mechanisms of Chl functioning, documented in thousands of publications, some aspects still remain to be uncovered. The amazing fact is that chemically the same entities are active in either energy or electron transfer in photosystems, depending on the structure of particular pigment-protein complex. This is achieved via a fine-tuning of Chl properties due to a system of weak intermolecular interactions that are experienced by these cofactors within the photosynthetic structures. As mentioned above, these tunings effects involve both pigment-pigment and pigment-protein interactions. Thus, in the photosynthetic antenna, (B)Chls act first as absorbers of photons. In most antenna complexes, Crts complement this function of (B)Chls in the spectral region between 400 and 500 nm, where light absorption by (B)Chls is minimal (see Fig. 2). Therefore, (B)Chls are very efficient as acceptors of excitation energy from Crts. A large number of pigment molecules bound per a single copy of antenna and assembled in arrays, serves to increase the effective cross-section for photon capturing. Indeed, the number of (B)Chl molecules contained in the antenna sometimes by orders of magnitude exceeds the number of pigment cofactors bound to RC (Roszak et al., 2003; Umena et al., 2011). In further steps, the excitation energy migrates among antenna pigments, practically in a loss-free manner, and then it is transferred either to another antenna complex, or directly to the RC complex, again in a loss-free process. The high quantum and energetic efficiency of energy transfer in the antenna is due to the specific oligomerization of (B)Chl molecules, which form super-complexes of excitonically coupled mers (Scherz and Parson, 1984). Smaller ensembles of (B)Chls are present in RCs, where they facilitate an ultrafast electron transfer along the chain of similarly coupled electron carriers (Deisenhofer and Michel, 1989). The electron transfer is initiated on the special pair of (B)Chls, which, after receiving the excitation from the antenna, becomes a strong electron donor (a reductant). An electron moves then from the special pair to the primary (accessory (B)Chl) and secondary ((bacterio)pheophytin) electron acceptors. The electron transfer along the chain of these cofactors results in a stable charge separation across the photosynthetic membrane, which drives further chemical reactions and thus the light energy is converted into the energy of chemical bonds. Here, with the primary charge separation, ends the direct role of (B)Chls as photoactive cofactors in photosynthesis (Blankenship, 2002).

Structural role of (bacterio)chlorophylls. Chls and BChls as the major photoactive cofactors of photosynthetic pigment-protein complexes at the same time remain the major structural cofactors of

these assemblies. As a rule, they are bound to the apoproteins exclusively via non-covalent bonds which belong to weak interactions but their presence is crucial for the assembly of all pigment-protein complexes. The structural features of Chls involved in structural stabilization of photosynthetic pigment-proteins comprise the central Mg^{2+} ion, phytyl moiety and the aromatic π-electron system (Allen et al., 1987). Energetically, the strongest binding interaction occurs between the coordinatively unsaturated central Mg^{2+} ion and the side groups of some amino acids which act as axial ligands (Kania and Fiedor, 2006). In most cases it is a monoligation via the imidazole moiety of His and sometimes the side carboxyl of Asp. However, in some cases, e.g. in bacterial LH1 complex, the binding of two axial ligands may take place (Fiedor, 2006). The other contacts of (B)Chl molecules to apoproteins concern the highly hydrophobic phytyl chain, which seems to be involved in close interactions with hydrophobic side chains of amino acids. Also the extensive aromatic π-electron system of (B)Chls may interact (π–π interactions) with aromatic amino acids. These two interactions seem to contribute to a proper positioning of pigment molecules within the protein scaffold, and this way they provide means to control the modes of pigment aggregation, strictly related to the functioning of each type of pigment-protein complex (Allen et al., 1987; McDermott et al., 1995).

Photoprotective function of (bacterio)chlorophylls. Being the primary absorbers of photons and carriers of excitation energy and electrons, Chls are also involved in the early photoprotection of the photosynthetic apparatus against harmful effects of the absorbed excess energy. The most damaging effects are associated with excellent photosensitizing properties of (B)Chls, i.e. the generation of (B)Chl excited triplet state, which may interact with molecular oxygen, the very product of oxygenic photosynthesis, yielding chemically very reactive singlet oxygen and other (ROS). Depending on the function of the particular pigment-protein complex, this photoprotection by (B)Chls takes place at several levels. During the energy transfer in PSII, the excess excitation energy is partly dissipated by fluorescence emission from Chl *a*, while in PSI, especially in its trimeric form, the «red emitters» seem to play such a «safety valve» function (see the Chapter 2). Another way to avoid the ROS formation is an efficient energy transfer from (B)Chls in the triplet state to Crts present at specific sites in the vicinity of (B)Chls, in virtually all types of photosynthetic pigment-proteins. Then, this energy is safely dissipated to the surrounding in a non-radiative way and the Crt molecules return to their ground states (see the Chapter 10).

In the electron transfer in the PSII RCs, the special Chla molecule, Chl_Z, participates in cyclic electron flow which is thought to be another «safely valve». Additionally, the oxidized Chl_Z acts as the excitation energy trap near the P680 site (Blankenship, 2002).

9.3. Distribution, formation and biodegradation of chlorophylls

Distribution of (bacterio)chlorophylls. The Chls are named *a*-*d*, and the BChls *a*-*g* (Blankenship, 2002). Chl *a* is found in all known eukaryotic photosynthetic organisms. Among prokariotes, it is found in large quantities only in the cyanobacteria. An important isomer of Chl *a* is its epimeric isomer (a diastereoisomer at the 13^2 chiral center) named Chl *a'*, found in the PSI reaction center, where together with Chl *a* it forms the special pair termed P700. Chl *b* is the major accessory light-absorbing (antenna) pigment found in most of eukaryotic photosynthetic organisms, with the exception of the red and brown algae. In photosynthetic prokaryotes it occurs only in the prochlorophytes. Chl *c* is perhaps the most unusual of all the Chls. It is chemically classified as a porphyrin, and not as a chlorin. Chl *c* is found in various groups of marine algae, such as diatoms and dinoflagellates, and functions as an accessory light-harvesting pigment. Chl *d* was known only as a trace constituent of certain algae, though it is the major pigment in cyanobacterium *Acaryochloris marina*. Chl *d* can be considered as a structural intermediate between Chl *a* and BChl *a*. Also its spectral properties fall in between those of these two pigments. BChls (indexed from *a* to *h*) are the principal Chl type pigment in the majority of anoxygenic photosynthetic bacteria. As the research in the field of photosynthesis and Chls goes on, a new Chl-related species, based on chlorin, has recently been discovered in stromatolites located in Shark Bay (Western Australia). The newly found pigment, named Chl f, has been structurally identified as [2-formyl]-Chl *a*. Intriguingly, the maximum of its Q_Y transition was found to be at 706 nm (in methanol), which is singnificantly red-shifted with respect to all other Chls of oxygenic photosynthesis (Chen et al., 2010).

Chlorophyll and bacteriochlorophyll formation. Synthesis of Chl involves the conversion of a porphyrin-type molecule in the magnesium branch of the tetrapyrrole pathway to a dihydroporphyrin (chlorin). This is achieved by the reduction of protochlorophyllide (PChlide) to chlorophyllide (Chlide). Dark-grown plants contain PChlide, which is transformed to Chlide by light. Most of the PChlide can be transformed by a short (ms) irradiation. The PChl(ide) present in dark-grown plants is characterized as short-wavelength and long-wavelength PChlide forms (Schoefs, 2001). The short-wavelength forms have fluorescence emission peaks between 631 and 643 nm whereas the main long-wavelength

form emits at 656 nm. The short-wavelength PChlide forms can function as precursors during the regeneration of the long-wavelength PChlide forms (Frank et al., 1993). The 656-nm emitting PChlide form represents a large aggregate of the ternary complex of NADPH, PChlide and NADPH-PChlide oxidoreductase (POR) located in prolamellar bodies (PLBs) (Boddi et al., 1989). The PLB is the distinctive feature of the etioplast. It consists of tubular membranes connected into highly regular three-dimensional lattice (Selstam and Wigge, 1993) in which NADPH: protochlorophyllide oxidoreductase (POR) is the main protein (Ryberg and Dehesh, 1986). This photoenzyme catalyses the photoreduction of protochlorophyllide (PChlide) into chlorophylide (Chlide) (Schultz and Senger, 1993). POR is a monomolecular light-dependent enzyme present in all organisms which synthesize Chl.

The second type of enzyme able to catalyze the reduction of PChlide, known as the light-independent NADPH:protochlorophyllide oxidoreductase (DPOR), consists of three subunits (Armstrong, 1998). It allows cyanobacteria, green algae, and the majority of gymnosperms to synthesize Chl in the dark. There is an opinion that angiosperms are unable to synthesize Chl in the dark. However, there are reports that mature green leaves of some angiosperms can synthesize Chl in the dark (Adamson et al., 1997). It is shown that evergreen coniferous species possess genes coding for light-dependent (LPOR) and dark-active (DPOR). Recent studies revealed the presence of DPOR genes in *Ginkgo biloba* whose leaves are similar to leaves of angiosperms (Skribanek et al., 2008). In our experiments with shade-tolerant species *Ajuga reptans* (Dymova et al., 2010) have shown that in overwintered leaves the Chl content was restored in darkness after exposure to strong light. The question about the presence of DPOR genes in overwintering *Ajuga reptans* leaves surviving under snow remains open. One may also suppose that green pigment accumulation was initiated by light during exposure of winter-green leaves to intense PAR (2000 μmol/(m^2 s)), as it occurs in etiolated leaves (Belyaeva, 2009). The capacity of restoring Chl synthesis in overwintered leaves as a source of assimilates during early spring period requires further investigations.

The phototransformation of PChlide leads to the removal of the newly formed Chlide from the active site of POR while the short-wavelength PChlide is transformed into long-wavelength form (Schoefs and Frenk, 1993). During a prolonged dark period, new PChlide, contributing to the formation of different spectral forms, is synthesized. During irradiation, the PLBs disperse within a few hours and at the same time the prothylakoids (PTs) increase in length and thylakoids with photosynthetic activity are gradually formed (Virgin, 1993). The fluorescence peak of newly formed Chlide undergoes a spectral shift (the Shibata shift) seen as a change in the fluorescence emission from

695 to 680 nm (Le Lay et al., 2001). Newly formed Chlide is esterified to form Chl *a* (Sunqvist and Dahlin, 1997). During continued irradiation, a certain part of the newly formed Chl is converted to Chl *b* (Tanaka et al., 1998). Chl accumulated in leaves during continuous irradiation, has a low temperature fluorescence emission spectrum with peaks at 685, 695 and 735 nm. The peaks at 685 and 695 nm represent fluorescence from CP43 and CP47 of photosystem II (PSII) (Krause and Weiss, 1991). The photosystem I (PSI) peak at 735 nm comes from at least three long-wavelength Chl forms (Kochubey and Samokhval, 2000).

In contrast to most eukaryotic photoautotrophs, in many species of purple photosynthetic bacteria, the genes coding for the proteins involved in pigment biosynthesis and the formation of the photosynthetic apparatus are regulated by partial pressure of oxygen (Zeistra-Ryalls and Kaplan, 2004). Thus, the pigment biosynthesis is switched on only under low oxygen. The biosynthetic pathway of BChl formation resembles the one of Chls and up to the stage of protoporphyrin IX the biosynthetic steps are common to heme and vitamin B_{12}. The next biosynthetic stages are unique to BChl. The energetically-demanding insertion of Mg^{2+} into protoporphyrin IX is catalyzed by magnesium chelatase at the expense of ATP. As in the Chl biosynthetic pathway, the conversion of protoporphyrin IX to Chlide requires the formation of the isocyclic ring and the reduction of ring III, but the latter step is catalyzed by protochlorophyllide reductase (DPOR) in the absence of light. The genes encoding this enzyme have sequence homologies to the bacterial nitrogenases. The next reduction step, from chlorin to bacteriochlorin, is catalyzed by another enzyme, chlorin reductase (Willows and Kriegel, 2009).

In fact, because Chlide is among the intermediates in this pathway, until not long ago it was assumed that BChls evolutionary evolved from Chl (Granick hypothesis), and that the purple photosynthetic bacteria branched from the main line of oxygenic photoautotrophs. Recently, thanks to the phylogenetic analysis of genes encoding photosynthesis-related proteins in various organisms, this view has been challenged. It appears that the Chl biosynthesis pathway, which is simpler than that of BChl, evolved via the shortening of the latter, and that purple photosynthetic bacteria seem to be evolutionary the oldest group of phototrophs (Xiong et al., 2000).

Biodegradation of chlorophylls. A massive turnover of Chls takes place in the biosphere, amounting to over 10^9 tons biosynthesized and biodegraded per annum (Brown et al., 1991). Since the characterization of several intermediate catabolites of Chl it is seen as a sequence of well synchronized steps in the safe withdrawal of Chl molecules (Matile, 2000; Oberhuber et al., 2003), which, once released from photosynthetic

complexes, become photocytotoxic due to the photodynamic effect (Fiedor et al., 1993, 2001). Chlorophyllase (Chlase), the first plant enzyme discovered a century ago by Willstatter and Stoll, and already then suggested to be an integral part of the Chl biodegradation pathway (Willstatter and Stoll, 1913), is among the key enzymes involved in this process. The enzyme catalyzes, with a high degree of stereospecificity, the splitting of the Chl molecule into the carboxylic acid Chlide and phytol (Fiedor et al., 1992, 1996). After this step, polar Chlide may leave the lipid membrane environment and undergoes pheophytinization catalyzed by magnesium dechelatase and then it is oxidatively converted to non-fluorescent water-soluble compounds which end up in vacuoles (Oberhuber et al., 2003). Recently, it has been proposed that the step of Mg removal is catalyzed by another enzyme called pheophytinase at the stage of intact Chl (Schelbert et al., 2009). The information of how the degradation process is triggered and controlled is still scarce. We have recently found that in *Arabidopsis* the expression of enzymes involved in Chl catabolism, the products of the *AtCLH1* and *AtCLH2* genes, is light-controlled, both during regular Chl turnover and senescence. The light effect on *AtCLH1* is mediated by a complicated interplay of photoreceptors while up-regulation of *AtCLH2* is mediated by cryptochromes and modulated by phototropin1 and phytochrome A (Banas et al., 2011).

The molecular structure of Chls incorporates a large portion of total leaf nitrogen and several studies have found that foliar Chls concentration provides an accurate, indirect estimate of plant nutrient status (Filella et al., 1995; Moran et al., 2000). Chls generally decrease under stress and during senescence and the ratio of Chl *a* to Chl *b* changes with abiotic factors (see below) such as light (Fang et al., 1998), therefore, measurements of total Chl can provide useful insights into plant-environment interactions (Richardson et al., 2002).

9.4. Factors influencing the level of chlorophyll in leaves

In nature the Chl content and ratio are controlled by many external and internal factors. Integrated action of these factors determines the activities of Chl biosynthesis and degradation. Some changes in environmental conditions such as quality and intensity of photosynthetically active radiation (PAR), UV-radiation and temperature can be unfavourable and thus potentially damaging to the photosynthetic apparatus. Irradiance is the energy source for all photosynthetic organisms, which are finely tuned to harvest it efficiently. Only the photosynthetically active part of the spectrum (PAR; 400 to 700 nm) directly drives photosynthesis. Low light irradiance limits photosynthesis. High light intensities may also be a stress for plants. Exposure of leaves to exces-

sive irradiance is a well-known cause of photoinhibition, which decreases the capacity for photosynthesis in many plants (Long et al., 1994; Osmond and Forster, 2006). This inhibition of photosynthesis is supposedly due to damage of photosystem II (PSII) (Murata et al., 2007). The acclimation of higher plants to contrasting light regimes involves specific features of leaf structure and chloroplast composition (Anderson et al., 1995; Dymova and Golovko, 1998; Maxwell et al., 1999; Pandey et al., 2003).

The plants having traits that are adaptive in a shady environment, are called *shade plants*. The term *sun plant* similarly refers normally to a plant grown in high-light conditions, but it is also used to indicate a shade-avoiding species. The terms *sun leaf* and *shade leaf* refer to leaves that have developed at high and low irradiance, respectively.

Shade leaves minimize light limitation through the increases in capacity for light capture on the expense of photosynthetic capacity. It is found (Chow et al., 1991; Krall et al., 1995; Dymova and Golovko, 1998, 2007; Burritt and Mackenzie, 2003) that some shade-adapted species (e.g., *Ajuga reptans*, *Alocasia macrorriza*, *Begonia erythrophylla*, *Hedera helix*, *Tradescantia albiflora*) may have higher Chl levels per unit leaf area in shade environments in comparison to sun. This might be due to the fact that their leaves do not get much thinner in the shade; however, there may also be some photodestruction of Chl in high light in such species.

The Chl *a*/*b* ratio varies from 2.0-2.8 for shade-adapted plants to 3.5-4.9 for plants adapted to full-light-conditions. This variation in Chl *a*/*b* ratios is due to differences in the ratio of photosystem I (PSI) to photosystem II (PSII) and the size and composition of the light-harvesting complexes (LHCs) associated with each photosystem. The photosystems contain Chl *a* but not Chl *b*, whereas the LHCs contain significant amounts of Chl *b*. Shade-adapted plants tend to have more LHCs associated with their photosystems than the sun-adapted plants and thus have lower Chl *a*/*b* ratios than the sun-adapted plants (Anderson, 1986; Porra, 2002).

Daily changes in chlorophyll content. The studies of daily changes in the pigment pool of a shade-tolerant bugle plant (*Ajuga reptans* L.) (Dymova and Golovko, 2007) and a hoary plantain (*Plantago media* L.) (Golovko et al., 2011, 2012) were carried out. Two groups of plants were selected for experiments. The first group grew on the open site (sun plants). The second group grew on the shady environment (shade plants). The leaves of sun plants had reduced Chl content (Table 1). In shade plants, daily changes in the pigment content were more expressed. The Chl *a*/*b* ratio were nearly equal in sun and shade plant leaves and remained almost constant throughout the day. Our data showed that the pigment content was lower in plants grown under high irradiance

Table 1

Characteristics of the pigment complex in leaves of shade and sun *Ajuga reptans* and *Plantago media* plants, mg/g dry weight

Time, h	*Ajuga reptans* L.		*Plantago media* L.	
	Chl *a+b*	Chl *a/b*	Chl *a+b*	Chl *a/b*
		Sun plants		
5:00-6:00	4.05±0.24	2.8±0.1	1.90±0.09	2.1±0.1
10:00-12:00	4.37±0.27	3.1±0.1	3.66±0.27	2.4±0.2
16:00-18:00	4.06±0.18	2.7±0.1	3.11±0.20	2.2±0.1
23:00-24:00	4.59±0.38	2.8±0.2	1.98±0.21	2.2±0.2
		Shade plants		
5:00-6:00	13.50±0.45	2.6±0.1	3.14±0.12	2.1±0.2
10:00-12:00	12.53±0.78	2.7±0.1	7.27±0.74	2.4±0.2
16:00-18:00	9.90±0.53	2.4±0.1	7.09±0.53	2.8±0.2
23:00-24:00	11.56±0.83	2.7±0.2	2.91±0.27	2.0±0.1

Source: Dymova and Golovko 2007; Golovko et al. 2011.

as compared to plants under a forest canopy. In this case, the accumulation level of green pigments did not change over 24-h cycle indicating that the rate of their formation *de novo* and degradation was balanced. In shade plants, a decrease in the Chl content in the afternoon and in the evening may be related to endogenous physiological rhythms and/or may be due to exogenous factors. It is known that in the morning hours solar radiation is enriched in infrared and red light which seems more effective for biosynthesis of green pigments, especially Chl *a*.

Seasonal changes in chlorophyll content. Early we studied seasonal changes in photosynthetic pigment contents in the three conifers: *Abies*

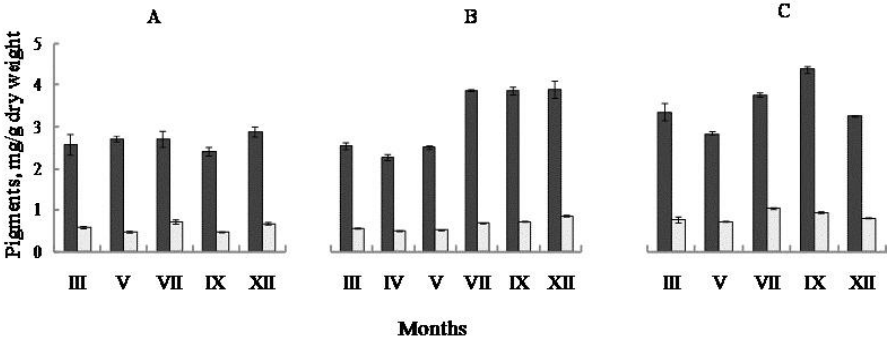

Fig. 3. Seasonal changes in the chlorophyll (dark symbols) and carotenoids (light symbols) contents in *Picea abies* (A), *Abies sibirica* (B) and *Juniperus communis* (C) needles. *Source*: Yatsco et al. 2009.

Table 2

Chlorophyll and carotenoids composition of evergreen conifers needles sampled in spring (III – March), summer (VII – July) and winter (XII – December), n = 5

Species	Chlorophyll *a*/*b*			LHC-Chl, %		
	III	VII	XII	III	VII	XII
Abies sibirica	2.4±0.1	2.5±0.1	2.7±0.1	65	64	59
Picea abies	2.4±0.1	2.5±0.3	2.9±0.1	66	63	57
Juniperus communis	2.8±0.1	2.8±0.1	2.9±0.1	60	57	56

Note: LHC-Chl – Chlorophyll belonging to light-harvesting complex.
Source: Yatsco et al. 2009.

sibirica, Picea abies and *Juniperus communis* (Yatsco et al., 2009; Golovko et al., 2011). The concentrations of Chl were relatively low, 2.4-4.3 mg/g DW (Fig. 3). In the winter a reduction of photosynthetic pigments pool was maximal in *Juniperus communis* needles. In *Picea abies* and *Abies sibirica* the pool of the green pigments is more stable during the year. The essential seasonal differences in Chl *a*/*b* ratio were not found (Table 2). In spring and summer all species had a tendency to higher LHC-Chl level. We noted already that the *Picea abies* needles were distinguished by the greatest stability of the Chl pool during the year among the studied species. In *Abies sibirica* and *Juniperus communis* needles, a portion of the Chls in winter and spring had been destructed. Several investigators have reported the loss of Chl in the needles of the conifers in winter (Ottander et al., 1995; Ensminger et al., 2004; Martz et al., 2007). It seems the different opinions about the character of the seasonal changes in Chl composition in conifers depend on the species traits and the climatic conditions of the habitats. In *Abies sibirica* and *Juniperus communis* the winter and spring loss of Chl was repaired in summer and autumn. In *Pinus sylvestris* spring accumulation of Chls occurred after photosynthesis was almost fully recovered (Ottander and Oquist, 1991). This fact can be regarded as the adaptive reaction which is directed to the reducing of the light absorption in winter and spring when photosynthetic consumption of excitation energy is blocked.

Changes in chlorophyll content during development. The Chl content in leaves varied depending on the stage of leaf development. The maximum pigment content generally occurs in a generative phase (Sestak, 1985). It is shown that the major difference between the high-light- (2400 μmol/m^{2}s) and low-light-grown (300 μmol/m^{2}s) tobacco plants was the reduced levels of Chl in the youngest leaves of the high-light-grown plants (Bugos et al., 1999). The youngest leaf from the high-light-grown plants had almost half the Chl content of the low-

light-grown plants. The Chl levels (about 60 nmol/cm^2) in both plants were similar in mature leaves. The loss in Chl in the oldest leaves most likely indicates that these leaves were starting to undergo senescence. The Chl *a*/*b* ratios were similar for both plants, with the ratios continuously declining: from 3.7 in the youngest leaves to 3.0 in the oldest leaves. Kukavica and Jovanovic (2004) found that Chl *a*+*b* content in ginkgo (32 mg/cm^2) and birch (28 mg/cm^2) leaves measured during autumn was constant until the beginning of October; when it started to decrease to about 50% and 37% of the initial level by the end of month in ginkgo and birch leaves respectively. Degradation of Chl during natural senescence was not accompanied either by an increase of H_2O_2 or by a decrease of reduced ascorbate. A rapid loss of Chl, associated with degradation of the chloroplast structure, has been widely used as a biomarker for the progression of senescence.

UV-irradiance. UV-B irradiance (290-320 nm) is potentially damaging to terrestrial plants. It inactivates the photosynthetic apparatus by inflicting damages at multiple sites (Bornman, 1989). UV-B acclimation involves accumulation of UV-absorbing compounds, which minimizes deleterious UV-B effects on the photosynthetic apparatus (Searles et al., 2001; Krause et al., 2007). Another component of solar UV radiation (UV-A; 320-400 nm) exhibits both positive and negative effects on plant photosynthesis (Wellmann, 1983). Whereas UV-A activates gene expression for photosystem II (PSII) reaction centre proteins (Christopher and Mullet, 1994), it inflicts damage to photosynthetic apparatus (Joshi et al., 1997). Plants develop strategies to alleviate the UV-induced damage of chloroplasts. The strategies include the shielding of the organelle by inducing the accumulation of UV-absorbing phenolic compound (Jansen et al., 1998), repair mechanism involving both DNA repair and *de novo* synthesis of UV-sensitive proteins, especially D1 and D2 proteins of PSII (Bornman, 1989).

Recently Kreslavskii et al. (2012) shown that irradiation of spinach leaves with UV-A reduced the content of Chl *a*+*b* and suppressed the activity of PSII. It was found (Gartia et al., 2003) that in clusterbean (*Cyamopsis tetragonoloba* L.) leaves UV-B irradiation decreased the content of total Chl and the decrease was furthered with the progress of UV-B dose. The degree of damage was reduced when UV-A accompanied the UV-B radiation.

9.5. Conclusion

The chlorophylls and bacteriochlorophylls are the most important biological chromophores as they are indispensible for photosynthesis. These pigments are responsible for light absorption and conversion of its energy into biochemically useful energy in photosynthetic organelles

of bacteria, cyanobacteria and plant cells. They act as both energy and electron transfer cofactors, depending on the type of photosynthetic complex. In addition, they are essential structural cofactors of all photosynthetic pigment-proteins, non-covalently bound to the apoproteins. On global scale, large quantities of Chls, estimated as 10^9 tones annually, are biosynthesized and in a controlled manner biodegraded in biosphere. The level of Chls in plants undergoes not only these seasonal changes but is influenced by several abiotic and biotic factors.

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Chapter 10. CAROTENOIDS AS PHOTOPROTECTORS

Wieslaw I. Gruszecki[1], Renata Szymanska[2], and Leszek Fiedor[2]
[1] Institute of Physics, Maria Curie-Sklodowska University, Lublin, Poland
[2] Jagiellonian University, Krakow, Poland

Contents

10.1. Introduction

The photosynthetic apparatus of higher plants and algae is where the molecular oxygen pool originates in the biosphere, as a result of light-driven water splitting reaction. At the same time, it is a site rich in chlorophyll molecules, which belong to the cyclic tetrapyrrolic group, one of the most efficient photosensitizers. Obviously, the photosynthetic apparatus is also exposed to light and therefore it is hard to imagine better conditions for the photo-sensitized oxidation of biological structures, especially of photosynthetic membranes rich in poly-unsaturated lipids and protein complexes binding photosynthetic pigment molecules based on conjugated double C=C bond chains. To protect themselves against light-induced oxidative damage, photosynthesizing organisms have developed in the course of biological evolution several defense strategies. One of the most common and, at the same time, most efficient photoprotective strategies of photosynthesizing organisms seems to be the binding of carotenoids, molecules which can work as accessory pigments transferring their singlet excitation energy to chlorophylls, being at the same time able to efficiently quench chlorophyll triplet excitations and singlet oxygen through both physical and chemical mechanisms (Krinsky, 1979; Landrum, 2010). Since early reports from the Stanier group it is known that carotenoids also have an important function in the protection of the photosynthetic apparatus against the photo-oxidative damage (Griffith et al., 1955). It has been shown that a *Rhodobacter sphaeroides* strain mutant lacking carotenoids is not able to survive under combined light and oxygenic conditions even though light or oxygen alone were not essentially toxic to the bacteria. Carotenoids are also capable of scavenging other reactive oxygen species by physical and chemical mechanisms (Krinsky and Yeum, 2003; Edge

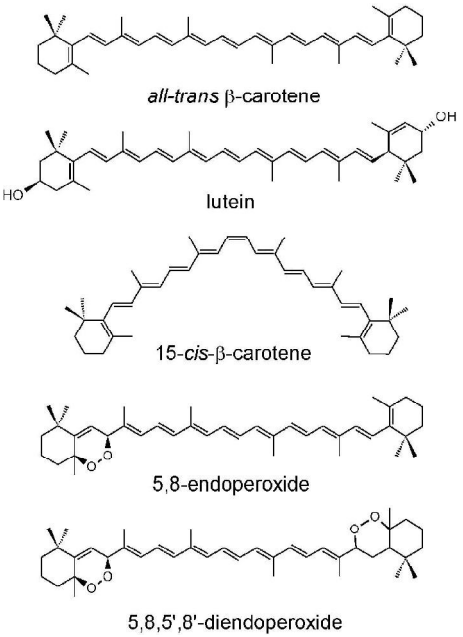

Fig. 1. The formulae of major carotenoids which take part in photoprotective processes in plant and the products of the chemical quenching of singlet oxygen ($^1O_2^*$) by all-trans-β-carotene.

and Truscott, 1999; Fiedor et al., 2001, 2005). This property makes them important constituents of the photosynthetic apparatus, as accessory antenna pigments (see the chapter on Photosynthetic pigment-protein complexes) and especially due to their ability to protect against light-induced oxidative damage (Britton, 2008). The structures of the major carotenoids involved in photoprotection in plants and the products of chemical quenching of singlet oxygen by carotenoids are shown in Fig. 1.

10.2. Physical mechanisms

To describe more precisely the reactions of photosensitization and photoprotection let us draw, at the beginning, a Jablonski diagram of a typical pigment, which could be possibly a chlorophyll (Chl) molecule (Fig. 2). The basic photophysical processes that follow photoexcitation are marked on the graph, with a typical timescale for

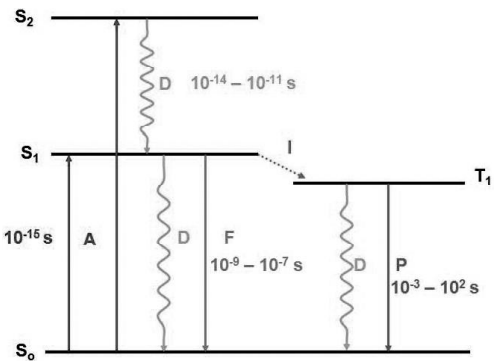

Fig. 2. A simplified Jablonski diagram of the energetic states of a pigment molecule (e.g. Chl) with basic photophysical processes and the typical timescales indicated. A stands for light absorption, F fluorescence, D thermal dissipation, I intersystem crossing, P phosphorescence. See the text for further explanations.

them to take place being indicated. The light absorption (A) from the ground state (the spin multiplicity of which equals 1, the singlet state) can transfer the molecule to one of the states from band B (blue light absorption) or to band Q (red light absorption). The pigment molecule at higher singlet energy levels relaxes rapidly to the lowest excited singlet energy state, from which the excess energy may be emitted as fluorescence (F) or dissipated (D) as heat. Despite the spin multiplicity conservation rule, Chl in a singlet excited state may also undergo conversion to the triplet state via what is known as the intersystem crossing reaction (I). This process, which is considered an exception to the rule, is in fact quite frequent. For example, the efficiency of intersystem crossing in Chla molecules in diethyl ether and in other organic solvents is higher than 60% (Fiedor et al., 1993; Drzewiecka et al., 2005). Chl in the triplet excited state (^{3}Chl*) is long-lived compared to the lifetimes of singlet excited states, primarily due to the fact that the ground energy level is a singlet state and a transition between triplet and singlet states is again forbidden owing to the spin multiplicity conservation rule. This means that ^{3}Chl* "has enough time" to react with other molecules in its surrounding, including molecular oxygen. The latter, unlike most other molecules, is a paramagnetic molecule and its ground state is a triplet state ($^3O_2$), which makes an exchange of excitation energy with ^{3}Chl* probable:

$$^3\text{Chl}^* + {}^3O_2 \rightarrow {}^1\text{Chl} + {}^1O_2{}^* \quad (1)$$

Such an energy transfer is called the photosensitized generation of the excited oxygen in which Chl plays the role of photosensitizer. This process leads to the formation of singlet excited molecular oxygen ($^1O_2{}^*$, the formal notation of its lowest excited state $^1\Delta_g$) called also "singlet oxygen". The energy of the chlorophyll in triplet state corresponds to 10 000 cm^{-1} (in spectroscopic units) and the difference between its ground state and the singlet excited state of $^3O_2$ corresponds to 7870 cm^{-1} which makes the reaction of oxygen photosensitization by ^{3}Chl* not only possible but also highly probable. $^1O_2{}^*$ is exceptionally reactive and its lifetime in water is relatively long (~$2\cdot10^{-6}$ s) which makes it possible to diffuse over considerable distances in solution and react with other molecules in the cell interior. In many cases, such reactions lead to the formation of free radicals, which are also potent reactive oxygen species (ROS). Owing to these facts, singlet oxygen is considered one of the most cytotoxic species. The most effective protection against $^1O_2{}^*$ attack would be its deactivation immediately at the site of its generation, e.g. via collisions with triplet excited Chl molecules, and even better, by quenching of the triplet excitations of the photosensitizer to prevent the formation of ROS. It so happens

that in the proximity of Chl molecules in the photosynthetic apparatus there are carotenoid molecules which are active in both $^1O_2{}^*$ and the chlorophyll triplet quenching. This is possible due to the fact that the photosynthetically active Crts possess a system of 9 or more conjugated double bonds (e.g. violaxanthin n = 9, lutein n = 10, β-carotene and zeaxanthin n = 11). The energy of a triplet excited Crt (E_T) depends on the length of the conjugated double bond system (n). For example, E_T equals 7200 cm^{-1} and 6000 cm^{-1} in the case of n = 9 and n = 11, respectively. These energies are lower than the energy of a Chl excited triplet (E_T = 10000 cm^{-1}) and also of singlet oxygen (E_T = 7800 cm^{-1}), which means that both photosensitizer and singlet oxygen can be efficiently quenched via an energy transfer to Crts, associated with the generation of excited Crt triplets (Fig. 3). It appears that there is a structural specialization among Crts in terms of the molecular conformation to the end of fulfilling their roles in the quenching of the excited triplet states of Chls in natural photosynthetic systems. Thus in antenna complexes, Crts are present mainly in the all-trans conformation. *cis*-Crts are relatively rare in nature, but for instance 15-*cis*-Crts are often found at specific sites in plant (PSI and PSII) as well as in bacterial photosynthetic RCs (Bialek-Bylka et al., 1995, 1996, 1998), where they are involved in quenching triplets of the primary donor. 15-*cis*-Crts have also been found in the RCs of oxygenic organisms (Kamiya et al., 2003; Ferreira et al., 2004).

Fortunately, the energy of an excited Crt triplet is easily and efficiently dissipated to the environment via heat emission. This kind of Crt activity protects the photosynthetic apparatus against oxidative damage, which can be initiated not only by singlet oxygen but also by other ROS (e.g. free radicals), which can be formed as a product of singlet oxygen reactions with other molecules (Youhg et al., 2004). To this end, Crts are also known as efficient free radical scavengers.

Carotenoids are located mostly within specific binding sites of pigment-protein complexes as structural components of them, and are involved both in light harvesting and the photoprotection of photosys-

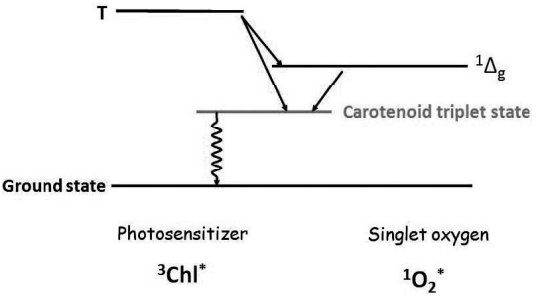

Fig. 3. A schematic representation of typical energy transfer processes responsible for photosensitized singlet oxygen generation and for chlorophyll triplet and the singlet oxygen quenching by carotenoids. See the text for a detailed explanation.

tems. β-Carotene is bound to RC subunits in both PSI and PSII, while xanthophylls are bound to peripheral light harvesting complexes (LHCs). In addition to their role as structure stabilizing components (Fiedor et al., 2004), carotenoids are involved in photoprotective mechanisms. Their essential role in photoprotection in plants was shown by studying the phenotype of carotenoid-less plants, photoauthotrophically non-competent (Kim et al. 2009). An analysis of an *szl* (*suppressor of zeaxantin less*) mutant of *Arabidopsis* with decreased activity of lycopene-β-cyclase revealed a lower carotene content than in the wild type (Cazzaniga et al., 2012). When this mutant was exposed to high-light at a low temperature, the photoinhibition of PSI was stronger than that of PSII and the rate of $^1O_2$ release from the isolated PSI LHC was higher than in wild type plants (Cazzaniga et al., 2012). These observations suggest that carotene depletion impairs photoprotection and that β-carotene in PSI has a crucial photoprotective function, especially at low temperatures. Moreover, those Crts bound to LHCI, namely lutein, violaxanthin and β-carotene, can participate in triplet and/or ROS scavenging, and probably occupy specific binging sites and have distinct functions (Cazzaniga et al., 2012). Changing one of these functions through mutation in the biosynthetic pathway leads to photosensitivity, similar to that observed within the PSII antenna system (Dall'Osto et al., 2007).

In PSII, Crts play a crucial role in the deactivation of triplet Chl and $^1O_2^*$ (see above) as well as dissipating excess energy as heat (non-photochemical quenching, NPQ). All these processes prevent the formation of ROS. In the PSII reaction center, the scavenging of $^1O_2^*$ is executed by β-carotene (Telfer, 2002), whereas the NPQ of excitation energy in antenna complexes is carried out by xantophylls such as antheraxantin, zeaxanthin and lutein (Horton et al., 2005; Latowski et al., 2011) (see chapter 12 for the role of xantophylls in photoprotection). β-Carotene molecules located in the PSII reaction center can be distinguished by their spectroscopic properties, i.e. β-carotene$_{507}$ (absorption maxima at 507, 473 and 443 nm) and β-carotene$_{489}$ (absorption maxima 489, 458 and 429) (Kwa et al., 1992). It has been proved that these molecules are unable to quench Chl triplets in PSII, but that they do scavenge $^1O_2^*$ (van Gorkom et al., 1993). It should be noted that the scavenging ability of β-carotene has one limitation – the distance between β-carotene and the oxidized chlorophyll cannot be larger than 18-20 A (Telfer and Barber, 1995).

β-Carotene bound to D2 protein is the primary electron donor for P^+_{680} when the electron transport from water is limited. Under such conditions, electrons are cycled around PSII *via* Q_B, cytochrome b_{559} (HP), β-carotene and P^+_{680} (Faller et al., 2001). When the electron supply from cytochrome is limited, β-carotene forms a bridge between

P^{+680} and ChlZ located at the edge of the PSII (Wang et al., 2002). It is highly probable that β-carotene can oxidize ChlZ, which in turn can quench fluorescence and reduce the photochemical pressure on PSII (Stewart et al., 1998). Thus, β-carotene located at D2 protein, as the electron donor, protects PSII against photoinhibition. On the other hand, when the electron supply from water to P^{+680} is limited, β-carotene located at D1 protein is oxidized. This is a signal for proteases which selectively degrade D1 protein to prevent further charge separation (Szymanska et al., 2012). These findings also explain why D1 protein is more sensitive to degradation than D2 protein.

10.3. Chemical quenching

Clearly, the major photoprotective activity of Crts involves the physical quenching of singlet oxygen (and other ROS) and triplet states of (B)Chls, but more and more evidence is being obtained that Crts also act as chemical quenchers of ROS (Foote, 1968; Burton and Ingold, 1984; Edge and Truscott, 1999). As model studies in solution show, photoprotective activity is strongly medium-dependent (Fiedor et al., 2001; Fiedor et al., 2005). For instance, β-carotene in methanol has no protective effect on the self-sensitized photodegradation of Chl a whereas in acetone it prevents the degradation of Chl *a*, at the expense of the rapid photodegradation of the Crt. There is a growing body of evidence that these products of Crt oxidation can themselves act as oxidants. Such pro-oxidant activity of Crts was also reported in lipid peroxidation studies (Burton and Ingold, 1984). However, neither the mechanisms nor the products of these reactions of Crts are well established, nor the contribution of these pro-oxidants to light-induced cellular damage and oxidative stress in living tissue (Young and Lowe, 2001). Recently, it has been found in the leaves of *Arabidopsis thaliana* that high-light stress results in an accumulation of β-carotene endoperoxides, the products of the chemical quenching of $^1O_2{}^*$, in the reaction centers of PSII, the site of $^1O_2{}^*$ generation (Ramel et al., 2012).

In earlier studies (Foote, 1968; Burton and Ingold, 1984), to account for the formation of endoperoxides of β-carotene, a primary attack of peroxyl radicals on the C-7 position was suggested, which would be favored by the relatively high electrophilicity of this site. It was proposed that the resulting radical of β-carotene would react with molecular (triplet) oxygen to produce cyclic endoperoxide (Fig. 1). In a more recent study, a cascade of oxidation products of β-carotene, generated in a BChl-sensitized reaction in acetone, has been observed. The identification of these products by mass spectrometry showed that β-carotene sequentially accumulates up to six oxygen atoms while its C_{40}-skeleton remains intact. It was shown that the photosensitized de-

gradation of β-carotene is strongly inhibited by the $^1O_2^*$-quencher, 1,4-diazabicyclo-[2.2.2]octane (DABCO), while the radical quencher, 2,6-di-*t*Bu-*p*-cresol (BHT), showed only little effect, which confirmed the crucial role of $^1O_2^*$ as the reactive intermediate. In another study based on the structural assignment of the products by NMR as cyclic endoperoxides of β-carotene (Fig. 1), a 2:4 cycloaddition has been proposed for the oxygenation mechanism of β-carotene. The concerted mechanism requires the presence of an s-*cis*-diene conformation of the Crt adduct. The 5,8-endoperoxides can proceed without any prior conformational change in all-*trans*-β-carotene because of the sterically favored 6-s-*cis* conformation, while the formation of 7,10-endoperoxides requires an s-*trans* to s-*cis* conversion at the C-8/C-9 single bond.

10.4. Conclusions

Carotenoids play a photoprotective role in both PSI and PSII. Carotenes protect PSI against photoinhibition while in PSII these pigments play a crucial role in the deactivation of triplet chlorophyll and $^1O_2^*$ and dissipating the excess energy as heat. Singlet oxygen in the photosynthetic apparatus is generated via the photosensitization reactions with triplet excited chlorophyll molecules:

1. Singlet excitation of chlorophyll via light absorption:
$^1Chl + h\nu \rightarrow {}^1Chl^*$
2. Chlorophyll triplet state formation via the intersystem crossing:
$^1Chl^* \rightarrow {}^3Chl^*$
3. Photosensitized singlet oxygen generation:
$^3Chl^* + {}^3O_2 \rightarrow {}^1Chl + {}^1O_2^*$.

Carotenoids are recognized as efficient photoprotectors in the photosynthetic apparatus owing to their ability to quench singlet oxygen and excited triplet states of chlorophyll:
1. Quenching of Chl triplet state:
$^3Chl^* + {}^1Car \rightarrow {}^1Chl + {}^3Car^*$
2. Physical quenching of singlet oxygen:
$^1O_2^* + {}^1Car \rightarrow {}^3O_2 + {}^3Car^*$
3. Thermal deactivation of the excited carotenoid triplet:
$^3Car^* \rightarrow {}^1Car + heat$
4. Chemical quenching of singlet oxygen:
$^1Car + {}^1O_2^* \rightarrow$ oxidized products.

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Chapter 11. THE ROLE OF CAROTENOIDS IN THE PROTECTION AND ASSEMBLY OF THE PHOTOSYNTHETIC APPARATUS IN A MODEL UNICELLULAR CYANOBACTERIA *SYNECHOCYSTIS* PCC 6803

Kinga Klodawska, Przemyslaw Malec, and Kazimierz Strzalka
Jagiellonian University, Krakow, Poland

Contents

11.1. Introduction

Carotenoids constitute a very broad group of pigments that can be found in practically every living organism. Animals are the only organisms that do not synthesize carotenoids, containing them exlusively as a result of the accumulation of food compounds. In cyanobacteria, the first step in carotenoid biosynthesis is phytoene synthesis by the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) (Hirschberg and Chamovitz, 1994; Martinez-Ferez et al., 1994). Later steps lead to differentiation of pigment molecules in two subgroups: carotenes – hydrocarbons with cyclic ionone rings at one or at two sides, and xanthophylls – oxygenated carotenes with ionone rings complemented by epoxy, keto or hydroxy functional groups. Various physiological functions fulfilled by carotenoids depend on the structural features of the molecules. In photosynthetic organisms carotenoid pigments can serve as accessory photosynthetic pigments, as photoprotective agents (Cogdell and Frank, 1987; Hirschberg and Chamovitz, 1994) and as modulators of membrane properties (Gruszecki and Strzalka, 2005). Additionally, carotenoid pigments play an important role in the assembly of multisubunit photosystem complexes. More than 700 naturally occurring carotenoid species have been identified (Britton et al., 2004).

Cyanobacteria are gram-negative prokaryotes, which conduct oxygenic photosynthesis in a mode similar to higher plants. Two multisubunit photosystems (PSI and PSII) and a set of protein and low molecular mass electron carriers comprise the photosynthetic apparatus. Instead of a chlorophyll-containing light harvesting complex (LHC), cyanobacteria contain distinctive antenna type – phycobilisome comp-

lexes (PBS), that contain phycocyanobilin and phycoerythrobilin as light harvesting pigments (Glazer, 1985; McColl, 1998; Adir, 2005).

Synechocystis sp. is a unicellular coccoid cyanobacterium that lacks gas vesicles or a sheath. The strain called "*Synechocystis* PCC 6803" was derived from a strain isolated originally from freshwater in California (Stanier et al., 1971, Rippka et al., 1979). It was the first phototrophic organism to be fully sequenced. The availability of the genomic sequence together with molecular biology tools established PCC 6803 as a versatile model to study the function of particular genes in photosynthetic organisms (Ikeuchi and Tabata, 2001). In particular, analysis of the genome made it possible to identify the genes which encode key enzymes involved in the biosynthesis of carotenoids in *Synechocystis* and other cyanobacteria (see: Takaichi and Mochimaru, 2007 for extensive review). A simplified scheme of the carotenoid biosynthesis pathway in *Synechocystis* is presented in Fig. 1.

This chapter briefly summarizes recent progress in carotenoid biology made by the use of mutants with impaired genes encoding the enzymes involved in carotenogenesis in *Synechocystis* PCC 6803.

11.2. Photoprotection

In their natural habitats, organisms are continuously exposed to changing photon flux density (Bjorn, 1994). For photosynthetic species light is necessary for survival, but can also be very dangerous when provided in excess. Over-excitation may lead to photooxidation, damage to photosynthetic machinery components, peroxidation of membrane lipids and finally cell death. Mechanisms protecting against excess illumination are indispensable. In cells there are enzymatic systems consisting of catalases and peroxidases, as well as antioxidant compounds. Carotenoid pigments are one of the most significant lipophilic antioxidants (Niyogi, 1999). It was possible to determine a quantitative relationship between the carotenoid content of the photosynthetic apparatus and degradation of thylakoid proteins and chlorophyll in the light (Sandmann et al., 1993; Komenda and Masojidek, 1995).

Higher plants and algae undergo a zeaxanthin/violaxanthin epoxidation/de-epoxidation cycle to dissipate excess energy from photosensitized chlorophylls (Demming-Adams and Adams, 1996; Latowski et al., 2004; Jahns et al., 2009). Cyanobacteria do not exhibit any type of pigment cycling, but they contain carotenoid molecules in the majority of cell compartments: photosynthetic membranes, cytosol (bound to specific proteins), plasma membranes and the cell wall. The carotenoid pattern varies among different species. In *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), the most abundant ones are myxoxanthophyll

(myxol 2'-dimethyl-fucoside), zeaxanthin, β-carotene and echinenone (Hirschberg and Chamovitz, 1994; Takaichi and Mochimaru, 2007).

Photoprotection is assured by carotenoid pigments by means of different mechanisms:

– quenching singlet and triplet excited states of chlorophyll (^{1}Chl* and ^{3}Chl*) and scavenging singlet oxygen (Kowalczyk-Schroder and Sandmann, 1992; Young and Frank, 1996)

$^1CHL^* + CAR \rightarrow CHL + CAR^*$

$^3CHL^* + CAR \rightarrow CHL + {}^3CAR^*$

$^3CHL^* + O_2 \rightarrow CHL + {}^1O_2^*$

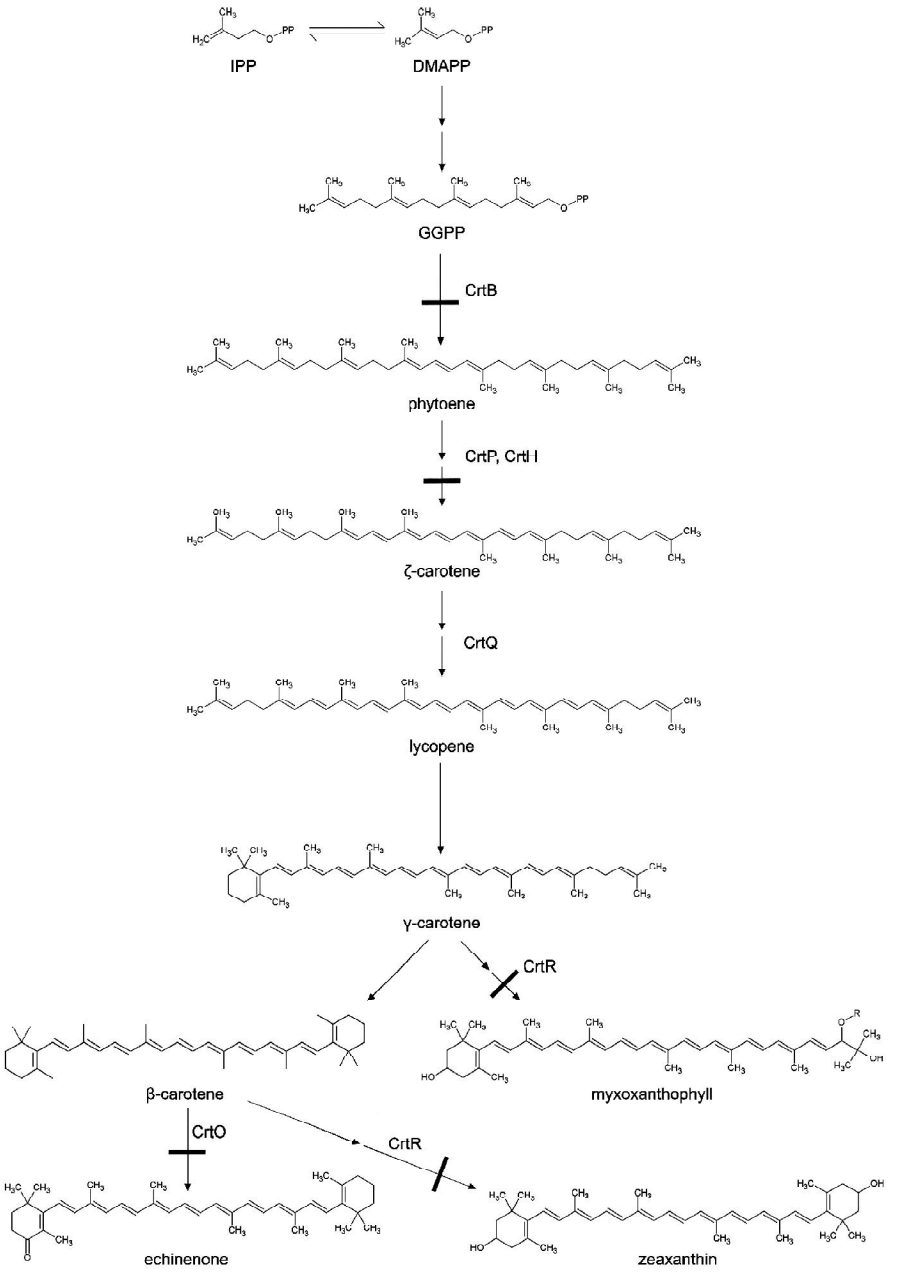
$^1O_2^* + CAR \rightarrow {}^3O_2 + {}^3CAR^*$

– inactivating peroxy radicals (Edge et al., 1997)

$RO^{\cdot}{}_2 + CAR \rightarrow CAR^{\cdot +} + R^- + O_2$

– orange carotenoid protein (OCP) – dependent phycobilisome fluorescence quenching (Kirilovsky, 2007).

The capacity to quench excited chlorophyll and oxygen states relies on the fact that the carotenoid triplet state (^{3}CAR*) energy level is sufficiently low to effectively perform its thermal dissipation (Polivka and Sundstrom, 2004). Functional groups attached to β-carotene carbon chain determine the type of antioxidative properties. Carotenoids with hydroxy groups are efficient peroxy radical inactivators while keto-carotenoids are good singlet oxygen quenchers (Woodal et al., 1997a, b). The dependence between the degree of desaturation and the capability of protection against photooxidation and $^1O_2$ quenching was described (Hirayama et al., 1994). Myxoxanthophyll, the *Synechocystis* pigment with the biggest number of conjugated double bonds, proved to have the best efficiency in protection against lipid photooxidation and radical oxidation (Steiger et al., 1999). It has also been found that the biosynthetic activity of myxoxanthophyll is upregulated in phosphatidylglycerol-depleted cells in response to oxidative stress and the concomitant destabilization of thylakoid membranes (Domonkos et al., 2009). The same carotenoid species was found to be preferentially accumulated upon exposure to high light stress, in a strain tentatively named *Aphanocapsa* (Nonnengiesser et al., 1996). Although myxoxathophyll has been found to be required for the normal membrane and cell wall organization and stability in *Synechocystis* (Mohamed et al., 2005), its photoprotective activity has not been proved so far based on the analysis of the mutant completely lacking myxoxanthophyll. *In vivo* experiments on *Synechocystis* mutants with altered pigment compositions showed that it is the zeaxanthin level that has the biggest impact on the stability of photosynthesis in high light conditions (Schafer et al., 2005). Studies on *Synechococcus* and *Oscillatoria agardhii* showed an increased accumulation of zeaxanthin in high light conditions (Millie et al., 1990; Masamoto and Furukawa, 1997, Masamoto et al. 1999). In *Synechococ-*


IPP
DMAPP
GGPP
CrtB
phytoene
CrtP, CrtI
ζ-carotene
CrtQ
lycopene
γ-carotene
CrtR
β-carotene
myxoxanthophyll
CrtO
CrtR
echinenone
zeaxanthin

cus transformants with enhanced zeaxanthin production, photosynthesis was protected from high light stress to a grater extent than in wild type cells (Gotz et al., 1999). The introduction of the gene for canthaxanthin (β-carotene-4,4'-dione) synthesis at the expense of zeaxanthin led to even higher photosynthetic tolerance of strong light and better protection against the photooxidation of chlorophyll by UV-B radiation (Albrecht et al., 2001).

In cyanobacterial cells a 35 kD cytosol localized protein that non-covalently binds a single carotenoid molecule was found and named orange carotenoid protein (OCP). It is encoded by the *slr1963* gene in *Synechocystis*. The presence of this protein was found to be crucial for a blue-green light-dependent mechanism of excess energy dissipation from phycobilin-containing PS II external antenna – phycobilisome (PBS) (Wilson et al., 2006). In a cell OCP exists in two distinct forms. In darkness or in dim light it is in a stable, inactive dark orange form (OCP^o) which upon illumination with blue-green light, takes a metastable red active form (OCP^r) (Wilson et al., 2008). This active form exhibits an altered absorption spectrum. Only OCP^r can bind to phycobilisome and mediate energy dissipation as heat (Gwizdala et al., 2011). A spontaneous reversion to the inactive OCP^o form takes place in the dark, although this process was accelerated by the addition of what is known as a fluorescence recovery protein (FRP). This 15 kD protein is a product of the *slr1964* open reading frame in *Synechocystis*. It was found to be strongly associated with the photosynthetic membranes (Boulay et al., 2010).

Blue-green light causes changes in both protein and carotenoid conformation in orange carotenoid protein. 3'hydroxyechinenone which is bound to OCP in physiological conditions, seems to gain one conjugated double bond upon high intensity blue-green irradiation, but retains its all-trans conformation (Wilson et al., 2008). It was shown in mutant studies that echinenone and zeaxanthin can also bind to OCP. Zeaxanthin-OCP proved not to have any quenching capability.

Fig. 1. A simplified scheme of the carotenogenesis pathway in *Synechocystis*. The first step in carotenoid biosynthesis is phytoene (C_{40}) synthesis by the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP, C_{20}) (Martinez-Ferez et al. 1994), catalyzed by phytoene synthase encoded by the crtB gene. The sequential desaturation steps and cyclization of the ends of the molecules to generate carotenes are catalyzed by phytoene desaturase (crtP), ζ-carotene desaturase (crtQ) and lycopene cyclase. The resulting carotenes are further modified by enzymes such as β-carotene ketolase (crtW or crtO) and β-carotene hydroxylase (crtR) to generate various xantophylls. The major carotenoid species in *Synechocystis* are β-carotene, myxoxanthophyll, zeaxanthin and echinenone (Takaichi and Mochimaru, 2007).

Bold lines striking through arrows indicate the sites of enzyme knock-outs in mutants described in text.

Thus it seems that the presence of carbonyl groups is essential for preserving the functioning of energy dissipation (Puginelli et al., 2009).

The mechanism by which PBS fluorescence is quenched is still a matter for debate. It is known that the photo-converted OCP^r form binds to phycobilisome. It was also shown that carotenoids are able to quench excitation energy from tetrapyrrole molecules *in vitro* through energy transfer to their optically forbidden S1 state, coupled with an intramolecular charge-transfer state (Berera et al., 2006). Recent studies confirm that this is the photophysical mechanism underlying the protective function of OCP (Polivka et al., 2005; Berera et al., 2012)

11.3. Photosystem assembly

Carotenoidless mutants have been previously reported for the green algae *Scenedesmus obliquus*, and the purple bacteria *Rubrivivax gelatinosus* (Romer et al., 1995; Ouchane et al., 1997). Recently a cyanobacterial carotenoid-deficient *crtH/B* mutant was described by Sozer et al. (2010). All these mutants carry a disruption of the phytoene syntase encoding gene. Interestingly, mutants of *R. gelatinosus* were viable in the light while both *S. obliquus* and *Synechocystis* could grow only in the dark (Romer et al., 1995; Sozer et al., 2010). An *S. obliquus C-6E* strain was carotenoid and chlorophyll b-deficient, while all the chlorophyll *a* was accumulated in a fully active photosystem I complexes. No activity of photosystem II was detected (Romer et al., 1995). A cyanobacterial carotenoidless strain exhibited similar characteristics. In a *Synechocystis crtH/B* mutant there was a normal form of PSI, but not of PSII.

In *Synechocystis* mutants with β-carotene production impaired by an inactivation of ζ-carotene desaturase and/or phytoene desaturase genes a compound structurally related to β-carotene with one β-ionylidene ring (β-zeacarotene) was found to be sufficient for the accumulation of PSII (Bautista et al., 2005a, b). In these mutants, photosystem I complexes exhibited a complete loss of the electrochromic band shifts centered at 500–510 nm, indicating that β-carotene functions in the oxidation kinetics of P700 reaction centers (Bautista et al., 2005b). It is believed therefore, that carotenoids are indispensable for the formation of the photosystem II complex, but not that of photosystem I. However, the carotenoid composition may influence the electron transport around PSI in cyanobacteria.

The transcription levels of genes encoding D1, CP47, D2 and D2 with CP43 were examined and the efficiency of the transcription of these genes in *crtH/B* cells was similar to that of wild type. In contrast, the protein pattern of a mutant strain was seriously altered, as revealed by two-dimensional electrophoresis. While in wild type the most inten-

sively stained protein of PS II, the D1 protein, was found mainly in fully assembled monomeric and dimeric reaction centers of PSII, in a carotenoidless mutant the majority of the D1 protein was accumulated in RC47 complexes that lack CP43 inner antenna. When investigated by a semi-quantitative Western blot method PsaA and PsaB subunits of PSI, along with cytochrome b_6f subunits, were found to be present in similar amounts in wild-type and *crtH/B* mutant cells. In contrast, the levels of PSII subunit proteins D1 and D2 in the *crt H/B* strain were much lower than those in wild type. Protein CP47 was hardly detectable and protein CP43 was completely absent (Sozer et al., 2010). The photosystem I monomer prevailed in the mutant strain while in wild type the PSI trimer was the main form. This may suggest that the presence of carotenoids stabilizes the photosystem I trimer (Grotjohann and Fromme, 2005).

11.4. Perspectives

The data presented above indicate that the analysis of *Synechocystis* mutants with impaired genes encoding the enzymes of the carotenoid biosynthesis pathway reveal novel physiological functions of particular carotenoid pigments in cyanobacteria. Beyond their "classical" role as protective agents and auxiliary antenna pigments, the carotenoid function in the assembly and the functioning of photosystem I and photosystem II complexes has been clearly demonstrated. The *Synechocystis* mutants used in the analysis of carotenoid function are summarized in Table 1. So far, a detailed analysis has been performed for only a few enzymes of the carotenogenesis pathway. There are other physiological effects in cyanobacteria associated with carotenoids that could be explored by the use of mutants of the carotenogenesis pathway. For example, carotenoid influence on the physical properties of thylakoid membranes was investigated in several mutants including *crtR* and *crtO*. It was shown that in native thylakoid membranes the elimination of xanthophylls decreased fluidity in the inner membrane region under optimal growth conditions and increased fluidity under sublethal heat stress. Thus, the overall fluidity of native photosynthetic membranes in cyanobacteria may be influenced by the ratio of polar to non-polar carotenoid pools under different environmental conditions (Klodawska et al., 2012).

In summary, *Synechocysis* carotenogensis mutants offer a unique research tool to explore both the physiological function of particular carotenoid derivatives as well as the functioning of the carotenogenesis pathway as a whole. It is expected that the introduction of new mutants together with their physiological analysis may facilitate future studies on carotenoid biology.

Table 1

Mutants of carotenogenesis pathway in *Synechocystis* PCC 6803

Mutant	Genetic manipulation	Phenotype	Reference
Δ*crtO*	Inactivation of the gene encoding β-carotene ketolase CrtO	Not able to synthesize echinenone and 3'-hydroxyechinenone	Fernandez-Gonzalez et al. 1997; Schäfer et al. 2005;
Δ*crtR*	Inactivation of the gene encoding β-carotene hydroxylase CrtR	Not able to synthesize zeaxanthin, myxoxanthophyll and 3'-hydroxyechinenone	Schäfer et al. 2005
Δ*crtRO*	1. Inactivation of the gene encoding β-carotene hydroxylase CrtR 2. Inactivation of the gene encoding β-carotene ketolase CrtO	Not able to synthesize xanthophylls	Schäfer et al. 2005
Δ*crtH*	Inactivation of *cis* to *trans* isomerase CrtH	Not able to synthesize β-carotene in darkness;	Masamoto et al. 2001
Δ*crtHB*	1. Inactivation of the gene encoding *cis* to *trans* isomerase CrtH 2. Inactivation of the gene encoding phytoene synthase CrtB	1. Carotenoidless, not able to synthesize any carotenoid species 2. Defective in PS II assembly	Sözer et al. 2010
Δ*crtP*	Inactivation of the gene encoding phytoene desaturase CrtP	Not able to synthesize β-carotene	Bautista et al. 2005a,b
Δ*crtQ*	Inactivation of the gene encoding ζ-carotene desaturase CrtQ	Not able to synthesize β-carotene	Bautista et al. 2005a;
Δ*sll1213*	Inactivation of the gene encoding fucose synthetase	Not able to synthesize myxoxanthophyll	Mohamed et al., 2005
Δ*OCP*	Inactivation of the *slr1963* gene encoding OCP	1. Impaired in chlorophyll fluorescence quenching 2. Increased sensitivity to high intensities of white light	Wilson et al. 2006
Δ*FRP*	Inactivation of the *slr1964* gene encoding FRP	Decreased fluorescence recovery in low light conditions or darkness	Boulay et al. 2010

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Chapter 12. THE XANTHOPHYLL CYCLE AND ITS PHYSIOLOGICAL FUNCTIONS

Dariusz Latowski[1], Olga Dymova[2], Tamara Maslova[3], and Kazimierz Strzalka[1]

[1] Jagiellonian University, Krakow, Poland
[2] Institute of Biology, Russian Academy of Sciences, Syktyvkar, Russia
[3] Komarov Botanical Institute, Russian Academy of Sciences, Saint-Petersburg, Russia

Contents

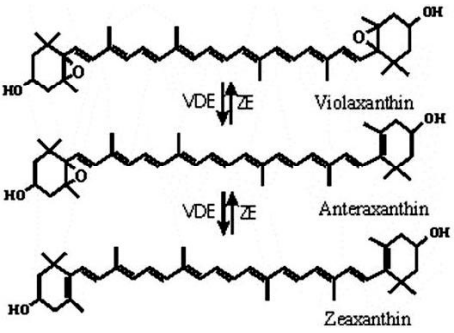
The study of the xanthophyll cycle has a long history. Sapozhnikov et al. (1957) were the first to observe that the violaxanthin (Vx) level in plant leaves responds dynamically to light-dark treatments. Later the violaxanthin cycle (Vx-cycle) sometimes also called the xanthophyll cycle was described in lettuce by Yamamoto et al. (1962). The leading role of prof. Sapozhnikov in this discovery is recognized by the plant physiologists of the world (Pfundel and Dilley, 1993; Eskling et al., 1997; Latowski et al., 2000).

12.1. What is the xanthophyll cycle?

The xanthophyll cycle is one of the most efficient mechanisms for optimising the amount of light necessary for photosynthesis and thus protecting plants and other photosynthesizing organisms under overexcitation conditions. The term "xanthophyll cycle" means the process which involves the enzymatic removal of the epoxy group from epoxy-xanthophylls (violaxanthin) under strong light. This creates de-epoxidised pigments (zeaxanthin), and the reverse reaction when de-epoxidised xanthophylls are re-epoxidised in low light or darkness. The first reaction is catalyzed by de-epoxidases and the second one by epoxidases (Fig. 1).

The xanthophyll cycle is associated with the membranes of thylakoids (Siefermann-Harms et al., 1978; Latowski et al., 2004, Jahns et al.,

Fig. 1. Xanthophyll cycle.



2009). De-epoxidases are located in the thylakoid lumen and epoxidases are postulated to be stromal enzymes, operating at rather neutral pH.

There are four xanthophylls known to undergo de-epoxidation in nature. These are violaxanthin, antheraxanthin (Ax), diadinoxanthin (Ddx) and lutein-epoxide (Lx). Depending on what kind of xanthophyll is de-epoxidised and what pigment is the product of this de-epoxidation, five different xanthophyll cycles have been described (Stransky and Hager, 1970; Bungard et al., 1999; Garcia et al., 2007, Jahns et al., 2009, Latowski et al., 2011). Four of them are based on β-xanthophylls and one on α-xanthophylls (Table 1).

The most commonly occurring type of xanthophyll cycle, and the most intensively studied, is the violaxanthin cycle. This type of xanthophyll cycle consists of the light-driven de-epoxidation of the diepoxide Vx through the intermediate monoepoxide Ax into zeaxanthin (Zx). This is carried out by the enzyme violaxanthin de-epoxidase (VDE). The dark epoxidation of Zx via Ax into Vx is carried out by the enzyme zeaxanthin epoxidase (ZE). This cycle is present in the chloroplasts of all higher plants studied to date, as well as in ferns, mosses, lichens, and some algae (Phaeophyta, Chlorophyta and Rhodophyta) (Hager, 1980; Siefermann-Harms, 1985; Pfundel et al., 1994; Kramer et al., 1999).

In the green alga *Mantoniella squamata* (I. Manton & M. Parke) T.V. Desikachary a shortened Vx-cycle has been described (Goss et al., 1998; Gilmore and Yamamoto, 2001). This cycle is also catalysed by VDE but this enzyme probably has reduced affinity for Ax, so that the de-epoxidation of Ax to Zx proceeds very slowly, whereas ZE rapidly drives the Ax back to Vx.

In the red algae *Gracilaria gracilis* (Stackhouse) Steentoft, L.M. Irvine & Farnham and *Gracilaria multipartite* (Clement) Harvey (Rmiki et al., 1996)

Table 1

Types of xanthophyll cycles

Xanthophyll cycle based on	
β-xanthophylls	α-xanthophylls
Violaxanthin cycle	Lutein epoxide cycle
Shortened violaxanthin cycle	
Antheraxanthin cycle	
Diadinoxanthin cycle	

another modification of the Vx-cycle has been proposed, although not fully demonstrated. This version of the xanthophyll cycle was postulated on the grounds of differences in pigment composition in these species of algae. No Vx is present in these algae and the xanthophyll cycle is postulated to be restricted to the interconversion between Ax and Zx.

In several algal groups (diatoms, phaeophytes, dinophytes and haptophytes) a different type of xanthophyll cycle is present (Stransky and Hager, 1970). It is called the diadinoxanthin cycle (Ddx-cycle). In this cycle monoepoxide Ddx is de-epoxidised to diatoxanthin in high light by an enzyme homologous to VDE called diadinoxanthin de-epoxidase (DDE). In the diatom *Phaeodactylum tricornutum* (Bohlin) simultaneous operation of the Vx- and Ddx-cycles has been described (Goss et al., 1998, Lohr and Wilhelm, 1999).

Another kind of xanthophyll cycle is based on the α-xanthophylls: lutein and lutein epoxide (Lx) and it is called the lutein-5,6-epoxide cycle (Lx-cycle). This cycle was first reported in green tomato fruits (Rabinowitch et al., 1975). Since Bungard et al. (1999) the lutein-epoxide cycle has been recognized in many diverse taxa (Garcia-Plazaola et al., 2003, 2007; Matsubara et al., 2003, 2008, 2012; Kruk and Szymanska, 2008; Forster et al., 2009, 2011).

The Lx-cycle involves the de-epoxidation of Lx which is monoepoxide to L and the reverse epoxidation of L to Lx (Ladygin, 2008). It has been proposed that the presence of Lx (and the Lx-cycle) could be the result of a recurrent mutation in the epoxidase gene that bestows the advantage of increasing its affinity for L (Garcia-Plazaola et al., 2007).

In the green alga *Caulerpa racemosa* (Forsskal) J. Agardh the operation of a new cycle has been recently suggested (Raniello et al., 2006). In this cycle another α-xanthophyll, siphonaxanthin is converted to L in response to light. However, this cycle is a rather nontypical xanthophyll cycle, because both pigments involved are non-epoxy xanthophylls.

12.2. Enzymes of the xanthophyll cycles

De-epoxidases. Three different groups of xanthophyll cycle enzymes have been indentified up to now. The first group involves violaxanthin de-epoxidases. These enzymes are strictly specific to 3 OH, 5,6-epoxy-carotenoids in configuration 3R, 5S, 6R, which are all-trans in the polyene chain (Yamamoto, 1979; Grotz et al., 1999).

Violaxanthin de-epoxidases (VDE) are encoded in nuclear DNA. In 1996 the cDNA of VDE from romaine lettuce was cloned for the first time and expressed in *Escherichia coli* (Bugos and Yamamoto, 1996). These experiments allowed a determination of the number of amino

acids (348 residues), and the calculation of the molecular mass of VDE as 39.9 kDa, which was close to the 43 kDa which resulted from its determination using PAGE (Rockholm and Yamamoto, 1996; Havir et al., 1997). The calculated isoelectric point amounted to 4.57, as compared with the 5.4 found experimentally (Rockholm and Yamamoto, 1996). Other genes of VDE indentified later encode proteins ranging from 446 to 478 amino acids with calculated molecular masses of 50 to 55 kDa. Each of the eight known VDE homologues possesses an N-terminal transit peptide that targets the protein to the chloroplast. Considering the cleavage of the transit peptide, the calculated molecular mass of the mature VDE proteins would be in the range of 39 to 40 kDa. VDE homologues show a conserved putative N-glycosylation site and 14 conserved Cys residues. Of those 14 Cys residues, 11 form a Cys-rich region in the N-terminal portion. The C-terminal portion contains 47% charged residues, most of which are Glu residues forming a Glu-rich region. It has been suggested that the xanthophyll cycle enzymes are lipocalin proteins, although recent research showed that these enzymes should be classified as lipocalin-like proteins (Bugos and Hieber, 1998; Hieber et al., 2000; Grzyb et al., 2006; Coesel et al., 2008). In lipocalin proteins highly conserved sites can be found for three short regions, called short conservative regions (SCR) (Flower et al., 1993). All VDE proteins possess the first lipocalin signature SCR1 next to the Cys-rich region. In six of the eight VDE sequences, SCR1 falls within the norm, while three VDE sequences show differences. All VDEs exhibit two invariant amino acids G and W that are key features of SCR1 (Flower et al., 2000). SCR2 is not present in the VDE sequences and SCR3 can be found in the Glu-rich C-terminal region, along with the conserved R residue (Charron et al., 2005).

Besides the lipocalin domain, there are also two other domains in the VDEs. One of them is the N-terminal region, enriched in Cys moieties, containing 11 of the 13 Cys found in VDE. This region most probably contains a-helices. The other, C-terminal, domain is charged and enriched in glutamyl residues and it probably contains long a-helices (Charron et al., 2005). A comparison of amino acid sequences (deduced from cDNA) indicates a high degree of homology among VDE proteins from different plants. VDEs from *Arabidopsis thaliana*, *Nicotiana tabacum* and *Lactuca sativa* differ just in nine amino acid residues (Hieber et al., 2000). The transit peptides of VDE do not show such high homology. Their structure is similar but the reported amino acid sequences agree in eight residues only (Bugos et al., 1998). Knowledge of the amino acid sequence helps in understanding some particular properties of VDE. A cysteine enriched domain is responsible for the inhibitory effect of dithiothreitol (DTT), which reduces the disulphide bonds in the enzyme molecule (Yamamoto and Kamite, 1972; Bugos

and Yamamoto, 1996). This inhibition (at pH 5.2 and 5.7) is reversible, but it is not reversible after iodoacetamide treatment (Arvidsson et al., 1996). After treatment at pH 7.2, the chemicals do not influence enzyme activity what means that in such conditions disulphide bonds are not exposed to the environment. This finding shows that the pH-dependent conformational changes in the VDE molecule are necessary for enzymatic activity.

VDE was identified as a water-soluble and lumen localised protein (Hager, 1969). VDE can be either unbound or bound to the thylakoid membrane depending on the lumenal pH. Connection to the membrane is important for enzymatic activity (Hager and Holocher, 1994). At pH lower than 6.0, all VDE molecules are associated with the membrane. If pH increases to 7.0 or more, the VDE exists in an unbound form. In pH 6.6, half of VDE molecules was found to be linked to the membrane (Bratt et al., 1995). These properties of VDE are useful for its purification (Yamamoto and Higashi, 1978). Membrane binding occurs by the C-terminal, charged domain of the enzyme. At low pH, glutamic acid residues are protonated, which can facilitate their association with the membrane (Bugos and Yamamoto, 1996; Hieber et al., 2000). The optimum pH for VDE activity *in vivo* is 4.8 and it increases to 5.2 after isolation (Hager, 1969). Recently, it was postulated that protonation of the histidine residues at low pH induces a conformational change in VDE, and hence indirectly regulates the binding of the enzyme to the thylakoid membrane (Emanuelsson et al., 2003; Gisselsson et al., 2004).

The VDEs in the primitive prasinophycean green algae *Mantoniella squamata* and *Ostreococcus tauri* constitute a special type of VDE. These enzymes show reduced affinity not only for Ax, as reported by Frommolt et al. (2001), but also for other tested mono-epoxides such as Ddx, Lx, cryptoxanthin epoxide (CxE), and neoxanthin (Nx). On the other hand, some xanthophylls with two epoxy-groups, e.g. cryptoxanthin-di-epoxide (CxDE), can be de-epoxidized with higher efficiency.

Although the overall amino acid sequences of the VDE from higher plants and the VDE from the prasinophycean algae show a high degree of homology, there are significant amino acid exchanges visible. The first exchange concerns threonine-112 which is changed to an aspartate in *O. tauri*. According to Saga et al. (2010) this amino acid plays an important role in the binding of ascorbate, the co-substrate of the VDE. The second exchange occurs within the DDW motif which is modified to a DTW sequence in the *Prasinophyceae*. These three amino acids are located in close vicinity to the binding site of the Vx molecule and the first aspartate residue of this motif (aspartate-177) was shown to be essential for enzyme activity, most probably by acting as a proton

donor to the Vx epoxy group (Saga et al., 2010). The exchange of aspartate-178 against an alanine reduced the activity of the mutated VDE to around 50% of wild-type activity. Since in the *O. tauri* sequence the second aspartate (aspartate-178) is exchanged to threonine, the alteration of the motif may have an important impact on the de-epoxidation of one of the two epoxy groups of the Vx molecule, which is observed as a higher level of Ax in the prasinophycean algae.

The second group of de-epoxidases involves Ddx de-epoxidases (DDEs). The genes of these enzymes were identified in diatoms. It was shown that they, like VDEs, consist of a cysteine-rich N-terminal domain, on SCR1 lipocalin domain, and a C-terminal glutamic acid-rich domain with on SCR2 sequence.

Both cysteine-rich N-terminal domains and SCR1 lipocalin domains are generally well conserved both among diatoms and plants, indicating that these proteins show similar folding and can bind the same molecules. The C-terminal Glu-rich domain is considerably less conserved among diatoms and plants. Whereas the C-terminal domain of plant VDEs contains on average 47% charged residues of which about 25% are glutamic acid residues, the percentage of charged amino acids in the DDE domains from *Phaeodactylum tricornatum* is 29 (37% Glu) and 13 (18% Glu) from *Thalassiosira pseudonana*, respectively. The divergence in the C-terminal domain most likely affects the pH-dependent binding of the diatom DDE to the thylakoid membrane (Arvidsson et al., 1996, Wehner et al., 2006). In comparison with spinach violaxanthin de-epoxidase (VDE) a much higher affinity of the DDE for the co-substrate ascorbate was found. The Km value of DDE at pH 5 (0.7 mM) is significantly lower than that observed for VDE (2.3 mM). The pH optimum of DDE activity was found at pH 5 at low ascorbate concentrations. At high ascorbate concentrations a strong shift of the pH optimum towards higher pH values was observed, and significant DDE activity was still present at almost neutral pH values. This is in contrast to VDE, where despite an ascorbate-induced slight shift towards higher pH values, enzyme activity was never observed above pH 6.5. The pH optimum of VDE was always found in a narrow range between pH 5 and 5.2, irrespective of the presence of high or low ascorbate concentrations. The high affinity of DDE for ascorbate indicates that, even with a limited availability of reduced ascorbate, high enzyme activity is possible at low pH values. On the other hand, at high ascorbate concentrations DDE activity can be shifted towards neutral pH values, thereby facilitating a very fast and strong response to small pH changes in the thylakoid lumen (Grouneva et al., 2006).

The activity of all isolated de-epoxidases can be measured *in vitro*. The reaction mixture has to contain additional components apart from Vx or Ddx – monogalactosyldiacylglycerol (MGDG) and ascorbic acid

(Hager, 1969; Siefermann and Yamamoto, 1975; Yamamoto and Higashi, 1978; Muller-Moule et al., 2002), which is thought to be an endogenous electron donor for de-epoxidation and a regulator of the activity of de-epoxidases (Bratt et al., 1995). There is strong evidence that the de-epoxidation of Vx and Ddx takes place in inverted hexagonal phase domains (Latowski et al., 2004; Goss et al., 2005) which are likely to be formed by the galactolipid MGDG in the thylakoid membrane.

Epoxidases. Another group of xanthophyll cycle enzymes are xanthophyll epoxidases (XEs). They are mainly called zeaxanthin epoxidases (ZEs) even if a xanthophyll different from Zx is used by these enzymes as a substrate. 12 complete gene sequences of different XEs have been identified. They are proteins ranging from 626 to 763 amino acids with a calculated molecular mass of 68 to 80 kD. As in the case of the VDE proteins, they also possess an N-terminal transit peptide that targets the protein to the chloroplast. After the cleavage of the transit peptide, the calculated molecular mass of the mature XE proteins ranges from 60 to 72 kD. XE homologues possess a conserved putative N-glycosylation site and two conserved Cys residues. The two invariant amino acids G and W that are key features of SCR1 are also present. XEs possess neither SCR2 nor SCR3 motifs. Additionaly, XEs contain an ADP-binding domain in their N-terminal portion and a FAD-binding domain in their C-terminal portion (Marin et al., 1996). It is also known that for their activity XEs require NADPH, FAD, ferredoxin and oxygen (Buch et al., 1996; Bouvier et al., 1996).

XEs are thought to be a monooxygenases, catalysing reaction of epoxidation in positions 5 and 6 of the xanthophyll rings (Hieber et al., 2000). XEs show 28% identity and 44% similarity with other monooxygenases and oxidases found in bacteria and cyanobacteria that also contain ADP-binding and FAD binding domains (Charron et al., 2005).

It was found that the *P. tricornutum* genome contains three copies of the XE gene. Proteins coded by these genes are marked here as XE1, XE2 and XE3 and they can catalyse the epoxidation of both Zx and Dtx. As compared to plant ZEs, the amino acid region covering lipocalin motif SCR1 is considerably larger in the diatom XE1 and XE2 proteins, but not in XE3 and the SCR1 motif consensus sequence is not conserved in any of the diatom XEs. All diatom XEs lack the C terminal forkhead-associated (FHA) domain that is normally found in plant ZEs. Interestingly, a transmembrane region is predicted in the C-terminal domain of XE3, which may have an effect on the localization and/or regulation of the diatom XEs. In this context it is worth noting that the epoxidation kinetics of diatoxanthin in diatoms under low light conditions is generally faster than the epoxidation of zeaxanthin

in higher plants and green algae (Goss et al., 2006), but in diatoms the presence of a proton gradient almost completely inhibits this reaction (Goss et al., 2006, Mewes and Richter, 2002). This is in strong contrast with the epoxidation reaction of higher plants, which occurs in the dark as well as in the light, and the level of zeaxanthin depends largely on the activity of VDE. The mechanism behind the peculiar light dependent XE activation/deactivation in diatoms is not yet understood, but it is possible that the divergent C-terminal domains of XE1, XE2 or XE3 may play a role. It is also worth to noticing that Zx epoxidation in higher plants is not only important for xanthophyll cycling, but also for abscisic acid (ABA) synthesis. Diatoms do not appear to synthesize this phytohormone (Coesel et al., 2008).

12.3. Functions of the xanthophyll cycle

Quenching of excess energy in PSII. This protective function is very essential when the absorption of light energy by chlorophyll exceeds its utilization during photosynthesis. Thorough and detailed studies by different research groups have shown a dependence between the content of Zx and non-photochemical quenching (NPQ) in chloroplasts (Demmig-Adams, 1990; Demmig-Adams and Adams, 1992; Ma et al., 2003). An even better correlation was found between NPQ and the total amount of Zx and Ax (Adams et al., 1995). An increase in NPQ after high light treatment and its correlation with Vx de-epoxidation in spinach (*Spinacia oleracea*) leaves, isolated chloroplasts and purified LHC complexes has also been observed (Ruban et al., 1999). Similarly, in diatoms, the NPQ level correlated well with the diatoxanthin (Dtx) amount, which was formed during the de-epoxidation of diadinoxanthin (Ddx) (Table 1) (Ruban et al., 1999, Lavaud et al., 2002a). In other experiments, the photoprotective action of Dtx during prolonged UV-A and UV-B illumination of diatoms (*Thalassiosira weissflogii*) has been demonstrated (Lavaud et al., 2002b). These UV-insensitive diatoms showed increased activity of the Dtx cycle as a response to light stress.

Studies of mutants of the green algae *Chlamydomonas* have also shown that L, similarly to the de-epoxidation products of Vx or Dtx, has a significant role in energy dissipation (Lavaud et al., 2002a). It has been also observed that the de-epoxidation of Lx to L facilitated the rapid engagement of NPQ, and that this process may be fine-tuned by concurrent Zx accumulation inducing strong energy dissipation in plants having both an Lx-cycle and Vx-cycle (Garcia et al., 2007).

On the other hand, the photoconversion of siphonaxanthin (Sx) to L detected in the green alga *Caulerpa racemosa*, showing as it does significant similarites to Vx–Ax interconversion, suggests a similar activation signal for these two mechanisms. In particular, both Ax

and L reached their highest values not only under high light, but also at sunrise when light intensity was lower (Young and Frank, 1996). This last feature suggests a similar and very high sensitivity of the two cycles to light. A photoprotective role of the interconversion between Sx and L may also be hypothesized, even if this hypothesis needs to be tested through detailed studies. In additional favour of this hypothesis are the biophysical similarities between L and Ax reported in the literature, the energetic state analysis revealing similar S_1 values for Ax and L (Young and Frank, 1996).

All of these observations show that the products of the xanthophyll cycles that are created under light conditions are effective quenchers of the chlorophyll excited states (Latowski et al., 2011).

Protection against lipid peroxidation. Degradation of lipids in pea leaves was observed after light stress (Havaux et al., 1991). In intensive light, the content of lipids in leaf cells decreased and the saturated/unsaturated lipid ratio increased. The lipid degradation was more significant when Zx formation was inhibited by DTT (Havaux et al., 1991). Similiar results came from experiments in which the lipid contents were measured in response to high illumination in *npq1* mutant (Havaux and Niyogi, 1999). In comparison to the wild *Arabidopsis* form, the *npq1* mutant had a significantly higher level of lipid photooxidation. Interestingly, in tomato leaves, the Zx level and lipid degradation (measured as ethylene formation) were also correlated. At 3 °C and in high light conditions (low level of Zx), ethylene production was intensive. But at 23 °C and in high light conditions, ethylene secretion was lower and the Zx content increased (Sarry et al., 1994). It is also possible that the xanthophyll cycle and Zx play a role in senescence, as a photoprotectant against lipid photooxidation (Munne-Bosch and Alegre, 2002).

Regardless of the differences among authors, the xanthophyll cycle is recognized as one of the main adaptation mechanisms responsible for a fast response to peroxidation and the creation of antioxidant substances in thylakoid membranes. These photoprotectants are Zx and Ax which can effectively quench singlet oxygen (Krinsky, 1979) and other reactive oxygen species (Burton and Ingold, 1984; Lim et al., 1992).

Blue light reception. Another postulated function of the xanthophyll cycle is blue light reception. Some researchers have suggested that Zx is responsible for blue light-dependent stomata opening (Srivastava and Zeiger, 1995; Quinones et al., 1996; Talbott et al., 2002), chloroplast movement (Tlalka et al., 1999), and phototrophism (Quinones and Zeiger, 1994). The HPLC measurement of Zx level as a function of chloroplast movement in strong and weak light in *Lemma trisulca* established a good correlation between these two variables (Tlalka et

al., 1999). Because of this finding, Zx is regarded as a photoreceptor in blue light stimulated chloroplast movement. Moreover, *Zea mais* coleoptiles, grown in darkness, did not show blue light dependent phototrophism. Interestingly, these coleoptiles did not contain Zx. The degree of increase in Zx level (regulated by red light, darkness periods, and use of DTT) correlated well with the blue light stimulated phototrophism of maize coleoptiles (Quinones and Zeiger, 1994). The DTT-related inhibition of Zx synthesis consequently inhibited blue light-dependent stomata opening in *Vicia faba* epidermis, which was thought to be proof of the photoreceptor function of Zx (Srivastava and Zeiger, 1995). Similar conclusions came from analysis of the blue light phototrophism or stomata opening activity spectrum and the absorption spectrum of Zx (Quinones et al., 1996). However, Palmer et al. (1996) showed in experiments correlating the level of protein phosphorylation and blue light induced phototropism that there is no connection between Zx, or any other carotenoid, and phototropism.

Regulation of membrane physical properties. The modulatory effects of carotenoids on the physical properties of model and natural membranes have been known since 70-ties. Soon after the characterisationof the xanthophyll cycle, there was a suggestion that this process may regulate the physical properties of thylakoid membranes (Siefermann and Yamamoto, 1975a; Yamamoto, 1979). This hypothesis has been confirmed by other studies (Gruszecki and Strzalka, 1991, 2005; Tardy and Havaux, 1997).

In natural and model membranes, zeaxanthin appears to have the strongest influence on such membrane properties as temperature of phase transition, molecular dynamics, permeability and polarity gradient (Gruszecki and Strzalka, 1991, 2005; Kostecka-Gugala et al., 2003). It was shown for two antarctic bacterial species, *Sphingobacterium antarcticus* and *Sphingobacterium multivorum*, that membrane fluidity is regulated mainly by Zx (Jagannadham et al., 2000). Zx is able to stiffen the membrane in the liquid-crystalline phase and this process is connected to the orientation of this xanthophyll in the membrane. The long axis of the Zx molecule is oriented perpendicularly to the membrane plane and ionone rings are anchored in peripheral, polar zones of the membrane (Gruszecki and Sielewiesiuk, 1991). In addition, Zx decreased the molecular dynamics of lipids, limiting heat-induced lipid mobility and preventing membrane disorganisation (Gruszecki and Strzalka, 1991; Strzalka and Gruszecki, 1997; Tardy and Havaux, 1997).

Regulation of abscisic acid synthesis. One of the xanthophyll cycle's function is its involvement in abscisic acid (ABA) synthesis. It has been shown (Marin et al., 1996; Pogson et al., 1996; Audran et al., 1998) that Vx is one of the intermediate products in this process. One

could suppose that conditions which cause an increase in VDE activity would cause a decrease in ABA production.

The existence of a relationship between xanthophyll cycle pigments and ABA synthesis was proven for *Nicotiana tabacum*. After ozone treatment the Vx level in leaves strongly decreased, while Ax and Zx contents changed insignificantly. This changed level was accompanied by a pronounced increase in ABA level (Pasqualini et al., 1999).

Oxygen evolution. Based on the chemical structures of xanthophylls and the experiments with the use of O^{18} isotope and some inhibitors some time ago, it was postulated that the violaxanthin cycle can directly participate in oxygen evolution (Maslova et al., 1978, 1996; Sapozhnikov et al., 1965). It was suggested that de-epoxidation of the epoxy groups of violaxanthin results in the evolution of molecular oxygen, and the photoreduction of these groups leads to the formation of water.

However, the hypothesis of the role of violaxanthin conversion in photosynthetic oxygen evolution has not yet been definitively proved.

12.4. Effects of temperature and irradiance on xanthophyll cycle

Factors like irradiance and temperature play an important role in the operation of the xanthophyll cycle. Some studies have investigated the effect of certain temperatures in combination with various irradiation intensities (Haldimann, 1995; Adams and Demmig-Adams, 2004).

Light-dependent conversion of violaxanthin. Light plays a decisive role in the regulation of biosynthesis and accumulation of Vx-cycle pigments by activating Zx formation through the light-induced expression both of violaxanthin deepoxidase (VDE) and zeaxanthin epoxidase (ZE) (Hieber e.a., 2000; Charron et al., 2005; Grzyb et al., 2006). Light also affects the rates of reactions in the xanthophyll cycle (Sapozhnikov et al., 1973; Maslova et al., 1996). When the light intensity was below a certain threshold level, there was no Zx production. The level of threshold intensity depends on the light-requirement of the plants.

Experimental comparison of the light curves of xanthophyll conversion and of photosynthesis have uncovered a great discrepancy between them. In leaves exposed to subthreshold light level, no marked changes in the content of individual xanthophylls of the cycle were observed, whereas photosynthesis had already reached 70% of its saturation value (Siefermann-Harms, 1985). The absence of visible changes in the xanthophyll content at subthreshold light intensities may be the result of equal rates of the direct and backward reactions of the Vx-cycle.

If the backward reaction of the cycle was stopped by salicylaldoxime (SA, inhibitor of epoxidation) similar light curves for photosynthesis and Zx content were obtained (Maslova et al., 1996). An analogous result was obtained for another inhibitor of epoxidation – glutathione at a temperature of +40 °C. The light curves of the de-epoxidation reaction of the Vx-cycle are similar to the light curves typical of photosynthesis (Maslova et al., 1996).

The action of light on the Vx-cycle may not be direct. The reactions of the cycle may be induced by red light, which is absorbed by chlorophyll and then transferred to xanthophylls.

On the illumination of thylakoids the transition carotenoids occur in a triplet state through the interaction of carotenoids with a triplet-excited sensibilizer (chlorophyll):

$^3Chl^* + {}^1Car \rightarrow {}^1Chl + {}^3Car^*$.

Such interaction is only possible when the carotenoid has nine or more conjugated double bonds. Zx, Ax and Vx have 11, 10 and 9 of these bonds respectively. Carotenoids deactivate the chlorophyll triplet state, which can produce a highly reactive oxident, singlet oxygen $^1O_2$. This «dangerous» singlet oxygen can also be effectively deactivated by carotenoids:

$^1O_2{}^* + {}^1Car \rightarrow {}^3O_2 + {}^3Car^*$.

Chlorophyll, oxygen and the carotenoids return to their ground state:

$^3Car^* \rightarrow {}^1Car$ + heat.

The specific protective function belongs to Zx, which accepts excess energy from excited chlorophyll. This results in the quenching of chlorophyll fluorescence and the thermal dissipation of surplus excitation energy in the antennae complexes of PSII as heat takes place (Demmig-Adams and Adams, 1992; Gilmore, 1997; Chen et al., 2011). Zx is believed to interact in a pH-dependent manner with CP22 protein encoded by the PsbS gene to facilitate energy dissipation (Li et al., 2002). In addition, Ax is also directly involved in the photoprotective dissipation process and is able to trap surplus excitation energy in the antennae complexes of PSII and to dissipate it harmlessly as heat (Gilmore and Yamamoto, 2001).

The amount of Vx, Ax and Zx (VAZ pool) have on several occasions been reported to increase when plants are grown under high light intensities (Demmig-Adams et al., 1995; Demmig-Adams and Adams, 1996; Logan et al., 1996). It has been shown by M. Eskling et al. (1997) that the VAZ pool doubles within 3-5 days in plants transferred from low to high light. As plants can upregulate the levels of Vx, one possible way of accumulating a higher amount of Zx in the membrane would be to increase the amount of VDE. However, it has been found that VDE is less active in spinach grown under high light intensities

compared to spinach in lower light (Eskling and Akerlund, 1998). This lower activity is not due to inhibition of the enzyme, but to a lower level of the enzyme even though other proteins in the thylakoid increase as in normal plant development. Bugos and Yamamoto (1996) found an increase in the level of the mRNA transcript of the gene for VDE in romaine lettuce during development. There is no obvious increase or decrease in the level of the transcript upon a shift from low to high light.

Temperature-dependent conversion of violaxanthin. At low temperatures Zx formation was found to be strongly suppressed (Demmig-Adams et al., 1989; Bilger and Bjorkman, 1991). It has recently been shown that in a tropical fruit crop (*Musa sp.*), and in a temperate spring flowering plant (*Allium ursinum* L.) only at temperatures above 30 °C did Zx increase and Vx decrease significantly. At nonlethal low temperatures the relative amounts of xanthophylls remained unchanged (Dongsansuk et al., 2013).

Temperature has a strong influence not only on the rate of Vx to Zx conversion but also on the degree of maximal conversion in thylakoids from non-cold acclimated spinach leaves (Arvidsson et al., 1997). In isolated thylakoid membranes at low pH in the presence of ascorbate, only 50% conversion of Vx was detected at 4 °C, whereas at 25 °C and 37 °C the degree of conversion was 70% and 80%, respectively. Raising the temperature further had no direct effect on the availability of Vx. The addition of extra VDE or the inhibition of endogenous VDE followed by the addition of VDE had no or little effect on the de-epoxidation rate at 25 °C and 37 °C, but at 4 °C the presence of both endogenous and added VDE increased the rate dramatically. This implies that at 4 °C the limiting factor in the de-epoxidation rate is enzyme activity. Although the reaction rate was changed, the final level was not changed by the addition of extra VDE. Koroleva et al (1995) observed faster de-epoxidation kinetics and a higher proportion of Vx that became de-epoxidised at higher temperature in non-acclimated leaves. Moderately elevated temperature has also been found to increase the accessibility of Vx in the membrane to VDE (Havaux and Tardy, 1996).

Under natural conditions plants are often grown under contrasting temperatures and light conditions. We earlier studied the activity of the Vx-cycle by comparing bugle plants (*Ajuga reptans* L.) grown under the canopy of European yew trees in low light (150 μmol m^{-2} s^{-1}) (shade plants) and plants grown in full sunlight (1000 μmol m^{-2} s^{-1}) (sun plants) (Dymova et al., 2010). To analyze the effect of temperature, overwintered (winter-green) and newly formed (summer-green) leaves were compared. The winter-green leaves were collected at the beginning of March, and the summer-green ones were sampled at the beginning of June. In our experiments the relative content of two xanthophyll

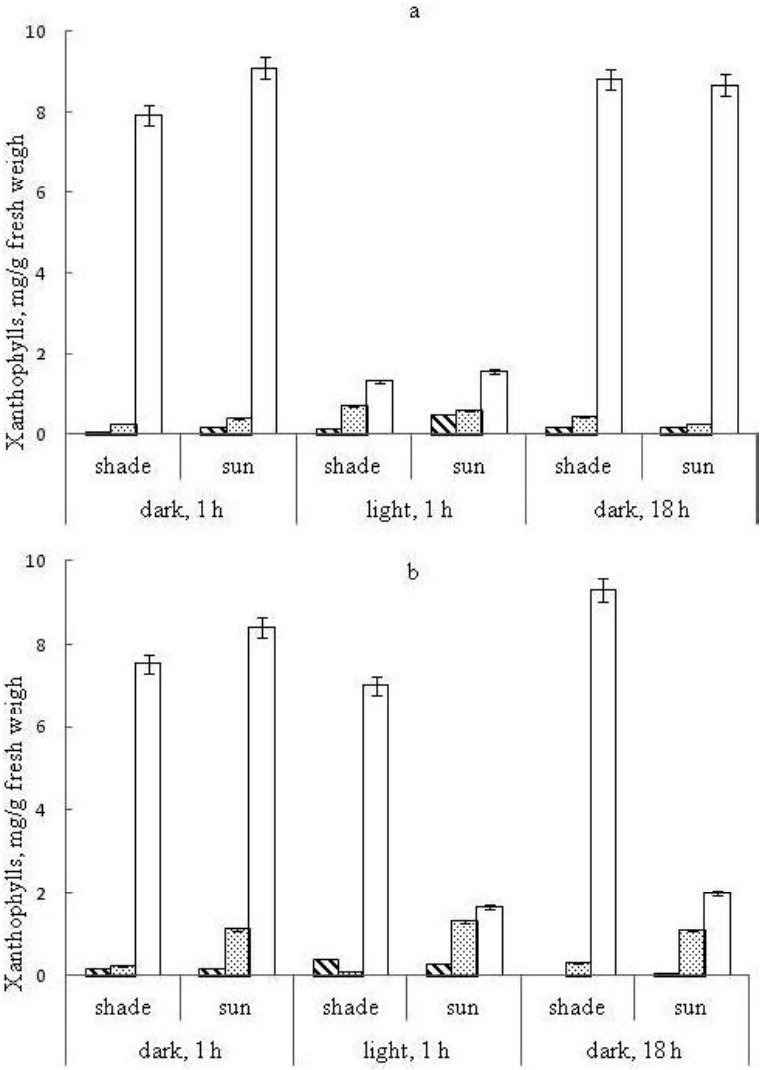


Fig. 2. Light-dependent changes in the content of xanthophylls (VXC components) in (a) winter-green and (b) summer-green leaves of shade-type (shade) and sun-type (sun) *Ajuga reptans* plants.

Dark, 1 h – 1-h dark incubation; Light, 1 h – 1-h exposure to light at PAR 2000 μmol/(m^2 s); Dark, 18 h – incubation in darkness for 18 h. 1 – zeaxanthin; 2 – antheraxanthin; 3 – violaxanthin. *Source*: Dymova et al. 2010.

pigments, Zx and Ax increased under high irradiance in overwintered leaves of sun and shade plants (Fig. 2). During the light-dependent carotenoid transformation, up to 85% of the Vx pool was converted to Zx. In summer-green leaves stimulation by strong light diminished the VAZ pool in sun plants but had little influence on the total content of the xanthophylls in shade plants. These results are in line with previous data (Demmig-Adams and Adams, 1992) and suggest that the extent of the VAZ pool can be reduced or remain unchanged at high irradiance. The reduction of the total VAZ pool is likely related to xanthophyll degradation under light stress.

The relative content of Ax and Zx in the total content of the VAZ pool characterizes the de-epoxidation state. We observed high capacity for de-epoxidation in the overwintered leaves of *Ajuga reptans*, which provides evidence that the Vx-cycle is active in the early spring through the combined action of high insolation and low temperatures. According to some studies (Demmig-Adams and Adams, 1992), the elevated content of VAZ pool pigments is characteristic of sun plant leaves and overwintering leaves. The increase in the extent of Vx de-epoxidation is beneficial for the retention of PSII activity in the light at low temperature. As we showed earlier (Dymova and Golovko, 2001), the overwintered leaves of *Ajuga reptans* were capable of active photosynthesis.

An increase in the extent of de-epoxidation under high irradiance and at low temperatures enables the thermal dissipation of excess energy, thus preventing the photodestruction of chloroplast pigment–protein complexes in overwintered leaves during the early spring period. It is supposed that the operation of the Vx-cycle plays an important protective role in overwintered leaves and promotes the adaptation of the photosynthetic apparatus to high light conditions. In the summer-green leaves of sun plants, the light-dependent accumulation of Zx and the comparatively high Ax content protect the photosynthetic apparatus against photoinduced destruction. The violaxanthin deepoxidation state (DEPS) level in summer-green leaves of shade plants remained low after the action of strong light. The low content of the VAZ pool and the low rate of interconversion can be explained by assuming that the carotenoids of summer-green leaves in shade plants perform the function of light harvesting.

Thus, the photosynthetic apparatus in the overwintered leaves of winter-green grassy plants, which start functioning in the early spring period, is characterized by high Vx-cycle activity. The operation of the the light-induced Vx-cycle largely controls the photoprotection of the photosynthetic apparatus in the summer-green leaves of sun bugle plants.

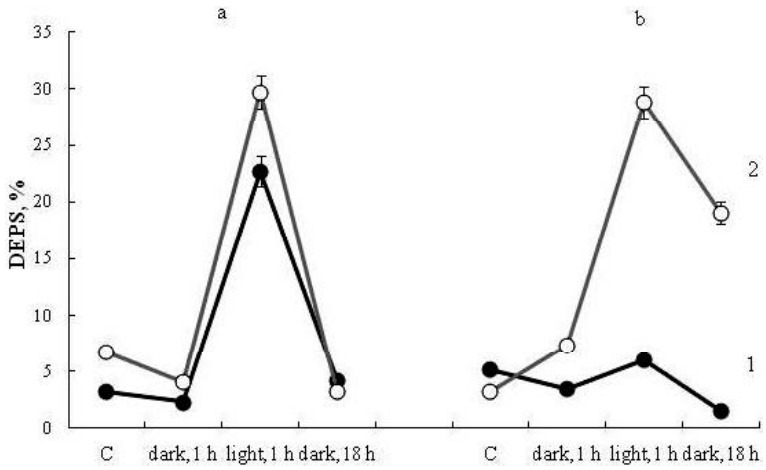


Fig. 3. Violaxanthin deepoxidation state (DEPS) in (a) winter-green and (b) summer-green leaves of (1) shade-type and (2) sun-type *Ajuga reptans* plants.

C – control plants; dark, 1 h – 1-h dark incubation; light, 1 h – 1-h exposure to light at PAR 2000 μmol/(m2 s); dark, 18 h – incubation in darkness for 18 h. *Source*: Dymova et al. 2010.

It should be noted that under unfavorable conditions (in winter) in the photosynthetic apparatus of evergreen conifers (*Taxus cuspidate*, *Thuja occidentalis*) Zx can carry out another protective function. This xanthophyll can be oxidized to the secondary carotenoid – rhodoxanthin, which forms a light filter under conditions of high insolation in winter (Maslova et al., 2009). Earlier Maslova et al. (2003) compared the pigment apparatus of two ecological groups – ephemeroids and summer-green plants. Ephemeroids grow and photosynthesize in early spring, when low temperatures and high insolation promotes the photoinhibition of photosynthesis. These results show that the content of carotenoids in the light-harvesting complex (LHC) of plastids and the active Vx pool are higher in ephemeroids than in summer-green species. Thus, potentially, the photosynthetic apparatus in ephemeroid leaves is better served by photoprotective mechanisms such as the functioning of the Vx-cycle. Under chilling temperatures of 5–10 °C and full insolation in early spring a high photosynthetic rate in ephemeroids was maintained.

12.5. Conclusion

Thus, the general roles for the xanthophyll cycle involve photoprotection, participation in the photochemical reactions of photosynthesis

(light harvesting), quenching of excess energy in photosystem II, protection against oxidative stress to lipids, the photoreception of blue light, the modulation of the physical properties of the thylakoid membrane, and the regulation of abscisic acid synthesis.

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Chapter 13. PIGMENTS AND PRODUCTIVITY OF THE CROP PLANTS

Tamara K. Golovko and Galina N. Tabalenkova
Institute of Biology, Russian Academy of Sciences, Syktyvkar, Russia

Contents

13.1. Introduction

Productivity of crop plants depends on genotypic and physiological-biochemical properties of the species and varieties, as well as on their interaction with the environment. Genetically determined growth and development processes form a foundation of crop productivity. Substrate and energy supply required for plant growth depends on photosynthesis, respiration and allocation of the assimilates.

Photosynthesis is the overall process whereby plants, algae and some prokaryotes use light energy to synthesize organic compounds. More specifically, light energy drives the synthesis of carbohydrates from carbon dioxide and water with generation of oxygen. In thylakoids of plant chloroplasts the light energy is converted into chemical energy by two photosystems. The end products of these thylakoid reactions are high-energy compounds ATP and NADPH, which are used for synthesis of sugars in carbon fixation reactions.

The energy of sunlight is absorbed by the pigments in plant chloroplasts. Chlorophylls and carotenoids associated with specific membrane-bound proteins form pigment assemblies for optimization of light energy absorption. Besides contribution to improve light harvesting in the pigment-protein complexes carotenoids may also participate efficiently in prevention of harmful photooxidative reactions related to the presence of oxygen.

The photosynthetic pigments are responsible for transferring and converting light energy. The process, called energy transfer, is an important vehicle for movement of the absorbed light energy through an array of the pigment molecules. The light energy is transferred from the antenna pigments to the specialized chlorophyll in the reaction center. This causes electron rearrangement in chlorophyll of the reaction center and reduction of the acceptor molecule by the excited pigment. The acceptor transfers its extra electron to the secondary acceptor and further down the electron transport chain. Hence, without any pigments light cannot be absorbed and, therefore, energy cannot be stored in biomass of the plants.

This chapter deals with the role of photosynthetic pigments in production of crop plants.

13.2. Physiological factors of productivity

A theoretical concept of photosynthetic plant productivity appeared in the middle of the past century (Nichiporovich, 1956, 1988). The concept related the crop yield to the leaf area and duration of leaf activity during the vegetation. The leaf photosynthetic potential (LPHP) is an integral index which accounts for the leaf area and for duration of leaf photosynthetic activity over the vegetation period.

The leaf area per a phytocenosis area unit or the Leaf Area Index (LAI, m^2/m^2) is an important indicator of the ability of phytocenosis to absorb light efficiently. The LAI value depends on the planting density. Crops with the LAI of 4 m^2/m^2 are able to assimilate about 90-95% of the incident photosynthetic active radiation (PAR). The LAI value of highly-productive crops is 5-6 m^2/m^2 (Dwyer et al., 1991; Grosse and Leon, 1992; Bondada, 2001). For the most agrocenoses optimal LAI is 4-5 m^2/m^2. The higher LAI may affect negatively on the yield due to cenotic interaction between the plants and strong competition for light, moisture, and mineral nutrition. The cost of the plant biomass maintenance increases as well.

To assimilate more light, plants tend to increase the leaf mass per the plant mass (LWR, g/g) or the leaf area per the leaf mass (specific leaf area, SLA, m^2/g) values. The SLA value varies within 0.01-0.06 m^2/g for agricultural plants. The SLA depends mostly on light intensity. The SLA is an integral structural tool of the plant for adjustment of its architecture within phytocenose for better use of PAR.

Specific leaf mass (SLM) characterizes the efficiency of the use of assimilates for leaf formation (per a unit of the leaf surface). SLM is a reciprocal value of SLA (leaf mass-to-area ratio, g/m^2). The SLM value varies considerably between the species and growing conditions (Potter and Jones, 1977; Muller and Moorby, 1990; Lambers and

Poorter, 1992; Hunt and Cornelissen, 1997). The crop plant SLM varies within the range of 40-100 g/m^2 and correlates with the net photosynthesis rate (Pn) and phytomass accumulation (Bedenko and Kolomeichenko, 2005).

Crop productivity depends on the allocation of photosynthetically assimilated carbon. Sink-source relations unite the plant parts and organs into an integral system (Geiger, 1969; Wareing and Patrick, 1973; Mokronosov, 1983; Kiriziy, 2004). According to the sink-source concept photosynthetic productivity depends on the epigenetic factors such as the growth rate (*de novo* synthesis of structural biomass), accumulation and storage of the assimilates. An increase of plant demand in carbohydrates and energy increases its photosynthetic activity appreciably. At the same time, the environmental conditions, influencing the growth and transport of assimilates, can significantly modify epigenetic regulation of photosynthesis.

13.3. Pigment system and photosynthetic productivity of plants

The main function of the pigments is the absorption of light energy. The pigment apparatus of plant chloroplasts contains two chlorophylls (Chl *a* and *b*) and carotenoids. Chlorophyll absorbs light in the visible region, mainly in the red and blue parts of the spectrum. Maxima of chlorophyll absorption spectrum fit maximum of the solar energy at the Earth's surface. Chl *a* in the reaction center is able to realize the energy transduction process. The rest photosynthetic pigments participate in absorption and transfer of the energy. Carotenoids participate in light absorption and photoprotection. These pigments protect plants against photooxidation, occuring when the reactive oxygen species (ROS) are formed via interaction of dioxygen with the excited chlorophyll molecules. These ROS can attack the biomolecules, destroy them and kill the cell eventually. Many herbicides kill the plants by blocking the synthesis of carotenoids, causing photooxidation of the plant itself.

In chloroplasts, the pigments form the stable complexes with proteins. The idea that chlorophyll is bound to proteins in the live leaves was proposed by V. Lubimenko (1963). Currently, four main protein-pigment complexes are known: the central (core) and the antenna complexes of the PSI and PSII. Light absorbed by carotenoids and Chl *b* bound to light-harvesting complex (LHC) proteins is rapidly transferred to Chl *a* and then to the other antenna pigments that are associated with the reaction centre.

Accordingly, there is no photosynthesis, no accumulation and transformation of solar energy into chemical energy without chlorophylls. The photosynthetic pigment content and composition reflect the condition of photosynthetic apparatus.

13.3.1. Photosynthetic pigments in leaves and non-leafy green organs of the main crop cultures

Generally, leaf chlorophyll content is dependent on the plant species, as well as the cultivar, life cycle, age and growing conditions. During leaf ontogenesis Chl is first accumulated to a certain maximum level; afterwards the pigments are degraded at the rate higher than that of their synthesis. The ontogenetic changes in Chl *a* and Chl *b* amount per a unit of fresh or dry weight or the leaf area of the herbs and deciduous trees are reviewed by Z. Shestak (1977). Among the crop plants, the leaves of legumes are characterized by the highest Chl content. A decrease of the leaf area in modern cultivars of pea is accompanied by a decrease of leaf Chl and an increase of Chl amount in stipules and tendrils (Kof et al., 2004).

The chlorophyll content in the leaves of the main crops cultivated at the northern border of the temperate zone varied from 1.5 to 3.5 mg/g FW (Kurenkova, 1998). At the heading stage of plant development, barley leaves (10 cultivars) accumulated 2.5-3.1 mgChl/g FW (Golovko et al., 2004). The chlorophyll amount in the leaves of 28 wheat genotypes and 10 maize genotypes varied from 1.7 to 2.6 mg/g FW and from 1.2 to 3.1 mg/g FW, respectively (cited from Zelensky, 1980). Shimko et al. (2009) showed that Chl amount in the leaves of 12 rye forms varied from 20 to 40 g/m^2, and no correlation between the Chl content and plant productivity was found. Other authors (Lu et al., 2001; Shadchina, 2010) revealed that high productivity of certain winter wheat cultivars resulted in the longer lifespan of the leaves. The Chl content in the leaves and electron transport in the PSII of highly productive cultivars were similar to those recorded in the low-productivity cultivars.

According to the data obtained by us (Golovko et al., 2004), the barley leaves are characterized by maximal contents of Chl (3 mg/g FW) and carotenoids (1.2 mg/g) at the earring phase. The bulk of barley chlorophylls (62-67%) is located in its leaf blades. Apart from the leaves, other green parts of the plants contained the photosynthetic pigments. However, the Chl content of the stems and heads of the cereals was less than that in the leaf blades. At grain ripening phase, the leaves contained 18-27%, the stems with the sheaths – 42-44%, and the ears – 28-30% of the whole plant Chl. The chlorophyll content in the winter barley leaves was made up 3-4 mg/g FW, that of the stems with bundle sheaths and the heads – about 1.0 and 0.4 mg/g FW, respectively (Derendovskaya and Josan, 2008). At the blooming stage, the oat panicles and leaves differed in the Chl content by 5 times. The Chl content in the panicles was only 0.5 mg/g FW.

Non-leaf green parts contribute to photosynthetic gas-exchange of the plants. Wheat and barley ear photosynthesis amounted to ca. 30%

of total photosynthesis at the earring stage and increased to 50-60% at the beginning of the grain ripening phase (Kumakov, 1980; Kumakov et al., 1983). The wheat ears and a flag leaf contributed 15 and 19%, respectively, into the daily carbon budget of the whole plant at the milky ripeness stage (Cupina et al., 1975). The contribution of non-leaf green organs of the winter wheat (Bezostaya 1, Mironovskaya 808 and Eritrospermum 7020 cultivars) to the total photosynthetic productivity was 31-38% for the whole vegetation period (Kumakov, 1980). The contribution of non-leaf parts to the winter wheat productivity increased under adverse environmental conditions, especially under drought.

The pigment content exhibits remarkable changes during leaf ontogenesis (Sestak, 1966, 1977; Mokronosov, 1981). Generally, Chl per a unit of leaf area is accumulated until maximum leaf area and the thickness is reached and declined thereafter. Usually, specific leaf weight increased slowly in the course of leaf growth and maturation. During ontogenesis of primary leaf of *Phaselus vulgaris* L. (cv. Jantar) maximum Chl content was about 4 mg/dm^2 after 17-18 days from sowing (Sestak, 1977). At this time Chl *a*/*b* ratio increased 3 times and then declined. The specific leaf weight increased slowly and reached 0.4 g DW/dm^2 by the end of the leaf lifespan.

According to Mokronosov (1981), maximum Chl content per chloroplast ($2.5 \cdot 10^9$ molecules) in the 7th leaf of the main potato shoot (Malakhit cultivar) was observed at the end of the growth period of the leaf. The growth of this leaf finished for 26-28 days upon unfolding, the leaf remained fully functional for two weeks after that time. At the beginning of its development the leaf grows due to the cell division, further an increase in the leaf area occurs due to the cell extension. The contents of Chl *a* and Chl *b* increased synchronously, and the Chl *a*/*b* ratio was equal to 1 during the first 10 days of the growth, increasing to 2.5 later on. The chlorophyll content of the spongy mesophyll chloroplasts was 15-20% higher than that in the palisade mesophyll chloroplasts.

Kurenkova (1998) showed that Chl is rapidly accumulated during the growth of potato leaves. Maximal Chl content in the 3-5th leaves was observed on the 10-12th day from appearance, and in the 12-14th leaves – on the 23-26th day. The first leaves grew more rapidly as compared to the leaves appeared later. Collectively, the Chl content of the leaves depends on the period of Chl accumulation and the lifespan of the leaves. In tree leaves with a long lifespan Chl accumulation often displays a plateau (Sestak, 1977).

At the beginning of the spring wheat earring phase, the Chl content of the flag leaf was 2-3 times higher than that in the senescent leaves of the lower layers (Andriyanova and Tarchevskiy, 2000). Dunaeva

and Galeeva (1980) found that Chl content in a wheat flag leaf increased till the beginning of the ear ripening; Chl *a* accumulated more rapidly before blooming, whereas Chl *b* – upon blooming. The green pigment content of the upper layer leaves in sunflower was 3 times higher as compared to the lower leaves (Kurenkova, 1998). The Chl *a*/*b* ratio was 2 and 3 in the lower and the upper leaves, respectively.

During annual plant ontogenesis maximal amount of pigments was observed mostly at the beginning of the reproductive phase. In spring barley and oat the leaf Chl content was 1.5-2.0 times higher in the blooming than that one in the other phases. In potato plants maximal pigment content was recorded at the beginning of the blooming phase. At the same time, the leaves of the late ripe cultivars accumulated more Chl (1.8-2.0 mg/g FW) relative to the early ripe cultivars (1.3 mg/g FW).

Changes in the content of leaf pigment in the course of perennial plant ontogenesis are similar to those of annual plants. Perennial forage grasses usually bloom in the second year after sowing. Maximal Chl content, 3.3-3.8 mg/g FW, was observed in the leaves of *Trifolium pratense* during the blooming phase. The pigment content was declined by 30% to the start of clover head maturation.

In functional leaves the chlorophyll fund is continuously regenerated. Since the daily pigment turnover involves a comparatively small proportion of their molecules, it is difficult to estimate its turnover rate. The Chl turnover rate varies from species to species and depends on the stage of plant (leaf) development. The half-life time of chlorophyll molecules in young soybean leaves was ca. 100 h (Sironval, 1963), and in barley leaf greened for 48 h it was about 58 h (Hendry and Stobart, 1986).

There are several reports indicative of the diurnal variation of Chl content (Hendry and Stobart, 1986; Buinova, 1988). Daily measurements of Chl content in strawberry showed that the amplitude of its oscillations depended on the stage of leaf development and the length of daylight (Sironval, 1963). S. Kurenkova (1998) could not obtain reliable data on diurnal variation of the leaf pigment content in plants cultivated at higher latitudes (under long day conditions). For example, at moderate temperatures (15-17 °C) and sufficient irrigation Chl content of the clover leaves was ca. 2.9 mg/g FW or 0.43 g/m^2 during the day period and decreased by 10-15% at night hours.

Under stressful conditions (high illumination and air temperature, water and mineral nutrition deficiency), the concentration of green pigments may decrease due to imbalance of chlorophyll synthesis and degradation.

The rate of Chl degradation increases in the senescing plant leaves. Many plant cultures (wheat, maize, oats, potato, etc.) contain conside-

rably less Chl in the leaves at harvest time, hence they turn yellow-green due to retention of carotenoids. One of the reasons for Chl loss in this case is believed to be degradation of nitrogen-containing compounds and reutilization of the nitrogen from the leaves before they die. Chlorophyll degradation is catalyzed by chlorophyllase and Mg-dechelatase, both enzymes are chloroplast localized (Shioi et al., 1991). Nitrogen and magnesium are exported from the leaves into the other plant organs, whereby the other chlorophyll catabolism products are accumulated in vacuoles of senescing leaves.

13.3.2. Assimilation index

Pigments are crucial for light energy absorption and transformation during the photosynthesis. Thus, numerous researchers looked for a quantitative relationship between the photosynthetic rate and the Chl content. K. Willstatter and A. Stoll (1918) introduced the index to characterize the efficiency of Chl. They suggested to calculate the photosynthetic rate per unit Chl (A, mg CO_2/(mg Chl·h)). If the same A value was recorded in different plant species, it could evidence on proportionality between the rate of photosynthesis and the Chl content. It was found, however, that A varies significantly with species and cultivar. Moreover, A varies in the same species depending on the leaf age and the stage of plant development. For instance, in case of spring wheat Kharkovskaya 46 the A value dropped by almost 30% between the tillering and earing stages (Andriyanova and Tarchevskiy, 2000). At the milky ripeness stage, the A value for the spring wheat leaves was 8 times lower as compared to that one at the earing stage. Potato leaves also endured essential changes in the A value during their ontogenesis (Mokronosov, 1981). A very young (3-day old) potato leaf had the A value of 30 mg CO_2/(mg Chl·h), whereas a mature (30-40-day old) potato leaf – only 3-5 mg CO_2/(mg Chl·h). The A value increased up to 12-15 mg CO_2/(mg Chl·h) in the senescing (60-day old) yellowing potato leaf. A decrease in the value of photosynthetic rate per unit Chl found in mature leaves was a result of the Chl accumulation in response to shading.

Breeding of domestic plants also did not reveal real dependence of photosynthetic rate on the content of leaf pigments. Positive relationship between the content of leaf pigments and the photosynthetic rate was found in different cabbage species and cultivars. However, domestic varieties of barley and wheat lacked such a relationship (Jifford and Jenkins, 1987; Kabanova et al., 2001). Nevertheless, dense-standing phytocenoses of new barley varieties bred for intensive agriculture accumulated Chl (per unit leaf area) by 15-18% higher than the leaves of the Viner 'extensive' variety (Chaika et al., 1995). The ratio between photosynthesis and the pigment content in leaves of feed cabbage and

sugar beet was affected by light conditions (Shestak, 1966). Close correlation between the photosynthetic rate (per unit leaf area) and the pigment content was characteristic of 15 trefoil clones under high light (Machler and Nosberger, 1978). Under lower light the correlation became insignificant. Shestak (1977) analyzed a great body of evidence obtained by different authors and concluded that direct dependence of photosynthetic rate on the Chl is observed only at low Chl contents.

Plant species and varieties differ by A value. Apparently, the Chl content is not a leaf photosynthetic rate-limiting factor in plant varieties adapted to certain agro-climatic conditions.

13.3.3. Chlorophyll index, chlorophyll photosynthetic potential and their relation to plant productivity

Special indices were proposed to describe the role of pigments in production process and crop formation (Tarchevskiy and Andriyanova, 1980; Andriyanova and Tarchevskiy, 2000). The chlorophyll index (ChlI) characterizes total pigment amount in the whole plant or per sown area unit at certain time. The chlorophyll photosynthetic potential (ChlPhP) is an integral index estimated as a sum of daily ChlI values for a certain time period or a whole vegetation period. These indices characterize the stage of development of plant photosynthetic apparatus and its potential. They are used for comparison of plant varieties and species, and for the study of contribution of the leaves and non-leaf green organs to photosynthesis and the yield.

During the earing phase, the ChlI equaled 2.6 and 1.6 mg/plant for the short-stem highly productive Italia 178 variety of spring wheat and long-stem Kubanka variety, respectively (Andriyanova and Tarchevskiy, 2000). Ears contributed 25%, leaf blades – 43%, leaf sheaths – 19% and stems – 13% to the ChlI of Italia 178 variety. For the low-productive Kubanka variety the contributions were 16, 58, 17, and 25%, respectively. In the milky ripeness phase, contributions of leaves and ears to total photosynthesis of Italia 178 wheat variety were 41 and 32%, and those in Kubanka variety – 33 and 20%, respectively. Despite the fact that Italia 178 plants contained Chl 1.6 times higher, they were characterized by lower efficiency of the Chl. At grain-formation stage, the A value (the ratio of photosynthetic rate to the Chl content) equaled 6.3 and 8.5 mg CO_2/(mg Chl·h) for the Italia 178 and Kubanka wheat varieties, correspondingly. Though the Chl efficiency appeared to be inversely dependent on its content, the difference in the A value between the varieties was less (1.3 times) than that in the Chl content (1.6 times).

Ju. Anriyanova and I. Tarchevskiy (2000) reported data on the Chl photosynthetic potential of wheat agrocenoses for a vegetation period. The ChlPhP value for the spring wheat Saratovskaya 29 variety was

2.75±0.14 t Chl/ha and for Kharkovskaya 46 variety – 3.58±0.28 t Chl/ha. The ChlPhP value for Kharkovskaya 46 variety was remarkably higher than that for Saratovskaya 29. This is in agreement with the data on biomass accumulation and yield of the wheat. The same authors revealed high correlation between the yield and the ChlPhP for different winter rye, millet, pea, and potato cultivars. High correlation was found between the yield and the ChlPhP value in beans and buckwheat too.

The ChlPhP value varies greatly depending on plant variety and growth conditions. According to the data obtained by us (Golovko et al., 2004), the ChlPhP value of barley agrocenoses comprised ca. 0.67-1.25 t Chl/ha for vegetation period depending on the variety. The Dina barley variety was characterized by the highest ChlPhP value and high grain yields.

The ChlPhP value was ca. 8% higher in the late ripening variety of spring wheat as compared to the early ripening variety (970 and 890 kg/(day·ha), respectively); the same was true for their production capacity (877 and 745 g/m^2, respectively) (Petrova et al., 2002). The whole-plant Chl content and the ChlI value were shown to correlate with the production capacity in barley (r = 0.70) (Derendovskaya and Josan, 2008).

Consequently, ChlI and ChlPhP represent a good measure of potential photosynthetic productivity and yield in cereals and other agricultural plants. The Chl content is an important factor of yield formation. Plant cultures with high ChlPhP normally produce rich yield. Chlorophylls of cereals and legumes reproductive organs contribute to their yields.

13.4. The effects of cultivation conditions on pigment accumulation and chlorophyll photosynthetic potential structure of plants

In agriculture, the established species and the varieties to the local climate are used. Nevertheless, crop productivity greatly depends on soil management, especially on mineral nutrition. Fertilizers affected the chlorophyll photosynthetic potential value and the contribution of different organs to ChlPhP value of the winter rye (Andriyanova and Tarchevskiy, 2000). Fertilizers applied in the amount of $N_{60}P_{60}K_{60}$ kg/ha increased the ChlPhP up to 2.2 t Chl/ha for a vegetation period, that was 1.7 times higher as compared to the non-fertilized control. The plant biomass increased by 20% and the grain yield - by 35%. The higher amount of the fertilizers reduced grain production and did not affect the ChlPhP value.

Application of the fertilizers increased the ChlPhP value of the spring wheat by 1.5-2.0 times and the grain crop – by 70-80% as compared to the non-fertilized plants (Pryadkina et al., 2011). The

grain productivity was directly proportional to the ChlPhP value. The ChlPhP of the non-fertilized wheat was 120-160 kg chlorophyll/(day·ha).

An improvement of mineral nutrition, especially by application of the nitrogen fertilizer, increased greatly the pigment accumulation in the fodder plants. The Chl amount in annual ryegrass leaves was 1.5 times higher on the well fertilized plots (90 kg N/ha) as compared to that one on poorly fertilized plots (30 kg N/ha) (Golovko et al., 1992). In addition, Chl *a* was accumulated more rapidly than Chl *b*, and the Chl *a*/*b* ratio increased from 1.8 up to 2.8. The leaf contribution to the whole plant pigment content was higher under well fertilization. The nitrogen supply affected the ChPhP value. Provided with enough nitrogen, annual ryegrass plants had the ChPhP value by 1.5-4.0 times higher than those suffering from nitrogen deficiency in the soil. The ChPhP value attained 1 t/ha for the period from sprouting till milky grain ripeness under favorable weather conditions. The ChPhP value of the annual ryegrass agrocenosis depended on the plant density. The Chl amount in plants grown in the low-density agrocenosis (300-400 plants/m^2) was 10-30% lower than in the thicken ones (900-1100 plants/m^2). The plants exposed to weaker cenotic interaction and competition in the low-density community. The ChlPhP values of the dense cenoses were higher, and they accumulated as much biomass as compared to the low-density cenoses. The Chl productivity varied within the range of 20-30 mg dry weight/(mg Chl·day), depending on weather, mineral nutrition, and crop density. The Chl productivity value of the thicken cenoses was 1.3-1.5 times lower as compared to the low-density cenoses.

Cereal species differ by photoperiodic sensitivity. Barley is known to grow better under long day. Short photoperiod did not exert a significant effect on the Chl content in barley plants, but increased the rates of photosynthesis and dark respiration (Ivanova et al., 1980). An increase in photosynthetic rate under short day compensated reduction of daily duration of barley leaf photosynthesis. Similar results we obtained with *Solanum stoloniferum* plants exposed to short day (11 h) conditions for a long time (20 days) (Markarov et al., 1993). Potato leaf photosynthetic capacity increased by 15% and the Chl amount – by 40%.

Drought induced changes in the ChlI of the whole plant. Under drought conditions the contribution of ear to the ChlI value of wheat of Saratovskay 29 variety increased from 10 to 24%, the contribution of stem – from 32 to 40%. At the same time the contribution of leaf laminas decreased from 33 to 14% (Andrijanova and Tarchevskiy, 2000). An increase of contributions of the non-leaf organs to the total amount of Chl in plants was also found in buckwheat. The increased leaves' contribution in winter rye under drought was observed. The treatment of plants with malic acid (10^{-4} M) increased their resistance

to drought and promoted Chl accumulation. The Chl content in the whole-plant and the ChlPhP value increased, on the average, by 1.5-1.8 times. We revealed that the ChlI value of barley plants increased by 40% and the ear biomass – by 20-25% when the seeds were treated with malic acid solution before sowing (Golovko et al., 2004). The inoculation of barley seeds by rezoagrin, a preparation of the free-living rhizospheric nitrogen-fixing bacteria, had a positive effect on Chl accumulation and crop yield.

Ju. Andrijnova and I. Tarchevskiy (2000) revealed that productivity of agrocenosis depends strongly on the ChlPhP index in the range of low ChlPhP values (to 600-800 kg/ha for the vegetation period). Remarkably, this relationship reveals no saturation at high ChlPhP values (Fig. 1).

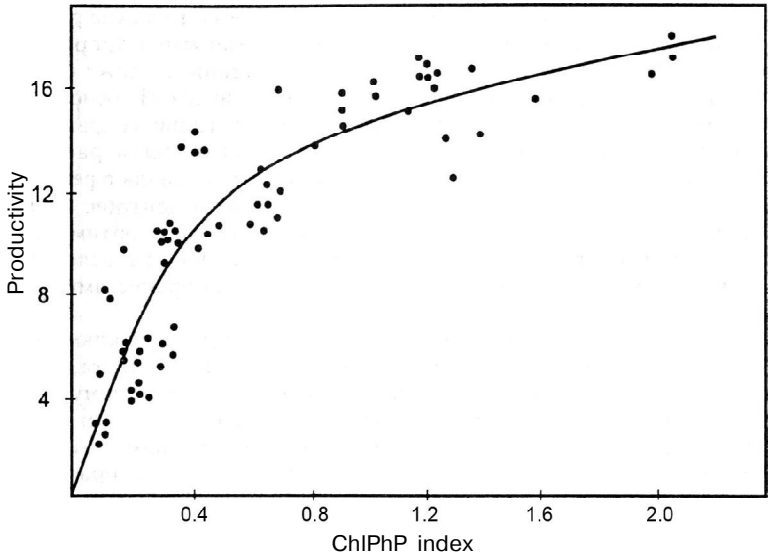

Fig. 1. Productivity of crops (t/ha) as function of the chlorophyll photosynthetic potential index (t/ha). *Source*: Andrijnova and Tarchevskiy 2000.

13.5. Summary and conclusion

Crop productivity has complex dependence on the absorption, transformation, and accumulation of light energy in form of plant biomass. Pigments influence directly on plant productivity. This chapter is focused on the role of photosynthetic pigments in this process.

Highly efficient phytocenoses accumulate defined quantities of the pigments in the special photosynthetic organs, *i.e.* leaves. The leaf

area index of the most agricultural plants varies from 3 to 8 m^2/m^2. The plant cultures with the LAI of 4-5 m^2/m^2 can absorb up to 95% of photosynthetic active radiation, accumulating 1-3 g Chl/m^2 sown area. Leaves usually contain 70-80% of the whole plant pigments. The contribution of green non-leafy plant organs and parts varies greatly, depending on plant species, variety, and the stage of development. The ChlPhP index takes into account contribution of non-leaf organs into yield formation. Cereals and legumes accumulate the required amounts of pigments in stems and reproductive organs. In most cases, the productivity of cultural plants depends on the ChlPhP value.

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Part 4. ECOLOGY OF PHOTOSYNTHETIC PIGMENTS AND PIGMENT-PROTEIN COMPLEXES

Chapter 14. PIGMENT CHARACTERISTICS OF THE PLANTS OF NORTHERN ECOSYSTEMS AND THEIR CORRELATION WITH PHOTOSYNTHETIC ACTIVITY

Olga Dymova, Igor Dalke, and Tamara Golovko
Institute of Biology, Russian Academy of Sciences, Syktyvkar, Russia

Contents

14.1. Introduction

In nature, plants frequently experience a wide range of stresses. Climate change is expected to influence plant photosynthesis, development and biomass growth in many regions of the world, including boreal conditions. An investigation of the functional plasticity is necessary for understanding the responses to environmental conditions, distribution of the species, predicting vegetation dynamics under global climatic changes. Leaves are the main plant organs optimized for photosynthesis. The indices of photosynthetic apparatus such as com-

position, the content and the ratio of pigments are considered to be the most informative for characterizing functional state of the plant (Lubimenko, 1963; Zalenskiy, 1977; Maslova and Popova, 1993; Pyankov and Mokronosov, 1993; Bazzaz, 1996; Larcher, 2003; Dymova and Golovko, 2007; Golovko et al., 2007). In addition, the assessment of photosynthetic pigments, and consequently their relationships, is an important indicator of senescence (Brown et al., 1991).

Chlorophylls and carotenoids are required for photosynthesis. Chlorophylls are necessary to capture the light energy and primary electron donors. Light received by the chlorophyll in a leaf is either absorbed or used in photochemistry (i.e. photochemical quenching; BolhaAr-Nordenkampf et al., 1989), lost as heat (i.e. non-photochemical quenching; Bjorkman and Demmig-Adams, 1995) or as fluorescence (Demmig and Winter, 1988; Schreiber et al., 1995). Carotenoids play crucial roles both in light harvesting and energy dissipation for protection of photosynthetic structures. Up to date, the spectral characteristics and biosynthesis of photosynthetic pigments have been studied, a concept of antenna complex and reaction centers has been developed, the fundamental mechanisms of photosynthesis have been revealed. At the same time, diversity of the plant species and ecological conditions of their habitat make investigation of the pigments' role in stability and regulation of the activity of photosynthetic apparatus an urgent issue. Qualitative content and quantitative composition of the pigments, their ratio changes are important and responsive characteristics of plants physiological state and their photosynthetic apparatus (Popova et al., 1989; Kornushenko and Solovjova, 1992; Maslova and Popova, 1993; Golovko et al., 2007). In addition, measuring gas exchange and chlorophyll content repeatedly on the same leaves in field may provide useful information on the relationship between these parameters (Schaper and Chacko, 1991). However, the relationship between photosynthesis and pigments' content has been rarely examined in plants from different botanical and geographical zones (Lukyanova et al., 1986).

The North-East European Russia is a unique region in continental Europe. Here, plant growth is restricted by lack of warmth, short vegetative period and poor soils. Photosynthetic apparatus, including pigment complex, is experienced additional stress at these severe conditions.

The current study focuses on the pigment contents and CO_2 assimilation of the plants within taiga cold climate environments of the North-East European Russia. This study examines an idea of increasing the role of pigments in tolerance and productivity of photosynthesis of the northern plants.

14.2. Photosynthetic pigment contents in the northern plants

We studied the pigment contents and gas exchange of more than 100 plant species inhabiting in three different sites on the European North-East of Russia: 1) the Sub-Polar Ural Mountains (65°22′ N, 60°46′ E), 2) the South Tyman (62°45′ N, 55°49′ E), and 3) the Middle Vychegda basin (61°38′ N, 50°43′ E).

The main climatic traits of these regions are shown in Table 1. The Sub-Polar Ural Mountains (the extremely north taiga subzone) is characterized by the most severe climate and the short vegetative period. The climate in the Middle Vychegda (the middle taiga subzone) is warmer, but the growing season in this region does not exceed 100-110 days. The average annual air temperature only slightly exceeds 1 °C. Though the region of the South Tyman belongs to the middle taiga subzone, it differs from the Middle Vychegda by temperature regime and duration of the vegetative period.

Table 2 shows the list of the examined plant species in each region. All 121 plant species have been investigated, most of them are boreal herbaceous plants and reflect the structure of floristic complexes in the regions under study.

Leaf samples for pigments analyses were collected from 20-30 plants of each species at the beginning-middle July (2004-2012). Analyses were carried out on mature healthy current-year leaves (herbs) or current-year shoots (*Empetrum nigrum* and *E. hermaphroditum, Lycopodium clavatum* and *L. annotinum, Diphasium complanatum*). The second-year needles were collected from evergreen conifers (*Abies sibirica, Juniperus communis, Picea abies, Pinus sibirica*). The majority of herbaceous plants flowered during the sampling.

Table 1

Mean traits of different places in the European North-East

Traits	Subpolar Ural Mountaines	The South Tyman	The Middle Vychegda basin
Mean annual air temperature, °C	–4.8	–1.5	+1
Mean July air temperature, °C	+13	+15	+17
Total of temperature above +5 °C	1070	1550	1800
The duration of growing season (above +5 °C), days	105–110	133	150
The duration of active growth period (above +10 °C), days	60–70	80–90	100–110
Mean annual precipitation, mm	600–685	660–750	650–765

Source: Courtesy of the Komi Republic Centre for Hydrometeorology and Environmental Monitoring, Russia.

Table 2

List of the species from different places in the European North-East. Species presented with note of its life form and geographical status

№	Sub-Polar Ural Mountaines	The South Tyman	The Middle taiga subzone
1	*Achillea nigrescens* (E.Mey) Rydb (H,B)	*Aconitum septentrionale* Koelle. (H, B)	*Abies sibirica* Ledeb. (T, B)
2	*Alchemilla murbeckiana* Buser (H,B)	*Antennaria dioica* (L.) Gaertn. (H, B)	*Achillea millefolium* L. (H, B)
3	*Amoria repens* (L.) C.Presl (H,B)	*Aster sibiricus* L. (H, A)	*Aconitum septentrionale* Koelle (H, B)
4	*Arctous alpina* (L.) Niedz. (SH, A+AA)	*Astragalus danicus* Retz. (H, C)	*Alchemilla* sp. (H, C)
5	*Artemisia tilesii* Ledeb. (H, A+AA)	*Calamagrostis epigeios* (L.) Roth (H, B)	*Alisma plantago-aquatica* L. (H, B)
6	*Astragalus frigidus* (L) A.Gray (H, A)	*Caltha palustris* L. (H, B)	*Antennaria dioica* (L.) Gaertn. (H, B)
7	*Astragalus norvegicus* Grauer (H, A+AA)	*Cortusa matthioli* L. (H, B)	*Bistorta major* S.F. Gray (H, B)
8	*Atragene sibirica* L. (SH, B)	*Cotoneaster melanocarpa* Lodd. (SH, B)	*Bromopsis inermis* Leyss. (H, B)
9	*Betula nana* L. (SH, A)	*Crepis sibirica* L. (H, B)	*Butomus umbellatus* L. (H, B)
10	*Bartsia alpina* L. (H, A+AA)	*Cypripedium calceolus* L. (SH, B)	*Calla palustris* L. (H, B)
11	*Calamagrostis purpurea (Trin.) Trin.* (H, B)	*Dactylorhiza fuchsii* (Druce) Soo (H, B)	*Chenopodium album* L. (H, B)
12	*Carex aquatilis* Wahlenb. (H, B)	*Dendranthema zawadskii* (Herbich) Tzvel. (H, R)	*Comarum palustre* L. (H, B)
13	*Cystopteris dickieana* R. Sim. (H, A+AA)	*Diphasiastrum complanatum* (L.) Rothm. (C-M, B)	*Deschampsia cespitosa* (L.) Beauv (H, B)
14	*Diapensia lapponica* L. (SH, A+AA)	*Dryas octopetala* L. (SH, AA)	*Dryopteris filix-mas* (L.) Schott. (F, B)
15	*Empetrum hermaphroditum* (Lange) (SH, A)	*Dryopteris filix-mas* (L.) Schott. (F, B)	*Elytrigia repens* (L.) Nevski (H, B)

№	Sub-Polar Ural Mountaines	The South Tyman	The Middle taiga subzone
16	*Hedysarum arcticum* B. Fedtsh. (H, A+AA)	*Equisetum palustre* L. (H, B)	*Empetrum nigrum* L. (SH, A)
17	*Larix sibirica* Ledeb. (T, B)	*Epipactis atrorubens* (Hoffm.) Besser. (H, B)	*Filipendula ulmaria* (L.) Maxim (H, B)
18	*Ledum decumbens* L (H, A)	*Geum rivale* L. (H, B)	*Galium boreale* L. (H, B)
19	*Lycopodium clavatum* L. (H, A)	*Gymnadenia conopsea* (L.) R.Br. (H, B)	*Gymnocarpium dryopteris* (L.) (H, B)
20	*Lycopodium annotinum* L. (H, B)	*Gymnocarpium robertianum* (Hoffm.) Newm. (SH, B)	*Hylotelephium triphyllum* (Haw.) Holub (H, B)
21	*Pedicularis verticillata* L. (H, A+AA)	*Juniperus communis* L. (SH, B)	*Hypericum maculatum* Crantz (H, B)
22	*Pentaphylloides fruticosa* (L.) O. Schwarz. (SH, B)	*Lathyrus pratensis* L. (H, B)	*Juniperus communis* L. (SH, B)
23	*Phyllodoce caerulea* (L.) Bab. (SH, A+AA)	*Lathyrus vernus* (L). Bernh. (H, B)	*Ledum palustre* L. (SH, B)
24	*Pyrola rotundifolia* L. (H, B)	*Lycopodium annotinum* L. (C-M, B)	*Leontodon autumnalis* L. (T, B)
25	*Rhodiola rosea* L. (H, A)	*Melica nutans* L. (H, B)	*Lycopodium clavatum* L. (C-M, B)
26	*Rosa acicularis* Lindley. (SH, B)	*Paeonia anomala* L. (H, B)	*Maianthemum bifolium* (L.) F.W.Schmidt (H, B)
27	*Rubus chamaemorus* L. (H, A)	*Paris quadrifolia* L. (H, B)	*Oxalis acetosella* L. (H, B)
28	*Salix dasyclados* Wimm. (T, B)	*Parnassia palustris* L. (H, B)	*Paris quadrifolia* L. (H, B)
29	*Salix nummullaria* L. (SH, A+AA)	*Pedicularis verticillata* L. (H, AA)	*Petasites spurius* (Retz.) Reichenb. (H, A)
30	*Salix reticulata* L. (SH, A+AA)	*Petasites radiatus* (J.F.Gmel.) Holub. (H, A)	*Pimpinella saxifraga* L. (H, B)
31	*Tanacetum bipinnatum* (L.) Sch. Bip. (H, A)	*Pinguicula vulgaris* L. (H, A)	*Picea abies* (L.) Karst. (T, B)
32	*Vaccinium myrtillus* L. (SH, B)	*Pinus sibirica* Du Tour (T, B)	*Pinus sibirica* Du Tour (T, B)

№	Sub-Polar Ural Mountaines	The South Tyman	The Middle taiga subzone
33	*Vaccinium uliginosum* L. (SH, B)	*Plantago media* L. (H, B)	*Plantago major* L. (T, B)
34	*Valeriana wolgensis* Kazak. (H, B)	*Polygonum viviparum* L. (H, AA)	*Polygonum aviculare* L. (H, B)
35	*Woodsia glabella* R.Br. (H, A+AA)	*Pyrola rotundifolia* L. (H, B)	*Pyrola rotundifolia* L. (H, B)
36		*Sanguisorba officinalis* L. (H, B)	*Rorippa amphibia* (L) Bess (H, B)
37		*Saussurea alpina* (L.) DC. (H, AA)	*Rosa majalis* Herrm. (SH, B)
38		*Saxifraga hirculus* L. (H, A)	*Rubus saxatilis* L. (H, B)
39		*Thymus talijevii* Klok. et Shost. (SH, B)	*Rubus chamaemorus* L. (H, A)
40		*Tofieldia pusilla* L. (H, AA)	*Taraxacum officinale* Wigg. (H, B)
41		*Tussilago farfara* L. (H, B)	*Thalictrum simplex* L. (H, B)
42		*Vaccinum uliginosum* L. (SH, A)	*Trifolium medium* L. (H, B)
43		*Valeriana capitata* Pallas. (H, B)	*Trifolium pratense* L. (H, B)
44		*Valeriana wolgensis* Kazak. (H, B)	*Veronica longifolia* L. (H, B)
45		*Vicia cracca* L. (H, B)	*Vaccinium myrtillus* L. (SH, B)
46		*Vicia sylvatica* L. (H, B)	*Vaccinium uliginosum* L. (SH, A)
47		*Woodsia glabella* R.Br. (SH, AA)	*Vaccinium vitis-idaea* L. (SH, A)

Note: life-form groups: H – herb, T – tree, SH – shrub; C-M – club-moss; latitudinal groups: A – arctic, AA – arctic and alpine, B – boreal species; S – steppe, R – mountain species.

Latin names of species given by Cherepanov (1995).

Leaf chlorophyll (Chl) and carotenoid (Car) contents were measured by the UV-1700 spectrophotometer («Shimadzu», Japan) in acetone extracts at 662 (Chl *a*), and 644 (Chl *b*), and 470 nm (total Car). Chlorophyll portion in LHC (LHC-Chl) was calculated, assuming that total Chl *b* was located in the LHC, and the Chl *a*/*b* ratio in this

complex was equal to 1.2 (Lichtenthaler, 1987). Leaf CO_2-exchange rate was measured with an infrared gas analyzer LCPro+ (ADC, UK).

14.2.1. The pigment complex of the Sub-Polar Ural Mountains plants

Differences in accumulation of photosynthetic pigments were revealed between the species (Fig. 1). The concentration of Chl and Car varied in the ranges of 1.5-14.0 and 0.5-5.0 mg/g dry weight (DW), respectively. The legume plants (*Astragalus norvegicus, A. frigidus, Hedysarum arcticum*) were characterized by the higher green pigments' content. Conifer (*Larix sibirica*), dwarf shrubs (*Empetrum hermaphroditum, Phyllodoce caerulea, Ledum decumbens, Vaccinium uliginosum*), club mosses (*Lycopodium clavatum* and *L. annotinum*) and herb (*Diapensia lapponica*) had a very low Chl content. In the leaves of more than 60% of the examined species, the content of green pigments consisted of 3-6 mg/g DW. These concentrations can be considered as relatively low.

The Chl *a*/*b* ratio varied from 2.0 to 3.5, but it was higher (3.8-4.0) in *Bartsia alpina, Salix dasyclados, Astragalus frigidus* and *Hedysarum arcticum*. In most of the species, Chl, belonging to the light-harvesting complex (LHC-Chl), consisted of 55-65% of total. In *B. alpina*, *A. frigidus, H. arcticum* and woody *S. dasyclados* leaves the LHC-Chl values were the lowest (43-45%). The leaves of *Arctous alpine* and *Pyrola rotundifolia* were characterized by the highest LHC-Chl level (70-80%).

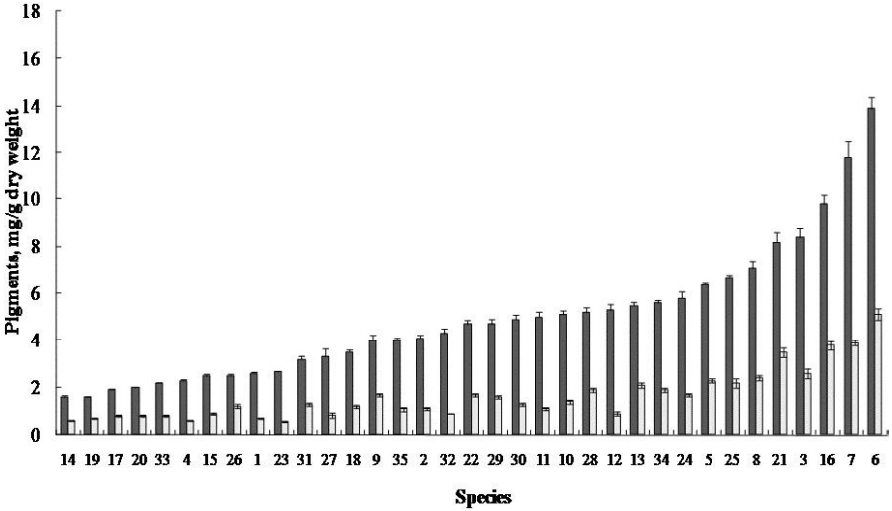

Fig. 1. Chlorophyll (dark symbols) and carotenoid (light symbols) contents in the leaves of the Sub-Polar Ural Mountains plants. Species names see according to the numbers in Table 2. *Source*: Golovko et al. 2011.

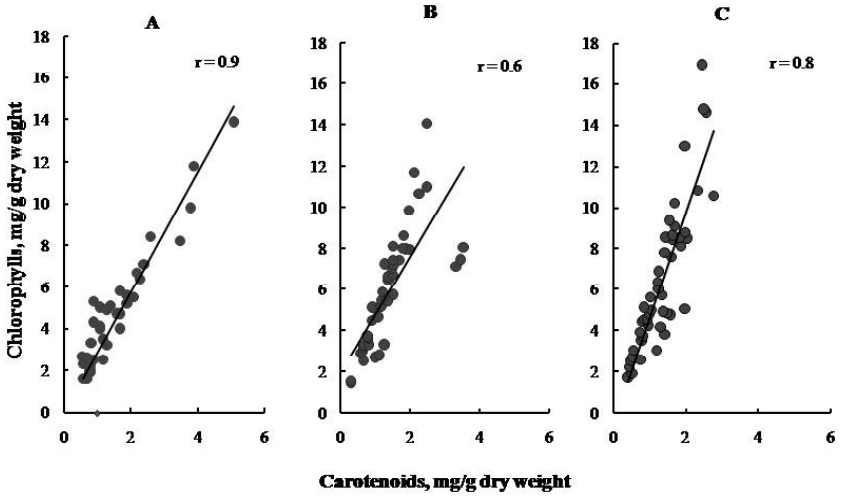

Fig. 2. The relationship between chlorophylls and carotenoids in the leaves of the Subpolar Ural Mountains (A), the South Tyman (B), the Middle Vychegda (C) plants. r – Pearson's correlation coefficient.

There are positive correlations found between the yellow and the green pigment concentrations (Fig. 2 A). The majority of the examined species had the Car content of 0.9-1.7 mg/g DW. *Diapensia lapponica, Arctous alpine, Phyllodoce caerulea, Achillea nigrescens, Lycopodium clavatum, L. annotinum* and *Vaccinum uliginosum* were distinguished by low accumulation of the yellow pigments (0.6-0.8 mg/g DW). Some of the legumes (*Hedysarum arcticum, Astragalus frigidus*) and *Pedicularis verticcilata* had high Car content, 4.5 mg/g DW. The Chl/Car ratios were 2.5-3.0 in most of the species, but these values were higher (4-5) in *Carex aquatilis, Calamagrostis purpurea* and *Arctous alpine*.

The plants with different levels of Chl and Car accumulation were revealed among the groups differed by life forms (herbs, ferns, trees and dwarf shrubs, and mosses). In the herbaceous plant group there were species with an extremely low (*Achillea nigrescens*) and high (*Astragalus frigidus*) content of photosynthetic pigments. However, overall perennial herbaceous plants, especially leguminous, contained more Chl and Car as compared to the trees and dwarf shrubs (Fig. 3 A). Among shrubs *Salix species, Betula nana* and *Pentaphylloides fruticosa* had comparatively high Chl content (2-5 mg/g DW). The ferns had the same Chl level. The mosses were characterized by the lowest pigment accumulation.

A comparison of the plants from different latitudinal groups (Fig. 4 A) showed that arctic, arctalpine, and hypoarctic species con-

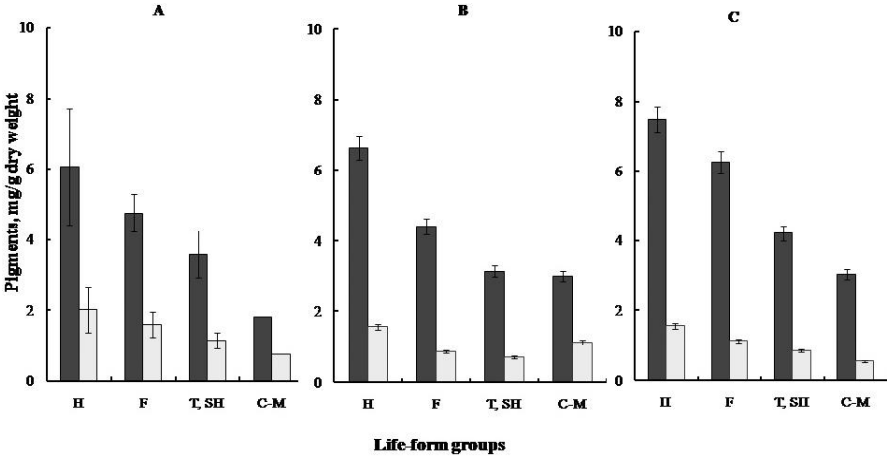

Fig. 3. Chlorophyll (dark symbols) and carotenoid (light symbols) contents in the leaves of the Subpolar Ural Mountains (A), the South Tyman (B), the Middle Vychegda (C); life-form groups: H – herbs, T – trees, SH – shrubs, F – ferns and C-M – club-mosses.

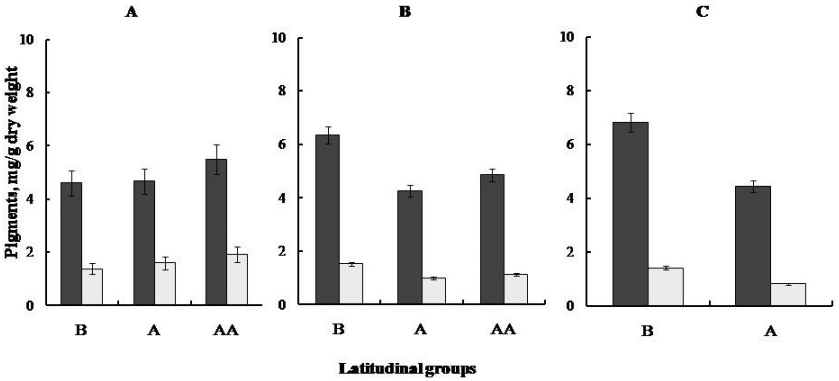

Fig. 4. Chlorophyll (dark symbols) and carotenoid (light symbols) contents in the leaves of the Subpolar Ural Mountains (A), the South Tyman (B), the Middle Vychegda (C); latitudinal groups: B – boreal, A – hypo-arctic, AA – arctic and alpine.

tained similar amount of photosynthetic pigments relative to that one of the boreal species. In the arctic and arctalpine species, the ratio of green and yellow pigments was lower (2.9) as compared to the boreal species (3.2). This indicates relatively high Car content in the pool of photosynthetic pigments of arctic and arctalpine species on the Sub-Polar Ural Mountains.

In general, the study carried out on the Sub-Polar Ural Mountains showed that the contents of photosynthetic pigments depended more on the plant species and its life form, whereas Chl/Car ratio depended on the geographical group. It should be noted that the pigment complex was characterized with relatively low Chl content.

14.2.2. The pigment complex of the South Tyman plants

Among 47 species examined on the South Tyman, 74% belong to the boreal latitudinal group. The other species are included in the arctic, arctalpine and hypoarctic groups. We found these plant species mostly in the mountain floristic complexes.

The South Tyman plants differed appreciably by the green pigments content. The Chl concentrations of these plant species varied from 1.5 to 14.0 mg/g DW (Fig. 5). The conifer (*Pinus sibirica*), shrub (*Vaccinum uliginosum*) and some herbs (*Pedicularis vercillata, Dendranthema zawadskii*) had a comparatively low pigment contents. Several species, including Trilliaceae (*Paris quadrifolia*), Orchidaceae (*Cypripedium calceolus*), Asteracea (*Crepis sibirica, Petasites radiatus*), Rosaceae (*Geum rivale*) and Fabaceae (*Vicia cracca* and *V. sylvatica, Lathyrus pratensis*) were characterized by the high Chl and Car contents.

The Chl and Car ratios were 2.4-3.1 in most of these species. These values were higher (3.6-3.9) in *Gymnadenia conopsea, Pedicularis ver-*

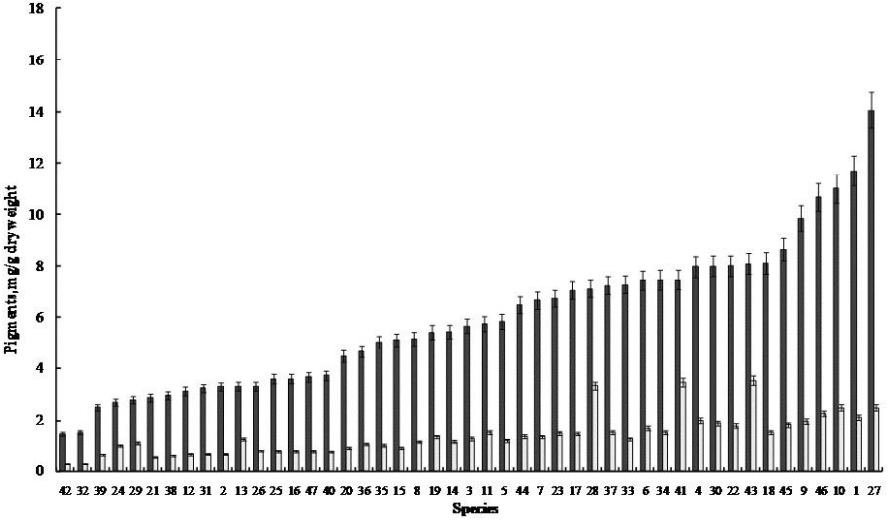

Fig. 5. Chlorophyll (dark symbols) and carotenoid (light symbols) contents in the leaves of the South Tyman plants. Species names see according to the numbers in Table 2. *Source*: Golovko et al. 2011.

ticillata, Thymus taljievii plants. The majority of the species had the LHC-Chl levels equaled to 50-65% of the total Chl. Among the examined species, only in leaves of *Pinguicula vulgaris*, the LHC-Chl was 75%. The high percentage of Chl in LHC could increase absorption of light energy and compensate a relatively low level of the green pigments in Pinguicula leaves. It should be noted that this plant was characterized by various types of nutrient deficiencies. It was the insectivorous plant.

As for the Sub-Polar Ural Mountains plants, a strong positive relationship between the content of the green and yellow pigments was revealed in the South Tyman plants (Fig. 2 B). Although the Car concentration varied strongly, from 0.3 to 2.5 mg/g DW, the ratio Chl/Car was rather similar for most of the plants and equalled 4-5.

The herbaceous plants were characterized by higher Chl and Car contents relative to the ferns, trees, and shrubs. The mosses had the lowest concentration of photosynthetic pigments (Fig. 3 B). A comparison of the plants from different latitudinal groups showed that arctic and hypoarctic species accumulated lower amount of the pigments as compared to the boreal plants (Fig. 4 B). Thus, arctalpine species were characterized by the low value of Chl/Car ratio (3.7) as compared to the boreal plants (4.1).

14.2.3. The pigment complex of the meadow and forest plants in the Middle Vychegda basin

Forty-seven species inhabited in the meadows and forests were studied. Our data showed (Fig. 6) that the leaf Chl content was relatively high (more than 4-5 mg/g DW) in the majority of these plants. In *Paris quadrifolia, Calla palustris* and *Aconitum septentrionale* the Chl content was about 14 mg/g DW. The LHC-Chl level was varied significantly, from 44 to 74%. Higher LHC-Chl was accumulated in the forest herbs as compared to the rest of the other species examined in this area. Although the species were distinguished by the Chl and Car concentrations, there was a positive correlation between the green and the yellow pigments accumulations. The content of the yellow pigments was 4-8-fold less than that of the green pigments. *Lycopodium clavatum, Vaccinum vitis-ideae, Vaccinum myrtillus, Abies sibirica* and *Pinus sibirica* contained a comparatively small amount of Car (0.4-0.5 mg/g DW). However, *Thalictrum simplex, Alisma plantago-aquatica, Aconitum septentrionale* accumulated the yellow pigments by 5 times higher in spite of the green pigments.

In the Middle Vychegda flora, like the other regions, it was observed that the herbs were characterized by maximal accumulation of photosynthetic pigments (Fig. 3 C). The Chl and Car contents were 1.5-2-times lower in the trees and mosses. A comparison of the plants from different latitudinal groups showed that hypoarctic species accumulated

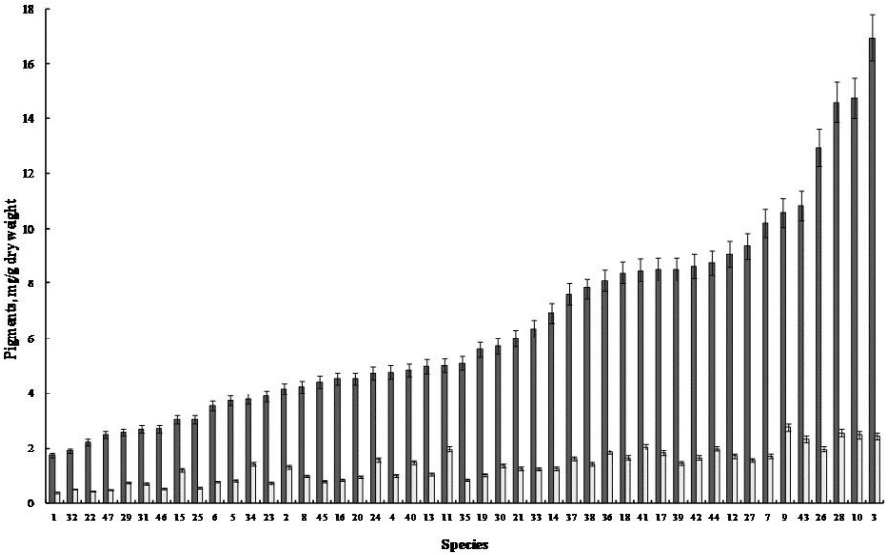

Fig. 6. Chlorophylls (dark symbols) and carotenoids (light symbols) content in the leaves of the Middle Vychegda basin plants. Species names see according to the numbers in Table 2. *Source*: Golovko et al. 2011.

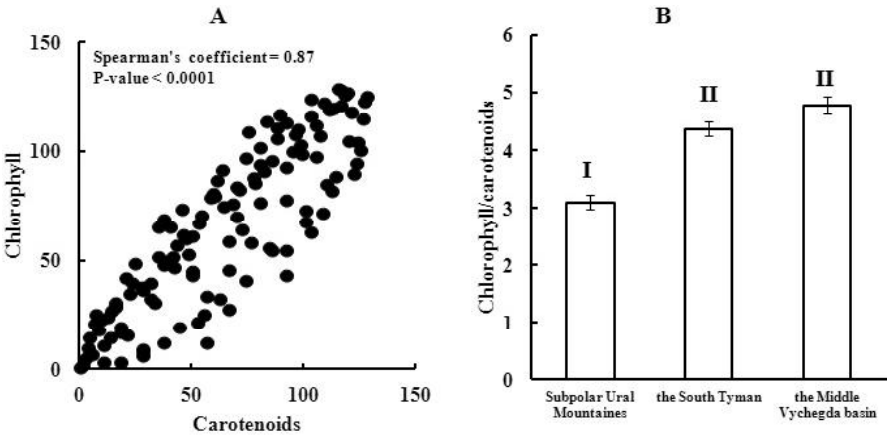

Fig. 7. Spearman's rank correlation between the content of chlorophylls and carotenoids (A), and their ratio (B) in the leaves of the plants from different regions. Differences between the groups I and II are significant (Kruskal-Wallis test, P-value <0.0001).

photosynthetic pigments by 1.3 times lower than the boreal species (Fig. 4 C).

The ratio between chlorophyll and carotenoids has been much less widely used diagnostically, although this ratio is said to be a sensitive marker distinguishing natural full-term senescence and senescence due to the environmental stresses such as desiccation in mosses (Buckland et al., 1991) and drought in flowering plants (Seel et al., 1992). Fig. 7 A shows high degree of correlation (Spearman's coefficient = 0.87) between the chlorophyll and carotenoid in plants from different regions. Our data showed that most of the examined plants from the Sub-Polar Ural Mountains region were characterized by relatively higher content of the yellow pigments (Fig. 7 B). The herbs accumulated pigments by 2-3 times higher relative to the trees and mosses. Photosynthetic pigments' pools in the leaves of the plants inhabited in the Middle Vychegda basin were larger as compared to the plants in the Sub-Polar Ural Mountains.

14.3. Net photosynthesis and its correlation with the pigment contents in the northern plants

Our data showed that the examined species differed on the CO_2 exchange rates significantly. At saturating PAR and 20 °C, the rate of net photosynthesis varied from 1.0 to 38 mg CO_2 g^{-1} (DW) h^{-1} for the Sub-Polar Ural Mountains plants, from 0.7 to 21 mg CO_2 g^{-1} (DW) h^{-1} for the South Tyman plants, and from 1.5 to 35 mg CO_2 g^{-1} (DW) h^{-1} for the Middle Vychegda flora.

The most of the natural flora species (60%) were characterized by net photosynthetic rate less than 10 mg CO_2 g^{-1} (DW) h^{-1}. Coniferous, dominating in a taiga zone, were able to uptake 3-4 mg CO_2 g^{-1} (DW) h^{-1}, herbs and shrubs – 4-6 mg CO_2 g^{-1} (DW) h^{-1}. The zone of optimum temperature of net photosynthesis (PN) was 10-20 °C. The rate of leaf photosynthesis was considerably depressed at the temperature of 35 °C. At 5-7 °C, the rate of net photosynthesis was equal to approximately 60-70% of the maximum values of PN. The shift in optimum temperature for PN according to environmental conditions was noted. It is important for successful adaptation of the studied northern plants.

Fig. 8 shows the relationships between the net photosynthetic rate and chlorophyll concentrations. In the examined species a capacity to assimilate CO_2 was positively correlated with the chlorophyll content. This relationship showed that the level of the chlorophyll content corresponded to the values of photosynthetic rates. Spearman's rank correlation coefficient (Spearman's rho), accounted for by the relationships between the photosynthetic rates and the chlorophyll content, was higher (0.62) for plants from the Sub-Polar Ural Mountains region as compared to the plants from the middle taiga subzone.

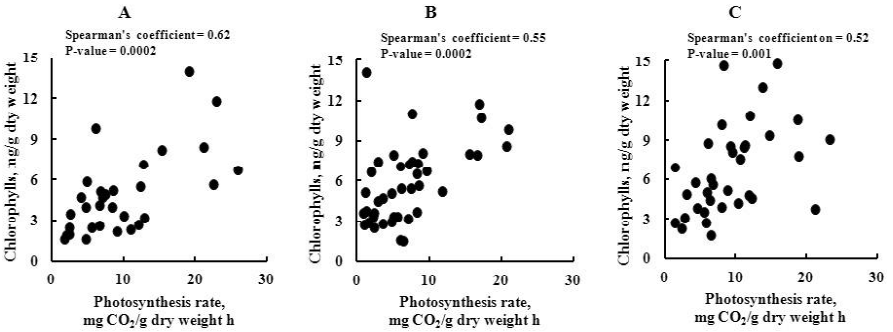

Fig. 8. Relationship between the content of chlorophyll and photosynthetic ability (the photosynthesis rate at optimum environmental conditions) of the plants from different regions: A – Subpolar Ural Mountains, B – the South Tyman, C – the Middle Vychegda.

For some arctic-alpine and hypoarctic species (*Arctous alpina, Phyllodoce caerulea, Vaccinum uliginosum, Rubus chamaemorus, Tanacetum bipinnatum, Woodsia glabella*) on the Sub-Polar Ural Mountains in this study, the species with lower chlorophyll contents appears to have possibly the highest CO_2 assimilation per dry weight. For the majority of the boreal herbs in the Middle Vychegda basin it was shown that the higher rates of photosynthesis were positively correlated with high chlorophyll content, and, on the contrary, the lower values of PN corresponded to the low chlorophyll levels.

Hence, our data showed that high correlation between the chlorophyll content and the photosynthetic rate was not obtained, and photosynthetic ability of the leaves of the plants studied changed insignificantly at advance to the North.

14.4. Summary and conclusions

The contents and the ratio of photosynthetic pigments and their relationships with CO_2 assimilation while growing in the Sub-Polar Ural Mountaines, the South Tyman, and in the Middle Vychegda basin, have been investigated. We found that the level of carotenoids' accumulation in plant leaves from all the regions studied closely correlated with the chlorophyll content. Comparing our data with the results reported by Maslova and Popova (1993) for the other regions, it has been found that the Sub-Polar Ural Mountains plants were closer to the alpine plants of the East Pamirs, the South Tyman plants – to the arctic plants of Taimir, and the middle Vychegda plants – to the species of the temperate zone on the Chl/Car index.

Carotenoids play an important role in the light harvesting complex and in photoprotection of the photosystems (Demmig-Adams, 1990; Young et al., 1997; Rmiki et al., 1999; Cuttriss and Pogson, 2004). Therefore, a relatively high level of the yellow pigments in the leaves of the northern plants, especially arctic species, can be regarded as an adaptive reaction which is directed to increase the stability of the pigment complex and to prevent its photodynamic destruction in cold climate. The pigment complex of the species from the middle taiga subzone was characterized by the higher Chl/Car ratio and the lower yellow pigment contents in contrast to the Sub-Polar Ural Mountains plants. This confirms an important role of carotenoids in the stability of the pigment complex of the extremely north taiga subzone plants. It should be noted that carotenoids not only have a protective role, but also absorb light in the near ultraviolet (UV), as well as the visible region. Carotenoids are bound together with Chl to proteins and participate in the light harvesting. As the components of an antenna complex they provide more effecient use of solar radiation during the short north summer.

As a whole, information on photosynthetic apparatus of different botanical and ecological groups of the northern plants was completed and summarized. Significant differentiation of the species on the green and yellow pigment contents was found. An increase of the relative content of carotenoids in row boreal – hypoarctic – arctalpine species was found. The role of carotenoids in resistance of photosynthetic apparatus and protection against photooxidative damage in the leaves of the plants from the northern regions was shown. The measurement of gas exchange and chlorophyll content allowed us to provide useful information on the relationship between these parameters. High correlation between the chlorophyll content and the photosynthetic rate was not obtained. Thus, our data showed that the level of carotenoids increased and photosynthetic ability of the leaves of the plants studied changed slightly at advance to the North. The results obtained extend our knowledge on the mechanisms of adaptation of pigment apparatus and fulfillment of photosynthetic function in plants under cold climate environments.

Acknowledgement

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Chapter 15. PHOTOSYNTHETIC PIGMENT-PROTEIN COMPLEXES OF WINTERGREEN HERBACEOUS PLANT *AJUGA REPTANS L.*

Olga Dymova[1], Michail Khristin[2], and Tamara Golovko[1]
[1] Institute of Biology, Russian Academy of Sciences, Syktyvkar, Russia,
[2] Institute of Basic Biological Problems, Russian Academy of Sciences, Pushchino, Moscow, Russia

Contents

15.1. Introduction

The phenomenon of overwintering with green leaves is characteristic of evergreen and winter-green plant species. The photosynthetic apparatus (PSA) of boreal evergreen conifer trees and winter-green plants is subject, throughout a significant part of the year, to the unfavorable conditions of hypothermia, often together with high insolation. Low temperature inhibits CO_2 assimilation in conifer leaves, but does not affect the capability of chlorophyll to absorb light energy (Ottander et al., 1995). The question of the fate of the absorbed energy and the mechanisms of its safe dissipation is especially important, because, when energy of the excited chlorophyll is not used in photochemical reactions, it can cause photoinhibition and lead to photodynamic damage of the PSA (Murata et al., 2007). Overwintering plants develop tolerance to freezing stress throught a cold acclimation process by which the cells provoke internal protective mechanisms against freezing. Trees employ a sustained form of photoprotection in which the dissipative pigments of the xanthophyll cycle are retained, and photosystem II (PSII) efficiency is maintained in a dissipative state throughout the winter, as has also been observed in other overwintering evergreens (Adams et al., 2002). It was shown that environmental conditions during the late spring – when air temperatures are warm but not hot, the soils have thawed, and there is a readily available supply of water from snowmelt – are ideal for photosynthesis in the conifer species of the subalpine forest (Zarter et al., 2006 a, b).

Two biennial mesophytes (*Malva neglecta* and *Verbascum thapsus*) maintained or upregulated photosynthetic capacity in the winter, and exhibited no upregulation of photoprotection (Adams et al., 2002). Winter-green herbaceous plants are able to uptake CO_2 after the first frost in autumn and in early spring. Earlier we showed (Dymova and Golovko, 2001) that photosynthetic capacity in overwintered *Ajuga reptans* leaves was twofold less than that in summergreen ones. About 60% of total pigments were preserved in overwintered leaves. Recent studies (Yatsco et al., 2011) revealed similar seasonal changes in pigment contents in conifer needles (*Abies sibirica*, *Picea obovata*, *Juniperus communis*). The decreases in PSII efficiency were accompanied by changes in chlorophyll fluorescence characteristics that indicated the increased levels of energy dissipation in the chlorophyll pigment complex (Adams and Demmig-Adams, 1994). The presence of sufficient amount of chlorophyll and changes in photosystem II (PSII) organization in overwintered needles of Scots pine allowed rapid recovery in photosynthesis in spring (Ottander et al., 1995). We can also assume that maintaining of the functional activity of photosynthetic apparatus of herbaceous winter-green plant leaves occurs due to the structural reorganization of PSII supercomplexes and its light harvesting system (LHCII), with their subsequent assemblies in early spring.

15.2. Pigment-protein complexes

The pigment-protein complexes in photosynthetic membranes exist mainly as aggregates (Rochaix, 2010). The transformation of light into chemical energy in photosynthesis takes place with the contribution of sequentially functining photosystems II and I (PSII, PSI). Each Photosystem is composed of two different moieties: (1) the core complex, responsible for charge separation and the first steps of electron transport, and (2) the peripheral antenna system, which plays a role in light harvesting and transfer of excitation energy to the reaction center. The antenna polypeptides in green algae and plants are all members of a multigenic family of proteins called LHC (Light harvesting complexes) (Jansson, 1999; Alboresi et al., 2008). Higher plants are highly enriched both with PSI and PSII. Chlorophylls in the light-harvesting antenna complexes of PSII (LHCII) or PSI (LHCI) of plants contribute to harvesting and migration of the absorbed energy to core antenna of both photosystems.

PSI is responsible for the light-induced electron transfer from plastocyanin to ferredoxin. The native PSI complex from higher plants has a core complex and a peripheral antenna (Jensen et al., 2003). In higher plants PSI complexes are monomers (Golbeck, 1992; Karapetyan et al., 1999). Each monomer (~300 kDa) contains one copy of 12 different

protein subunits. The core of the complex consists of two large subunits (products of genes PsaA and PsaB), which bind 96 Chl *a* molecules, 22 β-carotene molecules, two phylloquinone molecules, and an interpolypeptide iron-sulfur cluster F_X. The terminal iron-sulfur clusters F_A/F_B (of the 4Fe-4S type) are bound to the peripheral stromal subunit PsaC, which has a molecular mass of ~9 kDa (Scheller et al., 2001). The majority of Chl *a* molecules act as pigments of the antenna. It was found that the ratio between the number of the excited chlorophyll molecules of the antenna and of the reaction center depended on the spectral characteristics of the pump pulses (Semenov et al., 2012).

LHCI PSI consists of four different polypeptides denoted as Lhca1 to Lhca4 with mol. wts of 20-24 kD, which belong to the group of Chl *a*/*b*-binding proteins of the gene superfamily Lhc (Jensen et al., 2003). Lhca1 and Lhca4 are presented as heterodimers (LHCI-730), whereas Lhca2 and Lhca3 are likely occur as homodimers (LHCI-680) (Jensen et al., 2003; Scheller et al., 2001). LHCI contains about 70–110 Chl molecules, and each Lhca protein binds approximately 10 Chls (Schmid et al., 2002). Lhca4 subunit of LHCI-730 (Melkozernov et al., 2000), and also Lhca2, and Lhca3 of LHCI-680 contains the so-called low-energy Chl *a* (Schmid et al., 2001).

PSII is a membrane protein complex presented in all oxygenic photosynthetic organisms. PSII exists mainly in a dimeric form with the monomer containing at least 27 to 28 subunits (Dekker and Boekema, 2005). A large number of factors participate in the assembly of a functional PSII complex (Rochaix, 2010). PSII of green algae and higher plants contains more than 25 different polypeptides with the molecular masses ranging from 3 to about 50 kDa (Renger, 1992; Vermaas et al., 1993; Funk et al., 1995). Several of these are known to contain pigments, preferentially chlorophylls, but also carotenoids. At least two types of chlorophyll-binding proteins can be distinguished. The first group is represented by the pigment proteins forming the PSII core complex, i.e. the gene products of psbB (CP47) and psbC (CP43). CP47 and CP43, each containing 15-22 chlorophyll *a* molecules (Chang et al., 1994; Alfonso et al., 1994), are assumed to act as the inner or core antenna. The redox active groups that catalyze a stable charge separation (the photoactive chlorophyll *a* designated as P680 and the acceptors pheophytin and plastoquinone) are located in a heterodimer, consisting of polypeptides D1 (psbA) and D2 (psbD) that are closely associated with three smaller polypeptides, the two subunits of Cytb559 (psbE and psbF) and the psbI gene product. The D1/D2 heterodimer binds 4-6 chlorophyll *a* molecules, 2 pheophytins, and 1-2 carotenes (Kobayashi et al., 1990; Gounaris et al., 1990).

The second major type of chlorophyll-binding proteins is the family of CAB gene products forming the peripheral antenna system which

plays a key role in light-harvesting and is also involved in acclimation of the photosynthetic apparatus to different light environments. The proteins, belonging to the CAB gene family, contain chlorophyll *b* in addition to chlorophyll *a*. The dominant complex is the trimeric LHCII that forms the major part of the antenna system and accounts for about 50% of all chlorophylls in the thylakoid membrane (Jansson, 1994). The native and functional form of LHCII consist of 7-8 chlorophyll *a*, 5-6 chlorophyll *b*, 2 lutein, 1 neoxanthin, and variable amount of violaxanthin, zeaxanthin, and antheraxanthin (Jansson, 1994; Liu et al., 2004). The minor chlorophyll *a*/*b* antenna proteins are designated CP29, CP26, CP24, and CP14 according to the size (Dunahay and Staehelin, 1986; Bassi et al., 1987; Henrysson et al., 1989; Irrgang et al., 1993; Zucchelli et al., 1994). Dissipation of the excess absorbed energy in plants takes place mainly via LHCII (Horton et al., 1996; Rochaix, 2010). All LHCII complexes reveal dynamic behavior depending on light intensity (Janik et al., 2012). The LHCII trimers are more stable compared with the monomers.

Among photosynthetic complexes PSII is particularly prone to photooxidative damage, since the water-splitting reaction catalyzed by this complex inevitably leads to the formation of reactive oxygen species that damage the complex. Thus, PSII is constantly damaged and needs to be repaired. Indeed, a very efficient repair system has evolved in which the D1 subunit of photodamaged PSII is predominantly degraded and the complex moves from the grana to the stromal region for repair (Nixon et al., 2010). Possibly, chlorophyll released during this process is transiently stored in small CAB-like proteins of cyanobacteria containing a single transmembrane helix with high similarity to some transmembrane domains of the light-harvesting proteins (Kufrik et al., 2008; Nixon et al., 2010).

15.3. Fluorescence emission spectra at 77 K

The chlorophyll (Chl) fluorescence, both stationary and time-resolved, provides a powerful intrinsic probe for energy transfer and charge separation kinetics of the photosynthetic apparatus (Holzwarth, 1986). The fluorescence emission spectra at 77K gives information on the state of energy transfers, and more generally, on the state of chlorophyll-protein complexes in the photosynthetic membranes (Cho and Govindiee, 1970; Jeanfils et al., 1982; Somersalo and Krause, 1989; Krausz et al., 2005; Janik et al., 2012).

At low temperature the fluorescence characteristics of thylakoids and PS membrane samples are radically different from those at room temperature (Andrizhiyevskaya et al., 2005; Krausz et al., 2005). The emission attributed to PSI is very strong (λ = 720-735 nm) (Cho and

Godvindjee, 1970; Butler and Kitajama, 1975; Rijgersberg et al., 1979; Knoetzel et al., 1998) and occurs in addition to the PSII fluorescence bands F-680 and F-695. Moya and Garcia (1983) have compiled most of the time-resolved data available up to 1982 for the low temperature Chl fluorescence. These data are rather diverse. One of the important open questions is whether there exists a rise time for the long wavelength fluorescence. Moya and Garcia (1983) have resolved the total fluorescence into up to seven components. Two of them (688 and 698 nm maxima) are attributed to PSII and can be correlated with room temperature data. The low-temperature emission spectrum of the PSII core complex is thought to originate from the long-wavelength chlorophylls in the core antenna. It is well-known that 77K emission at 695 nm arises from one 690 nm chlorophyll in CP47, whereas 77K emission at 685 nm arises from several 683 nm chlorophylls in CP43 and/or CP47 (van Dorssen et al., 1987 a, b; Andrizhiyevskaya et al., 2005). The low-temperature PSII fluorescence is interpreted as arising from non-thermalised subsets of the CP47 and CP43 pigments, taken from their inherent inhomogeneous distributions, which are less well connected to the weak, homogeneously broadened underlying charge-separating state of the PSII (Krausz et al., 2005).

The 77K fluorescence emission spectrum obtained from intact PSI shows a characteristic long-wavelength band at 735 nm (F-735) (Mullet et al., 1980; Bassi and Simpson, 1987; Knoetzel et al., 1992). Removal of peripheral chlorophyll (Chl) *a*/*b* binding proteins (LHCI) leads to PSI reaction centre complexes that exhibit 77K emission of core antenna Chl at 720 nm (F720) (Mullet et al., 1980). The peripheral LHCI antenna can be isolated and was shown to fluoresce at 730 nm at 77K (Haworth et al., 1983). Croce et al. (1996) identified three Chl spectral forms in native PSI from maize thylakoids with 77K fluorescence emission maxima at 720 nm, 730 nm and 742 nm. LHCI-730 and LHCI-680 have the characteristic 77K fluorescence emission peak at about 730 nm and 680 nm, respectively (Knoetzel et al., 1998). The value of F730/F680 declined in an almost linear proportion to the increase in pH (Yang et al., 2009).

15.4. Seasonal changes in pigment-protein complex organization in *Ajuga reptans* leaves

We investigated seasonal changes of 77K fluorescence emission spectra of the photosystems (PSII and PSI) of bugle (*Ajuga reptans* L.) chloroplasts in order to receive information on the state of chlorophyll-protein complexes in the photosynthetic membranes. *Ajuga reptans* L. (Lamiaceae) – is a widely occuring species of the European nemoral complex. It is shade-enduring plants. *Ajuga reptans* plants were grown

in a flow of parvifoliate forest near Moscow (54°50′ N, 37°37′ E). Wintergreen leaves of *Ajuga* plants were collected in December and April, summer-green ones – at the beginning of July.

Chloroplasts were isolated from the bugle plants according to the following method. The leaves (3 g) were harvested and homogenized in 3 ml ice-cold extraction 100 mM HEPES buffer, pH 7.0, containing 80 mM 0.5 M mannitol, 100 mM NaCl. All subsequent steps were carried out at 4 °C. The suspension was filtered through the layer of gauze. The homogenate was centrifuged at 500 g to remove the cell debris; chloroplast pellets were collected by centrifugation of the supernatant at 10000 g for 10 min, and resuspended in HEPES buffer, pH 7.0. The chloroplast pellet were extracted with 0.2% Dodecyl-β-D-maltoside and centrifuged for 10 min at 15000 g. After centrifugation, the supernatant was loaded on the top of a 10-ml sucrose gradient (0.5, 1.0%). Gradients were centrifuged for 15 h at 4 °C at 208000 g (Type 70 Ti Rotor, Beckman Coulter, Inc, USA) and then fractionated into 150-μl aliquots. Fractions were subjected to low temperature (77K) fluorescence analyses. Fluorescence emission spectra under 435 nm excitation were recorded at 77K between 650 nm and 800 nm using a spectrofluorimeter Hitachi-850 (Japan). All measurements were made in three biological replicates.

The analysis of seasonal changes in pigment-protein complexes showed that fractions of free pigments, LHCII PSII monomer, LHCII

Table 1

Photosystem PS I / PS II ratio of the fractions from bugle chloroplasts, 2012

Month	Fraction	Relation		
		735/685	735/695	695/685
June	1	0.10	0.44	0.23
	2	0.11	0.38	0.28
	3	0.09	0.34	0.26
	4	0.28	0.78	0.35
	5	0.34	0.99	0.34
	6	0.79	1.50	0.53
December	1	–	–	0.19
	2	0.62	1.45	0.43
	3	0.11	0.42	0.26
	6	0.39	1.01	0.38
April	1	–	–	0.45
	2	–	–	0.32

Fractions: 1 – free pigments, 2 – LHCII PSII monomer, 3 – LHCII PSII trimer, 4 – monomeric form of PSI, 5 – PSII-LHCII complex and 6 – PSI-PSII-LHCII supercomplexes.

PSII trimer, monomeric form of PSI, PSII-LHCII complex and PSI-PSII-LHCII supercomplex were found by sucrose gradient centrifugation in summer (Table 1). Dimeric and monomeric forms of PSII occur in the stacked and stromal thylakoid membranes, respectively (Dekker and Boekema, 2005). The largest PSII antenna complexes (LHCII) may associate with the dimeric PSII core, yielding a PSII-LHCII complex (Nield and Barber, 2006). Steady state fluorescence measurements were performed at 77K. The emission spectrum measured at excitation wavelength of 435 nm is depicted in Fig. 1. It is shown that fraction 1, 2, 3, 5 was characterized by the main band peaking at 680 nm to 682 nm. These features are typical for chlorophyll *a*. The 77K emission spectrum of the PSII core complex emission shows the well-known relative fluorescence intensity at 685 nm (F-685) and 695 nm (F-695) emission bands.

In the beginning of winter (the end of November – mid-December) at freezing temperatures reorganization of PSII-LHCII complex to dimeric and monomeric forms was noted (Fig. 1B, Table 1). Earlier, we showed (Dymova and Golovko, 2001) that in the end of autumn (November) there was a decrease in the content both of chlorophyll *a* and *b* (by 25-30%) (Table 2). During winter the breakdown of chlorophylls continued, and, after a cold period, the chlorophyll levels retained 20% on an area basis. The overwintering bugle leaves contained substantial amount of total carotenoids (Table 2).

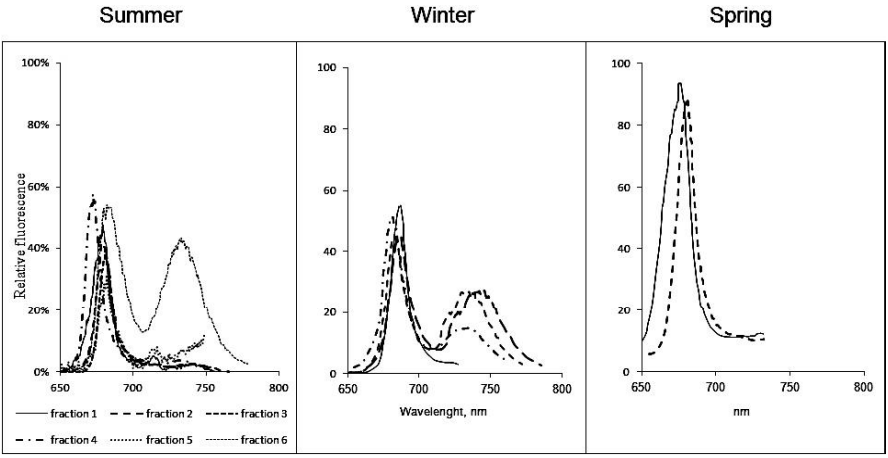

Fig.1. Seasonal changes in 77K fluorescence emission spectra of the chlorophyll-protein complexes in bugle chloroplasts, separated by sucrose gradient centrifugation. The excitation wavelength was at 435 nm.

Table 2

Pigment content in wintergreen (May) and summergreen (July-November) bugle leaves

Month	Chlorophyll *a*, mg/dm^2	Chlorophyll *b*, mg/dm^2	Chlorophyll *a*/*b*	Chlorophyll portion in LHC, %	Total carotenoids, mg/dm^2	Chlorophyll/ carotenoid ratio
May	1.79±0.01	0.81±0.06	2.2±0.2	69	0.64±0.01	4.0±0.1
July	1.50±0.05	0.65±0.04	2.4±0.2	66	0.46±0.04	4.4±0.2
August	1.72±0.02	0.65±0.04	2.6±0.2	60	0.52±0.03	4.6±0.3
September	2.27±0.06	0.81±0.02	2.8±0.1	57	0.64±0.03	4.8±0.2
November	1.62±0.02	0.55±0.04	2.9±0.2	54	0.48±0.02	4.3±0.1

Source: Dymova and Golovko 2001.

In spring under favourable conditions only free pigments and LHCII PSII monomer were observed (Fig. 1C). In the overwintered leaves pigment rearrangements and violaxanthin de-epoxidation occurred (Dymova et al., 2010). In early spring these leaves showed elevated photosynthetic capacity (Dymova and Golovko, 2001).

The effect of different light environments on the pigment-protein complexes of bugle chloroplasts was investigated in plants grown in shaded and sunny habitats. The 77K Chl fluorescence emission spectra both of shade and sun chloroplasts exhibited three peaks at 735 nm, and 685 nm, and 695 nm (Fig. 2), which has been attributed to the antenna pigment protein complexes of PSI and PSII (Krause and Weis, 1991). The Chl fluorescence yields of sun chloroplast were higher than those in shade chloroplasts at 685 and 695 nm. Thus, the chloroplasts of shade plants were enriched by LHCII PSII monomer, LHCII PSII trimer and monomeric form of PSI, PSII-LHCII complex and PSI-PSII-LHCII supercomplex. In sun chloroplasts the fraction 2 (LHCII PSII monomer) and the fraction 5 (PSII-LHCII complex) were absent. Sun chloroplasts have no LHCII monomers, since the LHCII trimers are more thermostable as compared with the monomers.

The fluorescence emission ratio of F735/F685 is a relative indicator of the excitation energy distribution between two photosystems (Duysen et al., 1984). The fluorescence emission spectra at 77K are often used to monitor ultrastructural changes in thylakoid membranes as a response to environmental condition variations. Thus, F735/F685 and F735/F695 ratios can be used as a probe for the amount of antenna Chls connected to

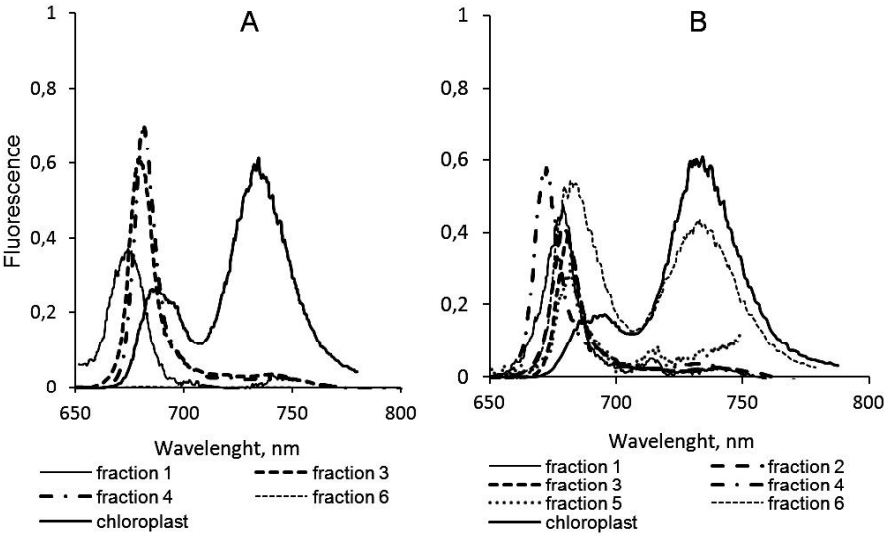

Fig. 2. Fluorescence 77K emission spectrum of the bugle chloroplasts (A – sun plants, B – shade plants) and the pigmented fractions obtained upon excitation at 435 nm, June 2012.

each photosystem (Van Dorssen et al., 1987a; Alfonso et al., 1994). Table 3 shows the ratios of PSII (F735)/PSI (F685 or F695) depending on light regime of bugle habitats. The PSI/PSII emission ratio of sun chloroplasts was lower than of the shade chloroplasts. F735/F685 and F735/F695 ratios decreased in fraction 4 (monomeric form of PSI) and fraction 6 (PSI-PSII-LHCII supercomplex) from sun leaves.

The antenna Chls of PSI emits maximum fluorescence at 735 nm, whereas those of LHCII surrounding PSII emit at 680 nm. CP43 antenna complex and PSII reaction center complex contribute to 685 nm fluorescence maximum, and the band at 695 nm is associated with CP47 antenna complex. It is common to use the ratio of F695 to F685 to characterize the level of aggregation. In our experiments the highest ratio of the long to the short wavelength fluorescence emission (F695/F685 = 1.11) was observed for the shade chloroplasts. The F695/F685 ratio was reduced up to 0.86 for the sun chloroplasts. According to V.Barzda et al. (1996) aggregation of LHCII complexes under excess light conditions leads to reversible structural changes that effect the energy migration and, probably, are involved in regulation of the pathways of energy dissipation in antenna. Our data showed that the strong macro-aggregation was visible in 77K fluorescence emission spectra of the shade chloroplasts. Apparently, thanks to the existence

of aggregated and trimeric with monomeric forms of LHCII PSII, the shade plants are able to react rapidly to a short-term increase in radiation intensity (sunflecks) under a forest canopy.

15.5. Summary and conclusion

Hence, seasonal changes in chlorophyll-pigment complex organization in *Ajuga reptans* chloroplasts were shown. For the first time the results on gradient sucrose centrifugation and fluorescence emission spectra at 77K for leaves of shade-enduring bugle plants are received. Our data show that in late autumn, before severe freezing stress begins, bugle plants lose PSII-LHCII supercomplex, since the contents of chlorophyll decrease. The 30% chlorophyll loss was almost complete by November. Similar chlorophyll loss (40-50%), together with a decrease in D1 protein and LHCII proteins, was observed in the evergreen Scots pine (Ottander et al., 1995). We suggest that during winter the light-harvesting complexes of bugle are reorganized mainly into monomeric forms of LHCII PSII. It is considered that the monomer was the most effective aggregating antenna form (Janik et al., 2012). Therefore, photoprotection connected with LHCII aggregation was more effective upon LHCII monomers in comparison to the trimer aggregation. In comparison with the herbaceous winter-green bugle during winter the PSII and light-harvesting complexes of the coniferous Scots pine are reorganised into large chlorophyll-pigment aggregates, capable to dissipate very efficiently excitation energy non-radiatively (Ottander et al., 1995). Xanthophylls played a significant role in protecting the photosynthetic apparatus of evergreen conifers from photodestruction. It is proved that there is strong connection between violaxantin cycle pigment de-epoxidation and light energy thermal dissipation (Yatsco et al., 2011). These results indicate that different types of pigment-protein complexes reorganization occur du-

Table 3

Photosystem PS I / PS II ratio of the fractions from bugle chloroplasts. Plants acclimating to shady and sunny environments, July 2012

Fraction	Ratio	Shade	Sun
1	735/685	0.10	0.08
	735/695	0.44	0.41
	695/685	0.23	0.20
2	735/685	0.11	–
	735/695	0.38	–
	695/685	0.28	–
3	735/685	0.09	0.07
	735/695	0.34	0.30
	695/685	0.26	0.24
4	735/685	0.28	0.06
	735/695	0.78	0.21
	695/685	0.35	0.26
5	735/685	0.34	–
	735/695	0.99	–
	695/685	0.34	–
6	735/685	0.79	0.38
	735/695	1.50	0.91
	695/685	0.53	0.42

Fractions are the same as for Table 1.

ring winter in evergreen coniferous and herbaceous winter-green species. In natural habitats, *Ajuga reptans* grow near the ground surface and has a prostrate life form, which allows it to benefit from leaf fall and snow cover, thus alleviating the effect of low temperatures in winter.

It is established that similar to conifers herbaceous winter-green species *Ajuga reptans* was able to recover rapidly the photosynthesis after melting of the snow cover in early spring due to changes in pigment-protein complex organization. Spring accumulation of chlorophylls in overwintering bugle leaves (Table 2) occurred after photosynthesis was almost fully recovered.

We found that in summer under low and high light conditions in natural habitats sun and shade bugle plants were characterized by different organization of the chloroplasts. We suggest that the existence of aggregated (PSII-LHCII complex and PSI-PSII-LHCII supercomplex) and trimeric with monomeric forms of LHCII complexes within the thylakoid membrane in leaves of shade bugle plant provides for the maximum efficiency of photosynthesis under a forest canopy.

To summarize, winter reorganisation of pigment-protein complexes allows *Ajuga reptans* plants to maintain large reserves of chlorophyll while avoiding severe photodestruction of pigments and thylakoids. The different organization and interaction of the pigment-protein complexes of the sun and shade chloroplasts leads to dissipation of excess absorbed energy increasing the stability of the photosynthetic apparatus under light stress.

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Chapter 16. PHOTOPROTECTIVE ROLE OF XANTHOPHYLL CYCLE IN CLUSIA PLANTS IN RELATION TO CRASSULACEAN ACID METABOLISM

Andrzej Kornas[1], Elzbieta Kuzniak[2], and Zbigniew Miszalski[1, 3]
[1] Institute of Biology, Pedagogical University, Krakow, Poland
[2] University of Lodz, Lodz, Poland
[3] Institute of Plant Physiology, Polish Academy of Sciences, Krakow, Poland

Contents

16.1. Introduction

In their natural environment, plants are often exposed to low and high light stresses and must acclimatize (adapt their metabolism) to daily and seasonal changes in the environmental conditions. Even under optimal conditions, in moderate light intensity, plants are able to use only a part of the light energy absorbed by antennae complexes of photosystems (PS) I and II. The amount of light energy harvested by plants in excess to that which they need for carbon assimilation and to get energy to perform living processes is termed excess excitation energy (EEE).

When absorbed light energy exceeds the capacity of light utilization not all electrons are used to produce the reducing equivalents and the excitation pressure results in excess production of reactive oxygen species (ROS), predominantly superoxide anion radical ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$) and secondarily hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) (Asada, 1999). In plants, ROS are produced in many oxidoreduction reactions as an inevitable consequence of the aerobic metabolism and their formation is greatly enhanced under environmental stress conditions of both abiotic and biotic origin. In plant cells chloroplasts are considered to be the main source of ROS. Apart

from chloroplasts, they are permanently generated in mitochondria, peroxisomes, endoplasmic reticulum, cytosol and apoplast (Mittler, 2002; Vranova et al., 2002; Foyer and Noctor, 2003). Under optimal conditions, there is a delicate balance between the processes of ROS generation and scavenging. The disturbance of the equilibrium between the pro- and antioxidative mechanisms in favour of the former has been referred to as oxidative stress (Foyer and Noctor, 2005). The overproduction of ROS can cause photoinhibition and photooxidation, in some cases leading to cell death. In recent years, ROS have received increasing attention as key signalling molecules in governing such processes as plant growth and development, defence responses to abiotic and biotic environmental stresses and programmed cell death (PCD) (Mazel and Levine, 2001; Overmyer et al., 2003).

16.2. Photoprotection in plants

Plants have evolved different mechanisms of dissipation and avoidance of EEE. The cells of aerobic and even anaerobic organisms are protected from ROS-induced damage by a complex antioxidative system. It serves to maintain intracellular ROS pool below a critical threshold, permitting also ROS-mediated signalling to function. Each photosynthesizing cell is equipped with photochemical mechanism of energy dissipation involving an integrated system of enzymatic and non-enzymatic antioxidants that acts by neutralizing excessive ROS. Its ROS-scavenging activity serves as a commonly accepted indicator of oxidative stress. Non-enzymatic antioxidants are low-molecular weight compounds reacting directly with ROS but they also mediate many enzymatic reactions which interrupt or slow down the free radical chain reactions and other oxidation processes. The non-enzymatic antioxidants prevent oxidation of vital biomolecules and they also mediate reduction of compounds oxidized by ROS. These antioxidants are often divided into hydrophilic antioxidants (e.g. ascorbate, glutathione, phenols and betalains) which operate in aqueous phase and lipophilic antioxidants protecting membranes from oxidative damage (e.g. α-tocopherol, carotenoids).

With respect to the photosynthetic apparatus, photoprotection is related to two essential mechanisms: (a) the ability of transferring electrons to acceptors in chloroplasts, i.e. photochemical quenching (q_p) and (b) dissipation of EEE as heat, i.e. non-photochemical quenching (NPQ). The processes related to photochemical quenching involve water-water and ascorbate-glutathione cycles (AsA-GSH), chlororespiration and photorespiration. The AsA- and GSH-dependent redox systems acting in an integrated co-operation with antioxidant enzymes, e.g. superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase

(CAT), regulate ROS level and ensure adequate protection from potentially deleterious effects of their action.

Electron transfer from PSI directly to O_2 results in the generation of $O_2^{\cdot-}$ (Mehler reaction) which is immediately converted into H_2O_2 mainly by SOD present in chloroplasts (CuZnSOD or FeSOD). Hydrogen peroxide may be reduced to water either by stromal or thylakoid-bound forms of APX using AsA as a specific electron donor. At the same time AsA is oxidized to monodehydroascorbic acid. Re-reduction of AsA is catalyzed by monodehydroascorbate reductase (MDHAR) at the expense of NAD(P)H. The water-water cycle is the photoreduction of O_2 to water in PSI by electrons generated in PSII from water (Asada, 1999). This cycle *via* ROS scavenging and EEE dissipation is crucial for the protection of photosystems from photoinhibition, and the consumption of reducing equivalents (NADPH) supports the proton gradient across thylakoid membranes and results in a balanced ratio of ATP to NAD(P)H (Ort and Baker, 2002; Kramer et al., 2004). Regeneration of ascorbate from its oxidized form can also be mediated by other enzymes located in the chloroplast, constituting the ascorbate-glutathione cycle. This cycle involves successive oxidations and reductions of ascorbate and glutathione catalysed by APX, MDHAR, dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Noctor, 2006). Monodehydroascorbate, the primary product of ascorbate oxidation, disproportionates spontaneously to dehydroascorbate that is reduced by DHAR using GSH as a reductant. The oxidized glutathione (GSSG) is reduced by GR at the expense of NADPH. Apart from its direct antioxidant role, the ascorbate-glutathione cycle is involved in ROS sensing and signalling. Modulation of the appropriate defense response under stress can be achieved by changes in the absolute concentrations of the total, reduced and oxidized forms of ascorbate and glutathione as well as their redox ratios (Foyer and Noctor, 2009).

Chlororespiration, similarly to the water-water and ascorbate-glutathione cycles, protects photosystems from photoinhibitory damage. This respiratory-like pathway operating in chloroplasts is linked to the activities of plastoquinone-reducing NAD(P)H dehydrogenase complex (Ndh) and plastid terminal plastoquinone oxidase (PTOX) (Jans et al., 2008). The Ndh complex, located in the thylakoid membranes with catalytic centre exposed toward the stroma, utilizes NAD(P)H to reduce plastoquinone (PQ). PTOX catalyses the oxidation of reduced plastoquinone (plastoquinol, PQH_2) and the reduction of O_2 to H_2O. The catalytic site of PTOX which resides in the thylakoid membranes is exposed to the stroma (Lennon et al., 2003; Kuntz, 2004).

Photorespiration is the consequence of oxygenation of ribulose-1,5-bisphosphate (RuBP) catalyzed by ribulose bisphosphate carboxylase-oxygenase (RuBisCO). This process occurs in chloroplasts, peroxisomes

and mitochondria. During this pathway ATP and reducing equivalents are consumed and CO_2, NH_3 and H_2O_2 are produced. Thus, photorespiration is traditionally recognized as a wasteful process. However, this process serves as an energy sink protecting the photosynthetic electron transport chain from overreduction and photoinhibition. Moreover, it is a source of compounds such as glycine and serine used in other processes. Glycine is used for biosynthesis of glutathione playing multiple roles in plant defenses against stress (Noctor and Foyer, 1998; Mike et al., 1998). H_2O_2 produced during photorespiration is a key signalling molecule involved in regulation of a variety of biological processes including photosynthesis and defense responses to different environmental stimuli (Van Breusegem et al., 2006; Pitzschke et al., 2006).

The basic principle of NPQ is the safe dissipation of EEE as heat. This process is related to the xanthophyll cycle and consists of the EEE transfer from excited chlorophyll molecules located in the light-harvesting pigment-protein complexes (LHC) to the nearby carotenoid molecules, e.g. β-carotene or lutein, and dissipation of excess absorbed light energy as heat emission. Carotenoids protect the photosynthetic apparatus through two different mechanisms: (1) carotenoid molecule, e.g. β-carotene, directly quenches both triplet chlorophyll (^{3}Chl*) and $^1O_2$ and (2) the xanthophylls lower the ^{3}Chl* formation by quenching excited singlet state of chlorophyll (^{1}Chl*). It has been established that zeaxanthin (Z), which is formed from violaxanthin (V) within the LHC through the xanthophyll cycle, plays a critical role in these processes. In excessive light, an elevated $[H^+]$ in the thylakoid lumen induces the activity of violaxanthin de-epoxidase generating zeaxanthin. These xanthophyll pigments protect not only from photoinhibition but they also prevent peroxidation of membrane lipids (Eskling et al., 1997).

Dissipation of EEE as heat emission mediated by carotenoids is one of the most important photoprotection mechanisms occurring in thylakoid membranes in plants and algae. The photoprotective quenching of both triplet chlorophyll (^{3}Chl*) and $^1O_2$ in reaction centres of photosystems and in light-harvesting pigment-protein complexes is conditioned by carotenoid molecules present at these sites. In addition to this light-regulated constitutive protection mechanism, the induction of xanthophyll cycle enabling thermal dissipation of EEE captured by photosynthetic antennae, occurs. This suppression mechanism prevents the formation of highly reactive $^1O_2$ which destroys D1 protein in the reaction centre complex of PSII. Moreover, prevention of lumen acidification (long-term lowering of pH under 6.0) contributes to the protection of environmental stress sensitive oxygen-evolving complex and PSII reaction centre. As a result the xanthophyll cycle may

counteract photoinhibition of photosynthesis (Bruce et al., 1997; Xu et al., 2000).

Under strong light, especially in combination with other stressors the protective regulatory mechanisms operating in plant cells may be insufficient to use excitation energy. When electrons are not effectively channeled away from the reaction center of PSII, photodestruction of the photosynthetic apparatus (photoinhibtion) may occur. Photoinhibition involves inactivation of the photosynthetic apparatus through the production of ROS, especially $^1O_2$ which promotes degradation of D1 protein and inactivation of the plastoquinone binding Q-B niche (Nixon and Mullineaux, 2001; Iwata and Barber, 2004) as well as of the PSII and enzymes of the Calvin cycle (Stieger and Feller, 1997). Photoinhibition leads to the impairment of the oxygen evolving complex from which Mn ions are released (Hakala et al., 2005). Rapid functional reconstitution of PSII consists in energy dissipation mechanisms, especially the xanthophyll cycle (Bilger and Bjorkman, 1990; Ruban et al., 2001).

The slower rate of PSII recovery results from a disturbed process of D1 protein turnover. Under photooxidative stress the rate of D1 protein damage may exceed cell repair abilities (Andersson and Aro, 2001). The latest data suggest that D1 protein production is controlled by direct inactivation of its translation and/or by inhibition of CO_2 assimilation (Takahashi and Murata, 2008).

In order to effectively use light in photosynthesis plants have to acclimatize quickly to changing light quality and quantity. Optimization of light absorption might be controlled by many signalling pathways which may be triggered both inside and outside of a chloroplast as a result of EEE absorption (Karpinski et al., 1999; Mullineaux and Karpinski, 2002; Muhlenbock et al., 2008). Adaptation of metabolism to variable light conditions is possible, among others, due to the structural rearrangements of the photosynthetic apparatus by state transition. This mechanism consists in the translocation of a mobile pool of LHCII between PSI and PSII. State transitions require several minutes (Dietzel et al., 2008). Due to different activities of PSI and PSII caused by the changes of intensity or quality of light reaching the photosynthetic antenna system, redox status of plastoquinone (PQ) pool is modified. When plants are exposed to illumination favouring PSII, the reduction of PQ is intensified (Karpinski et al., 1999; Pfannschmidt, 2003; Allen, 2005; Kruk and Karpinski, 2006) and the signalling pathway leading to STN7 protein kinase-dependent phosphorylation of LHCII subunits, is induced (Bellafiore et al., 2005). Due to this process the phosphorylated LHCII dissociates from PSII and migrates through thylakoid membranes towards PSI. Upon preferential excitation of PSI, the PQ pool is less reduced and the constitutively active LHCII phosphatase

dephosphorylates LHCII which returns to PSII (Allen, 2005; Dietzel et al., 2008). This results in optimization of the amount of energy absorbed by both photosystems and in balancing the stoichiometry of electron transport. Besides their well-known functions in light harvesting and photoprotection, carotenoids can also play a role through their non-enzymic oxidation in the sensing and signalling of reactive oxygen species and photooxidative stress in photosynthetic organisms (Ramel et al., 2013).

Plant reaction to EEE is accompanied by changes in absorption of photosynthetic electron flux, plant water relations and in leaf temperature which are related to the increase in the activity of heat shock protein transcription factors and in abscisic acid (ABA) level as well as to changes in glutathione redox state and reduction of stomatal conductance (Panchuk et al., 2002; Fryer et al., 2003; Ball et al., 2004; Chang et al., 2004).

The above described mechanisms are directly or indirectly involved in production/scavenging of ROS with the use of antioxidative enzymes as well as with the non-photochemical quenching. Certain biochemical parameters, e.g. the activities of superoxide dismutase and xanthophyll cycle, are characterized in order to describe changes resulting from oxidative stress.

16.3. General characteristic of crassulacean acid metabolism

Higher plants have evolved three types of photosynthetic carbon metabolism, i.e. C_3, C_4 and CAM (Crassulacean Acid Metabolism). The latter occurs in about 7% of vascular plant species belonging to 34 families (Winter and Smith, 1996a; Holtum et al., 2007). CAM species grow in extreme habitats, in arid or extremely wet regions and even in water ecosystems which proves biochemical flexibility of this metabolic adaptation of photosynthesis (Dodd et al., 2002; Silvera et al., 2009).

CAM basic function consists in concentration of CO_2 in a leaf, at the site of RuBisCO (Winter and Smith, 1996b; Taiz and Zeiger, 2002; Keeley and Rundel, 2003; Luttge, 2004). It results from PEPC specificity, as it exhibits 60-fold higher affinity to CO_2 in comparison to RuBisCO. Thus when CO_2 concentration in the atmosphere is low, phospho*enol*pyruvate carboxylase (PEPC) can effectively catalyze the fixation of CO_2. In CAM plants, CO_2 is fixed during the dark period and stored over the night in the form of organic acids. According to Luttge (2002) in CAM plants the intracellular concentration of CO_2 during some phases of the day is 2-to 60-fold higher than that in the atmosphere. It is believed that in terrestrial plants CAM metabolism is advantageous as it increases their water use efficiency (WUE) due

to the fact that stomatal opening at night results in lower transpirational water loss than opening during the day.

Generally, it seems that CAM may generate especially high metabolic costs resulting from the need to maintain the enzymatic machinery. Moreover, the whole CAM cycle is much more energy-consuming than C_3 photosynthesis. From eco-physiological point of view control of possible over-excitation of the photosynthetic apparatus and dissipation of excess energy seem to be the most important problems in photosynthesizing organisms. Thus, despite the fact that CAM is a more energy-consuming process it is advantageous over other physiological processes of harmless excess energy dissipation.

CAM metabolism may be regarded as a functional variation of C_4 photosynthesis in which reactions of primary and secondary carboxylation are separated both spatially and temporally. In CAM metabolism, primary CO_2 fixation by the carboxylation of phospho*enol*pyruvate (PEP) catalyzed by PEPC (β-carboxylation) takes place in darkness when stomata are open and results in oxaloacetic acid (OAA) production. OAA is then converted into other carboxylic acids (malic and citric acids) stored in vacuoles. During the day these organic acids are decarboxylated in reactions catalyzed by NADP- and NAD-dependent malic enzymes (NADP-ME, NAD-ME) or by phospho*enol*pyruvate carboxykinase (PEPCK). This process supplies CO_2 and makes the functioning of Calvin-Benson cycle possible in light when stomata are closed. Taking into account the circadian changes of PEPC and RuBisCO activities, four phases of CAM have been distinguished (Osmond, 1978): phase I – primary CO_2 fixation during the night with open stomata and vacuolar storage of organic acids (high PEPC activity); phase II – PEPC activity decrease, induction of RuBisCO, stomata gradually closing; phase III – decarboxylation of organic acids and assimilation of CO_2 in Calvin-Benson cycle behind closed stomata (high RuBisCO activity); IV phase, end of the day – RuBisCO activity decrease, induction of PEPC, stomata gradually open.

In the process of CO_2 fixation, the regulation of activities of PEP-carboxylating enzyme (PEPC) and of organic acids-decarboxylating enzymes is of crucial importance. These antagonistic reactions cannot proceed simultaneously since this would lead to a futile cycle in which the fixed CO_2 would be immediately released. In the mechanism regulating PEPC activity a significant role is played by reversible phosphorylation catalyzed by a specific PEPC kinase (Nimmo, 2003). This PEPC kinase is regulated at the gene expression level by a circadian oscillator. Induction of PEPC kinase gene transcription takes place at night then the synthesized enzyme catalyzes PEPC phosphorylation. The activated (phosphorylated) PEPC is much less sensitive to malate inhibition and it mediates PEP carboxylation (Nimmo, 2003).

It is widely accepted that CAM induction does not require any new enzymes but a different way of existing metabolic pathways control. The differences between C_3 and CAM photosynthesis consists in enzyme activity regulations at the transcriptional, translational and post-translational levels (Dodd et al., 2002). Diversity of the variants of photosynthetic carbon metabolism is of crucial importance for plant acclimatization to constantly changing environmental conditions. CAM metabolism should be considered as a natural and very useful model to study functional correlations occurring in all metabolic types of photosynthesis. Some bushes and trees of the *Clusia* genus have an extraordinary ability to shift the photosynthetic carbon metabolism from C_3 to CAM and from CAM to C_3.

16.4. *Clusia* genus characteristic

Neotropical *Clusia* genus grows only in Central and South America. It comprises 300-400 bush and tree species with very close typical morphotype characterized by great ecophysiological flexibility resulting from diversity of photosynthetic physiotypes (Pipoly et al., 1998; Luttge, 2000b). The species differ in the size and shape of leaves, however, from the morphological and anatomical point of view the leaves are similar, quite stiff and semi-succulent. Woody species from *Clusia* genus performing CAM metabolism are the only dicots with typical secondary thickening developing from a conventional cambial ring (Luttge, 2007).

Different types of metabolism, namely C_3, C_3-CAM and CAM were described in *Clusia* genus (Borland et al., 1992; Franco et al., 1992; Luttge, 1999). CAM, considered as the capability of night accumulation of dicarboxylic acids, can be strongly (e.g. in *Clusia rosea*) or weakly expressed (e.g. in *C. minor*) and some species show great flexibility of CAM expression depending on developmental stages or environmental conditions (Luttge et al., 1993).

Analysis of rDNA (nuclear DNA responsible for rRNA sequence) and of rDNA internal transcribed spacer (ITS) regions revealed that CAM evolution was polyphyletic. The studies of phylogenetic tree on the basis of ITS analysis in nearly 89 *Clusia* species suggest that CAM evolved in this genus at least twice (Gehrig et al., 2003; Gustafsson and Bittrich, 2003). It has been hypothesized that the occurrence of CAM in *Clusia* is a phylogenetically isolated phenomenon, and that CAM photosynthesis is rather unlikely to be present in other genera of *Clusiaceae* (*Guttiferae*) (Luttge, 2007).

The metabolic flexibility and plasticity of *Clusia* genus were best studied in *C. minor* where different types of C_3 and CAM metabolism may be realized even by the opposite leaves of the same whole or even

by different fragments of the same leaf. Moreover, all known CAM variants were observed in this species (Luttge, 1991) and the C_3-CAM metabolic shift is dynamic, reversible and may occur frequently and repeatedly. Thus *C. minor* is able to colonize different habitats ranging from arid savannah to rain forests in subtropics (Luttge, 2006). With reference to the variability of CAM metabolism observed in *Clusia* genus, it seems that the widely used assessment of CAM based on nocturnal accumulation of malate and citrate is a simplified explanation of this phenomenon.

A lot of ecophysiological data indicate that C_3 photosynthesis is more efficient than CAM under light stress (Luttge, 2007) and this may explain why many CAM species prefer shady areas (Luttge, 2000a). It is well documented that CAM is a survival strategy triggered when CO_2 assimilation by RuBisCO is limited.

16.5. The course of xanthophyll cycle in plants from *Clusia* genus

Many studies concern the possibility of light stress-induced shift from C_3 metabolism to CAM in *Clusia* genus. We studied the light stress-induced changes in epoxidation state (EPS) of xanthophyll cycle pigments and in the activity of xanthophyll cycle in *Clusia* species representing different types of the photosynthetic carbon metabolism.

The research was conducted on *Clusia* species grown under varied stressful conditions and differing with respect to the photosynthetic metabolism, namely *C. multiflora* (an obligatory C_3 species), *C. minor* and *C. fluminensis* (intermediate C_3-CAM plants) and *C. alata* and *C. hilariana* (obligatory CAM species). *C. minor* is the most studied species and is becoming a model plant for the whole *Clusia* genus whereas *C. multiflora,* an obligatory C_3 species, is often used as a reference. These two species were often used for research both under laboratory and natural conditions.

In the first variant *C. minor* and *C. multiflora* were exposed for 7 days to drought and HL (high light) stress (photosynthetic photon flux density (PPFD) 1200 μmol $m^{-2}s^{-1}$ for 12 h per day) (Miszalski et al., 2007). In the second variant *C. minor, C. multiflora*, *C. alata* and *C. hilariana* grown under optimal water conditions were exposed for 2 days to HL stress but with lower PPFD (650-740 μmol $m^{-2}s^{-1}$ for 12h per day) (Kornas et al. 2010). In the case of *C. alata* and *C. hilariana* the HL stress was prolonged to 7 days (Kornas et al., 2009; Miszalski et al., 2013). In the first variant the analysis of photon-use efficiency measured as epoxidation state (EPS) of xanthophyll cycle pigments in *C. minor* showed its lowering under combined drought and HL stress in the morning in comparison to well-watered plants (Fig. 1). Regardless of water availability, EPS in *C. minor* was relatively low in the after-

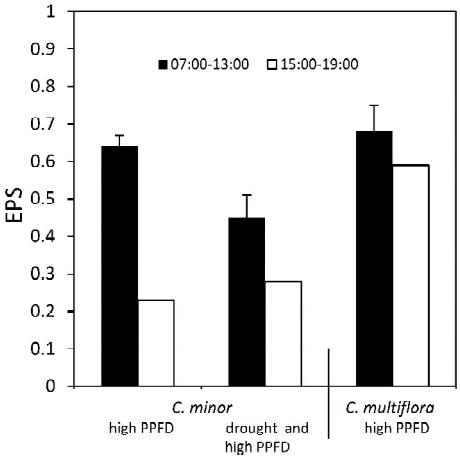


Fig. 1. Epoxidation state (EPS; V+0.5 A)/V+A+Z) of the xanthophyll cycle pigments of *Clusia minor* and *Clusia multiflora* exposed to high irradiance (1200 μmol $m^{-2}s^{-1}$) alone or to drought stress and high irradiance. Data are means ± SD, n = 3 for EPS values between 07:00 and 13:00. For the time period of 15:00 to 19:00 values represent the mean of two measurements (Miszalski et al., 2007*).

*Original published data in table form are presented in the graph.

noon. In *C. multiflora* EPS level was stable throughout the day and was similar to that observed in the morning in *C. minor* under optimal water conditions. This supports the earlier suggestion that *C. multiflora*, the obligatory C_3 plant, uses water and light better under high PPFD conditions than *C. minor* performing C_3-CAM. This is in agreement with observations that *C. minor* prefers shaded areas (Luttge, 2000a; Miszalski et al., 2007).

In *C. alata* on the 2nd day of HL stress a significant increase in the NPQ level was found in the afternoon. A similar tendency was observed at noon and dusk (data statistically insignificant). In plants exposed to HL for 7 days some NPQ increase was observed only at noon and at dusk. Thus it seems that a prolonged exposition to HL activated other defense mechanisms. It was observed that the maximum NPQ value on the 2nd day of intense light exposure correlated with the actual decrease in citrate content during phase III of CAM. Changes in these two parameters were parallel, thus it has been suggested that NPQ increase and citrate decarboxylation form the first line of defense before other photoprotective mechanisms are triggered (Kornas et al., 2009).

It is known that plants exposed to HL adapt the levels of xanthophyll cycle components and the activity of the antioxidant system. In our experiment, in *C. alata* exposed for 7 days to HL, a significant increase in the total pool of violaxanthin (V, doubly epoxidized) + antheraxanthin (A, mono-epoxidized) + zeaxanthin (Z, non-epoxidized) was observed at noon and in the afternoon (Fig. 2). Enhanced Z level was responsible for the increase in the total pool of xanthophyll pigments. The content of Z increased slightly after 2-day exposure to HL while after 7 days a significant increase in the afternoon was observed. It was found that the levels of V+A+Z did not differ significantly if the samples were collected in the morning and at dusk which suggests that during HL exposition not only total Z level increased but also the epoxidation/de-

epoxidation mechanism was induced. These processes require longer time (usually several days) to be activated. In *C. alata* they were accompanied by a slight increase in pigment contents in the morning and in the evening. Thus, it means that even in plants grown under low light (LL) conditions the level of xanthophylls is sufficient to control the HL stress. In *C. alata* on the 7th day of light exposure at noon a significant decline in EPS level was observed. It was accompanied by increment of Z content in the time when high levels of malate and citrate were available. Later during the day-course EPS level increased (Kornas et al., 2009).

In *C. hilariana* after one week of HL exposure Z, A and V contents and the EPS value had clearly changed during the light-phase resulting in stronger adaptations of xanthophyll cycle pigments which were much clearer than after 2 days of exposure (Fig. 3). This indicates high activity of the xanthophyll cycle in dissipating light energy and was observed at noon and during the evening when the

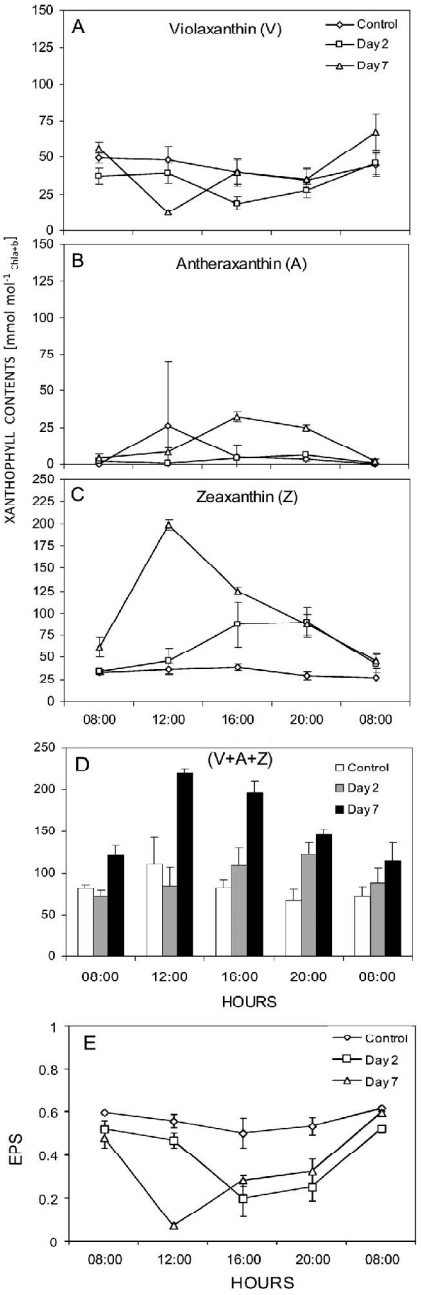
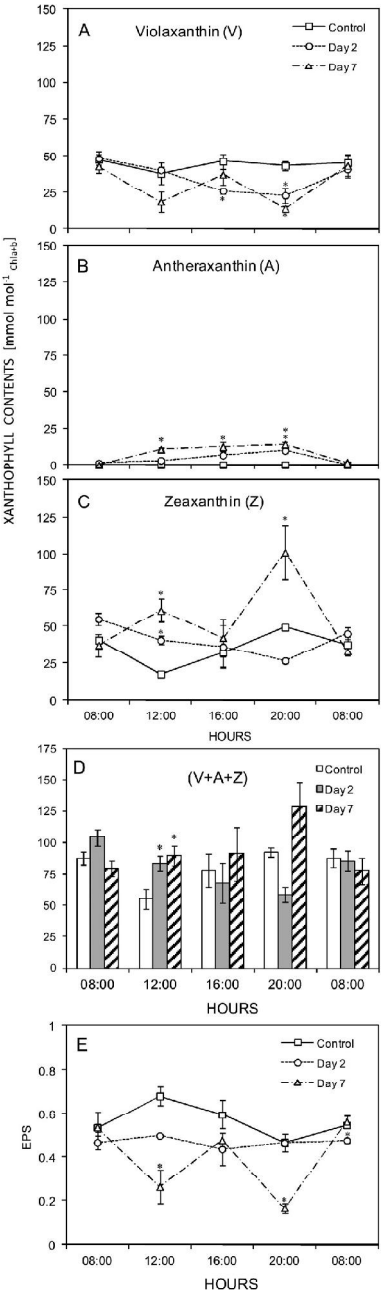


Fig. 2. Daily time-courses of the levels of violaxanthin, V (A); antheraxanthin, A (B); zeaxanthin, Z (C); violaxanthin+antheraxanthin+zeaxanthin (D) and the epoxidation state (EPS) (V+0.5 A)/(V+A+Z) of the xanthophyll cycle pigments (E) in *Clusia alata* leaves exposed to high light (650-740 μmol m^{-2} s^{-1}) under well-watered conditions. Samples were collected from control (day 0) and on days 2 and 7 after high light exposure; values represent means ± SD (n = 3).

Source: Kornas et al. 2009.



malate + citrate pool was depleted. The mechanism of interconversion of xanthophyll pigments (epoxidation/de-epoxidation) measured as EPS was activated, but the process took several days. Small daily fluctuations in the EPS value were observed also in plants growing at LL. Thus, to some extent this system responsible for dissipation of excess energy operated also at LL when no CAM symptoms were observed (Miszalski et al., 2013).

When *C. minor* and *C. multiflora* grew under optimal water conditions and they were exposed to HL (PPFD 650-740 μmol $m^{-2}s^{-1}$) for 2 days they performed C_3 metabolism. In both species on the 2nd day of HL exposition the level of V did not change significantly while the contents of A and Z increased significantly in the afternoon (Fig. 4) (Kornas et al., 2010). In *C. minor* these differences were statistically significant which indicated that in both species under HL stress de-epoxidation process, i.e. intensification of xanthophyll cyle, occurred. The changes of A and Z were not reflected in V pool. The total con-

Fig. 3. Daily time-courses of the levels of violaxanthin, V (A); antheraxanthin, A (B); zeaxanthin, Z (C); violaxanthin+ antheraxanthin+ zeaxanthin (D) and the epoxidation state (EPS) (V+0.5 A)/(V+A+Z) of the xanthophyll cycle pigments (E) in *Clusia hilariana* leaves exposed to low light (200 μmol m^{-2} s^{-1}) – control and high light (650-740 μmol m^{-2} s^{-1}) under well-watered conditions. Samples were collected from control (day 0) and on days 2 and 7 after high light exposure. Values represent means ± SD (n = 3). Asterisks indicate significance of differences from controls ($p < 0.01$; T-test).

Source: Miszalski et al. 2013.

tent of V was higher than those of A and Z, thus changes of A and Z seem to be in the range of fluctuations and oscillations of V pool indicated by high SD values. Slight decreasing tendency of V content on the 2nd day is accompanied by significant enhancement of A and Z which can be explained by changes of the total pigment pool. Both *C. multiflora* and *C. minor* effectively use xanthophyll cycle for photoprotection. It was proved by the analysis of A and V levels. In our experiment the levels of EPS in *C. minor* and *C. multiflora* were comparable both in the morning and in the afternoon. In previous studies with *Clusia* it was shown that HL (PPFD about 1200 μmol $m^{-2}s^{-1}$) and drought induced significant EPS level decrease (Miszalski et al., 2007). EPS level decline is characteristic of plants exposed to HL (Adams and Demmig-Adams, 1992) however, high PPFD applied in our experiment (650-740 μmol $m^{-2}s^{-1}$) did not influence this parameter. It seems that very dynamic changes and reorganization of PSII are not directly correlated with changes of CAM (Johnson et al., 2008; Horton et al., 2005).

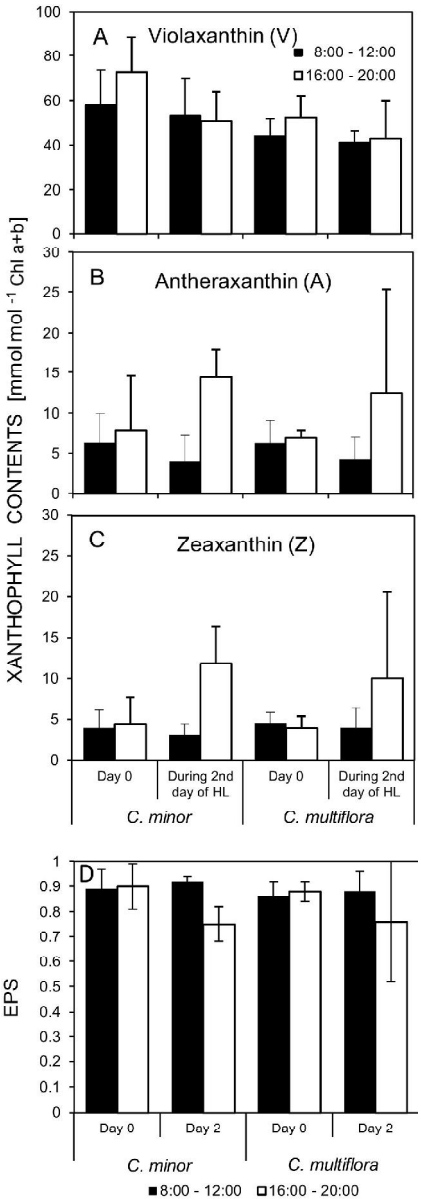


Fig. 4. Daily time-courses of the levels of violaxanthin, V (A); antheraxanthin, A (B); zeaxanthin, Z (C) and the epoxidation state (EPS) (V+0.5 A)/(V+A+Z) of the xanthophyll cycle pigments (E) in leaves of *Clusia minor* and *Clusia multiflora* growing at low light (day 0) and during the day 2 of stress application of high light (HL, 650-740 μmol m^{-2} s^{-1}) under well-watered conditions. Data represent means ± SD (n = 3).

Source: Kornas et al. 2010.

In *C. alata* transferred from LL to HL transient NPQ enhancement was first observed indicating strong photoinhibition. After prolonged (7 day) exposition to HL, photoinhibition decreased and the mechanism of V, A and Z interconversion (epoxidation/de-epoxidation) was activated. This process was accompanied by the increase in pigment levels at dawn and dusk. Our study indicated that *C. alata*, an obligatory CAM plant, is able to adapt well to HL conditions (Kornas et al., 2009). *C. minor* apart from xanthophyll cycle may use CAM for photoprotection but this mechanism is not activated under optimal water conditions (Kornas et al., 2010). *C. multiflora*, an obligatory C_3 species, is able to use water and light under high PPFD more efficiently than *C. minor*, a facultative C_3-CAM species (Miszalski et al., 2007). The role of xanthophyll pigments constituting the xanthophyll cycle in *C. hilariana* seems to be secondary and this cycle is not responsible for CAM induction. It is also clear that changes in total xanthophyll pigment levels are not very fast even when their daily fluctuations are visible (Miszalski et al., 2013).

16.6. Conclusions

Neotropical *Clusia* genus shows high ecophysiological flexibility resulting from diversity of photosynthetic physiotypes, i.e. C_3, CAM or C_3-CAM, found in these plants. Their physiological adaptations depend, at least partly, on photoprotective mechanisms including the xanthophyll cycle. It has been shown that even in *Clusia* plants grown under LL the levels of xanthophylls were sufficient to control HL stress. In plants exposed to HL, the contents of xanthophylls and their contribution to dissipation of excess light energy increased at noon and evening (during the CAM cycle), when malate and citrate pools were depleted. Despite the important function of xanthophyll pigments in the photosynthetic metabolism, their role in CAM induction seems to be of minor importance.

Acknowledgements

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Chapter 17. ALTERATION IN CHLOROPHYLLS AND CAROTENOIDS IN HIGHER PLANTS UNDER ABIOTIC STRESS CONDITION

Mirza Hasanuzzaman[1, 2], Kamrun Nahar[1, 2], and Masayuki Fujita[1]
[1] Kagawa University, Kagawa, Japan
[2] Sher-e-Bangla Agricultural University, Dhaka, Bangladesh

Contents

17.1. Introduction

Photosynthetic pigments or antenna pigments are present in chloroplasts and capture the light energy necessary for photosynthesis. The most abundant and important photosynthetic pigments in plants are chlorophyll (Chl) and carotenoids (Car). Photosynthesis in plants is dependent upon capturing light energy in the pigment Chl, and in particular Chl *a*. This Chl resides mostly in the chloroplasts and gives leaves their green color. Chlorophylls are necessary for the capture of light energy and also act as primary electron donors. Carotenoids play crucial roles in both light harvesting and energy dissipation for the protection of photosynthetic structures. The range of light absorption in leaves is facilitated by some accessory pigments such as the Car. In

addition, as an antioxidant Car plays a multitude of functions in plant metabolism including oxidative stress tolerance.

Plants are frequently exposed to a plethora of unfavorable or even adverse environmental conditions, termed abiotic stresses such as salinity, drought, heat, cold, flooding, heavy metals, ozone (O_3), ultraviolet (UV) radiation, nutrient deficiencies etc. and thus they pose serious threats to the sustainability of crop yield (Ahmad and Prasad 2012a, b; Hasanuzzaman et al., 2012). Abiotic stresses are the greatest constraint for crop production worldwide. It has been estimated that more than 50% of yield reduction is the direct result of abiotic stresses (Rodriguez et al., 2005; Acquaah, 2007). Most of the crops grown under field conditions are frequently exposed to various abiotic stresses. The complex nature of the environment, along with its unpredictable conditions and global climate change, are increasing gradually, which is creating a more adverse situation (Mittler and Blumwald, 2010). Abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang et al., 2001). One of the most visible changes in the plants due to abiotic stress is alteration of photosynthetic pigments i.e. Chl and Car.

Several reports confirmed that these photosynthetic pigments significantly altered by different stressors like salinity (Borghesi et al., 2011; Chookhampaeng, 2011), drought (Kannan and Kulandaivelu, 2011; et al., 2011), high temperature (Nxawe et al., 2011; Ornelas-Paz et al., 2011), low temperature (Aghaee et al., 2011; Mishra et al., 2011; Soares-Cordeiro et al., 2011), flooding (Cazzonelli, 2011), metal toxicity (Pant et al., 2011; Singh and Pandey, 2011), O_3 (Blum et al., 2011; Correa et al., 2011), UV-radiation (Kuster and Lage, 2011; Sudheer et al., 2011), and nutrient deficiencies (Fallovo et al., 2011; Multu et al., 2011; Zhou et al., 2011). Extreme environmental factors are having an ever greater effect on agriculture and thus plant biologists are faced with the urgent task of developing genotypes capable of tolerating environmental changes with the least possible damage. Considering the above facts one of the future strategies of plant stress tolerance would be the enhancement of the biosynthesis of photosynthetic pigments under stressful condition.

In this chapter, we have provided a comprehensive review on the recent reports on the alteration/changes in photosynthetic pigments in plants exposed to various abiotic stresses in the light of recent reports. We have also briefly discussed the biosynthesis and general functions of these pigments in plants.

17.2. Biosynthesis and functions of chlorophylls and carotenoids in plants

17.2.1. Chlorophyll

Leaf mesophyll is the most active photosynthetic tissue in higher plants containing numerous chloroplasts, which possess the particular light-absorbing green pigments, the chlorophylls. In higher plants, Chl is vital for photosynthesis that strongly absorbs light and may act as photosensitizers (Meskauskiene et al., 2001). Photosynthesis is a process by which the plant uses solar energy to oxidize water, thereby releasing O_2, and to reduce CO_2, thereby forming large carbon compounds, primarily sugars.

There are several forms of Chl identified so far (Fig. 1). There are five chemically distinct Chls known to date in oxygenic photosynthetic organisms, termed Chls *a*, *b*, *c*, *d* and *f* in the order of their discovery (Larkum and Kuhl, 2005; Scheer, 2006; Chen et al., 2010). Chlorophyll *a* and *b* are abundant in green plants, and *c, d* and *f* are found in some protists and cyanobacteria (Taiz and Zeiger, 2002). In fact, Chl *f* is a hypothesized form of Chl that absorbs further in the red (infrared light) than other Chls which was reported from cyanobacteria (Chen et al., 2010). All five pigments are present in light-harvesting complexes, though until recently only Chl *a* was thought to be indispensable for energy transduction in the photosystem reaction centers (Bjorn et al., 2009).

Chlorophyll biosynthesis in higher plants. Like all other biomolecules, Chls are produced by a biosynthetic pathway in which simple molecules are used as building blocks to assemble more complex molecules which consists of more than a dozen of steps (Taiz and Zeiger, 2002; Fig. 2). However, the biosynthesis of Chl is a very complex process requiring many enzymes to transform the first committed precursor, D-aminolevulinic acid (ALA) into the final product(s), i.e. Chl *a* or Chl *b* and so on. As reviewed by Beale (1999), the overall pathway of Chl synthesis from glutamate can be divided into several sections, each leading to a key intermediate or branch point: the initial steps (Fig. 2) that divert general metabolic intermediates into the formation of the first cyclic tetrapyrrole (uroporphyrinogen III); transformation of uroporphyrinogen III to protoporphyrin IX along the oxidative branch; insertion of Mg^{2+} into protoporphyrin IX to begin the branch leading to Chl; formation of the isocyclic 'fifth' ring that is present on all Chl; reduction of a peripheral vinyl group to an ethyl group, followed or preceded by reduction of the macrocyclic ring system to form a chlorine, the defining oxidation state of true Chl; and addition of a polyisoprene alcohol to the tetrapyrrole to complete the structure of Chl *a* or Chl *b* (Beale, 1999; Fig. 2).

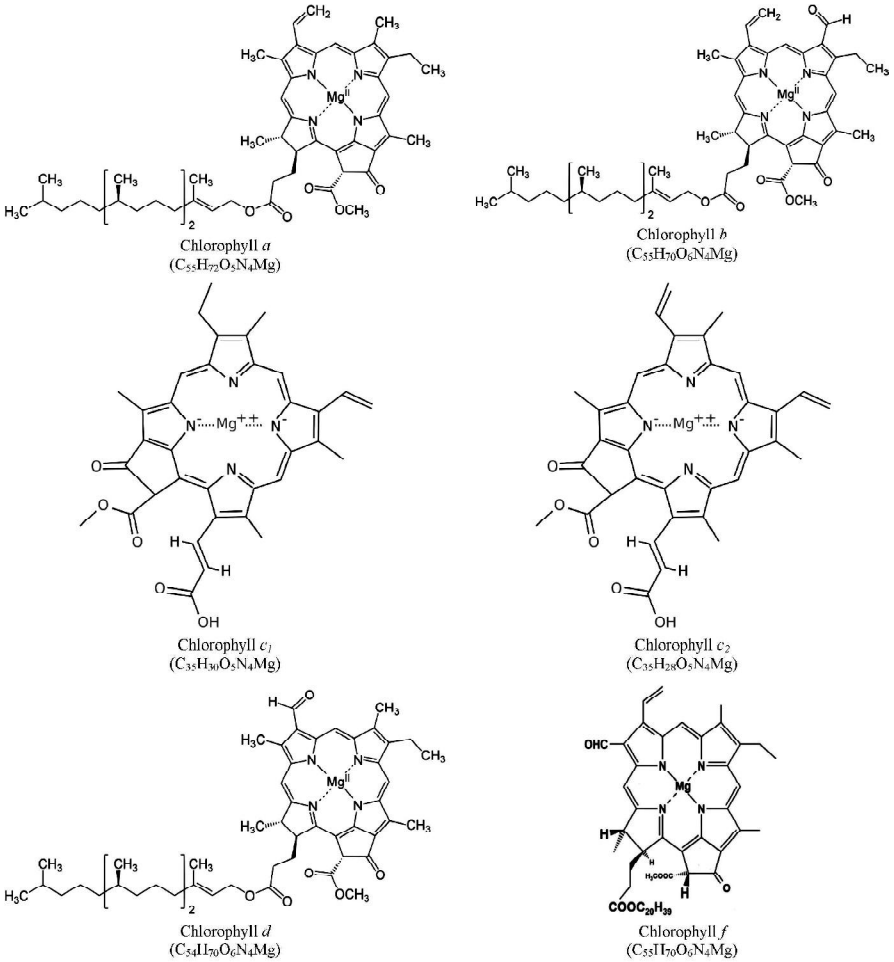

Fig. 1. Different forms of chlorophyll found in photosynthetic organisms.

In the first phase of Chl biosynthesis, the amino acid glutamic acid is converted to ALA (Fig. 2). Two molecules of ALA are then condensed to form porphobilinogen (PBG), which ultimately form the pyrrole rings in Chl. The next phase is the assembly of a porphyrin structure from four molecules of PBG. This phase consists of six distinct enzymatic steps, ending with the product protoporphyrin IX. In the next step, magnesium (Mg) is inserted by an enzyme called Mg-chelatase, and then the additional steps needed to convert the molecule into Chl take place.

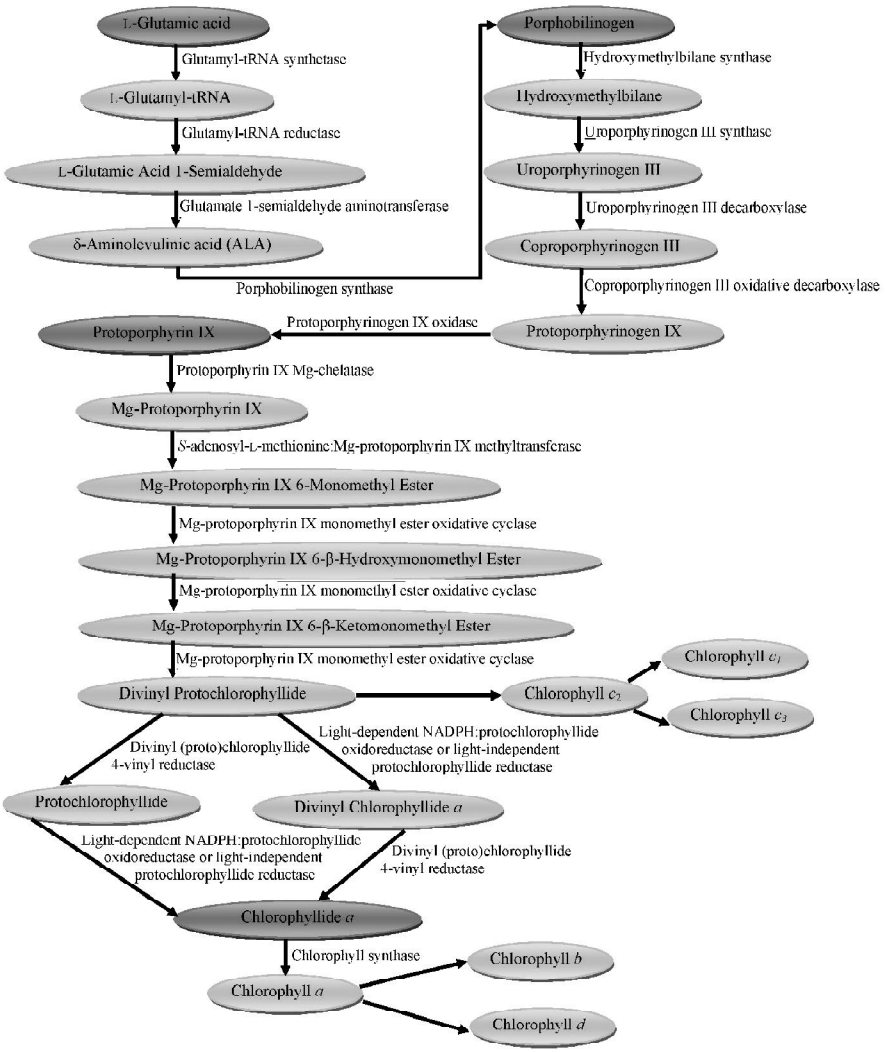

Fig. 2. Simplified outline of chlorophyll biosynthesis pathways in higher plants.

The next phase of the Chl biosynthetic pathway is the formation of the fifth ring (ring E) by cyclization of one of the propionic acid side chains to form protochlorophyllide. The pathway involves the light-driven NADPH-dependent reduction of one of the double bonds in ring D which is carried out by protochlorophyllide oxidoreductase (POR).

The final step in the Chl biosynthetic pathway is the attachment of the phytol tail, which is catalyzed by Chl synthetase (Malkin and Nyogi, 2000; Fig. 2).

The elucidation of the biosynthetic pathways of Chls and related pigments is a difficult task, in part because many of the enzymes are present in low abundance. Recently, genetic analysis has been used to clarify many aspects of these processes. In plants, Chl may be synthesized from succinyl-CoA and glycine, although the immediate precursor to Chl *a* and *b* is protochlorophyllide. Chlorophyll itself is bound to proteins and can transfer the absorbed energy in the required direction. Protochlorophyllide occurs mostly in the free form and, acts as a photosensitizer in light and generates highly toxic free radicals. Hence, plants need an efficient mechanism of regulating the amount of Chl precursor which is done at the step of ALA formation (Meskauskiene et al., 2001).

17.2.2. Carotenoids

Plant Car are 40-carbon tetraterpenoid organic pigments orisoprenoidorganic compound with polyenechains that may contain up to 15 conjugated double bonds. Because of their chemical properties Car are essential components of all photosynthetic organisms that are of chloroplast and chromoplast of plant, algae, some bacteria, and some types of fungus. Carotenoids constitute a much larger group of over 700 structures (Britton et al., 2004); they are split into two classes, xanthophylls (which contain oxygen) and carotenes (which are purely hydrocarbons, and does not contain oxygen) (Pizarro and Stange, 2009). Some major Car and their structures are given in Fig. 3.

Carotenoids biosynthesis in higher plants. The Car biosynthesis is an enzymatically catalyzed complex process. These enzymes are presented in the stroma, plastid membrane and cytosol and, therefore, the biosynthesis occurs in these organelles in a linear way from one organelle to another. Production of substrate isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) is the first step of the synthesis of Car. There are two different compartments where by two different pathways these substrates are synthesized in plant cell. First one is cytosol and endoplasmic reticulum located mevalonate (MVA) pathway and the second one is plastid located methylerythritol (MEP) pathway (Francis and Cunningham, 2002; Fig. 4). The MVA pathway begins with acetyl-CoA and proceeds to form hydroxymethylglutaryl-CoA (HMG-CoA) and then MVA. The last product of this pathway is IPP, which is then reversibly converted to DMAPP (dimethylallyl diphosphate) in a reaction catalyzed by IPP isomerase (IPI) (Bachet et al., 1999). The MEP pathway occurs in plant plastids, in cyanobacteria, and in certain other bacteria (Rohmer, 1999), and utilizes pyruvate

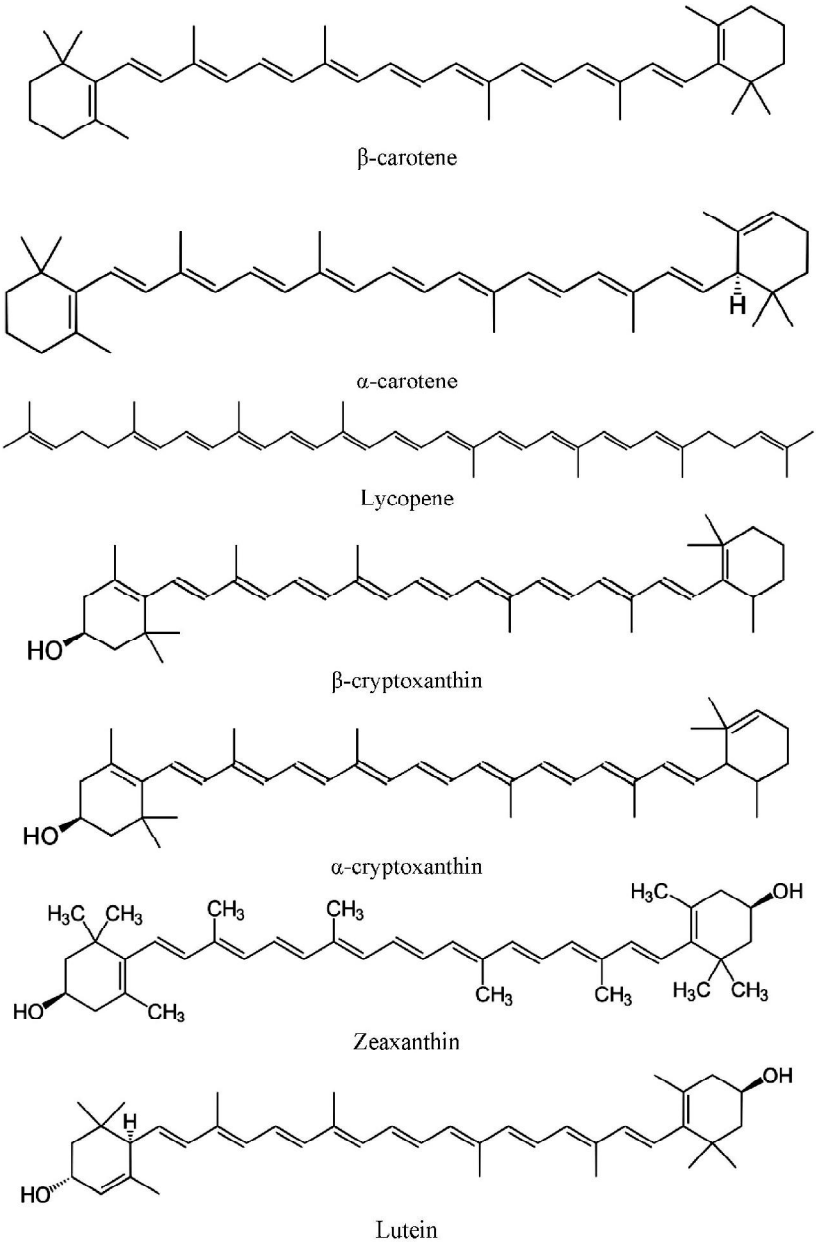

Fig. 3. Some common carotenoids in plants.

and glyceraldehyde-3-phosphate (GAP) as the initial substrates (Fig. 4). Through the action of enzyme deoxyxylulose-5-phosphate synthase (DXS) the product deoxyxylulose-5-phosphate (DXP) is produced. Methylerythritol is produced by the activity of deoxyxylulose-5-phosphate reductoisomerase (DXR) and then DMAPP and IPP are produced individually via a branching of the MEP pathway (Rodriguez-Conception et al., 2000).

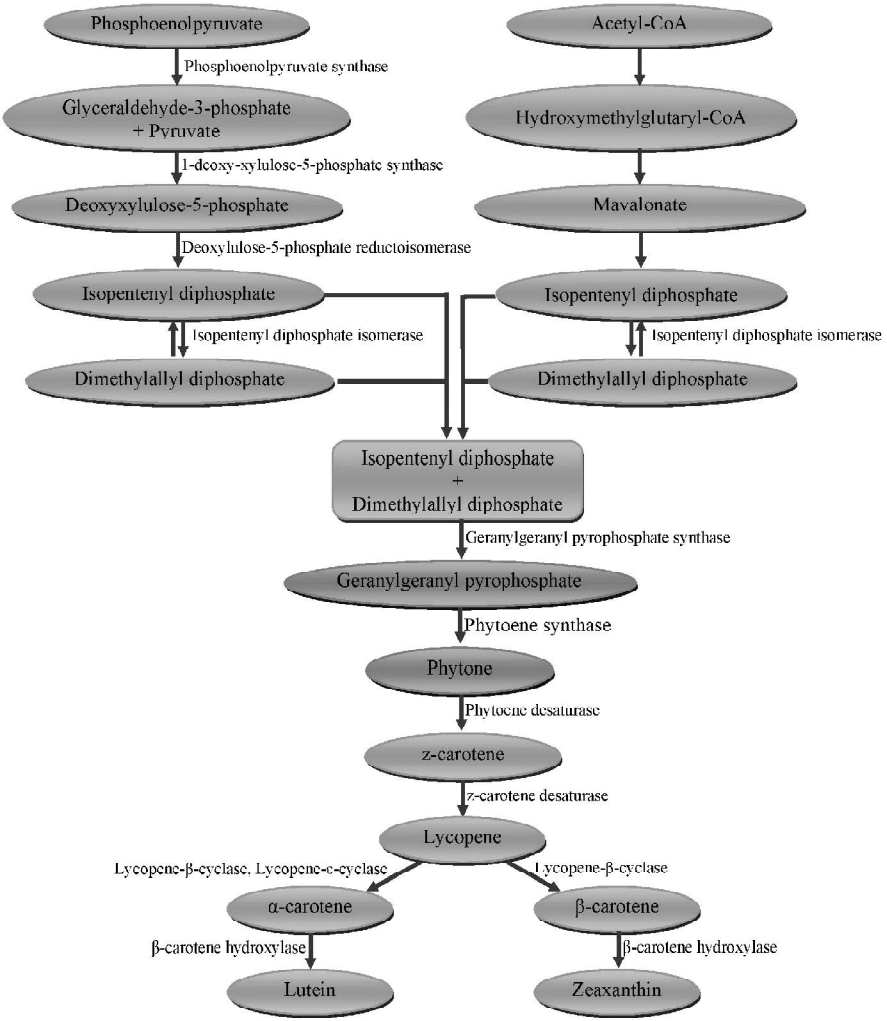

Fig. 4. Simplified outline of carotenoids biosynthesis pathway in higher plants.

The next step is the production of 20-carbon molecule geranylgeranyl pyrophosphate (GGPP) from three molecules of IPP and DMAPP. In this process GGPP synthase (GGPPS) catalyzes the reaction. All the DXS, IPI and GGPPS enzymes are located in the stroma of the chloroplast where they catalyze the formation of IPP, DMAPP and GGPP in higher plants (Cunningham, 2002). Phytoene biosynthesis is the first reaction specifically related to the Car biosynthesis pathway in higher plants which froms GGPP (Francis and Cunningham, 2002; Pizarro and Stange, 2009). Once the phytoene is produced, it can be easily converted into different types of Car by desaturation and hydroxylation reaction but several other steps may occur between phytoene and the final Car product.

Desaturation of the colorless phytoene produces the pink-colored trans-lycopene. These reactions are catalyzed by phytoene desaturase (PDS) that catalyzes the biosynthesis of z-carotene, z-carotene desaturase (z-CDS) which synthesizes pro-lycopene (7, 9, 9', 7'-tetra-cislycopene) and carotene isomerase (CRTISO) that transforms the prolycopene into lycopene (all-trans-lycopene) in plants (Park et al., 2002; Isaacson et al., 2004).This lycopene produces different Car by cyclilization. For instance, lycopene-β-cyclase (LCYB) converts lycopene into γ-carotene and subsequently to β-carotene. The other enzyme is lycopene-ε-cyclase (LCYE), which cyclizes one ends of the lycopene molecule with an ε-ring (δ-caroteno); the other ring is formed by LCYB, thus producing α-carotene (Cunningham et al., 2007). The β-carotene synthesized is utilized as a substrate for the enzyme β-carotene hydroxylase (CβHx) to produce zeaxanthin, while is formed by hydroxylation of α-carotene by the ε-carotene hydroxylase (CεHx) and CβHx.

17.2.3. Function of chlorophyll and carotenoides in plants

Both the Chl and Car are active in the absorption of light for photosynthesis and therefore contribute towards the productivity of plants. Chlorophyll is one of the major chloroplast components for photosynthesis, and relative Chl content has a positive relationship with photosynthetic rate. Chlorophyll captures the sun's radiant energy and converts it into chemical energy for the plant which plays an important role in photosynthesis, the process in which plants obtain materials and make the food they need. The increase of Chl content in plants and subsequent increase in the photosynthetic rate show better response to different types of abiotic stress and thus improve crop productivity (Fig. 5). In parallel with Chl, Car are also important for plant growth and development in various ways. Carotenoids are involved in photosystem assembly, light harvesting and photoprotection, photomorphogenesis,nonphotochemical quenching, lipid peroxidation and affect the size and function of the light-harvesting antenna and seed set (Lokstein

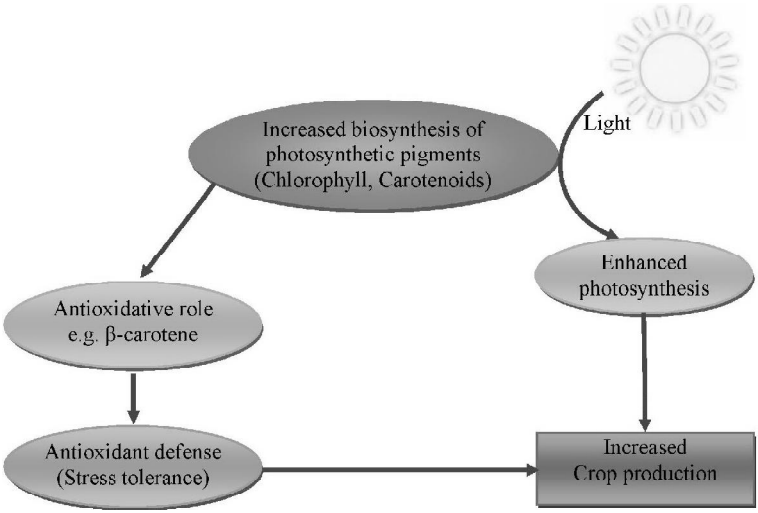

Fig. 5. Relation between photosynthetic pigments and crop production.

et al., 2002; Holt et al., 2005). Some kinds of Car absorb light energy for use in photosynthesis, and they protect Chl from photodamage (Armstrong and Hearst, 1996). In photosynthesizing species lutein and zeaxanthin and few several other Car, are integrally associated with the light harvesting Chl proteins (LHCP) of photosynthetic systems and regulate light harvesting in photosynthetic systems and increase the efficiency of light harvesting. Carotenoids are also involved in the distribution of light harvesting complexes between the two photosystems, PSI and PSII (Johnson, 2009).Thus, Car plays great roles in photosynthesis also. In addition, some major phytohormones, strigolactones and abscisic acid (ABA), are derived from Car precursors (Cazzonelli and Pogson, 2010) and in some cases gibberellins are produced as byproduct (Francis and Cunninghum, 2002). In flowers and fruits, the presence of these pigmented molecules serves to attract pollinators and seed dispersal agents to the intense yellow, orange and red colors that they provide (Grotewold, 2006). Carotenes and xanthophylls also accumulate in lipid bodies or in crystalline structures in the chromoplasts of flowers, fruits and reserve roots (Vishnevetsky et al., 1999).

17.3. Role of chlorophyll and carotenoids in abiotic stress tolerance

Both the Chl and Car absorb light for photosynthesis and thus the increase of the content in plants and subsequent increase in the

photosynthetic rate improves crop productivity and better response to different types of abiotic stress. There are numerous reports indicating the decrease in Chl content under abiotic stresses due to the result of pigment photo-oxidation and Chl degradation (Bertrand and Schoefs, 1999; Korkmaz, 2012). Photosynthetic pigments are important to plants mainly for harvesting light and production of reducing powers. Thus researchers are trying to develop the plants with more capable to synthesis photosynthetic pigments and observed that increased capacity of pigment synthesis confers stress tolerance.

It was proved that transgenic plants in which the Chl biosynthetic pathway was genetically modified are potentially useful for agricultural and horticultural applications (Tanaka and Tanaka, 2007). As Chl synthesizing enzymes are the targets of stressors, attempts were made to produce transgenic plants tolerant to those adverse situations (Tanaka and Tanaka, 2007). Zavgorodnyaya et al. (1997) introduced the gene encoding yeast ALA synthase into the tobacco genome, so that the transgenic plants could bypass the glutamate-to-ALA-synthesizing route, resulting in the tolerance of the transgenic plants to stress. Shen et al. (2006) identified the ChlH protein as a novel receptor of ABA, which regulates plant responses to stressful conditions and various developmental processes. Exogenous application of ALA at low concentrations increases Chl content of plants, promoting photosynthetic capacity and yield of crops. Recent studies have demonstrated that ALA has both growth promoting and stress mitigating capabilities and when applied in appropriate concentrations, ALA is also reported to provide enhanced tolerance to abiotic stresses (Korkmaz, 2012). The ability of ALA to boost abiotic stress tolerance is due to elevated activities of enzymatic or nonenzymatic antioxidant system providing significant protection to the membranes against harmful reactive oxygen species (ROS) within tissues (Korkmaz, 2012). Balestrasse et al. (2010) observed that ALA at low concentrations (5-10 μM) provided significant protection against cold stress in *Glycine max* compared to non-treated plants by enhancing Chl content and by preventing lipid peroxidation. In a recent study, it was indicated that application of ALA improved Chl content, but reduced malondialdehyde (MDA) content and ROS production significantly under water-deficit stress (Liu et al., 2011). Lower dosages of ALA also enhanced glutathione (GSH) and ascorbate (AsA) redox state as compared to the seedlings under water-deficit stress. Exogenous ALA treatment also considerably enhanced the activities of ascorbate peroxidase (APX), peroxidase (POD), catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) under water-deficit stress. Same treatment also induced the expression of POD, CAT and GR to a certain extent (Liu et al., 2011).

Recently, Chl fluorescence is widely used as a tool to investigate photosynthetic performance in plants (Baker, 2008; Woo et al., 2008). Many researchers used Chl fluorescence analysis to estimate PS II activity, which is an important target of abiotic stresses (Calatayud et al., 2008; Ehlert and Hincha, 2008; Massacci et al., 2008). The application of Chl *a* fluorescence has been extensively used in diverse areas, such as in the understanding of chloroplast structure-function relationship, and influence of various abiotic stress factors on photosynthesis. These reports strongly established the correlation between Chl synthesis and abiotic stress tolerance.

In line with Chl, Car also play crucial roles in both light harvesting and energy dissipation for the protection of photosynthetic structures. They serve three major functions in plants: (i) they absorb light at wavelength between 400 and 550 nm and transfer it to the Chl (an accessory light harvesting role) (Sieferman-Harms, 1987); (ii) they protect the photosynthetic apparatus by quenching a triplet sensitizer, $^1O_2$ and other harmful free radicals which are naturally formed during photosynthesis (an antioxidant function) (Havaux and Niyogi, 1999; Collins, 2001); (iii) they are important for the PSI assembly and the stability of light harvesting complex proteins as well as thylakoid membrane stabilization (a structural role) (Niyogi et al., 2001; Gill and Tuteja, 2010; Gill et al., 2011). Importantly, apart from their light harvesting role, Car also have antioxidative role in plants and therefore involved in stress tolerance. In all photosynthetic organisms, the Car viz. β-carotene and zeaxanthin serve important photoprotective role, either by dissipating excess excitation energy as heat or by scavenging ROS and suppressing lipid peroxidation (Gill and Tuteja, 2010). Carotenoids also serve as precursors to signaling molecules that influence plant development and abiotic stress responses (Li et al., 2008).

As an antioxidant Car play multitude of functions in plant metabolism including oxidative stress tolerance. Car within the photosynthetic apparatus are known to quench $^1O_2$ (Sieferman-Harms, 1987), but the photosynthetic growth of cells lacking Car suggests there are other mechanisms to protect cells from $^1O_2$ damage. It was found that inhibition of Car synthesis by various inhibitors/herbicides, results in rapid photo-oxidative bleaching of chloroplasts. Carotenoids are also able to react directly with $O_2^{\cdot -}$ and other free radicals (Young and Lowe, 2001; Krinsky and Yeum, 2003). It can also form resonance-stabilized carbon-centered radicals, e.g. by reaction with lipid peroxyl radicals (ROO·): CAR+ROO· → ROO→CAR·. In addition, Car could also react with ROO· to produce lipid hydroperoxides (LOOHs) and Car radicals (CAR·) (Smirnoff, 2005). Yildiz-Aktas et al. (2009) observed a higher content of Car and antiradical capacity and lower MDA level in the drought-tolerant genotype of cotton compared with the sensitive

genotype at normal water supply which might be due to the more effective defense system maintained by tolerant plants. They also indicated that the plants subjected to water deficit, a decline in the level of Car and antiradical capacity was observed which established a proof of a positive correlation between Car content and drought tolerance. Apart from the whole plants, Car are also considered as antioxidants protecting the seeds against oxidative stress. Carotenoids are precursors in the biosynthesis of ABA and this is involved in dormancy, maturation and differentiation of vegetal embryonic cells and in tolerance to abiotic stress (Crozier et al., 2000). Recently, Smolikova et al. (2011) studied the role of Car in seed tolerance to abiotic stressors. The seed Car were represented mainly by lutein and, in much smaller quantities, by β-carotene. Carotenoids were found to be accumulated in seeds during seed germination at high temperatures. However, the Car/Chl ratio was proposed to be a measure of seed tolerance to stress factors. The seeds with elevated Car/Chl ratio were characterized by higher tolerance to stress treatments.

In plants, zeaxanthin is considered as a regulator of light harvesting, and plays a fundamental role in the xanthophyll cycle of PSII. Zeaxanthin carries out three protective functions: (i) protection against photo-oxidation due to radical oxygen attack (physical quenching of oxygen singlet energy), (ii) absorption of Chl triplet energy (which, if not captured, leads to $^1O_2$ formation through a process in which ground state $^3O_2$ absorbs Chl triplet energy becoming a dangerously ROS and (iii) to absorb incoming photons and to transfer these photons to neighboring Chl molecules thereby increasing the overall absorption spectrum of the photosytem in which it is participating. Zeaxanthin synthesis in high light was found to prevent photooxidative stress and lipid peroxidation (Sarry et al., 1994; Havaux et al., 2000) by a mechanism that seems to be different from non-photochemical quenching (NPQ) (Havaux and Niyogi, 1999). In a number of cases, accumulation of zeaxanthin was shown to increase tolerance to photooxidative stress while NPQ was not substantially modified (Davison et al., 2002; Havaux et al., 2004; Johnson et al., 2007). Havaux et al. (2007) indicated that the antioxidant activity of zeaxanthin, distinct from NPQ, can occur in the absence of PSII light-harvesting complexes. Davison et al. (2002) showed that in *Arabidopsis thaliana* overexpression of the *chy*B gene that encodes CβHx (an enzyme in the zeaxanthin biosynthetic pathway) caused a marked increase in the size of the xanthophyll cycle pool. The plants are more tolerant to conditions of high light and high temperature, as shown by reduced leaf necrosis, reduced production of the stress indicator anthocyanin and reduced lipid peroxidation. Stress protection is probably due to the function of zeaxanthin in preventing oxidative damage of membranes.

17.4. Stress-induced alteration of chlorophyll and carotenoids contents in plants

Most crops grown under field conditions are frequently exposed to various abiotic stresses. Abiotic stresses modify plant metabolism leading to harmful effects on growth, development and productivity (Ahmad and Prasad 2012a, b; Hasanuzzaman et al., 2009, 2010, 2011a, b, 2012a-c, 2013a-c; Hasanuzzaman and Fujita, 2013). If the stress becomes very high and/or continues for an extended period it may lead to an intolerable metabolic load on cells, reducing growth, and in severe cases, result in plant death. However, in response to any kind of abiotic stresses, the alteration of Chl and Car content is one of the important indicators of stress. Plants exposed to stress showed different alteration in Chl and Car. Upon exposure to stress, the Chl and Car contents were found to be increased, decreased or remained unchanged as reported in many plant studies (Sabale and Kale, 2010; Aghaee et al., 2011; Agrawal et al., 2011; Babu et al., 2011; Chutipaijit et al., 2011; Khalifa et al., 2011; Oancea et al., 2011; Wang et al., 2011; Zhang et al., 2011a) (Fig. 6).

17.4.1. Salinity

Soil salinity, one of the most severe abiotic stresses, limits the production of nearly over 6% of the world's land and 20% of irrigated land (15% of total cultivated areas) and negatively affects crop production worldwide. Increased salinity has diverse effects on the physiology of plants grown in saline conditions and in response to major factors like osmotic stress, ion-specificity, nutritional and hormonal imbalance, and oxidative damage (Hasanuzzaman et al., 2013a, b). The decrease in Chl content under salt stress is a commonly reported phenomenon and in various studies and the Chl concentration were used as a sensitive indicator of the cellular metabolic state (Chutipaijit et al., 2011).

Many authors studied on the alteration of Chl content in response to salt stress. In *Oryza sativa* leaves, the reduction of Chl *a* and *b* contents of leaves was observed after NaCl treatment (200 mM NaCl, 14 d) where reduction of the Chl *b* content of leaves (41%) was affected more than the Chl *a* content (33%) (Amirjani, 2011). These reductions in Chl content led to a decrease in quantum yield of NPQ and net photosynthetic rate in salt stressed plants and as a consequence growth was retarded. In another study, *O. sativa* exposed to 100 mM NaCl showed 30, 45 and 36% reduction in Chl *a*, Chl *b* and Car contents compared to control (Chutipaijit et al., 2011) which retarded the growth efficiency. Saha et al. (2010) observed a linear decrease in the levels of total Chl, Chl *a*, Chl *b*, carotene and xanthophyll as well as the intensity

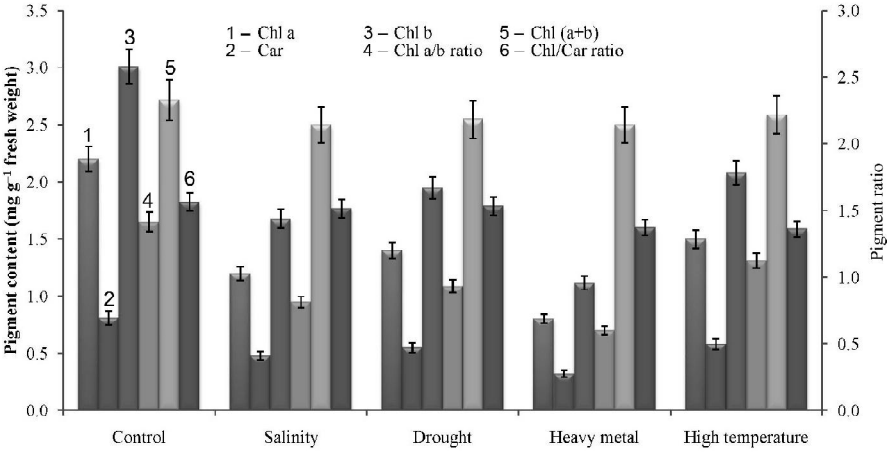

Fig. 6. Changes in Chl and Car content in rapeseed (*Brassica napus* L. cv. BINA Sharisha 3) leaves exposed to salinity (200 mM NaCl), drought (15% PEG-6000), heavy metal (0.5 mM $CdCl_2$) and high temperature (40 °C). Seedlings were grown in semi-hydroponic condition (light, 100 µmol photon m^{-2} s^{-1}; temp, 25±2°C; RH, 65-70%). Twelve-d-old seedling was exposed to each stress for 48 h. The pigment contents were measured by extracting the leaf tissues using 50% acetone. Mean (±SD) was calculated from three replicates for each treatment (Hasanuzzaman and Fujita, unpublished data).

of Chl fluorescence in *Vigna radiata* increasing concentrations of NaCl treatments. Compared to control, the pigment contents decreased on an average, by 31% for total Chl, 22% for Chl *a*, 45% for Chl *b*, 14% for carotene and 19% for xanthophyll (Saha et al., 2010). Associated with the decline in pigment levels, there was an average 16% loss of the intensity of Chl fluorescence as well. Azizov and Khanisheva (2010) grew twenty wheat (*Triticum aestivum*) genotypes under a naturally salinized (1.5-2.0%) soil conditions and studied during the ontogenesis and observed that the Chl content and photochemical activity of chloroplasts were declined in all the genotypes by salinity. To what was expected, salt stress (150 mM NaCl) did not seem to induce any effect on the content of Chl in durum wheat (Azzedine et al., 2011). However, in stressful situations, the plants undergo a quick decrease (about 8.33% of reduction) in the content of Chl compared to the controls.

The effects of salt stress on Car metabolic gene expression in tomato (*Lycopersicon esculentum*) leaves were studied by Babu et al. (2011). In their study, plants were subjected to different concentration of salt water. However, among the different concentrations 200 mM was most detrimental for the Car metabolic gene expression. Lycopene β-cyclase, the enzyme that converts lycopene to β-carotene was seen to be highly

affected compared to other genes studied showing a 1.87-fold inhibition in its expression at 200 mM NaCl. Effect of salinity levels (34, 68, 102, 136, 170 and 204 mM NaCl) on Chl and Car contents in cotyledons of four cauliflowers varieties (YN, SF, SL and YD) were studied by Zhu et al. (2011) and observed that Chl contents increased first and then decreased with increasing of salinity. The highest Chl contents were observed at 102 mM NaCl, whereas the highest inhibitions of Chl contents were observed at 170 mM NaCl. Similar trends were obtained in Car contents in cotyledons of these four cauliflower genotypes. Mane et al. (2011) observed a differential alteration of Chl and Car content in *Vetiveria zizanioides* to salinity. They observed that Chl content of the *Vetiveria* not much affected by the salinity up to 100 mM NaCl but reduction in Chl content was observed beyond 100 mM NaCl concentration. Consequently Chl *a*/*b* ratio was increased considerably in the leaves of *Vetiveria zizanioides* up to 100 mM NaCl level. Therefore it was evident that the highest salt concentration (300 mM NaCl) is certainly negatively influential on Chl *a*/*b* ratio, in grass species. Further it was also observed that the Chl *a* and Chl *b* contents have responded with the same trend of increase or decrease. However, Chl *a* appeared to be more sensitive to salinity than Chl *b*. Such increases in the Chl content at lower levels of salinity might be due to the osmotic adjustment mechanism developed by the plants while a decrease at higher levels might be associated with disruption in cellular functions and damage to photosynthetic electron transport chain due to accumulated ions (Mane et al., 2011). In addition, the drastic reduction in Chl *a*/*b* ratio at higher levels of salinity indicates that Chl *a* might be replaced by Chl *b*. In the same study, maximum increase in the Car content was observed as 23% (at 50 mM NaCl) while it was drastically reduced by 84% (at 300 mM NaCl) over the control. These decreases in Car content indicate that the higher concentration of salt acts as inhibitory and thus unable to prevent chloroplast from photo-oxidative damage. On the contrary at lower levels of salt Car performs protective role for chloroplast and acts as accessory pigments.

The alteration of photosynthetic pigments in response to salinity also differs among the plant species. Samiullah and Bano (2011) investigated 4 halophytes (*Suaeda fruticosa, Atriplex leucoclada, Haloxylon salicornicum, Salicornica verginica*) and found that plants growing in saline soils were significantly lower in Chl *a* and *b* contents. However, the Chl *a* content was significantly higher than Chl *b* saline condition. Among the selected halophytes Chl *a* was found maximum in *Salicornica verginica* while it was minimum in *Atriplex leucoclada*; whereas Chl *b* found maximum in *Haloxylon salicornicum*. The Chl *a*/*b* was found higher in *Atriplex leucoclada* but total Chl was found maximum in

both *Haloxylon salicornicum Salicornica verginica. Atriplex leucoclada, Haloxylon salicornicum Salicornica verginica* the higher Car content among all species, the minimum amount of Car was found in *Suaeda fruticosa*. Idrees et al. (2012) reported that the lemon grass (*Cymbopogon flexuosus*) exhibited considerable decline in photosynthetic pigments when exposed to salt stress (50, 100 and 150 mM NaCl). However, maximum reduction in photosynthetic pigments was noted in 150 mM NaCl treated plants, which resulted in 55% and 23% reduction in the content, respectively compared with control. In a recent study, Heidari (2012) found that by increasing NaCl levels from 0 to 6 ds m^{-1}, the content of Chl *a* and *b*, and Car reduced in *Ocimum basilicum*. Maximum reduction was observed at 6 ds m^{-1} of NaCl. It was also observed that the genotype showing maximum reduction of Chl *a*, *b* and Car was more susceptible to salt stress than other genotype.

17.4.2. Drought

Drought is one of the most devastating environmental stresses that affects the growth and development of plants. Decrease in Chl content under water deficit stress is a commonly observed phenomenon (Chaves et al. 2003; Renolds et al., 2005). Decrease in Chl *a, b,* under drought stress in plants were reported which might due to water deficit and mainly because of the damage to chloroplasts by ROS (Agastian et al., 2000). Drought stress not only causes dramatic loss of pigments but also leads to disorganization of thylakoid membranes, therefore reduction in Chl contents is expected.

Parida et al. (2007) investigated moderately drought tolerant (GM 090304) and drought sensitive (Ca/H 631) cotton (*Gossypium hirsutum* L.) genotypes against short-term drought stress and observed that total Chl and Car contents decreased significantly by drought induction in both the genotypes as compared to their respective controls. However, on re-watering, both Chl and Car contents of the stressed plants tended to increase. The total Chl content decreased by 21% and 23% in GM090304 and Ca/H 631 genotypes, respectively, after 7 days of drought induction. Re-watering in drought-stressed plants showed a 32% increase in Chl content of GM 090304 and 28% increase in Ca/H 631 compared to the drought-stressed plants. Similarly, a 31% decrease in Car content in GM 090304 and a 33% decrease in Ca/H 631 was observed as compared to their respective controls. Recovery from drought stress also significantly increased Car content of both the genotypes (39% in GM 090304 and 34% in Ca/H 631). The Chl *a*/*b* ratio of drought stressed plants decreased significantly in both the genotypes. More importantly, Chl *a*/*b* ratio of both the genotypes recovered to control values after 7-d of rehydration (Parida et al., 2007). In *Zea mays*, a marked reduction in Chl *a* and *b* contents was

observed under water deficit conditions (60% field capacity) while, in contrast, *a*/*b* ratio increased under water deficit conditions (Javed et al., 2011). pigments contents in leaves of common bean (*Phaseolus vulgaris* L.) decreased significantly with increasing level of drought stress (Abass and Mohamed, 2011). Bean plants showed 18% reduction in Chl *a*, Chl *b*, total Chl and Car content under 60% water holding capacity; whereas, 35% reduction of Chl *a* and total Chl and 35% reduction in Chl *b* and Car content were observed at 40% water capacity.

In *T. aestivum*, both Chl and Car content was significantly declined by drought stress (Chakraborty and Pradhan, 2012). The plants grown in soil after 3, 6 and 9-d of withholding water resulted in 45, 55 and 59% reduction in Chl content; whereas Car content did not show any significant change. Interestingly, the Chl *a*/*b* ratio showed an initial increase before declining, and this decrease was 39% after 9-d of withholding water. While investigating five canola cultivars viz., Shirale, Oscar, Con-II, Rainbow and 19H, Din et al. (2011) observed that drought stress reduced the total Chl contents in all the canola cultivars at flowering stages. The average reduction in total Chl was 24%. Drought caused highest relative reduction in total Chl contents of cultivar Oscar (45%) with respect to control whereas least reduction was observed in Rainbow and 19H (13%). The variation in Chl between the cultivars might be due to specific Chl synthesizing enzymes. More importantly, the varieties showing higher Chl content resulted better yield. In a recent study, Zhang et al. (2011a) observed a significant relationship photosynthetic pigment content with the level of water stress. Water stress decreased Chl *a*, Chl *b*, Chl (*a*+*b*) contents and the *a*/*b* ratioin oriental lily (*Lilium*) cv. Sorbonne. After 10- and 20-d of water stress, Chl *a* content decreased by 31 and 35%; whereas Chl *b* content decreased by 19 and 16%, respectively over control. Total Chl content decreased by 24 and 30% after 10- and 20-d of stress. The Chl *a*/*b* ratio decreased by 8 and 22% after 10- and 20-d of water stress, respectively. Saraswathi and Paliwal (2011) studied *Albizia lebbeck* and *Cassia siamea* seedlings under drought stress condition by withholding watering for 7, 14 and 25 d. The total Chl content of leaves was more in well-watered plants in both the species, while it decreased as the drought treatment increased. *A. lebbeck* showed 17, 38 and 62% reduction in Chl content at water stress for 7, 14 and 25 d, respectively. However, *C. siamea* was more prone to drought where total Chl content reduction was 26, 59 and 81% with 7, 14 and 25-d of drought.

17.4.3. High temperature

It is evident that the global average temperature of the air of the earth's surface could rise by 1.6 to 3.8 °C by 2100 (IPCC, 2007). Like

other physiological processes of plant temperature plays one of the most important roles in the rate and ability of a plant to photosynthesize effectively (Hasanuzzaman et al., 2013e). In general, there is a positive correlation between change in temperature and photosynthesis. When temperatures exceed the normal growing range of plants heat injury takes place. Most of the crop plants generally grow in the 15 °C to 45 °C temperature range. An increase in temperature of 10 °C to 15 °C above normal growth temperature leads to alteration of photosynthetic pigments and thus limiting photosynthesis. temperatures denature enzyme proteins complexs, pigments of photosystems and it is apparent in many researches. Chl and Car pigments are closely associated with photosystem proteins and any damage to photosystem will be evident in Chl as well as Car. Temperature stress caused reduction in Chl biosynthesis, disintegration of chloroplast membranes and disrupted the biochemical reactions of photosystems (Jones, 1992; Havaux, 1998). Mechanism and reasons of interpretation of unfavorable temperature and decrease in photosynthetic pigments may be due to inhibition of biosynthesis, changes in ultrastructure of chloroplast, especially the membrane, and photodeterioration (Smillie, 1983; Tewari and Tripathy, 1998; and Mandoura, 2011). This could be supported by significant differences in Chl fluorescence at high temperature in *T. aestivum* leaves for resistant cultivars and that of sensitive ones (Smillie, 1983). Photo deterioration of Chl as Car content decreased at treatment 55 °C and it is known that Car preserve Chl from photo-oxidation (Hall, 1987). Tewari and Tripathy (1998) showed that, in heat-stressed *T. aestivum* and *Cucumis sativus* seedlings, protochlorophyllide synthesis was inhibited, POR, ALA dehydratase and porphobilinogen deaminase were partially inhibited and thus Chl synthesis was adversely affected. Premature loss of Chl as a result of heat stress has been noticed in many crop species (Guo et al., 2006).

Chlorophyll accumulation capacity of *T. aestivum* decreased at temperatures over 35 °C (O'Mahony et al., 2000). Differences among net photosynthesis ratios of *T. aestivum* varieties exposed to high temperatures were related to changes in Chl *a* to Chl *b* ratio due to low Chl concentrations and rapid leaf senescence (Yildiz and Terzi, 2007). Heat stress has been reported to reduce Chl content, Chl *a*/*b* ratio and Chl:Car ratio in various plant and tree species like *Festuca arundinacea* (Langjun et al., 2006) and *Solanum spp.* (Aien et al., 2011), *T. aestivum* (and Mandoura, 2011). In another study, Chl *a* in the *L. esculentum* leaves was reduced by 10-32%, 10% in Chl *b* 5% reduction in the ratio of Chl *a* and Chl *b* observed after 2-d of treatment (Malgorzata, 2009). Liu and Huang (2008) experimented with 2 group of bentgrass plants (*Agrostis stolonifera* L.) in one group of plants was allowed to acclimate to increasing temperatures sequentially at 20, 25, 30, and 35 °C (day/

night) for 7 d in each temperature regime (acclimated treatment). Another group of plants were directly transferred from 20 to 40 °C without going through the gradual temperature increase (non-acclimated treatment). Chlorophyll content of heat-acclimated plants was 28, 37 and 52% higher than non-acclimated plants at 7, 14 and 28 d of heat stress, respectively. However, 33, 32 and 43% higher Car content than non-acclimated plants at 7, 14, and 28 d of heat stress (Malgorzata, 2009). De Padua et al. (2009) reported that the Chl content in *G. max* green seeds was inversely proportional to chlorophyllase activity, which was affected by high temperature stress. Arkus et al. (2005) reported that chlorophyllase catalyzed the initial step in the degradation of Chl and plays a key role in leaf senescence and fruit ripening. In cucumber 42 °C for 48 h reduced the Chl synthesis by 60% (Tewari et al., 1998). Recently, and Mandoura (2011) reported that for 30 minutes of high temperature stress at 55 °C and 4-d after the stress treatment the enzyme chlorophylase is still activated and lead to a decline in Chl in *T. aestivum* plant.

Efeoglu and Terzioglu (2009) observed that heat stress inhibited Chl accumulation in *T. aestivum* seedlings at 45 °C for 8 h and caused marked alterations in the Chl *a* fluorescence and photosynthesis in the primary leaves at 37 °C and 45 °C for 8 h. Later, Wang et al. (2011) investigated the effect of pre-anthesis high-temperature acclimation on leaf physiology of winter wheat in response to post-anthesis heat stress. The results showed that both pre- and post-anthesis heat stresses significantly depressed flag leaf photosynthetic pigments and photosynthesis. Pre-anthesis high-temperature treatment reduced ratios of Chl *a*/*b* and Chl/Car in the flag leaf of wheat at 7 days after emergence (DAE). Post-anthesis heat stress also decreased the Chl *a*/*b* but increased Chl/Car compared with the control; the changes were more marked in non-acclimated plants than in pre-acclimated ones. Leaf Chl *a*/*b* of both pre-acclimated and non-acclimated plants exposed to post-anthesis heat were 6.7% and 13.8% lower than that of control at 10 DAE, respectively; they were further decreased at 13 DAE and were 9.2% and 21.3% lower than the control plants. In contrast, leaf Chl/Car in pre-acclimated and non-acclimated plants exposed to post-anthesis were 4.7 and 11.1% at 10 DAE and 7.0 and 10.4% at 13 DAE greater than those of controls (Wang et al., 2011). In contrary, Pospisilova et al. (2011) did not find any effect of short-term heat stress on the pigment contents in transgenic tobacco.

In our recent study, we observed 15 and 31% decrease in Chl *a* content in *T. aestivum* seedlings (8-d-old) under 24 h and 48 h of heat stress (38°C) (Hasanuzzaman et al., 2012c). Similar to Chl *a*, Chl *b* content also decreased upon exposure to heat stress where the reduction was 25 and 22% at 24 and 48 h of heat stress, respectively compared

to control. As a result, total Chl content of wheat leaves decreased by 18 and 28% at 24 and 48 h of heat treatment, respectively compared to respective control (Hasanuzzaman et al., 2012c). Importantly, SNP (nitric oxide donor) supplementation in heat treatment significantly recovered the Chl depletion in heat stressed seedlings.

17.4.4. Low temperature

In most regions around the world, plants are exposed to low temperature at least part each year. Among the abiotic stresses, low temperature stress is a serious threat to the sustainability of crop yield. Photosynthesis is strongly reduced 18 °C (Ramalho et al., 2003), while temperatures 4 °C dramatically depress photosynthetic performance (DaMatta et al., 1997; Silva et al., 2004). The decline of photosynthetic capacity in low temperature is related to a decrease in the quantum efficiency of PSII and the activities of PSI, the ATP synthase and the stromal enzymes of the carbon reduction cycle (Allen and Ort, 2001). Chlorophyll biosynthesis is mostly reduced due to low temperature stress. Experimental evidences showed that protochlorophyllide synthesis in low temperature stressed seedlings was significantly inhibited due to the inactivation of all of the enzymes involved in protoporphyrin IX (Proto IX) synthesis, Mg-chelatase, and Mg-protoporphyrin IX monoester cyclase. At low temperature, the Chl biosynthesis was inhibited because these enzymes are related to biosynthesis of Chl (Tewari and Tripathy, 1998). Carotenoids were less affected than Chl in low temperature stress, leading to a decrease in Chl/Car ratio (Hola et al., 2007).

Partelli et al. (2009) experimented with coffee plant by decreasing temperature from 25/20° to 13/8 °C (day/night temperature) found 30% reduction in Chl *a*, 27% – in Chl *b*, 29% – of total Chl. For Car, 86% reduction of α-carotene, 57% – in β-carotene, 68% – in α/β-carotene ratio, 32% – in lutein, but 21% increase in zeaxanthin. These results were parallel with the results of Ramario et al. (2003) who found that *C. arabica* exposed to low temperature (15/4 °C, 3 d) caused 51% reduction in α-carotene, 30% – in β-carotene, 40% – in α/β-carotene ratio, 26% – in lutein, 139% increase in zeaxanthin, 18% reduction in neoxanthin. In *O. sativa*, the total Chl content was reduced by 50% due to exposure to low temperature (15/10 °C) for 2 weeks (Aghaee et al., 2011). Gonzales et al. (2002) described that pigments such as Car could be interacting with leaf morphology to the photosynthetic mechinary. In a recent study, Reda and Mandoura (2011) reported that even at low temperature stress of 3 °C the enzyme chlorophylase is still activated and lead to a decline in Chl in *T. aestivum* plant. Carotenoids contents were markedly increased after exposure to 3 °C temperature that protects Chl from degradation. The xan-

thophylls are believed to have function in the photoprotection the photosynthetic apparatus (Koroleva et al. 1994). Several xanthophylls are important photoprotective pigments that act by dissipation avoiding energy overpressure the photosynthetic apparatus (Demmig-Adams et al., 1995).

17.4.5. Waterlogging

Waterlogging stress may develop due to several direct (improper irrigation practices) and indirect (global warming) anthropogenic and natural consequences (meteorological) leading to altered plant metabolism, architecture and ecogeographical distribution depending upon a plant's responses. Gonzalez et al. (2009) observed a significant alteration of Chl content in *Chenopodium quinoa* subjected to waterlogging stress. After 50 d of waterlogging, Chl *a*, *b* and total Chl contents were 14, 14 and 19% lower than control. However, there was no significant difference in the Chl *a*/*b* ratios. In *Spartina alterniflora* and *Phragmites australis*, the responses of their photosynthetic pigments to durative waterlogging were studied by Gu et al. (2009) and observed that under durative waterlogging stress, *S. alterniflora* had the decreased contents of Chl *a*, *b* and Car but the increased Chl *a*/*b* and Car/Chl ratios, whereas *P. australis* had the increased pigment contents and rather constant Chl *a*/*b* and Car/Chl ratios. After the waterlogging stress relieved, the pigment contents of *S. alterniflora* increased and Chl *a*/*b* and Car/Chl ratios decreased to the levels of the control, whereas the pigment contents of *P. australis* were significantly higher than the control. Both *S. alterniflora* and *P. australis* showed a compensatory effect after the stress relieved. However, *Phragmites australis* could better adapt to the waterlogging stress than *S. alterniflora*, being able to be used as a substitutive plant for the restoration of coastal wetlands. Chen et al. (2008) reported that the Chl and Car contents and their ratio decreased significantly with the prolongation of waterlogging in *Salix variegate* seedlings. However, the ratio of Chl *a* to *b* increased significantly. While studying on the growth and physiological responses of two mangrove species viz. *Bruguiera gymnorrhiza* and *Kandelia candel* to waterlogging, Ye et al. (2003) found that *B. gymnorrhiza* plants waterlogged for 12 weeks had significantly higher concentrations of Chl *a*, Chl *b* and total Chl than control. However, for *K. candel*, both waterlogging for 12 weeks as well as drained for 4 weeks followed by waterlogged for 8 weeks treatments resulted in significantly higher Chl contents than the control, and in terms of Chl *a* contents. For both species, total Car contents were highest for 12 weeks of waterlogging and the values between other treatments were not significantly different. According to Sabale and Kale (2010) total Chl and Car contents declined by about 50% in *Coriandrum sativum* plants when grown under waterlogging condition.

17.4.6. Ozone

In many industrialized countries, tropospheric O_3 reaches to such high concentration which is harmful for the plant species. Due to increased emissions of nitrogen oxides and hydrocarbons surface O_3 levels are increasing (Agrawal et al., 2011). Deleterious effects of O_3 have been investigated through a number of experiments. Ozone is the reason for premature senescence, chlorosis and necrosis of leaf, decrease in leaf interception, Chl content, Car content and photosynthesis, reductions in assimilate availability, alterations in assimilate partitioning, alterations of metabolism, reduction in biomass yield (Biswas et al., 2008; Feng et al., 2008, 2009). However, pattern of effects of O_3 may be different from crop to crop. The effect of O_3 on the physiological properties of different plants have been studied since long time by different researchers and both the increase and decrease in the Chl by exposure of plants to O_3 was reported by many authors (Paoletti et al., 2009; Bohler et al., 2010; Singh and Agrawal, 2010). Agrawal et al. (2011) observed 37, 38, 38, 2 and 16% reduction in Chl *a*, Chl *b*, total Chl, Chl *a*/*b* ratio and Car, respectively from filtered to open plot after 50 days of leaf expansion due to increased O_3 concentration of 45.7 ppb during growth and 50.2 ppb after flag leaf development of *T. aestivum*. Previousl, in the same crop (*T. aestivum*), Singh and Agrawal (2010) found the reduction of 20 to 48% in total Chl and 18 to 30% in Car with 400 ppm of ethylenediurea (EDU) and without EDU treatment. Total Chl content was increased by 14, 11, 25 and 7% in plants treated with 200, 300, 400 and 500 ppm EDU, respectively at 60 days after growing. Carotenoids content also showed 30, 3, 32 and 15% increments in 200, 300, 400 and 500 ppm EDU-treated plants, respectively as compared to control plants. Chl *a*, Chl *b* and Car content was reduced by 61, 56 and 61% respectively in NFA+O_3 (non-filtered+additional O_3) as compared to CFA (charcoal filtered O_3 free air) in *Lactuca sativa L. esculentum* also showed significant effect when exposed to O_3 stress. Shrestha and Grantz (2005) found 35% and 50% decline in Chl content in exposure to 78 and 142 nl l^{-1} O_3 for 12 h. Mina et al. (2010) observed that Chl reduced by 7-39%, Car reduced by 5-42% at 75 and 150 ppb O_3 in closed top dynamic chamber for 2 hd^{-1} for 12-d. The similar results were previously investigated in many other crop plants *N. tabacum* (Saitanis et al., 2001), *Solanum tuberosum* (Bindi et al., 2002); and in many trees *Fraxinus ornus* (Paoletti et al., 2009), *Populus tremula* (Bohler et al., 2010), *Fagus sylvatica* (Cascio et al., 2010), *Liriodendron chinense* (Zhang et al., 2011b), *Pinus tabulaeformis* (Xu et al., 2012) etc.

The mechanism how plant cell is injured by O_3 can be expressed in brief that the O_3 enters the leaves through the stomata and, upon contact with the apoplast, the molecule degrades into ROS (Rao et al.,

2000). In the chloroplast these ROS could directly or indirectly impair the light- and dark reactions of photosynthesis (Fiscus et al., 2005). The O_3 or ROS may alter the properties of thylakoids, thereby affecting the Chl *a* fluorescence leading to an over reduction of photosystem reaction centers (Singh et al., 2009; Feng et al., 2011).

17.4.7. UV radiation

Enhanced UV radiation has many effects on plant morphology, physiology and development, and its impacts on growth and development are seen in many plant species (Frohnmeyer and Staiger, 2003). UV radiation targets three important features of plant cells viz. the genetic system (e.g., DNA), photosynthesis, and membranes (Bjorn, 1996). In plants, Chl contents were found to be increased (Poulson et al., 2006), decreased (Qaderi et al., 2007) or unchanged (Cechin et al., 2007) due to UV radiation exposure. It is found that both Chl *a* and *b* contents of leaves dropped in *P. vulgaris* leaves grown under UV-B stress (Michaela et al., 2000). Most of the plant studies evaluating the effects of UV-B and UV-A on plants have reported a reduction of Chl and Car contents and a decrease in photosynthetic efficiency (Vass et al., 2002; Boeger and Poulson, 2006; Agrawal and Mishra, 2009). Some studies have verified a high production of Car after low-level UV-A exposure (Jahnke 1999). The increase (61%) in Car under UV-A was also found by Victorio et al. (2011) in *Phyllanthus tenellus* cultured in vitro, while Chl *a* and *b* decreased by 19 and 40%, respectively. Musil et al. (2002) investigated seventeen herb, shrub and tree species exposed at one location to UV-B (280-315 nm). They observed that leaves of trees had altered Chl *a*, *b* and Car contents, but those of shrubs or herbs did not. Seeds of *Z. mays* and *T. aestivum* were irradiated during 20 min and 40 min, respectively, using a UV laboratory source and the content of pigments for irradiated plants were examined by Oancea et al. (2011) that showed no significant differences in examined pigment contents which was thought due to the fact that the control plants germinated faster and they have been grown better than the other plants, the plants have the capacity to revert after irradiation.

Brassica napus plants grown under zero UV-B had relatively lower concentration of Chl *a* compared to those grown under other UV-B levels (Sangtarash et al., 2009). In addition, plants grown under ambient or enhanced UV-B produced higher amount of Car than those grown under zero UV-B. In greenhouse, eight green leaf and eight red leaf lettuce varieties were grown under control, receiving supplemental UV-A (320–400 nm) and supplemental UV-A plus UV-B (290–320 nm) radiation and the photosynthetic pigments were measured (Caldwell and Britz, 2006). The results showed that depending upon the cultivar and light treatment, all the Car (neoxanthin, lutein and β-carotene)

varied significantly. Similar to the Car levels, supplemental UV-B increased the Chl concentration of green leaf lettuce, while reducing the levels of these compounds in red leaf lettuce. However, the effect of UV-B radiation was more prominent than UV-A. Salama et al. (2011) investigated the effect of enhanced UV radiation in some annual desert species viz. the *Malva parviflora, Plantago major, Rumex vesicarius* and *Sismbrium erysimoids* and observed that Chl contents decreased due to enhanced UV radiation. The impact of UV-B radiation on photosynthetic related parameters was studied in *O. sativa* exposed to UV-B irradiation (1 h d^{-1} for 7 d) with a ten narrow-band (311 nm). The results showed that UV radiation caused a decline of xanthophylls, carotenes, Chl *a*, Chl (*a* + *b*) and Chl *a*/*b* ratio values (Lidon and Ramalho, 2011). The pool of the xanthophyll cycle pigments (violaxanthin, antheraxanthin, zeaxanthin) was moderately affected 1 day after the end of UV-B treatment, but suffered a much stronger decline (61%) by the 7th day after the end of the irradiation. UV-B irradiation also affected the carotene content, more accentuated by the 7th day of recovery. Carotenes were similarly affected, as reflected in the small fluctuations of the (*a*/*b*) ratio values. In fact, among the light capturing pigments, Chl *a* was strongly affected in the leaves subjected to direct UV-B radiation, decreasing 28% and 41%, 1 and 7 days after the end of irradiation, respectively. That decrease was the sole responsible by the significant reduction of total Chl, since, Chl *b* showed no significant variations on these leaves. That led to decreases in the Chl *a*/*b* ratio. Singh et al. (2011) reported that supplemental UV-B radiation induced photosynthetic pigments of *Dolichos lablab* under field conditions. The plants exposed to UV-B (280-315 nm: 7.2 kJ m^{-2} d^{-1}) radiation caused significant decrease in total Chl and Chl *a*/*b* ratio at both 15 and 30 days after germination (DAG). Total Chl content was reduced by 28 and 5.3% at 15 and 30 DAG, while Car showed reductions of 9 and 40% at 15 and 30 DAG (Singh et al., 2011). They also concluded that the reduction in Car content might result either from inhibition of synthesis or from breakdown of the pigments or their precursors. It was suggested that decline in Chl level might be due to inhibition of *cab* gene, which codes for Chl protein.

17.4.8. Nutrient deficiencies

Improving the mineral nutritional status of plants under marginal environmental conditions is of great importance for maintenance of crop productivity as worldwide. About 60% of cultivated soils facing plant growth limiting problems caused by mineral nutrient deficiencies and toxicities. As a general rule, nutrient deficiency induces plant senescence, the appearance of necrosis, and chlorosis (Dietz and Harris, 1997). As a consequence of these phenomenon, pigment composition is altered.

Physiological investigations of Chl and Car content in the uppermost internode of several *T. aestivum* cultivars were studied by Bojovic and Stojanovic (2005). They observed a dependency of these pigments to the fertilization treatments. The results indicate that Chl and Car contents depended on the presence and ratio of mineral elements in the substrate. This is demonstrated by the variant with unfertilized soil, where Chl and Car content in all cultivars was lowest. The variant of fertilization with nitrogen (N) and phosphorus (P) turned out to be most favorable. Zhao et al. (2005) reported that starting from 20 days after N was withheld from the nutrient solution, the N-deficient plants had significantly lower leaf Chl content than the control plants of *Sorghum bicolor*. Chu et al. (2006) reported that the increased Chl and Car contents in N and P supplemented plants were due to the enhanced ALA biosynthesis and activities of PBG synthase. They observed that ALA synthetic rate increased as nitrate (NO_3^-) concentrations supplied to larch seedlings increased from 1 to 8 mM. Under phosphate (PO_4^{3-}) treatments, ALA synthetic rates were similar to those under NO_3^- treatments. Rate of ALA biosynthesis and activities of PBG synthase were affected by phosphate supply, but contents of Chl and Car were not affected. Rice (*Oryza sativa* L. subsp. *indica*) seedlings when exposed to N-deficient nutrient solution for 10-d showed 34% decrease in total Chl content as compared to the control (Huang et al., 2004). There was a transient increase in Chl *a*/*b* in N-deficient plants in the beginning and then the ratio decreased as the N-deficiency lasted. The Car content was decreased by 36% in N-stressed plants. The speed of decline in total Chl was faster in N-stressed plants than in the control ones and consequently, the ratio of Car/Chl began to rise significantly. The trend of loss of Chl was more pronounced until the N-deficient leaves. In *Urtica dioica* L. leaves, increases in N levels were found to be positively correlated with Chl and Car content (Biesiada et al., 2009). The plants supplemented with 50 and 100 kg ha^{-1} N showed 31 and 16% decrease in total Chl content compared to 150 kg N ha^{-1}. Similar to Chl, Car content was also decreased by 11, 24 and 11% with 50 and 100 kg ha^{-1} N, respectively. In a recent study, Ordog et al. (2012) found N became a limiting factor for photosynthetic pigments in algae. They observed a negative relationship with N levels and pigment concentrations in *Chlorella minutissima*. In 7, 21 and 35 mg L^{-1} N treatments in the growing media, all pigment concentrations decreased in time-depending manners. In the 70 and 700 mg L^{-1} N treatments, Chl *a* and Chl *b* concentrations initially decreased (day 4), then recovered to levels above their initial concentrations (days 6–8) and then gradually decreased again. In contrast, Car concentrations never increased even in cultures with the highest N levels.

While measuring Chl content in cotton plants, at different stages of plants Onanuga et al. (2012) observed that after 83, 91 and 104 days of transplanting, Chl *a* content in P-deficient plants decreased by 76, 73 and 26%, respectively compared to high P supplemented plants. However, at later stage Chl content increased in P-deficient plants which were 16 and 52% higher at 120 and 148 days after transplanting (DAT). In case of Chl *b*, it decreased by 87 and 82% at 83 and 91 DAT with low P; whereas it markedly increased at later stages. Total Chl content also decreased at earlier stages and then increased by low P. However, in their study potassium (K) deficit plants did not show significant differences in Chl content in cotton plants. Alikhani et al. (2011) investigated the changes of photosynthetic pigments in *Aeluropus lagopoides* seedling under K deficiency. Among applied treatments, Chl contents of the seedling that treated by 1.75 or 100 mM K^+ was similar to the control. However, in the presence of 100 mM K^+ and other treatments, amount of Car clearly increased. Maximum amount of the Car was observed in treatments that were supplemented with 1.75 or 100 mM K^+. These pigments were raised only to 3.5 and 2.75 times more than the control by supplemented media without K^+ or with high concentration of K^+ up to 100 mM. Amount of the Chl *a* was reduced to 38% by low concentration of K^+ compared to control. Kaya et al. (2001) showed that Chl degradation in tomato leaves could be significantly reduced by foliar application of K fertilizer. Tewari et al. (2010) reported that Chl *a*, Chl *b*, total Chl and Car contents were decreased significantly in the leaves of sulfur (S)-deficient plants. While there was a marked increase in the ratio of Car/Chl in S-deficient plants, Chl *a*/*b* ratio was not affected. The loss of photosynthetic pigments and development of pale young leaves in S-deficient plants may be the consequences of poor mobility of S from older to young developing parts. Tewari et al. (2006) observed 46 and 24% decreases in the concentrations of Chl and Car in young leaves of Mg-deficient mulberry plants. Mg-deficiency did not affect Chl *a*/*b* ratio but increased Car/Chl ratio by 41%.

While studying the effects of N, Fe, Mg, manganese (Mn) and molybdenum (Mo) deficiencies, Afrousheh et al. (2010) found that Chl content significantly altered in *Pistacia vera* seedlings when grown under nutrient deficient condition. The seedlings receiving complete nutrient solution had higher contents of all fractions of Chl. The Chl content of the leaves was significantly influenced by the deficiency of various nutrient elements. All the fractions of Chl of treated seedlings, particularly N-deficient and Mn-deficient seedlings, reduced considerably which was because of inadequate supply of N for chloroplast protein synthesis (chlorosis of the older leaves). In their study, N deficiency appeared as yellow chlorotic patches in the older leaves and severe

chlorosis of the entire seedling which was due to the reduced amount of Chl. Decreasing in Chl content in N-deficient seedlings have also been reported in *Tectona grandis* (Gopikumar and Varghese, 2004), *G. max* (Paliwal et al., 2004) and *Spinacia oleracea* (Ronaghi et al., 2002). The functions of Mn are regarded as being closely associated with those of iron and as being concerned with Chl formation. Hence, when Mn is deficient, chlorosis is a common symptom (Govindjee, 2007). In their study, Kosesakal and Unal (2009) investigated the effect of Zn deficiency on pigment content in early growth phase of *L. esculentum* seedlings. In 8-d-old plants, Zn deficiency promoted Car accumulation in hypocotyl and cotyledon tissues of seedlings. While comparing the level of Chl in the cotyledon tissues of plants grown in control medium and in Zn-deficient medium, the total amount of Chl was almost equal, but there was some difference in the Chl a/b ratio. However, in another experiment, it is shown that the application of exogenous Zn to *L. esculentum* plants caused accumulation of Chl content of leaves at both low and high concentrations (Kaya and Higgs, 2002). In *Mentha pulegium*, the Chl and Car contents in the Zn-deficient plants were significantly lower than those of the control (Candan and Tarhan, 2003). Singh et al. (2005) reported that total Chl content, and Car were significantly altered under Zn-deficient condition in *P. sativum* genotypes. In a recent study, Khalifa et al. (2011) found that in *Iris* plants, Zn-deficiencies cause 11, 54 and 26% reduction of Chl *a*, Chl *b* and total Chl, respectively but no changes in Car contents. In same experiment they showed that the plants grown without boron (B) caused 41, 9, 31 and 20% reduction in Chl *a*, Chl *b*, total Chl and Car content, respectively compared to the optimum level (Khalifa et al., 2011).

17.5. Conclusions and future perspectives

The existence of all organisms in the world is directly or indirectly dependent on the photosynthetic pigments. Among the photosynthetic pigments Chl and Car are the most important. Both of them are involved in the plant pigment systems and contribute in photosynthesis and abiotic stress tolerance. Biosynthesis and functioning of these pigments are very complex and strictly regulated by defined enzymatic system and require definite favorable atmosphere. Abiotic stresses cause significant alteration of the biosynthesis of Chl and Car in different ways. The use of Chl fluorescence is considered now-a-days as a new tool for determining the rate of flow of electron through the photosystem as well as the total photosynthetic performance is a very reliable one. This technique can be efficiently used to assess the changes in the rate of electron flow or reactions due to changes in the environmental alterations like different stresses. Application of these kinds of tools

Table 1

Alteration of photosynthetic pigments in plants under abiotic stresses

Types of stress	Plant	Treatment and duration	Effects	References
Salinity	*Vigna radiata* L. Wilczek	150 mM NaCl, 7 d	22%, 45% and 31% decrease in Chl *a*, Chl *b* and total Chl, respectively 14% and 19% decrease in Car and xanthophylls contents	Saha et al. (2010)
	Oryza sativa L.	200 mM NaCl, 14 d	33% decrease in Chl *a* content 41% decrease in Chl *b* content	Amirjani (2011)
	Triticum aestivum L.	150 mM NaCl, 5 d	13% decrease in Chl content	Erdal (2012)
	Triticum durum Desf.	150 mM NaCl, 14 d	Decrease in the Car content	Azzedine et al. (2011)
	Vetiveria zizanioides (L.) Nash	25, 50, 100, 200 and 300 mM NaCl, 4 week	Chl *a*: 13, 43 and 52% increase with 25, 50 and 100 mM NaCl; but 26 and 57% decrease with 200 and 300 mM NaCl, respectively Chl *b*: 7, 9, 10, 32 and 11% increase with 25, 50, 100, 200 and 300 mM NaCl, respectively Total Chl: 10, 29 and 35% increase with 25, 50 and 100 mM NaCl; whereas 1 and 19% decrease with 200 and 300 mM NaCl, respectively Car: 10 and 23% increase with 25 and 50 mM NaCl, whereas 17, 36 and 84% decrease with 100, 200 and 300 mM NaCl, respectively	Mane et al. (2011)
	Cymbopogon flexuosus Steud. Wats.	150 mM NaCl, 60 d	55% reduction in total Chl content 23% reduction in Car content	Idrees et al. (2012)
	Ocimum basilicum L.	6 ds m^{-1} NaCl, 20 d	Decrease in Chl and Car content	Heidari (2012)
	Oryza sativa L. spp. *indica*	100 mM NaCl, 4 d	30, 45 and 36% reduction of Chl *a*, Chl *b* and Car content	Chutipaijit et al. (2011)
Drought	*Gossypium hirsutum* L.	Withholding irrigation, 7 d	Total Chl decreased by 21% and 23% in drought tolerant and sensitive genotypes, respectively Car content decreased by 31% and 33% decrease in drought tolerant and sensitive genotypes, respectively Significant decrease in Chl *a*/*b* ratio	Parida et al. (2007)
	Phaseolus vulgaris L.	46 and 40% field capacity	18% reduction in Chl *a*, Chl *b*, total Chl and Car content under 60% water holding capacity 35% reduction of Chl *a* and total Chl and 35% reduction in Chl *b* and Car content under 40% water capacity	Abass and Mohamed (2011)

Types of stress	Plant	Treatment and duration	Effects	References
	Brassica napus	Withholding water up to wilting	23.8 % reduction in total Chl	Din et al. (2011)
	Brassica napus L.	PEG-600 at 0.3 MPa, 4d	20, 22 and 21% reduction in Chl *a*, Chl *b* and total Chl, respectively	Liu et al. (2011)
	Triticum aestivum L.	3, 6 and 9-d of withholding water	45, 55 and 59% reduction in total Chl content No change in Car content	Chakraborty and Pradhan (2012)
	Albizia lebbeck	3, 6 and 9-d of withholding water	17, 38 and 62% reduction in Chl content at water stress for 7, 14 and 25 d	Saraswathi and Paliwal (2011)
	Cassia siamea	3, 6 and 9-d of withholding water	26, 59 and 81% reduction in Chl content at water stress for 7, 14 and 25 d	Saraswathi and Paliwal (2011)
	Oriental lily (*Lilium*)	Withholding irrigation, 10 and 20-d	31 and 35% decrease in Chl *a* content 19 and 16%,decrease in Chl *b* content 24 and 30% decrease in total Chl content 8 and 22% decrease in Chl *a*/*b* ratio	Zhang et al. (2011a)
High temperature	*Cucumus sativus*	42°C for 48 h	Chl biosynthesis reduced by 60%	Tewari et al. (1998)
	Pisum sativum L.	38°C for 72 h	37% reduction in the ratio of Chl *a* and *b*	Georgieva and Lichtenthaler (2006)
	Triticum aestivum and *Triticum turgidum*	37°C and 45°C, 8 h	Marked alterations in the Chl *a* fluorescence and photosynthesis	Efeoglu and Terzioglu (2009)
	Lycopersicon esculentum L.	Gradual increase of temperature from 30 to 35 and 40°C for 2 d	Chl *a* in the leaves of the studied plants is reduced by 10-32%, 10% in Chl *b* and 5% reduction in the ratio of Chl *a* and Chl *b*	Malgorzata et al. (2009)
	Triticum aestivum	45 and 55 °C for 30 min	50% decrease in Car content	Reda and Mandoura (2011)
	Triticum aestivum L.	34/30°C, 7 d	Decrease flag leaf Chl *a*/*b* ration and Chl/Car ratio	Wang et al. (2011)
Low temperature	*Cucumus sativus*	7 °C for 48 h	Chl biosynthesis reduced by 90%	Tewari and Tripathy (1998)
	Coffea arabica	15/4ºC, 3 d	51% reduction in α-carotene, 30% reduction in β-carotene, 40% reduction in α/β-carotene ratio, 26% reduction in lutein, 139% increase in zeaxanthin, 18% reduction in neuxanthin.	Ramalho et al. (2003)
	Camellia sinensis	15°C for 1 year	58% reduction in Chl *a*, 63% reduction in Chl *b*, 60% reduction in total Chl, 15% increase in the Chl *a*/*b* ratio.	Du et al. (2008)
	Coffea canephora	13/8 °C day and night temperature for 3 days	30% reduction in Chl *a*, 27% reduction in Chl *b*, 29% reducing total Chl, 86% reduction of α-carotene, 57% reduction in β-carotene, 68% reduction in α/β-carotene ratio, 32% reduction in lutein, 21% increase in zeaxanthin	Partelli et al. (2009)

Types of stress	Plant	Treatment and duration	Effects	References
	Cichorium intybus L.	4 to 16°C for 2 weeks	Increase in xanthophyll by 9.6 % and 26.3%, respectively	Devacht et al. (2009)
	Oryza sativa L.	15/10°C for 2 weeks	50% reduction in total Chl	Aghaee et al., 2011
Waterlogging	*Chenopodium quinoa* Willd.	Waterlogging (-0.20 MPa soil water potential), 50 d	14, 14 and 19% reduction in Chl *a*, Chl *b* and total Chl contents	González et al. (2009)
	Coriandrum sativum L.	Waterlogging, 8 d	50% reduction in Chl and Car contents	Sabale and Kale (2010)
Ozone	*Lactuca sativa*	Non-filtered + additional O_3 @83 nl l^{-1}, for 12 h	Chl *a*, Chl *b* and Car content was reduced by 61, 56 and 61% respectively	Calatayud and Barreno (2004)
	Lycopersicon esculentum L.	78 and 142 nl l^{-1} O_3 for 12 h	35% and 50% decline in Chl content in exposure to 78 and 142 nl l^{-1} O_3	Shrestha and Grantz (2005)
	Betula pendula	Elevated O_3	11% decrease in Chl *a*, 13% decrease in Chl *b* 9% decrease in total Car 10% decrease in total pigment.	Silfver et al. (2008)
	Triticum aestivum	EDU (400 ppm) and	Reduction of 20.5 to 47.6% in total Chl and 18 to 30% in Car	Singh and Agrwal (2010)
	Lycopersicon esculentum L.	75 and 150 ppb O_3 2 h d^{-1} for 12 d	Chl content reduced by 7-39% Car reduced by 5-42%	Mina et al. (2010)
	Triticum aestivum	45.7 ppb during growth and 50.2 ppb after flag leaf development	Reduction in Chl *a*, Chl *b*, total Chl, Chl *a*/*b* ratio and Car by 37%, 38%, 38%, 2 and 16%, respectively	Agrawal et al. (2011)
	Picea abies L. (Karst.)	40 ppb	4% and 21% increase in Chl *a* and Chl *b* 17% reduction in Chl *a*/*b* ratio	Utriainen and Holopainen (2001)
UV radiation	*Vigna unguiculata*	Enhanced UV-B	15 and 21% decrease in Chl *a* and Chl *b* content No change in Car content	Musil et al. (2002)
	Phylica pubescens	Enhanced UV-B	27, 31 and 19% increase in Chl *a*, *b* and Car content	Musil et al. (2002)
	Colophospermum mopane	Enhanced UV-B	42, 36 and 46% increase in Chl *a*, Chl *b* and Car content	Musil et al. (2002)
	Lactuca sativa L.	UV-A ((320–400 nm)	53, 27 and 26% increase in Chl *a*, Chl *b* and β-carotene content	Caldwell and Britz (2006)
	Lactuca sativa L.	UV-B (290–320 nm)	235, 34 and 74% increase in Chl *a*, Chl *b* and β-carotene content	Caldwell and Britz (2006)
	Brassica napus	5 and 10 kJ m^{-2} d^{-1}	25-50% increase in Chl *a*, Chl *b* and Car contents No changes in Chl *a*/*b* ratio	Sangtarash et al. (2009)

Types of stress	Plant	Treatment and duration	Effects	References
	Zea mays and *Triticum aestivum*	125W, 20 and 40 min	No significant changes in pigment contents	Oancea et al. (2011)
	Plantago major	UV-B (302 nm)	67, 63, 89 and 56% decrease in Chl *a*, Chl *b*, total Chl and Car contents, respectively	Salama et al. (2011)
	Phyllanthus tenellus	UV-A (320–400 nm)	Chl *a* and Chl *b* increased by 19 and 40%, respectively Chl *a*/*b* ratio and Car content increase by 31 and 61%, respectively	Victório et al. (2011)
	Oryza sativa L.	UV-B (311 nm) 1-7 d (1 h d^{-1})	28% and 41% decrease in Chl *a* content at 1 and 7 d after the end of irradiation	Lidon and Ramalho (2011)
	Dolichos lablab	UV-B (280-315 nm: 7.2 kJ m^{-2} d^{-1})	By 28 and 5.3% at 15 and 30 DAG	Singh et al. (2011)
Nutrient deficiencies	*Sorghum bicolor* (L) Moench	N-deficiency	50% reduction in total Chl content	Zhao et al. (2005)
	Gossypium hirsutum	P-deficiency	Decrease in Chl content at earlier stages but increased at mature states.	Onanuga et al. (2012)
	Gossyplum hlrsutum	P-deficiency	No significant differences in Chl content	Onanuga et al. (2012)
	Aeluropus lagopoides	K-deficiency	38% decrease in Chl *a* content	Alikhani et al. (2011)
	Morus alba L.	S-deficiency	Decrease in *a*, Chl *b*, total Chl and Car Increase in the ratio of Car/Chl	Tewari et al. (2010)
	Morus alba L.	Mg-deficiency	46 and 24% decreases in Chl and Car contents	Tewari et al. (2006)
	Iris germanica	Zn deficiency	11, 54 and 26% reduction of Chl *a*, Chl *b* and total Chl, respectively No changes in Car contents	Khalifa et al. (2011)
	Lycopersicon esculentum Mill.	Zn deficiency	11% decrease in Chl *a* content 17% increase in Chl *b* content. No changes in total Chl	Kösesakal and Ünal (2009)
	Iris germanica	B deficiency	41, 9, 31 and 20% reduction in Chl *a*, Chl *b*, total Chl and Car content	Khalifa et al. (2011)

together with others like genetic engineering can also help to find out the enzymes as well as the genes responsible for the biosynthesis and functioning of pigments of photosystems. Thus, the efficiency of Chl and Car will be possible to alleviate to a desired level to increase total photosynthesis even under stressful conditions. In fact the Chl and synthesis under stressful condition is not completely elucidated yet. Therefore, further study should be done emphasizing the molecular basis of the alteration of these pigments and their manipulation towards the development of highly productive plants.

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Chapter 18. PHOTOSYNTHETIC PIGMENTS AND PIGMENT-PROTEIN COMPLEXES OF AQUATIC PLANTS UNDER HEAVY METAL STRESS

Maibam Dhanaraj Meitei[1], Abhay Kumar[1], Majeti Narasimha Vara Prasad[1], Przemyslaw Malec[2], Andrzej Waloszek[2], Maria Maleva[3], and Kazimierz Strzalka[2]

[1] University of Hyderabad, Hyderabad, India
[2] Jagiellonian University, Krakow, Poland
[3] M. Gorky Ural State University, Ekaterinburg, Russia

Contents

18.1. Introduction

Photosynthesis, which forms the base of all the food chains in the world, is either directly or indirectly affected by the action of the heavy metals (HMs). Photosynthetic pigments play an important role in plants because they enable the process of photosynthesis, which converts the solar energy into chemical energy, making it available to all other living creatures. Chlorosis, retardation of growth and necrosis are the prominent symptoms observed in aquatic plants during HM stress. HMs directly affect photosynthetic activities in aquatic plants by damaging the reaction centers (RC) of photosystems (PS) I and II, interrupting electron (e^-) flow at the oxygen evolving complex (OEC), destroying the light harvesting complex (LHC) II antenna complex, the cytochrome b_6f protein complex and various other components of the electron transport chain (ETC) (Aravind et al., 2004). Indirectly,

HM stress inhibits chlorophyll (Chl) biosynthetic pathways in many aquatic plants, destroys pigments, chloroplast membranes and thylakoid membranes leading to a reduction in photosynthetic efficiency.

Inhibition of the biosynthetic pathways and the degradation of photosynthetic pigments are one of the main effects observed during HM stress in aquatic plants. As a result, aquatic plants show lower photosynthetic efficiency, retarded growth and reduced biomass. Cadmium (Cd), lead (Pb), nickel (Ni), copper (Cu), zinc (Zn), iron (Fe), chromium (Cr), manganese (Mn) and magnesium (Mg) among others, are found to inhibit as well as degrade photosynthetic pigments in many aquatic plants (Aravind et al., 2004; Paiva et al., 2009; Malec et al., 2010; Singh et al., 2010; Xing et al., 2010; Delmail et al., 2011; Dhir et al., 2011; Xu et al., 2011; Monferran et al., 2012; Sapci et al., 2012; Wang et al., 2012).

Among the toxic metals, some have been investigated thoroughly with respect to their influence on photosynthetic pigments and pigment-protein complexes in aquatic plants. The content of Chl *a* and *b* pigments are considered more prominently in the present study. In this chapter, an effort is made to give an idea on the impact of HM stress on the pigment-protein complexes involved in the light reactions of photosynthesis in aquatic plants, with special emphasis on Chl fluorescence parameters.

18.2. Photosynthetic pigments and pigment-protein complexes

Chlorosis of leaves, reduction in the length of roots, retarded growth and necrosis are the visible symptoms of HM toxicity in aquatic plants. Inhibition of the activities of Chl biosynthetic enzymes, the substitution of Mg ions by target metal ions in the Chl, increased chlorophyllase activity, peroxidation of the chloroplast membrane, inhibition of the pigment-protein complexes and degradation of Chl, reduces photosynthetic efficiency (Appenroth et al., 2001; Aravind et al., 2004; Mishra et al., 2006).

18.2.1. Heavy metal stress and chloroplast structure

HMs cause structural or ultrastructural changes in the chloroplast through the peroxidation of membrane lipids. Changes in the ultrastructure of chloroplast were observed in *Ceratophyllum demersum* (Aravind et al., 2004) when exposed to Cd. The most prominent modifications seen in the chloroplast and vacuole involve the increased number of small starch grains occupying almost the whole chloroplast. Cytoplasmic vacuolization and the appearance of various types of vacuoles were observed with non membranous, electron-opaque deposits of an undefined structure occupying almost whole vacuoles along with

myelin-like figures, whereas the cell wall structures along with the nucleus structure were unaffected.

18.2.2. Heavy metal stress and pigment content

HM stress has been reported to reduce the Chl content in aquatic plants (Babu et al., 2001; Aravind et al., 2004; Delmail et al., 2011; Dhir et al., 2011; Sapci et al., 2012; Wu et al., 2012). HM-induced inhibition of photosynthetic pigment biosynthesis and its degradation in aquatic plants have been well studied (Table 1). The photosynthetic activity of aquatic vascular plants under HM stress has attracted a lot of attention, towards the generation of natural bioindicators and potential phytoremediants of various HMs from various aquatic environments. In either of the two cases, avoidance of metal toxicity or the easy, fast detection of injury to photosynthesis and its related pathways are the main topic of interest for scientists. It is difficult to specify in a general way the dose of a metal which will produce toxic effects on chloroplast content in aquatic plants, since the experimental conditions used by researchers all over the world vary.

Cadmium inhibits the light harvesting Chl *a*/*b* protein complex in *Salvinia natans* (Dhir et al., 2011), which significantly decreases the rate of photosynthetic efficiency. In *C. demersum,* Cd inhibits the e^- flow at each of the protein complexes involved in e^- transport starting from the OEC, PSII-RCs, plastocyanin (PC) and PSI-RCs, resulting in a reduced photosynthetic rate (Aravind et al., 2004). In addition, excessive amounts of micronutrients such as Zn, Cu and Mn showed metal toxicity dependent on concentration and duration, in *Spirodela polyrhiza* (Upadhyay et al., 2010), *Lemna minor* (Kanoun-Boyle et al., 2009), *Hydrilla verticillata* (Srivastava et al., 2006; Wang et al., 2009), *Pistia stratiotes* (Tewariet al., 2008), *Fontinalis antipyretica* (Rau et al., 2007), *Zostera capricorni* (Catriona et al., 2002) and *Lemna trisulca* (Prasad et al., 2001). Reduced pigment content, the main toxicity symptom of Cd might be due to the inhibition of Chl biosynthesis and degradation of Chl leading to a sharp decline in the net photosynthetic rate. Aravind et al. (2004) showed Chl *a* and *b* concentrations reduced by 23 and 40% respectively in *C. demersum* treated with Cd (10 μM) for 7 days. This might be due to Cd inhibited Chl biosynthesis through δ-aminolevulinic acid dehydratase (ALAD) and protochlorophyllide reductase by its interference with the sulphydryl site leading to lower production of δ-aminolevulinic acid (ALA).

It was observed that Chl content reduced by up to 15% after 300 μM Cd treatments for 7 days as compared to control plants in *Egeria densa*. This also supports the toxic effect of Cd in the Chl biosynthetic process (Malec et al., 2009b). Cadmium at a 10 μM concentration decreased the Chl *a*, *b* and carotenoid concentrations in *L. trisulca* fronds,

Table 1

The impact of heavy metals (HMs) on the photosynthetic pigment and pigment-protein complexes in aquatic plants

Plant taxon	HMs	Experimental observations	Reference
Echinodorus amazinocus	Cu, Ni, Zn, Mn, Fe, Cr, Ca	Translocation of Mg (II) into the chloroplast is inhibited	Sapci et al. 2012
Potamogeton pusillus	Cr, Cu	Inhibited Chl biosynthesis; Chl degradation; Disruption of membrane with leakage of ions	Monferrán et al. 2009 and 2012
Vallisneria natans	Pb	Disturbed uptake of Mg, Fe and Mn or increased chlorophyllase activity that catalyze Chl degradation	Wang et al. 2011
Typha angustifolia	Cd	Reduction of NPR and stomatal conductance	Xu et al. 2011
Myriophyllum alterniflorum	Cd	Inhibition of Chl biosynthesis; Xerophytic characters developed	Delmail et al. 2011
Salvinia natans	Cr, Fe, Ni, Cu, Pb, Cd Co, Zn, Mn	Reduced efficiency of ALAD; Decrease in availability of Fe and Mn; Chloroplast membrane peroxidation; Formation of metal substituted Chl; F_v/F_m ratio decline in Cu, Pb, Cd and Zn exposed plants due to binding of HMs to manganoprotein complex of chloroplast; Substitution of Mg(II) by the metal ions in RUBISCO; PSI activity increased; Build up of the trans thylakoidal proton gradient which acts as a driving force for ATP synthesis	Dhir et al. 2008 and 2011
Spirodela polyrhiza	Cu, Zn	Chl degradation; Change in the antenna size of PSII due to the change in the number of LHC per PSII-RC	Upadhyay et al. 2010
Callitriche cophocarpa	Cr	Oxidative damage of chloroplast structure; decrease F_v/F_m ratio	Augustynowicz et al. 2010
Najas indica	Pb	Inhibition of ALAD enzymes and protochlorophyllide reductase activities	Singh et al. 2010
Elodea nuttallii	Fe	No significant change in Chl *a*, *b* and carotenoids concentration; Photosynthetic rates and Chl fluorescence decreased	Xing et al. 2010
Lemna minor	Cu	Break down of pigment and chloroplast membrane lipids by ROS; Increased membrane ion leakage as a result of lipid peroxidation	Kanoun-Boyle et al. 2009.

Plant taxon	HMs	Experimental observations	Reference
Hydrilla verticillata	Zn	Zn inhibits Chl production by interfering with Fe metabolism	Wang et al. 2009
Hydrilla verticillata	Cu	Down regulation of genes coding Chl biosynthesis enzymes, proteins of PSI and II; EC value increased 122%	Srivastava et al. 2006
Elodea canadensis	Ni	Substitution of Mg (II) by metal ions in the chlorophyll molecules	Maleva et al. 2009
Egeria densa	Cd	Photosynthetic apparatus damage	Malec et al. 2009b
Eichhornia crassipes	Cr	Reduction in the size of the peripheral parts of the antenna complex; F_v/F_m ratio didn't change, compared to F_v/F_o ratio, indicating its importance in Cr stress monitoring	Paiva et al. 2009
Pistia stratiotes	Pb, Ni, Cu, Zn, Mn, Cr	Substitution of Mg of Chl by the metal ions; Carotenoids content increased	Tewari et al. 2008
Alternanthera philoxeroides	Cd	Chlorophyll degradation; protochlorophyllide reductase activities inhibited	Ding et al. 2007
Fontinalis antipyretica	Cd, Pb, Zn, Cu	Degradation of Chl; F_v/F_m changed drastically indicating the photoinhibition of PSII	Rau et al. 2007
Ceratophyllum demersum	Pb	Inhibition of ALAD, Impaired uptake of Mn and Fe; Chl degradation by increased chlorophyllase activity; EC increased upto 142%	Mishra et al. 2006
Bacopa monnieri	Cd	Interaction of Cd to –SH group of enzymes of chlorophyll biosynthesis as well as lipid peroxidation mediated degradation	Singh et al. 2006
Ceratophyllum demersum	Cd	Lower production of ALA; 39% reduction in the NPR; Cd (II) ions repaced Mn (II) ions at the OEC, the primary source of e^- from water to PS II, thereby inhibiting the PS II; Reduction in PSI, PSII and whole chain ETS	Aravind et al. 2004
Zostera capricorni	Cd, Cu, Pb, Zn	Chl degradation; NPR declined as metal ions inhibits transport of e^- at the donor and acceptor sites of PSII	Catriona et al. 2002
Lemna trisulca	Cd, Cu	Lipid peroxidation of the chloroplast membranes; Decreased NPR; Metal substituted Chl fails to fluoresce	Prasad et al. 2001

Note. Chl – chlorophyll; EC – electrical conductivity; ETS – electron transport system; NPR – net photosynthetic activities; PS – photosystem; RC – reaction center; ROS – reactive oxygen species.

whereas Chl *b* was found to be less sensitive compared to Chl *a* and carotenoids (Prasad et al., 2001). Alterations in photosynthetic pigment composition as induced by externally applied Cd were observed in the photosynthetic organs of several aquatic macrophytes: *Elodea canadensis*, *E. densa*, *L. trisulca* (Malec et al., 2009a, b, 2010) and *Myriophyllum alterniflorum* (Delmail et al., 2011).

An excess amount of Cu was observed to reduce the Chl content in aquatic plants (Srivastava et al., 2006; Monferran et al., 2009; Upadhyay et al., 2010). Increasing concentrations of Cu decrease the content of Chl *a* and carotenoids in *L. minor* while Chl *b* content seemed to be less affected. The loss of pigment is due to direct peroxidative damage to the chloroplast membrane (Kanoun-Boyle et al., 2009). Prasad et al. (2001) reported an elevated level of Chl pigment in *L. trisulca* exposed to 1 to 10 μM Cu in the medium. The pigment content decreased when the concentration of Cu reached 25 μM and above due to Cu induced degradation of the chloroplast membrane. A dose dependent decrease in pigment was observed in *H. verticillata* during Cu exposure. No effect was observed up to 1 μM till 4 days, beyond which their content declined with a more severe effect on Chl *a* than Chl *b* (Srivastava et al., 2006). Similar to Cd and Cu, Pb was also reported to reduce the photosynthetic pigment content (Wang et al., 2011). A concentration dependent reduction of Chl and carotenoids were observed in *Najas indica* (Singh et al., 2010) and *C. demersum* (Mishra et al., 2006) after Pb treatment. The capability of Pb to bind thylakoid membranes and the resulting loss of function might account for the toxic effect of Pb on photosynthetic light reactions (Heng et al., 2004).

The aquatic plant *E. canadensis* tolerates elevated levels of Ni (up to 10 μM) without significant change in the photosynthetic pigment content whereas after treatment with 50 μM of Ni, the accumulation of Chl *a* reduced remarkably (Maleva et al., 2009). This effect correlates with a significant decrease in the net photosynthesis rate. An increase in the accumulation of carotenoids has been considered to play an important role in the scavenging of reactive oxygen species (ROS) and in the recovery of cells from Ni stress (Maleva et al., 2009). An increase in the total Chl content were observed at 0.05-0.5 mg L^{-1} Zn exposed *H. verticillata*, it decreased significantly with 10 and 30 mg L^{-1} Zn treatments (Wang et al., 2009). Augustynowicz et al. (2010) observed that Chl *a* and carotenoids were more sensitive to Cr stress as compared to Chl *b* in *Callitriche cophocarpa*.

18.2.3. Heavy metal stress and photosynthetic light reactions

A reduction in the rate of photosynthesis has been observed in many aquatic plants under HM stress (Aravind et al., 2004; Ding et al., 2007; Rau et al., 2007; Paiva et al., 2009; Wang et al., 2009;

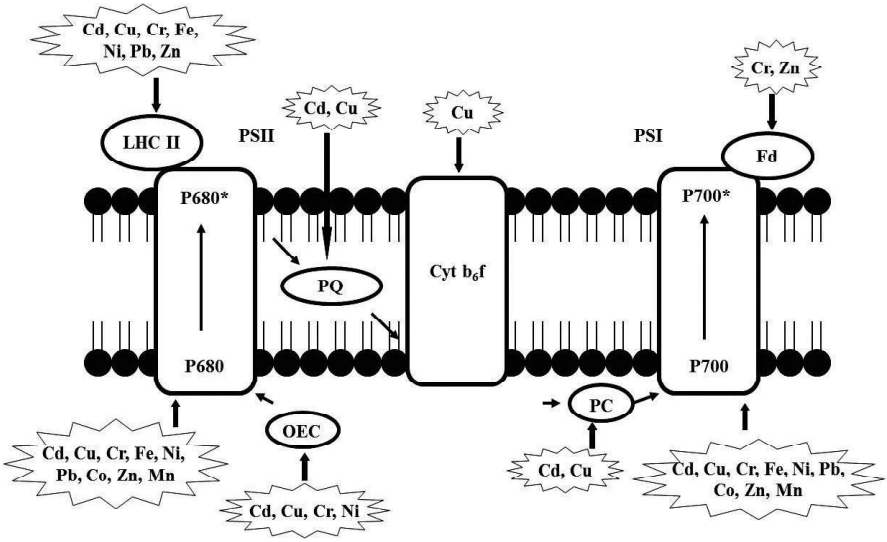

Fig. 1: Site of action of HMs on the photosynthetic electron transport. OEC – oxygen evolving complex; LHC II – light harvesting complex II; PSII – photosystem II; PSI – photosystem I; Cytb_6f – cytochrome b_6f; PQ – plastoquinone pool; Fd – ferredoxins.

Augustynowicz et al., 2010; Monferran et al., 2012). The effects of HM toxicity on the photosynthetic ETR can be either the inhibition of the OEC, the light harvesting Chl *a*/*b* protein complex, the degradation of the D1 protein of PSII, the inhibition of plastocyanin or the reduction of NADP (Fig. 1). In addition to this, degradation of Chl *a* and *b* also results into reduced net photosynthesis (Catriona et al., 2002; Dhir et al., 2008; Xing et al., 2010; Wu et al., 2012). The sites of inhibition in the photosynthetic ETC caused by various HMs have been thoroughly described (Table 2).

Cadmium is found to inhibit the light harvesting Chl *a*/*b* protein complex, the OEC, PSII, the plastoquinone (PQ) pool, the PC and PSI protein complex in many aquatic plants (Aravind et al., 2004; Dhir et al., 2011). Cadmium reduced the rate of photosynthesis in many aquatic plants such as *Typha latifolia* (Xu et al., 2011), *M. alterniflorum* (Delmail et al., 2011), *E. densa* (Malec et al., 2009b), *A. philoxeroides* (Ding et al., 2007) and *C. demersum* (Aravind et al., 2004). The majority of the experiments have concentrated on the inhibitory effect of Cd on the PSII of the light chain reactions. The toxic effect of Cd either on PSII or PSI is dose dependent and increases with the exposure period (Delmail et al., 2011). In *S. natans*, PSI activity increased with Cd exposure in order to cope the additional flow of e^- during the stress

Table 2

Heavy metals and the site of action in the photosynthetic electron transport chain

HMs	Site of action	References
Cr, Fe, Ni, Cu, Pb, Zn, Cd	Light harvesting chlorophyll *a*/*b* protein complex	Appenroth et al. 2001; Aravind et al. 2004; Paiva et al. 2009; Dhir et al. 2011, 2008
Ni, Cd , Cu, Cr	Oxygen evolving complex	Appenroth et al. 2001; Prasad et al. 2001; Aravind et al. 2004; Maleva et al. 2009
Cr, Fe, Ni, Cu, Pb, Cd Co, Zn, Mn	Photosystem II	Appenroth et al.2001; Babu et al. 2001; Prasad et al. 2001; Catriona et al. 2002; Aravind et al. 2004; Rau et al. 2007; Paiva et al. 2009; Augustynowicz et al. 2010; Xing et al. 2010; Dhir et al. 2008; 2011
Cd, Cu	Plastoquinones and Cytochrome b_6f	Babu et al. 2001
Cd, Cu	Plastocyanin	Catriona et al. 2002; Aravind et al. 2004
Cr, Fe, Ni, Cu, Pb, Cd Co, Zn, Mn	Photosystem I	Babu et al. 2001; Aravind et al. 2004; Dhir et al. 2008; 2011
Cr, Zn	Ferredoxins $NADP^+$ oxidoreductase	Dhir et al. 2008

period (Dhir et al., 2011). The inhibition of photosynthesis in *C. demersum* intact chloroplasts treated with Cd is due to the peroxidation of lipid membranes, the loss of thylakoid membrane integrity and major fatty acids around the light harvesting Chl *a*/*b* protein complex (Aravind et al., 2004).

Copper is an essential micronutrient, but at high concentrations it becomes toxic and inhibits many sites at the photosynthetic ETC (Table 2) (Babu et al., 2001; Prasad et al., 2001; Srivastava et al., 2006; Dhir et al., 2011). Photosystem II and PC-protein complexes are the main sites susceptible to for ample amounts of Cu in aquatic plants. Copper forms a main constituent of the PC, which helps in the transport of e^- between the cytochrome b_6f complex and PSI, which is highly sensitive to excessive Cu concentrations. On the contrary, a deficiency of Cu inhibits the photosynthetic ETC, by reducing the transfer of e^- by the PC between PSII and PSI (Babu et al., 2001; Mysliwa-Kurdziel et al., 2002). Degradation of Chl due to an increase in chlorophyllase activity under Pb treatment was observed to disturb the photosynthesis in *Vallisneria natans* (Wang et al., 2011). Augustynowicz et al. (2010)

observed that PSII activity is inhibited in *Callitriche cophocarpa* due to Cr stress as it leads to oxidative damage to chloroplast structure.

18.2.3.1. Photosystem I (PSI)

The effect of HMs on PSI has been thoroughly studied in many aquatic plants such as *S. natans* (Dhir et al., 2008 ; 2011), *P. stratiotes* (Tewari et al., 2008) and *C. demersum* (Aravind et al., 2004). Photosystem I is a membrane-bound protein complex which performs the function of catalyzing the oxidation of the PC and reducing ferredoxins under light conditions. During the process, a photon of light is captured by the Chl of PSI reaction centres (P_{700}), which passes an excited e^- to the acceptor called A_o and then to ferredoxins with the help of several other e^- carriers (Mysliwa-Kurdziel et al., 2004).

Cadmium inhibits the e^- flow at the e^- donor side of PSI in *C. demersum*, as an impaired PC remains as a block (Aravind et al., 2004). It reduces the activity of PSI by 42%. The toxic effects of Cd on an aquatic plant can be overcame by supplements of Zn from the environment, where Zn supplemented plants are able to recover nearly 36% of the damaged PSI activity (Aravind et al., 2004). Dhir et al. (2011) reported that the activity of PSI increases with metal stress in *S. natans*. The increase in the PSI mediated ETR might be an adaptation of the plant to cope the additional flow of e^- during HM stress. In general, a decrease in the linear e^- flow and activation of cyclic ETC has been reported as a response of plants exposed to various forms of abiotic stress (Golding et al., 2004). The same phenomenon was observed in *S. natans* in Cr and Zn stress environments (Dhir et al., 2008). Ferredoxins, situated on the acceptor side of the PSI were observed to be a target of Cr and Zn action (Dhir et al., 2008).

Plants have developed certain strategies to maintain homeostasis under HM stress during photosynthesis. These may include the activation of thiol regulated chloroplast enzymes such as NADP-malate dehydrogenase (MDH). It catalyzes the formation of malate from oxaloacetate by using NADPH to generate an e^- acceptor (Scheibe et al., 2005). The activation of the enzyme provides a path for reducing equivalents to allow e^- transport and proton pumping. *S. natans* did not exhibit a significant increase in NADP-MDH activity during metal stress indicating that the stromal status has adequate levels of NADPH and reducing equivalents for the assimilation of CO_2.

18.2.3.2. Photosystem II (PSII)

The majority of the studies on the impact of HMs on aquatic plants are restricted to the light reactions of PSII. The photosynthetic activities in many aquatic plants are blocked at the site of PSII (Table 2), both on the reducing and acceptor side of e^- due to HM toxicity. Heavy

metals are found to inhibit the rate of photosynthesis at the PSII site in many aquatic plants (Catriona et al., 2002; Aravind et al., 2004; Rau et al., 2007; Paiva et al., 2009; Augustynowicz et al., 2010; Xing et al., 2010; Dhir et al., 2011). PSII is located either in the appressed lamellae or the grana in the chloroplasts. It is a multi-protein complex consisting of nearby 25 different subunits (Aro et al., 1993).

The efficiency of PSII was reduced in *S. natans* exposed to Cu, Cd, Pb and Zn due to insufficient transfer of energy from the LHC to the RC-Chl molecules or due to the binding of HMs to the mangano-protein complex of chloroplast (Dhir et al., 2011). In another study, PSII photochemical efficiency showed no significant change in Cr-exposed *S. natans* while Zn-treated plants showed a decline in PSII efficiency. A Zn-induced decrease in the efficiency of photosynthesis might have resulted from damage to RC molecules and/or loss of Chl content (Dhir et al., 2008). Xing et al. (2010) demonstrated that the photosynthetic rate in *Elodea nuttallii* decreased under iron toxicity, which is caused mainly by the disturbed integrity of the thylakoid membranes and their fatty acid composition.

Copper is well known to regulate the pigment content and composition of the pigment-protein complexes of PSII (Mysliwa-Kurdziel et al., 2002). It inhibits the photosynthetic ETC at the PSII e^- acceptor and donor site. An excess of Cu (II) causes the photoinhibition of PSII and lowers Chl concentration in *F. antipyretica* (Rau et al., 2007).

Aravind et al., (2004) reported that Cd affects the lipid structure around PSII, mainly the light harvesting Chl *a*/*b* protein complex II leading to a loss of major fatty acids and the production of lipid hydroperoxides in *C. demersum*. This ultimately induces the release of several Chl *a*-protein complexes and thylakoid membrane proteins including Mn-stabilizing protein and PC. An impaired PC in turn affects the ETC thereby reducing the rate of photosynthesis. Catriona et al. (2002) demonstrated the effects of Cu, Zn, Pb and Cd exposure inhibiting photosynthesis in *Z. capricorni*. Copper exposure inhibited the transport of e^- at the donor and acceptor sites of PSII, leading to a decrease in the quantum yield as Cu is an essential constituent of the PC protein complex. Zinc interacts with the donor side of PSII, inhibiting photosynthetic CO_2 fixation and the Hill reaction. Lead has been found to inhibit PSII by blocking electron transport on the oxidizing side of PSII (Catriona et al., 2002).

18.2.3.3. Chl fluorescence parameters

The technique of Chl fluorescence has emerged as one of the favored methods for the detection and investigation of the HM stress in aquatic plants. The main advantage of this method is that it is simple and non-destructive in nature. The Chl fluorescence technique can be used to

give an overview of the activity of the PSII-protein complex of the ETC during metal stress, including energy dissipation by the antenna complex and photoinhibition. Fluorescence originates from the Chl in photosynthetic tissues when exposed to light. Chl fluorescence gives an insight into the physiological state of the photosynthetic apparatus on the thylakoid membrane.

Dhir et al. (2011) investigated the toxic effects of various HM exposures such as Cr, Fe, Ni, Cu, Pb, Cu, Zn, Co and Mn on the photosynthetic activity of *S. natans*. It was observed that insufficient energy transfer from the LHC to the RC-Chl molecules in Cu, Cd, Pb and Zn exposed plants reduces photo-chemical efficiency (PSII activity) (F_v/F_m). Inhibition in the activity of PSII might result from the binding of HM ions to the mangano-protein complex of the chloroplast. However, PSII-mediated electron transport measured as F_v/F_m remained more or less unaffected in *S. natans* exposed to Cr in contrast to that observed in Zn-treated plants (Dhir et al., 2008). In Cr exposed plants, the absorption of light energy and electron transport per RC was equal. In contrast, Zn-treated plants showed a reduction in F_v/F_m, which might have resulted from damage to RC molecules and/or loss of Chl content. HMs not only affect the analysed parameters by altering the energy transfer efficiency from the light harvesting complex to PSII reaction centers but also by altering the water splitting site (Aravind et al., 2004).

Appenroth et al. (2001) demonstrated the toxic effects of Cr on the photosynthetic activity of *S. polyrhiza*. The JIP test suggested that there was a reduction in the efficiency of PSII due to the damage to RCs and the OEC as the main targets of Cr toxicity. *E. nuttallii* showed remarkable tolerance to Fe toxicity up to concentrations of 0.1-500 mg L^{-1}, where no significant changes in Chl fluorescence were observed. All Chl fluorescence parameters reduced significantly at 1000 mg L^{-1} suggesting ROS caused by the excess iron concentration severely damaged the photosynthetic apparatus. This implies that metal toxicity is concentration dependent (Xing et al., 2010). Prasad et al. (2001) reported that in Cd and Cu treated *L. trisulca* fronds, Chl *a* and Chl *b* fluorescence declined drastically in a dose-dependent manner due to Chl degradation, indicating that Cd and Cu affect photosynthetic energy transfer in *L. trisulca* fronds. The substitution of Mg by Cu in the Chl of the antenna complexes and RCs might be the cause of the observed fluorescence decay induced by Cu, as Cu-substituted Chl fails to fluoresce.

Paiva et al. (2009) investigated the tolerant nature of a metal hyperaccumulating aquatic macrophyte, *Eichhornia crassipes* during Cr (III) and Cr (VI) stress. The results showed that the plants were more sensitive to Cr (VI). There was a reduction in the F_v/F_m and F_v/F_o ratios

caused by Cr (VI) due to the damaged photosynthetic apparatus. The decrease in the F_v/F_o ratio is an indicator of the structural damage which occurs in the thylakoid membrane and in turn affects the photosynthetic transport of e^-. Decrease in F_v/F_o ratios were observed in other treatments in addition to 10 mM Cr (VI), as also seen in the presence of 1 mM Cr (VI). The decrease in the F_v/F_o ratio was mainly due to a decrease in F_v, and not to an increase in F_o. The decrease in the F_m value is due to changes in the ultrastructure of the thylakoid membrane, which in turn affect the ETR. In some point, Cr (III) also increases the rate of photosynthesis and Chl contents in treated plants. Thus, the above method can be used for the monitoring of Cr (VI) toxicity or pollution with the help of *E. crassipes*.

The pattern of Cu ions affecting plant growth, photosynthesis and the metabolism is different. However, Cu (II) access causes a reduction of the relative amount of active PSII-PCs as well as a decrease in F_v/F_m ETR (Babu et al., 2001). Rau et al. (2007) demonstrated that Chl fluorescence changes significantly in *F. antipyretica* during Cd and Cu stress but were less affected in Pb and Zn treated samples. Cu concentrations from 25–50 μM resulted in a decline in fluorescence to 0.54 and 0.55, where the controls gave a reading of 0.72-0.75 (F_v/F_m) after 7 days of exposure. On the other hand, increasing Cd concentrations up to 100 μM caused a decrease in fluorescence to 0.52-0.56. An excess of Cu (II) causes photoinhibition of PSII which is dose and time dependent, lowering the Chl concentration and reducing the thylakoid membrane composition.

18.2.3.4. Oxygen evolving complex (OEC)

The OEC, which is located on the thylakoidal luminal side acts as the donor of e^- from water to PSII-RCs. The OEC is a target site for a number of HMs such as Cd, Ni, Cu and Cr in the aquatic plants, *E. canadensis* (Maleva et al., 2009), *C. demersum* (Aravind et al., 2004), *S. natans* (Appenroth et al., 2001) and *L. trisulca* (Prasad et al., 2001). In *E. canadensis*, impact of respiration in the total oxygen evolution (OE) was enhanced after the treatment of the plants with Ni concentration of 1 μM. Treatment with higher concentrations of Ni leads to a gradual decrease in the rate of respiration. This shows that the dark respiration rate increases in plants exposed to mild metal stress, whereas O_2 consumption decreased under increasing metal stress, which indicates metabolic damage caused by stress (Maleva et al., 2009). Aravind et al. (2004) reported that Cd (II) ions replaced Mn (II) ions at the OE centers, inhibiting the photosynthetic activity of PSII and associated protein complexes of the PSII-RCs especially the D1 polypeptide in *C. demersum*. The rate of OE reduced significantly with increasing concentrations of Cd (from 0.1 mM to 10 mM and Cu from 1 μM to 50 μM) in *L. trisulca* (Prasad et al., 2001).

18.2.3.5. Photoinhibition

This can be defined as the phenomenon caused when the rate of photodamage exceeds the rate of repair, leading to a decrease in photosynthetic yield. PSII is the main target of photodamage in the photosynthetic ETC. During the process, damage can occur either on the acceptor or the donor side of PSII. Donor side inhibition is noticed when e^- is unable to deliver to P_{680} RCs from the donor side, and acceptor side inhibition is seen when the PQ acceptor pool is lost from its binding site on PSII. As a result, this causes changes in the conformation of the D1 protein leading to its degradation, resulting in a lower photosynthetic yield (Aravind et al., 2004; Augustynowicz et al., 2010; Xing et al., 2010, 2011; Monferran et al., 2012).

Cadmium toxicity was found to reduce photosynthetic efficiency in *C. demersum* by inhibiting the activity of PSII (Aravind et al., 2004). It was observed that Cd formed complexes with aromatic amino acid residues such as tryptophan and the PS-D1 protein. The Cd-D1 complex then impairs the normal activity of the PS-protein complex by interrupting the normal process of D1 protein degradation by a protease. Prasad et al. (2001) reported a reduction in PS activity due to Cd toxicity in *L. trisulca* leading to a decrease in the net photosynthetic rate. Dhir et al. (2008) demonstrated that there is a significant reduction in the activity of PSII in Zn treated *S. natans* due to degradation of the Chl molecule and damage to the RC molecule.

18.3. Conclusions

HMs are effective in inhibiting photosynthetic activities, leading to the disruption of normal physiological activities in aquatic plants. They interact directly with the lipid membrane of the thylakoid resulting in membrane damage and destruction of the chloroplast structure. HMs inhibit a number of pigment-protein complexes associated with the light reactions of photosynthesis such as the OEC, the light harvesting protein complex, PSII and PSI, the PQ pool, the PC, the cytochrome b_6f complex and ferredoxins resulting in reduced photosynthetic efficiency (Fig. 1, Table 1).

All the above studies showed that HM stress inhibits several enzymes in the Chl biosynthesis pathway, disturbs the structure of the chloroplast membrane and increases the activities of pigment degrading enzymes, which results in the reduction of the pigment content in aquatic plants during the stress period. Inhibition of the photosynthetic pathway leads to photoinhibition which ultimately leads to necrotic changes in the exposed plants.

The various studies performed related to the influence of HMs on photosynthetic pigments and pigment-protein complexes are of great

value. Reduction in the pigment content will eventually lead to a decrease in the photosynthetic efficiency of an aquatic plant which in turn will reduce the biomass, which is a serious matter for aquatic crop plants. The study of aquatic plants susceptible to HM stress makes available model aquatic plants which can be used as tools for biomonitoring HM polluted environments. On the other hand, the study of resistant plants which can take up HMs and survive, without showing visible symptoms, provides us with aquatic plants which can be used for phytoremediation of HM polluted aquatic wastewater environments.

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Chapter 19. STRUCTURAL AND FUNCTIONAL ASPECTS OF PHOTOSYNTHETIC APPARATUS UNDER UV-B STRESS

Rima Kumari[1], Majeti Narasimha Vara Prasad[1], Andrzej Waloszek[2], and Kazimierz Strzalka[2]

[1] University of Hyderabad, Hyderabad, India

[2] Jagiellonian University, Krakow, Poland

Contents

19.1. Introduction

Ultraviolet-B radiation (UV-B; 280–320 nm) constitutes a minor part of the solar spectrum and most the UV solar radiation is absorbed by the UV-screening ozone layer in the stratospheric region of the atmosphere. In the past few decades, global depletion of the stratospheric O_3 layer, largely due to the release of chlorofluorocarbons caused by human activities, has resulted in an increase of solar UV-B radiation on the earth's surface (Madronich et al., 1998). This phenomenon, first described over the Antarctic polar region, is now extending significantly even to temperate regions (Kerr and McElroy, 1993). Global ozone is still lower than in the 1970s and as the changes in ozone impinge directly on UV radiation, elevated UV radiation due to

reduced ozone is expected to continue for decades (McKenzie et al., 2007). The prediction of future changes in UV-B due to ozone depletion is difficult to verify through direct measurement. Observational evidence suggests that climate change factors i.e. changes in cloud cover, aerosol emissions, albedo and surface reflectivity can also affect UV-B irradiance. The ozone layer is not expected to recover until 2070 due to a decrease in the temperature in the stratosphere as an effect of climate change (Caldwell et al., 2007). Despite the uncertainty of long term predictions, there will be an estimated UV-B increase of 5–10% over temperate latitudes within the next 15 years (Lidon and Ramalho, 2011).

Enhanced solar UV-B radiation reaching the Earth's surface has received considerable attention from scientists because of its potential adverse effects on the biosphere, including photosynthetic organisms. Since UV-B wavelengths (290–320 nm spectral ranges) are biologically active, it can be readily absorbed and drive photo excitation or photo modification reactions of various important macromolecules, including DNA, proteins and lipids, and can impair various cellular processes. It is a major abiotic stress factor, which has potentially deleterious effects on agricultural production and natural plant ecosystems (Bornman, 1989). UV-B radiation affects either directly or indirectly the photosynthetic performance of plants (Teramura and Sullivan, 1994; Ranjbarfordoei et al., 2011). UV-B inflicts damage on the photosynthetic apparatus of higher plants at multiple target sites structural damage to chloroplast (i.e. disruption and disorganization of granal stacks and rupturing of chloroplast envelope), the inactivation of the oxygen evolving complex, damage to PS II reaction centre protein and the impairment of the thylakoid electron transport chain. Furthermore, UV-B radiation was reported to induce indirect effects on the photosynthetic mechanism including the suppression of chlorophyll synthesis and the regulation of stomatal conductance (Vass et al., 2005). Change in these processes ultimately leads to lower quantum yield of PS II, which would contribute to the inhibition of photosynthesis.

19.2. Effect of UV-B on photosynthesis Inhibition

Several reviews point to the fact that the influence of UV-B radiation on photosynthesis is not straightforward, probably depending upon intra or interspecific variation, growth conditions, environmental factors, UV-B doses etc., (Rozema et al., 2002; Kakani et al., 2003). The inhibition and acclimation mechanisms of photosynthetic organisms to UV-B stress have been extensively studied. Previous studies showed that UV-B induced inhibition of photosynthesis only appears at high UV-B radiation levels (Bassman et al., 2002). However, a wide range

Table 1

Effects of UV-B radiation on photosynthetic pigments and physiological function in various plant species under different environmental conditions

Plants	UV-B $_{BE}$ dose (kJ m^{-2} d^{-1})	PAR (µmol photons m^{-2} s^{-1})	Exp. condition	Growth parameters	UV-B induced response	References	Country
Triticum aestivum & *Vigna radiata* L.	A +UVB (+7.2)	1100-1200	FC	Total chlorophyll	↓ (10.9 to 31.1%) *T. aestivum* ↓ (5.1 to 23.4%) *V. radiata*	Agrawal and Rathore (2007)	India
Spiraea pubescens L.	A +UV-B (+9.4)	A	FC	Gs WUE	↓ (max. 24.7%) no effect	Lan and Shouren (2007)	China
Zea mays L.	A (6.84) +UV-B (+3.16)	A	FC	Pn, Gs and E	↓ (by 21%, 32% and 13% respectively)	Correia et al. (2005)	Portugal
Pseudotsuga menziesii L.	A +UV-B (2x and 3x of A)	ranged from 600-1800	GC and FC	Net photosynthesis	↑at 2x (in Ist & IInd yr) ↓at 3x (in Ist year)	Bassman et al. (2002)	USA
Helianthus annuus L.	A (4 Wm^{-2}) +UV-B (+0.6 Wm^{-2})	1800	GH	Pn, Gs and E Total chlorophyll Fv/Fm ratio $C0_2$ assimilation	Decrease in all the parameters No change No change ↓ (28.5%)	Cechin et al. (2008)	Brasil
Pisum sativum L.	A (1.89) +UV-B (2.44)	1250	FC	Net photosynthesis	No change	Allen et al. (1999)	U.K.
Sorghum vulgare L.	A +UV-B (+7.1)	1000-1200	FC	Pn, Gs and E Total Chl. and Carotenoids	Pn ↓ by 13%; Gs ↑ (65.2%); E ↓ (24%) ↓ (23 and 27.6%)	Ambasht and Agrawal (1998)	India
Triticum aestivum L.	A +UV-B (+7.1)	990	FC	Total chl. and carotenoids Pn and Gs	↓ (24.4 and 17.9%) No significant effect	Ambasht and Agrawal (2003b)	India

Plants	UV-B $_{BE}$ dose (kJ m^{-2} d^{-1})	PAR (µmol photons m^{-2} s^{-1})	Exp. condition	Growth parameters	UV-B induced response	References	Country
Glycine max L. Cv. Heidou and Jindou)	A (8.85) +UV-B (13.1)	–	FC	Photosynthetic parameters	Significant ↓ (Heidou) No change (cv. Jindou)	Feng et al. (2003)	China
Picea asperata L.	A (11.02) +UV-B (+3.31)	–	FC	Photosynthetic parameters Total chl. and Carotenoids	↓ by 19.4, 43.7 and 42.4% in Pn, Gs, E. ↑ by 12.8% in Ci ↓ (22.2% and 50%)	Yao and Liu (2007)	China
Glycine max L. (20 cultivars)	A (10) +UV-B (+5)	NA	FC	Tot. chlorophyll	↓ in 12 cutivars (max. upto 42.6%), ↑ (max. upto 50.4%) in 7.	Yanqun et al. (2003)	China
Saussurea superba L. *Gentiana straminea* L.	A +UV-B (15.8)	1800	FC	Photosynthetic CO_2 uptake photosynthetic O_2 evolution	Increase in both species	Shi et al. (2004)	China
Pisum sativum L.	A +UV-B (+7.1)	A	FC	Tot. chlorophyll and carotenoids	↓ (12.7 and 7.6%)	Singh et al. (2009)	India
Spinacea oleracia L.	A (8.6) +UV-B (+7.1)	1100-1200	FC	Tot. chlorophyll and carotenoids	Significant ↓	Mishra and Agrawal. (2006)	India
Glycine max L.	A (8.6) +UV-B (+7.1)	1010	FC	Pn and Gs Tot. chlorophyll and carotenoids	↓ (14.7% and 40%, respectively) ↓ (21.1%) in Tot. Chl. No change in carotenoids	Ambasht and Agrawal (2003a)	India
Vigna unguiculata L.	-UV-B UV-B (5, 10, 15)	0-2000	CC	Photosynthetic rate Total chl. and chl.a/b Carotenoids	↓ with UV-B doses (max. 43%) ↓ (max. up to 24%) No significant change	Surabhi et al. (2009)	USA

Plants	UV-B $_{BE}$ dose (kJ m^{-2} d^{-1})	PAR (µmol photons m^{-2} s^{-1})	Exp. condition	Growth parameters	UV-B induced response	References	Country
Phaseolus mungo L	A +UV-B (+.5 Wm^{-2})	–	FC	Total chl. and carotenoids	↓ (40 and 32.6%)	Jayakumar et al. (2004)	India
Vigna unguiculata L. *Crotalaria juncea* L.	C (0) UV-B (1.0, 1.4, 4.7, 6.0)	–	FC	Tot. Chl. Carotenoids	↓ (max. 49 and 20%) in *V. unguiculata* and *C. juncea* respectively No significant variation	Selvakumar, 2008	India
Triticum aestivum L.	A +UV-B (30% above A)	–	FC	Fv/Fm, chlorophyll, carotenoids	Significant ↓	Lizana et al. (2009)	Chile
Picea abies L.	A +UV-B (17%	–	FC	Fv/Fm	Significant ↑	Sedej and Gaberscik (2008)	Slovenia

Abbreviations and symbols used: C: control; A: ambient level; NA: near ambient; +UV-B (supplemental UV-B above ambient); −UV-B (UV-B excluded).

FC: field condition; CC: control chamber; GC: growth chamber; GH: Green house; – informations not available.

↓ represent decrease and ↑ increase in the parameter due to elevated levels of UV-B radiation, compared to their control values.

Pn: photosynthetic rate; Gs – stomatal conductance; E – transpiration rate; Ci –intracellular CO_2 concentration.

of responses have been uncovered in different studies (Table 1). Some plants are sensitive to the mere presence ambient UV-B fluences, whereas many plants appear quite tolerant to even high fluences of UV-B (Sullivan et al., 2003). A study simulating 15–25% ozone depletion or a 30% increase in UV-B radiation reported no significant changes in plant growth and photosynthetic performance in crops (Allen et al., 1999). A lack of harm by UV-B to photosynthesis under ambient field conditions was also reported in tundra vegetation plants (Newsham et al., 2009, Haapala et al., 2010) and Antarctic ice microalgal communities (McMinn et al., 2003). On the other hand, damage to photosynthesis in response to UV-B has been reported in crop plants such as maize (Corriea et al., 2005), pea (Singh et al., 2009), sunflower (Feng et al., 2003), in trees (Laposi et al., 2009, Krause et al., 2003), arctic herbal shrubs (Albert et al., 2010; Albert et al. 2011, Albert et al. 2012,), bryophyte (Ren et al., 2010) and macro- and microalgae (Bischof et al., 2003). Moreover, Hanelt et al. (2009) and Figueroa et al. (2009a, 2009b) show that in chlorophycean algae acclimated to high solar radiation (e.g. *Ulva* and *Zygnemopsis*) high light depleted of UV-B is more harmful than these of full spectral range.

The balance between energy absorption by PS II and its utilization by the terminal electron acceptor system (photostatic balance [Huner et al., 2002]) in a photosynthetic organelle is essential to maintain optimum photosynthetic functioning. Plants stressed by UV-B often display low photosynthetic rates due to the influence on the down regulation of photosynthetic gene expression, detrimentally affecting UV sensitive parts of the photosynthetic machinery (Caldwell et al., 2007). UV-B radiation can impair all major processes of photosynthesis, including the photophosphorylation reactions of the chloroplast thylakoid, the CO_2 fixation reaction of the Calvin cycle and the stomatal regulation of CO_2. Photosystem II is considered the most sensitive component of the photosynthetic apparatus targeted to UV-B. Accordingly, the impact on various photosynthetic components has been reported as an alteration in the redox potential of energy transfer between the two photosystems, and/or a decline in electron acceptance capacity. Furthermore, a decrease in Rubisco content and activity would also contribute to a loss of photosynthesis (Surabhi et al., 2009).

19.2.1. Genetic regulation of photosynthetic functions in response to UV-B

UV-B radiation elicits multilevel oxidative stress on the photosynthetic organelle. When a plant is exposed to stress, it induces free radical generation, mostly hydroxyl and peroxy ions in UV-B irradiated thylakoid membranes and activates UV-B signaling pathways in plants (Kovacs and Keresztes, 2000). These reactive oxygen species (ROS)

become involved in the regulation of UV-B signal transduction pathway(s) leading to the down-regulation of photosynthetic genes in response to UV-B radiation in green plants (Mackerness et al., 1999). In most experiments, the genes that encode the enzymes involved in photosynthesis showed a lower transcriptional response in plants exposed to UV-B. Changes in photosynthetic gene expression reported in response to UV-B radiation include a reduction in the expression and synthesis of key photosynthetic proteins, such as D1 polypeptide of PSII (*psb*A), chlorophyll *a*/*b* binding protein (*Lhcb* and/or *cab*) (Izaguirre et al., 2003; Mpoloka, 2010), the mRNA transcript for the smaller and larger subunits of Rubisco (*rbc*S and *rbc*L). Strid et al. (1994) researched the effects of UV-B irradiation on gene expression and mRNA transcript levels for chloroplast proteins in pea plants, and finding that amounts of mRNA transcripts declined for all the photosynthetic genes studied. Futhermore, it has been found that the decline in mRNA transcripts seems to be more rapid for nuclear coded genes than that of the chloroplast localized genome. For example, it was more rapid for nuclear coded *cab* genes than for the mRNA of plastid localized *psbA*. The decreases in mRNA transcripts for photosynthetic complexes and other chloroplast proteins are among the very early events of UV-B damage (Agrawal et al., 2009). Down-regulation of these chloroplast and nuclear coded photosynthetic genes would clearly affect protein synthesis and substantially lead to inhibition of photosynthetic functions.

19.2.2. UV-B induced change in photosynthetic structure: (Chloroplast)

Chloroplast provides the structural basis for photosynthesis and the anabolism of chlorophyll. It has also been well demonstrated that chloroplast is a major target site of UV-B (Bornman, 1989). In chloroplast, the integrity of thylakoid membranes and their organization seem to be more sensitive to distortion by UV-B stress stimuli. UV-B induced ultra structural damage to chloroplast includes the dilation of thylakoids, a progressive disruption and disintegration of the double membrane envelope surrounding the chloroplast, the destruction and disorganization of the stacking of granal and stromal thylakoids (Kovacs and Keresztes, 2002). He et al. (1994) observed that UV-B distorted the thylakoid lamellar system and caused the disruption of the outer membrane envelope in the chloroplast of pea plants. Ultra structural analysis of UV-B irradiated and control plant tissue was also researched by other investigators to determine the effect of UV-B irradiance on the structural integrity of chloroplast membranes (Hollosy, 2002; Santos et al., 2004). Brandle et al. (1997) reported the dilation of the thylakoid membranes of chloroplast in some cells in pea leaves. Excessive UV-B

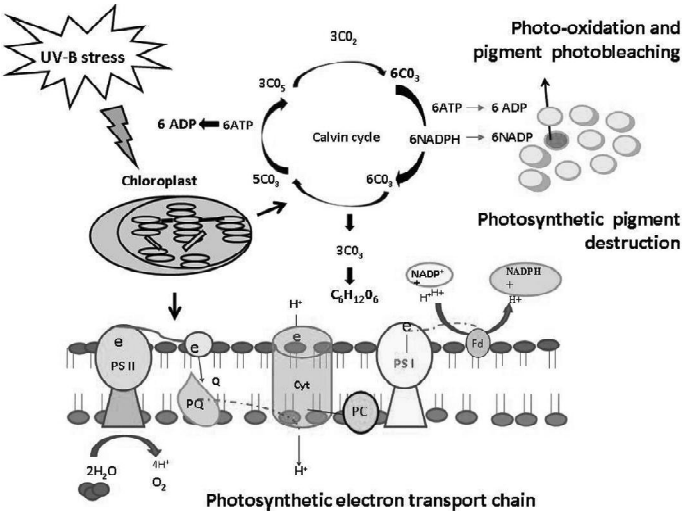

Fig. 1. Effect of UV-B on photosynthetic structure and function. UV-B induces structural damage of photosynthetic orgenelles i.e. disrurption of thylakoid membranes; granal stacks, pigment destruction, destruction of photosynthetic electron transport system at PSII site; QB binding site; O_2 evolution site, Inhibition of amount and activity of key enzymes of Calvin cycle.

exposure time led to vesiculation in the chloroplast stroma and endoplasmic reticulum (Hollosy, 2002). Santos et al. (2004) reported a response in potato plants differeing from reports for other species. They reported only minor ultra structural changes such as a reduction in guard cell size, changes in the epidermal layer and palisade cells under UV-B exposure, while it did not affect the structural integrity of chloroplasts. Zhu et al. (2002) studied the effects of UV-B irradiation on the chloroplast of several seedlings of woody plants, and reported that UV-B irradiation damaged the chloroplast structure in leaves, and that the damage varied with different species. In a recent publication, Sarghein et al. (2011) reported on UV-B changes to the chloroplast structure of pepper plants and showed that at the ultrastructural level, chloroplast thylakoids were dilated and that there was a reduction in starch. The relevant literature reveals that UV-B radiation inflicts damage on the photosynthetic apparatus of green plants at multiple sites such as the donor and accepter sites of PSII binding protein, the O_2 evolving site (OEC), the D1/D2 site of the pigment protein complex. The sites of action of UV-B in the photosynthetic system are diagrammatically represented in Fig. 1.

19.2.3. UV-B effect on photosynthetic pigment pool

The decrease in photosynthetic pigment content during UV-B irradiation is caused by the intensive production of reactive oxygen species (ROS), which can destroy many types of susceptible molecules in the cell (Hader and Worrest, 1991). Photobleaching of these chlorophyll pigments hinders the penetration of the photosynthetic optical cross-section – the amount of light available for photosynthesis. A decrease in chlorophyll content was the general response observed on exposure to enhanced UV-B radiation in most of the plant species reviewed (Table 1). UV radiation may induce photo-oxidation of chlorophylls resulting in modification of these pigments as oxygenated chlorophyllide forms. The decline in chlorophyll level might also be due to the inhibition of the *cab* gene, which codes for the chlorophyll a/b binding protein of the LHC and it may lead to the functional breakdown of the LHC from PSII (Jordan et al., 1994). Literature regarding the effects of UV-B treatment on the pigment pool produced conflicting conclusions. Some studies have shown a substantial lack of effect (Barsig and Malz, 2000), while others have emphasized a decrease in pigments, particularly chlorophyll and carotenoid contents, (Ambasht and Agrawal, 2003 a, b; Feng et al., 2003; Yao and Liu, 2007, DeLong, 1997; Malanga, 1997). Laposi et al. (2009) studied European beech and found that UV-B enhancement resulted in the activation of photoprotective pigments (significant increases in xanthophyll cycle pigments and in the total of carotenoids), but significantly reduced the concentration of chlorophylls. A similar effect of UV-B on carotenoid content was observed by Sangtarash et al. (2009) in *Stellaria longipes*. Carotenoids act as antioxidants, protecting chlorophyll from photo-oxidative destruction (Agrawal and Rathore, 2007). Under high UV-B stress an inhibition of the xanthophyll cycle (XC) was observed in *Ulva* (Bischof et al., 2003, Fredersdorf and Bischof, 2007). In the bacillariophycean alga *Thalassiosira* the XC was also UV-B inhibited during short UV-B+PAR illumination, whereas in the same conditions in *Dunaliella* (Chlorophyceae) UV-B addition has no effect (van de Poll, 2010). Additionally, in the same paper an increase in xantophyll cycle pigment synthesis was reported for *Dunaliella* under PAR+UV-B exposure in comparison to PAR only exposure.

19.2.4. UV-B effects on photosystem I and II

The light energy converting complex of photosystem II (PSII) is considered the most sensitive component of the photosynthetic apparatus to UV-B exposure. PSII is a multifunctional protein pigment complex of thylakoid membranes and contains over 20 protein subunits and a light induced electron transport component. It functions as a water plastoquinone oxidoreductase catalyzing the light driven transfer of electrons from water to plastoquinone. The core factors of PSII

electron transport are bound to or contained in D1 and D2 protein subunits, which form the redox reaction center of PSII (Barber et al., 1995). The mechanism of damage induced by UV-B light to the PSII reaction centre (RC) protein structure has been documented in higher plants by many investigators, both in vitro and in vivo (Renger et al., 1989; Frisco et al., 1995; Rajagopal et al., 2000). UV light may cause the degradation of PSII-RC proteins and the inhibition of semiquinone anion formation at Q, thus affecting the electron transport in PSII. Segui et al. (2000) in their study reported that even a short term irradiation of the PSII complex with UV280 light (40 min) of low intensity (2.0 W/m^2) gave rise to a complete loss of oxygen evolution and disrupted the structural stability of the functional centers in the PSII complex. A detailed analysis of different PSII properties revealed that both the donor and acceptor sites of photosystem II reaction centres were affected by UV-B. Crucial targets inside the PSII complex for UV-B damage are the QA, QB, and PQ quinone electron acceptor side and on the donor side, at the level of primary donor of electrons in the electron transport chain, the Tyr-Z and Tyr-D redox active tyrosine residues, as well as the Mn cluster of the water oxidation complex (Melis et al., 1992). Rodrigues et al. (2006) reported that plastosemiquinones are photosensitizers for UV-B radiation and that absorption of UV-B by these quinones initiates reactions leading to damage to photosystem II. A UV-B driven decrease in the activity and content of PSII ultimately corresponds to a net inhibition of photosynthetic CO_2 assimilation (Krause et al., 2003).

Conversely, PSI damage is seldom reported as a site of UV-B induced photosynthetic inhibition. Krause et al. (2003) found a decrease in PSI efficiency in shade acclimated seedlings of tropical trees under sudden exposure to high solar PAR+UV-B irradiation. They presume that this is the result of enhanced charge recombination in reaction centers.

19.2.4.1. Impacts on PSII reaction centre protein (D1/D2) subunits

The D1 and D2 proteins constituting photosystem (PS) II reaction center are extremely sensitive targets of UV-B radiation and can be used as an in situ sensor for UV penetration into photosynthetic tissue. Based on the action spectrum of protein degradation, plastosemiquinon is suggested to be the main UV activated compound responsible for D1/D2 protein cleavage (Spetea et al., 1996).

A UV-B driven degradation and a reduced rate of the turnover of D1 polypeptides of PSII has been reported in several studies even under very low fluences of UV-B as low as 1 μmol m^{-2} s^{-1} (Jansen et al., 1996b; Chaturvedi and Shyam, 2000), and can be accelerated under high intensity PAR 1000 μmol m^{-2} s^{-1} (Jansen et al., 1996 a). The D2 protein during photoinhibition of photosynthesis is relatively stable and its degradation *in vivo* is quite low compared to the D1 protein.

Barbato et al. (1995) investigated the *in vivo* and *in vitro* effects of ultraviolet-B radiation (280-320 nm) on the PSII activity in spinach leaves and reported that irradiation of isolated thylakoids with ultraviolet- B light brings about the breakdown of the D1 protein into detectable 20-kDa and 13-kDa fragments. Barbato et al. (2000) studied the effect of UV-B radiation on D1 protein turnover in PSII in barley leaves and reported more D1 protein degradation and the inhibition of protein D1 synthesis at a UV-B dose of 10 μmol m^{-2} s^{-1} with respect to visible light. They explained that there are two possible mechanisms behind this. One is damage to the PSII donor side through impairment of the Mg cluster bound at its catalytic site(s), which leads to cleavage of the D1 protein. The other mechanism is dephosphorylation of PSII proteins, which favors increased turnover of protein D1.

19.2.4.2. Water oxidation complex (WOC)

Water oxidation occurs at the Mn cluster located on the luminal side of PSII (Sproviero et al., 2008). According to the most accepted view the Mn cluster of the water oxidation of PSII is reported as a highly sensitive target site of UV-B damage (Szilard et al., 2007). Since the Mg cluster of the water oxidation complex (WOC) is the most fragile component of the electron transport chain, UV-B absorption may lead do conformational changes and the inactivation of the Mg cluster, by inducing the formation of reactive oxygen species (hydroxyl radicals) through interaction with peroxides and/or hydroxyl ligands, thereby actively disrupting the water oxidation process. Vass (2012) in a recent publication made clear that though the precise mechanism of the UV-B induced impairment of the Mn^4Ca cluster is not quite clear, this effect is most likely related to the absorption of UV quanta in the higher valency states of Mn (S2 and S3 oxidation states) in which the water oxidizing complex is most prone to UV damage.

19.3. Calvin cycle

The inhibition of photosynthesis in response to UV-B radiation could potentially be caused either by effects on PSII performance (source side) as discussed above, or by effects on the performance of the Calvin cycle, CO_2 diffusion changes, etc. (sink side) at the functional site of photophosphorylation or CO_2 carboxylation. A reduced electron transport rate between PSII and PSI would induce a decrease in ATP concentration in chloroplast, affecting the steps of the Calvin cycle in which ATP acts as a substrate. UV-B stress could potentially disturb CO_2 fixation, decreasing ribulose bisphosphate (RuBP) regeneration and decreasing the maximum rate of Rubisco carboxylation. Besides Rubisco, glyceraldehyde phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) are other important key regulatory enzymes

involved in the regeneration of ribulose bisphosphate and $NADP^+$. It has been reported that in chloroplast, several important enzymes of the Calvin cycle are extremely sensitive to a high ROS level (particularly H_2O_2). UV-B stress inhibits $NADP^+$ regeneration by the Calvin cycle, and consequently the photosynthetic electron transport chain becomes over-reduced (photoinhibition of photosynthesis). Under such conditions, highly oxidized radicals – superoxide radical ($\cdot O_2^-$) and singlet oxygen (O_2^-) – form in the photosystems (Xu et al., 2008). These can be formed by photo excitation of chlorophyll and its reaction with O_2. Izaguirre et al. (2003) also found that some genes for Calvin cycle enzymes were down-regulated by solar UV-B. In several studies it has been indicated that UV-B inhibition of photosynthesis is associated with the enzymatic reactions of the Calvin cycle rather than PSII limitations (Allen et al., 1998).

19.3.1. Rubisco and other Calvin cycle enzymes

Decreases in photosynthesis on the exposure of plants grown in the presence of UV-B to high UV-B doses have been attributed to the deactivation or loss of Rubisco activity (Allen et al., 1997). Levels of mRNA coding for both the larger (i.e. 55 kDa) and smaller subunits (14.5 kDa) of Rubisco have also been reported to decline during UV-B exposure, which would contribute to a large reduction in the expression and abundance of Rubisco (Jordan, 1996). The inhibition of photosynthetic CO_2 assimilation due to specific UV-B effects on the activity, synthesis and degradation of Rubisco were reported in a number of plant species as in *Oryza sativa* (Huang et al., 1993), *Populus deltoides* (Bassman et al., 2001), *Glycine max* (Feng et al., 2003). Allen et al. (1997) reported that the decrease in the rate of the CO_2 assimilation of an oilseed rape plant under enhanced UV-B radiation is a consequence of the loss of Rubisco content and its activity. Takeuchi et al. (2002) have also demonstrated the suppression of Rubisco synthesis during early age of leaf development in a UV sensitive (Norin 1) cultivar of *Oryza sativa*.

Calvin cycle enzymes also are involved in photosynthetic decreases under UV-B. Allen et al. (1998) reported a large reduction in the content of sedoheptulose-1,7- bisphosphatase (SBPase), a key regulatory enzyme in the Calvin cycle during UV-B exposure.

Wilson et al. (1995) in *Brassica napus, Lycopersicon esculentum* and *Nicotiana tabacum* plants under UV-B stress observed a novel 66 kDa protein, which was identified as a product of the photomodification of the large subunit of Rubisco. The similarity of the structure of this protein among different species and further investigation suggest that it is a product of some specific UV-B induced photoreaction in the Rubisco holoenzyme complex.

19.4. Stomatal regulation

Changes in stomatal function are other important regulatory processes in the UV-B induced inhibition of leaf photosynthesis. Enhanced UV-B irradiance has negative effects on photosynthetic efficiency due to a decrease in stomatal conductance and/or the closing of the stomatal aparture of plant leaves which permits less CO_2 to enter into plants (Zheng et al., 1996). Due to a decrease in stomatal density and functioning, a reduction in transpiration, water use efficiency and carboxylation efficiency of plant leaves was observed in soybean under elevated UV-B radiation (Gitz et al., 2005). On the other hand, in some species, such as *Vicia faba* a high fluence rate of UV-B reported to stimulates both stomatal opening and stomatal closure, which perhaps depends upon the metabolic stage of guard cells (Jansen and Noort, 2000).

Although the response of stomata to UV-B radiation is well known, the underlying mechanism behind the UV-B effect on stomata is not clear. On exposure to elevated UV-B radiation, the stomata of plants exhibit complex responses. One possible mechanism is an inhibition of ATP synthesis by electron transport in guard cell thylakoids. A second mechanism may involve the direct inhibition by UV-B of the plasmalemma ATPase proton pump.

19.5. ATPase activity

The ATPase complex is involved in the hydrolysis of ATP to ADP and orthophosphate, as well as functioning as an exchanger or transporter for Na^+, K^+ or Ca^{2+} (Jordan, 1996). The action spectrum for the inactivation of plasma membrane bound ATPase peaks at 290 nm, showing their sensitivity towards increased UV-B radiation (Caldwell, 1993). The inactivation of ATPase probably results from the singlet oxygen mediated destruction of tryptophan residues in the ATPase protein (Stapleton, 1992). Long and Jenkins (1998) demonstrated the role of UV-B and UV-A/blue light in regulating cellular calcium via a stimulated calcium efflux from the cytosol, partly via the action of specific Ca^{2+}-ATPase.

19.6. Monitoring tool for UV-B Induced PSII damage: Chl *a* fluorescence parameters

Analysis of the fluorescence properties of chlorophyll *a* (Chl *a*) in photosystem II (PSII) is a rapid and versatile tool for monitoring the functioning of the photosynthetic apparatus as well as the vitality of photosynthetically active organisms (Lichtenthaler and Rinderle, 1988;

Schreiber, 2004; Strasser et al., 2004; Lichtenthaler et al., 2005). One reason for photosynthetic efficiency loss is a decrease in the primary photochemistry quantum yield of PSII reaction centers – a value that is directly measurable by chlorophyll fluorescence analysis as F_V/F_M (Maxwell and Johnson, 2000; Strasser et al., 2004; Lichtenthaler et al., 2005). A decrease in F_V/F_M is associated with an increase in excitation energy dissipation in PSII reaction centers and is generally considered indicative of an inhibition of electron transport (Nogues and Baker, 2000) and possibly the inhibition of photosynthesis (Bjorkman and Demmig, 1987). In particular the F_V/F_M ratio is often used as an indicator of UV-B stress (Tevini, 2004), being in many cases the only parameter that reflects photosynthetic electron transport decay in the field or laboratory investigation of algae and higher plants (eg. Herrmann et al., 1996; DeLong and Steffen, 1997; Ghetti et al., 1999; Hanelt et al., 1997, 2009; Haapala et al., 2010; Ren et al., 2010 and others). A very interesting paper by Newsham and Robinson (2009), which bears an analysis of data from 34 field studies of the UV-B effect on plants of Polar regions, quotes F_V/F_M as a widespread parameter of photosynthetic electron transport used in field investigation – but they show its low sensitivity in comparison to other physiological parameters measured in the field. Moreover, Andreasson and Wangberg (2006) show that F_V/F_M underestimates the inhibition of photosynthesis by UV-B in comparison to ^{14}C-fixation measurements.

On the other hand, for deeper analysis of data, both main techniques of chlorophyll fluorescence measurement – Continuous Light-induced chlorophyll fluorescence analysis (CL) and the Pulse Amplitude Modulation (PAM) method were used for particular investigation of UV-B effects in photosynthetic apparatus. The CL technique with OJIP-analysis were used as a very subtle tool for the description of energy transfer functioning inside PSII (e.g. Gilbert et al., 2009; Albert et al., 2011, 2012; Pan et al., 2011) whereas broadened PAM measurements can investigate changes in the electron flow system of photosynthetic membranes (e.g. McMinn et al., 2003; Roncarati et al., 2008; Figueroa et al., 2009a, b,) as well as in adaptation of the system to light conditions (e.g. Altamirano et al., 2000, Surabhi et al., 2009; van de Poll et al, 2010).

19.7. Conclusion

Ultraviolet B radiation alters the structural and functional organization of the photosynthetic system at multiple target sites. At the structural level of organella, thylakoid morphology is modified upon exposure to ultraviolet B radiation. Thylakoids appear more vesiculated, with a disorganized and disrupted granum structure. The destruction

of thylakoid membranes causes damage to chlorophyll protein complexes and accelerates the photo-destruction of chlorophyll under UV-B stress. Ultraviolet radiation specifically alters PS II reaction centre photochemistry. The ultraviolet-B degradation of proteins D1 and D2 leads to the disassembling of PS II centers. It impairs primarily the Mn^4Ca catalytic site of the water oxidizing complex of PS II. In conclusion, inhibited photosynthesis under UV-B stress is likely the result of the inactivation of photosystems, the closure of stomata, the transcriptional down-regulation of photosynthetic related genes and the effects on the expression and/or activity of the Calvin cycle enzymes. Further research will be needed in order to improve our understanding of the role of UV light in signal transduction events and the underlying molecular mechanism in the cells of photosynthetic organisms to assess its possibility it connection with adaptation and resistance response under UV-B exposure.

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Chapter 20. FUNCTIONAL TUNING OF PHOTOSYNTHETIC PIGMENTS IN RESPONSE TO TRACE ELEMENTS

Abhay Kumar[1], Abin Sebastian[1], Majeti Narsimha Vara Prasad[1], Przemyslaw Malec[2], and Kazimierz Strzalka[2]
[1] University of Hyderabad, Hyderabad, India
[2] Jagiellonian University, Krakow, Poland

Contents

20.1. Introduction

Plant pigments are organic compounds that absorb certain wavelengths of light. The diversification of plant groups, in terms of their pigment composition has a central place in the classification of plant taxa. In higher plants, pigments are crucial for various processes, including for instance light absorption during photosynthesis, the protection of plant cells against photo-oxidative stress and the process of attracting pollination agents in the course of pollination. The mechanism of photosynthesis aided by pigments has been evolutionarily successful and is one of the most studied among these processes. Photosynthetic pigments such as chlorophylls, carotenoids and phycobilins are widely distributed in plants and other photoautotrophic organisms. These pigments play a crucial role through their physicochemical properties which ensure the functioning of photosynthetic electron transfer and give coloration to cells and plant organs.

Chloroplast development as well as the biosynthesis of photosynthetic pigments are processes sensitive to trace elements (TEs). Trace elements, such as cadmium (Cd), lead (Pb), nickel (Ni), mercury (Hg), chromium (Cr) and copper (Cu) as well as metalloid arsenic (As) contaminate the environment and potentially lead to threats to biota due to their toxicity. Biochemical and biophysical analytic studies have revealed that the inhibition of pigment biosynthesis and, in consequence, the depletion of pigment accumulation in plant tissues and organs are primary effects of TEs in plants. Trace element accumulation depends on the developmental stage of the plant. Hence, the pigment metabolism is highly dynamic over the growth period of plant under TE stress. Particularly, TEs accumulated at early stages of the life cycle (i.e. during seed formation) cause a decrease in the content of photosynthetic pigments in emerging plants (Cherif et al., 2011; Kumar et al., 2012), which results in the reduction of the net photosynthesis level (Ekmeci et al., 2008). In this chapter, the influence of TEs on the biosynthesis and function of photosynthetic pigments are described. TE interactions with plant photosynthetic pigments and their effects on chlorophyll fluorescence are especially emphasized.

20.2. Photosynthetic pigment biosynthesis

The major photosynthetic pigments in autotrophic organisms, including plants, are chlorophylls (Chl), carotenoids and phycobilins. Toxic concentrations of TEs inhibit the synthesis of these pigments (Fig. 1). Among photosynthetic pigments, Chls are compounds whose principal function is to absorb light energy and use it in photosynthesis. The Chl content varies dependently on the species of plant, light conditions, and the availability of minerals such as magnesium (Mg) (Rissler et al., 2002). The diversity of Chl molecules is generated through functional group substitutions and double bonds in the tetrapyrrole rings of a Chl molecule. For example, the structure of Chl *b* differs from that of Chl *a* by the substitution of a formyl group for a methyl group in ring-2 through an oxygenase enzyme. Chl *c*1, *c*2, and *c*3 are accessory light-harvesting pigments in many algae. These pigments differ from Chl *a* and *b* in that the double bond in ring-4 is not reduced, indicating that they are most likely derived from protochlorophyllide, without its prior reduction (Suzuki et al., 1997). Chl *d* differs from Chl *a* by substitution of a 2-formyl group for 2-vinyl and Chl *f* is (2-formyl)-Chl *a*. The loss of Mg ion from Chl often starts the formation of various Chl breakdown products: pheophytins and their derivatives (Oberhuber et al., 2003).

The chlorophyll biosynthesis pathway is completed in four main phases i.e. synthesis of porphobilinogen (PBG), protoporphyrin IX,

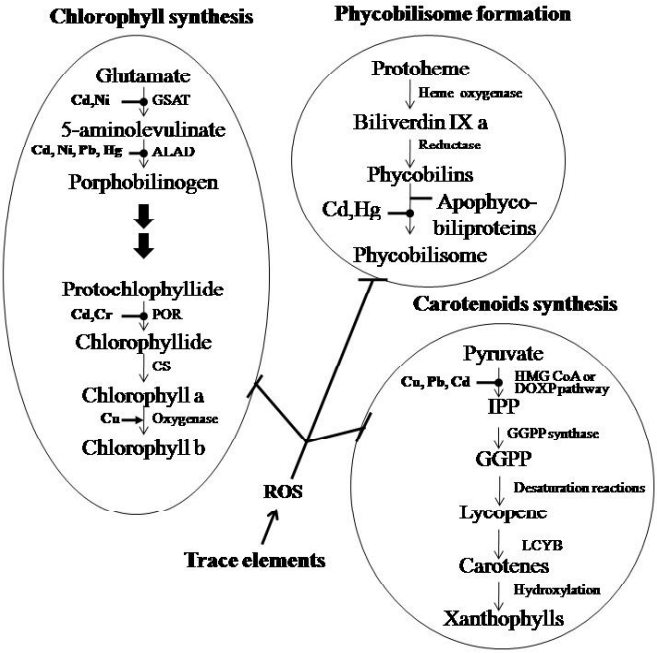

Fig. 1. Site of action of toxic trace elements (TEs) on photosynthetic pigment biosynthesis. Reactive oxygen species (ROS), resulting from the malfunctioning of the electron transport chain (ETC) by TEs, have an inhibitory effect on the enzyme catalytic steps of pigment synthesis. Copper has a stimulatory effect on the conversion of Chl *a* to Chl *b* while the majority of other elements have inhibitory effects on enzyme catalysis. The effect of Cd and Hg on the phycobilisome complex in cyanobacteria is reported during the incorporation of apoproteins to the pigment part (Dudkowiak et al., 2011).

chlorophyllide *a* and Chl *a*, respectively (Fig. 1). The first phase, catalyzed by glutamate 1-semialdehyde aminotransferase (GSAT), includes the conversion of glutamic acid into δ-aminolevulinic acid (ALA) – the first intermediate and common precursor for all porphyrins. In the next phase, two ALA molecules are asymmetrically condensed to porphobilinogen. This reaction is catalyzed by δ-aminolevulinic acid dehydratase (ALAD; E.C. 4.2.1.24), a sulphydryl-containing enzyme. A PBG molecule is converted into uroporphyrinogen III and then coproporphyrinogen III, through linear subsequent reactions which are catalyzed by the enzymes porphobilinogenase and uroporphyrinogen III decarboxylase, respectively. The next anabolic step of Chl biosynthesis is a conversion of coproporphyrinogen III into protoporphyrinogen III and next into protoporphyrin IX, by oxidizing enzymes

coproporphyrinogen III oxidase and protoporphyrinogen III oxidase, respectively. Protoporphyrin IX is the first coloured intermediate due to the presence of conjugated double bonds. In the successive pathway, Mg is incorporated into the protoporphyrin IX ring and forms Mg-protoporphyrin IX through the Mg chelatase enzyme, which is followed by the formation of monovinyl-protochlorophyllide *a*. The subsequent reduction of monovinyl-protochlorophyllide *a* in the presence of the reducing agent nicotinamide adenine dinucleotide phosphate (NADPH) and the enzyme protochlorophyllide oxidoreductase (POR) leads to the formation of chlorophyllide *a*, which after esterification is converted into Chl. The latter process is catalyzed by the enzyme Chl synthetase (CS). In angiosperms, the reduction of protochlorophyllide into chlorophyllide is a light-triggered process and constitutes an important step during developmental conversion to photoautotrophy (see: Masuda and Takamiya, 2004 for a review).

Carotenoids are a group of plant pigments comprised of more than 600 compounds, which play key roles in the photosynthetic apparatus as both accessory light-harvesting pigments and photoprotecants in pigment-protein complexes. Additionally, the accumulation of carotenoids in various plant organs is often responsible for their coloration. Also, carotenoids are precursors of plant hormone abscisic acid. The well known carotenoids which play a crucial role in photosynthesis are carotenes and their oxygenated derivatives, xanthophylls. Isopentenyl diphosphate (IPP) formed through the plastid-specific DOXP (1-deoxyxylulose-5-phosphate) or HMG CoA (3-hydroxy-3-methyl-glutaryl-CoA) pathway is the five-carbon building block for carotenoids (Tanaka et al., 2008). The isoprene IPP constitutes the initial compound and the four IPP molecules are converted into the geranyl geranyl pyrophosphate (GGP) by a condensation reaction (Fig. 1). Then two molecules of GGP are condensed into the first carotenoid compound phytoene, with the enzyme phytoene synthase. The addition of conjugated double bonds to phytoene by two structurally similar enzymes, phytoene desaturase and carotene desaturase leads to the formation of intermediate lycopene which is characterized by having 11 conjugated double bonds. The *cis* configured intermediate products such as 7′, 9′-di-cis-lycopene formed during the pathway are converted to *trans* form such as all *trans*-lycopene by enzyme carotenoid isomerase. In plants, the formation of lycopene constitutes a junction where the carotenoid synthetic pathway diverges to a two step enzyme-mediated reaction (lycopene β-cyclase; LCYB and lycopene ϵ-cyclase) leading to the formation of α-carotene or a LCYB alone-mediated reaction leading to formation of β-carotene. Hydroxylation of the β and ϵ rings of α-carotene is catalyzed by β-hydroxylase (CHYB) and ϵ-hydroxylase (CHYE), which leads to the formation of such compounds as lutein. In contrast, β-carotene oxidation

leads to the formation of zeaxanthin, catalyzed by β-hydroxylase. The enzyme zeaxanthin epoxidase catalyzes the formation of violaxanthin in an epoxidation reaction at C5, 6 and C5′, 6′ of the β-ring of zeaxanthin. Violaxanthin is further converted in to neoxanthin by enzyme neoxanthin synthase (NSY). Violaxanthin as well as neoxanthin on cleavage leads to xanthoxin, which is a precursor of ABA.

Carotenoids in cyanobacteria function both in light harvesting and in protection from photooxidative stress. These organisms are characterized by an abundance of glycosylated carotenoids. In addition to the prevailing β-carotene and zeaxanthin, cyanobacteria contain unique keto-carotenoids such as echinenone and canthaxanthin. They do not synthesize ε-rings and therefore contain β-, but not α- or δ-, carotenes and their oxygenated forms (Takaichi and Mochimaru, 2007). Genes encoding several carotenogenic enzymes cloned from cyanobacteria show that the primary structures of the polypeptides are conserved with homologous enzymes as in algae and plants but are distinct from those of other microorganisms. Each of these enzymes is single gene coded and functional in an autonomous manner in heterologous cells.

In all oxygenic photoautotrophic organisms, including plants, algae and cyanobacteria, carotenoids are pigments essential to the photosynthetic apparatus. They are involved in photoprotection, mostly by quenching excited Chl *a* triplet states which can generate toxic singlet oxygen (Cogdell et al., 2000). The role of carotenoids in the maintenance of the integrity of the photosynthetic apparatus is also established (Szabo et al., 2005). The dynamic changes in carotenoid composition can be sensitive indicators of various stress conditions in plants (Netto et al., 2005; Sytar et al., 2012).

Phycobilins (Phy) are a class of water-soluble pigments of the photosynthetic apparatus in cyanobacteria and red algae. In cyanobacteria, they are covalently bound to the polypeptide chains forming phycobiliproteins and are organized into supramolecular complexes called phycobilisomes. The biosynthesis of phycobilins starts from the conversion of protoheme to biliverdin IXa. This process is catalyzed by heme oxygenase, followed by a reduction of biliverdin IXa to free phycobilins which subsequently form covalent bonds with apophycobiliproteins (Brown et al., 1999).

20.3. Trace elements and photosynthetic pigments

TE interaction and site of action on the biosynthetic pathways of photosynthetic pigments are shown briefly in Fig. 1. Trace elements act on pigment synthesis predominantly by affecting the activities of the various enzymes involved in their biosynthetic pathways. In particular, elevated TE concentrations affect the photosynthetic electron

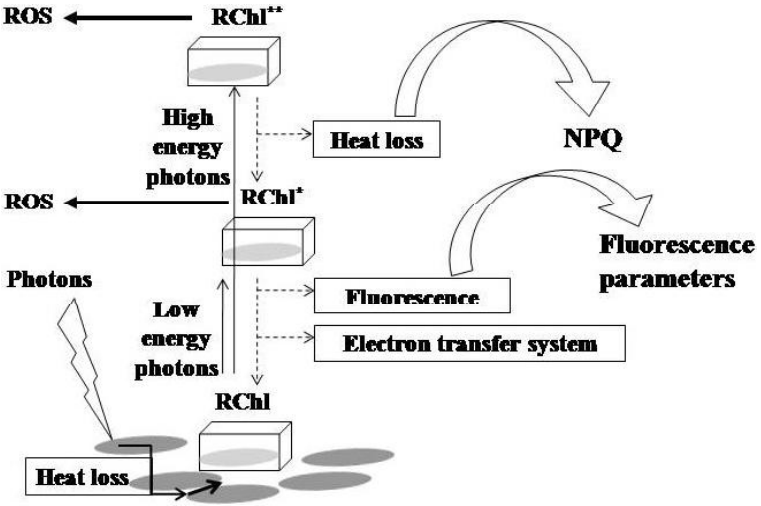

Fig. 2. The ways of energy dissipation of absorbed photons in photosynthesis and its significance in the generation of ROS with TE toxicity. As absorbed light energy is transferred via resonance transfer from the antenna complex to reaction centre chlorophyll (RChl), the energy is lost in the form of heat. The excited RChl may be in the high energy state (RChl**) or in the lower energy state (RChl*), depending on the energy flux from antenna complexes. In the first case energy is lost as heat and RChl** reaches the RChl* state, which subsequently achieves ground state (RChl) either by fluorescence or by the involvement of photochemistry. The action of TEs often leads to disturbances in photochemistry which will shift energy dissipitation as fluorescence. Otherwise if there is no decrease in photosynthetic pigment content, excited Chls react with oxygen and form reactive oxygen species (ROS). The fluorescence parameters such as the quantum yield of the photosystem, electron transport rate (ETR), photochemical and non-photochemical quenching, are used to analyze the photosynthetic activity of plants.

transfer chain promoting the formation of reactive oxygen species (ROS), which accelerate the inhibition of Chl synthesis enzyme machineries (Qian et al., 2009) (Fig. 2). In several studies with terrestrial plants the site of action of TEs on the activity of various key enzymes of photosynthetic pigment biosynthetic pathways are well documented (Table 1). Research dealing with the effects of TEs on Chl *a*, *b*, total Chl and carotenoid content in terrestrial plants with relevant examples is summarized in Table 2.

20.3.1. Mercury (Hg)

Hg is a heavy metal lethal to plants even at low concentrations. A substantial decrease in both Chl *a* and Chl *b* contents during Hg treatments has been observed (Backor and Dzubaj, 2004). The site of

Table 1

TEs effects on the activity of various enzymes of the chlorophyll biosynthetic pathway

Plant species	Tes	Concentration	Enzymes	Reference
Zea mays	Cd	0.1-0.5 mM	ALA, ALAD	Sarangthem et al., 2011
Brassica rapa	Pb	0.5-5 mM	ALAS, ALAD	Cenkci et al., 2010
Zea mays	Pb	25-200 μM	ALAD	Gupta et al., 2009
Cucumis sativus	Cd	1 mM	ALAD	Gonçalves et al., 2009
Glycine max	Cd	200 μM	ALAD	Noriega et al., 2007
Cucumis sativus	Al	1-2000 $\mu molL^{-1}$	ALAD	Pereira et al., 2006
Zea mays	As	0.01-1.0 $mmolL^{-1}$	ALAD	Jain and Gadre, 2004
Triticum aestivum	Hg	0.1-1 mM	NADPH-POR	Lenti et al., 2002
Triticum aestivum	Cd, Fe, Cr	10-0.1 μM	NADPH-POR	Berska et al., 2001
Nymphaea alba	Cr	1-200 μM	ALAD	Vajpayee et al., 2000
Hordeum vulgare	Fe, Co, Ni, Mn	0.01 M	ALAS, ALAD, PPBG	Shalygo et al., 1999

action of Hg covers both photosystems (PS) I and II, including the oxygen evolving complex. Elevated Hg concentrations were found to induce both the formation of Chl triplet states and a leakage of electrons from the electron transport chain (ETC) leading eventually to the generation of reactive oxygen species (ROS) (Palatnik et al., 1999), which may cause the degradation of Chl and other pigments during Hg stress. Also, Hg shows an affinity for functional groups like -SH, C=O and C–N, which possibly results in functional alterations in the proteins involved in the photosynthetic electron transport chain (Nahar and Tajamir-Riahi, 1996). The replacement of Mg in Chl by Hg caused a loss of function in light harvesting complexes (LHC) and led to the malfunctioning of photosynthesis (Kupper, 1996).

20.3.2. Cadmium (Cd)

Cd is well known to cause both chlorosis and damage to photosynthetic reactions. The loss of photosynthetic pigments in Cd-treated plants is due to the inhibitory action of Cd on the enzymes involved in Chl biosynthesis (Noriega et al., 2007; Sarangthem et al., 2011; Sytar et al., 2012) (Fig. 1). The damage of photosynthetic electron transfer through the action of Cd on the D1 subunit of PSII (Giardi et al., 1997) promotes the formation of excited states of Chl which ultimately bleach pigments by generating ROS (Somashekaraiah et al., 1992; Sarvari, 2005; Qian et al., 2009). The affinity of Cd to -SH groups of enzymes involved in the synthesis of chlorophyll was reported to be responsible for the inhibition of their activities (Griffiths, 1975; Gonsalves et al., 2009). In particular, Cd has been demonstrated to inhibit ALAD, porphobilinogen deaminase and protochlorophyllide oxidoreduc-

Table 2

TE effects on chlorophyll and carotenoid contents in selected plant biota

Plant species	TEs	Concentrations	Function				Reference
			Chl *a*	Chl *b*	Total Chl	Carotenoids	
Talinum triangulare	Pb	0.25-1.25 mM	↑↓	↑↓	↑↓	↑↓	Kumar et al., 2012
Jatropha curcas	Pb	0.5-4 mM	↓	↓	↓	↓	Shu et al., 2012
Solanum lycopersicum	Cd	10 μmol L^{-1}	↓	↓		↓	Cherif et al., 2011
Allium sativum	Cd, Co, Cu, Ni	0.05-0.25 mM	↓	↓		↓and ↑(Co)	Soudek et al., 2011
Myriophyllum alterniflorum	Cd	10 μg L^{-1}	↓	↓		↓	Delmail et al., 2011
Zea mays	Cd	0.1-0.5 mM				↓	Sarangthem et al., 2011
Kandelia candel	Cd, Pb, Hg	0.1-15 mg L^{-1}		↓		↓	Huang and Wang, 2010
Brassica rapa	Pb	0.5-5 μM	↓	↓		↓	Cenkci et al., 2010
Cucumis sativus	Al	1- 2000 μM			↓		Pereira et al., 2010
Zea mays	Ni	100-200 μM	↓	↓			Drążkiewicz and Baszyński, 2010
Brassica juncea	Cd	10-160 μM	↓	↓		↓	Seth et al., 2008
Triticum aestivum	Ni	50-100 μM			↓		Gajewska and Skłodowska, 2007
Brassica napus	Cd	10-100 μM			↓		Nouairi et al., 2006
Helianthus annuus	Cu	80 μM			↓	↓	Ouzounidou and Ilias, 2005
Brassica juncea	Cr	0.2- 20 μM			↑	↑	Pandey et al., 2005
Sedum alfredii	Cd	0.2-1.0 μM	↑	↑		↑	Zhou and Qiu, 2005
Cucumis sativus	Cu, Cd, Pb	20-50 μM				↓	Burzynski and Kolbus, 2004

tase (Stobart et al., 1985; Padmaja et al., 1990; Noriega et al., 2007; Sarangthem et al., 2011). In terrestrial plants, a Cd-induced decrease in both Chl and carotenoid contents has been reported in *Solanum lycopersicum* (Cherif et al., 2011), *Allium sativum* (Soudek et al., 2011), *Myriophyllum alterniflorum* (Delmail et al., 2011), *Brassica juncea* (Seth et al., 2008). The activity of the ALAD enzyme and the accumulation of both ALA and PBG were analyzed after the Cd treatment of soybean leaves. In this plant, Cd at a concentration of 200 μM produced an approximate 100% inhibition of ALAD activity as compared with control. Inhibition of ALAD activity increased the ALA accumulation by 104% and drastically diminished the PBG concentration in a Cd-treated soybean leaf (Noriega et al., 2007). The loss of Chl after long term exposure to Cd has also been found to accelerate the degradation of carotenoids (Cherif et al., 2011; Sarangthem et al., 2011). The toxic effect of Cd depends on multiple factors such as concentration, exposure time, age of the plant and the tolerance mechanisms active in a particular organism. That is why the results obtained in different experimental systems are not always directly comparable. For example, a stimulatory effect of Cd, in concentrations of 0.2–1.0 μM, on chlorophyll and carotenoid accumulation has been observed in *Sedum alfredii* (Zhou and Qiu, 2005). Simultaneously, the substitution of Mg^{2+} in Chl molecules by Cd has not been detected in this plant. As Cd-treatment has been accompanied with an enhanced accumulation of Fe, important co-factor and regulatory agent many enzymes, including those involved in the biosynthetic pathway of chlorophylls (Marschner, 1995), this effect could be a consequence of a Fe-induced increase in Chl content (Zhou and Qiu, 2005). In cyanobacteria, Cd-treatment has been shown to severely affect the accumulation of phycocyanin. However, the detailed mechanisms of this process remain unknown (Atri and Rai, 2003; Surosz and Palinska, 2004).

20.3.3. Lead (Pb)

Lead is a toxic heavy metal known to suppress plant growth through its cumulative action on various metabolic pathways (Gupta et al., 2009; Cenkci et al., 2010; Sytar et al., 2012). In general, Pb is known to cause a decrease in the accumulation of plant Chl pigments (Table 2). Pb caused a decrease in total Chl and carotenoid concentrations in maize (Gupta et al., 2009) and *Brassica rapa* (Cenkci et al., 2010) leaves along with a decrease in ALAD enzyme activity. These effects suggest the toxic nature of Pb to photosynthetic pigments and their biosynthetic processes (Table 1). Studies on mash bean showed that Chl *a*, *b* and total Chl content drastically decreased under Pb treatment, while the Chl *a*/*b* ratio remained unaffected. These results suggest the equal sensitivity of both Chl *a* and *b* to Pb stress (Hussain, 2006).

Similarly, a decrease, induced by externally applied Pb, in Chl and carotenoid contents have been observed in *Talinum triangulare* (Kumar et al., 2012), *B. rapa* (Cenkci et al., 2010), *Jatropha curcas* (Shu et al., 2011), *Kandelia candel* (Huang and Wang, 2010) and *Cucumis sativus* (Burzynski and Kolbus, 2004). The diminished chlorophyll accumulation may be attributed to reduced Chl synthesis because Pb interferes with heme biosynthesis and Chl formation by interacting with functional –SH groups of enzymes such as ALAD (Gupta et al., 2009; Cenkci et al., 2010). Also, Pb was reported to inhibit both PSII and PSI activity, which leads to the subsequent formation of ROS and oxidative damage to the photosynthetic apparatus (Kumar et al., 2012; Kalaji and Loboda, 2007; Joshi and Mohanty, 2004). It has been reported that in *Talinum triangulare* leaves, Chl and carotenoid contents increased at 0.25–0.50 mM Pb, thus thereafter decreased at 0.75–1.25 mM Pb over control. This reduced pigment accumulation was accompanied by the significant increase in ROS, enhanced cell death and the decrease in relative water content (Kumar et al., 2012).

20.3.4. Nickel (Ni)

Ni is important to the plant metabolism mainly with regard to urease enzyme activity (Srivastava and Kayastha, 2000), but excess Ni induces a degradation of Chl as well as the inhibition of the biosynthesis of Chl (Kupper, 1996). In Ni-treated plants and microorganisms, the reduction in Chl content may be due to impaired ALA utilization (Singh and Pandey, 2001). In cyanobacteria, Ni at a moderate concentration (10 μM), was reported to increase phycocyanin (Phyc) content, whereas at higher concentrations (>10 μM) it led to a decrease in Phyc accumulation (Pandey and Gautam, 2009). The nickel ion is also reported to decrease the permeability of cell membranes, which alters nutrient uptake resulting in chlorosis, being a consequence of a decrease in total Chl content (Pandey and Gautam, 2009). Hence, the accumulation of Chl *a* can be a sensitive marker for Ni stress in plants. In particular, the crop plant *Triticum aestivum* when exposed to elevated levels of Ni (up to 100 μM) showed significant changes in the concentrations of Chl and carotenoids. This effect correlated with a significant decrease in the net photosynthesis rate and an increase in tocopherol level (Gajewska and Sklodowska, 2007).

20.3.5. Chromium (Cr)

Chromium is a toxic trace element that causes damage to plants through the production of ROS (Pandey et al., 2005). The impaired ALAD activity leading to reduced accumulation of Chl is one of the reasons for the decreased Chl contents observed during Cr stress (Table 1) (Vajpayee et al., 2000). At Cr concentrations of 10, 50 and 100 μM,

(Somashekaraiah et al., 1992) and 0.1–10.0 μg ml^{-1} (Vajpayee et al., 2002) a reduction in carotenoid contents occurred, which represented metal-induced ROS production. Interestingly, Cr in concentrations up to 20 mg l^{-1} promoted the growth of the halophilic cyanobactria *Nostoc linckia* HH-203, increasing the accumulation of Chl, carotenoids and accessory pigments (phycocyanin, allophycocyanin), whereas in the related *Nostoc spongiaeforme*, chromium treatment caused a decline in both phycobilins and carotenoids (Kiran et al., 2008), indicating the existence of the complex regulation of the biosynthetic pathways of key photosynthetic pigments.

20.3.6. Copper (Cu)

Cu is a plant micronutrient essential for the biosynthesis of Chl, carotenoids, quinones, plastocyanin and superoxide dismutase (Yruela, 2005). However, an excess of Cu has a deleterious effect on various biosynthetic pathways (Fig. 1). The presence of Cu in excess led to a reduction in total Chl content and in the net photosynthetic rate along with stomatal conductance and the internal CO_2 concentration of cucumber leaves (Burzynski and Kolbus, 2004). The inhibition of the synthesis of ALA as well as protochlorophyllide oxidoreductase activity by Cu can also be attributed to a decrease in Chl accumulation (Table 2) (van Assche and Clijsters, 1990). Also, nutrient deficiency caused by excessive Cu uptake blocks the synthesis of protochlorophyllide *a* and phytoene, thus decreasing the contents of Chl and carotenoids respectively (Lidon and Henriques, 1992). The inhibitory action of Cu in the carotenoid biosynthesis pathway possibly occurs during terpenoid synthesis prior to the formation of C 20 geranyl-geranyl pyrophosphate, since the plastoquinone content has also been found to be reduced during Cu stress (Goodwin, 1965; Henriques, 1989; Droppa and Horvath, 1990). This Cu-induced decrease in carotenoid concentration leads to enhanced ROS formation in the photosynthetic apparatus (Mishra and Biswal, 1981). The replacement of Mg in Chl by Cu may also lead to a decrease in photosynthetic activity due to an impaired energy transfer to photosynthetic RCs (Malec et al., 2008).

20.3.7. Arsenic (As)

Arsenic is a metalloid growing in importance as a technogenic contaminant. Since As is not a redox metalloid, the free radical formation by this element is limited in comparison to that of heavy metals (Szivak et al., 2009). However, an increase in the activity of some antioxidant enzymes (superoxide dismutase, catalase, peroxidases) during As treatment suggests that this toxicant induces oxidative stress in plants (Shri et al., 2009). It has been found that As-induced disruption of the membrane system in chloroplasts leads to ROS-induced alterations in

the photosynthetic apparatus (Miteva and Merakchiyska, 2002). It has also been reported that As enhanced Chl degradation in oat plants indicating free radical production in response to As (Stoeva and Bineva, 2003, Stoeva et al., 2003).

20.4. Trace element toxicity and light reactions of photosynthesis

Trace element enhanced photoinhibition is a result of damaged photosynthetic machinery (Mysliwa-Kurdziel and Strzalka, 2002; Malec et al., 2008). Trace element-mediated malfunctioning of ETC increased ROS production resulting in increased lipid peroxidation of the cell and chloroplast membranes, thereby damaging the light phase of photosynthesis (Fig. 2) (Mysliwa-Kurdziel and Strzalka, 2002; Ekmekci, 2008; Huang and Wang, 2010; Soudek et al., 2011). Once energy is harvested by the LHC, the Chl- reaction centres (RCs) of photosystems are excited to a higher energy level and transfer the e^- to components in the ETC (Fig. 3). Photosystem II and I are protein-pigment super-

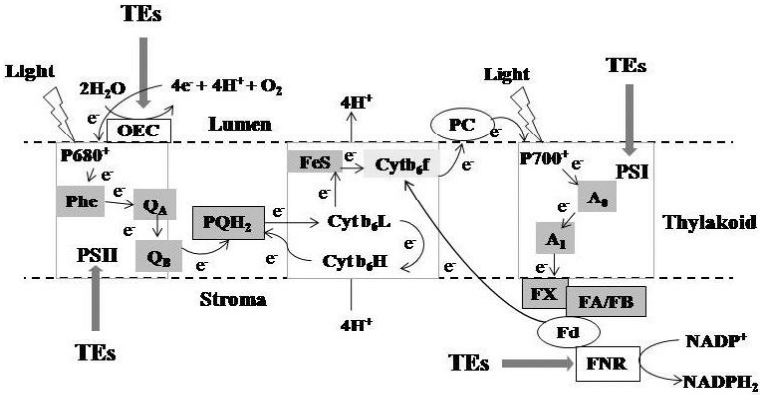

Fig. 3. A simplified scheme of the plant photosynthetic electron transport chain (ETC) and key elements of the ETC sensitive to an excess of TEs. Upon excitation of chlorophylls in PSII and PSI reaction centers ($P680^+$ and $P700^+$), electrons (e^-) released in the water splitting system (OEC) are transferred eventually to ferredoxin-NADP reductase (FNR) which reduces $NADP^+$ (the linear e^- transport). Partially, electrons may return back to the cytochrome $b_6$complex through plastohydroquinone (PQH_2) (the cyclic e^- transport). The e^- from PSII are transferred through the ETC carriers in the following order: pheophytin *a* (Phe*), two PSII-bound plastoquinones (Q_A and PQH_2 forming Q_B), cytochrome complex, where the transfer of e^- cytochrome b_6 (Cyt b_6) can follow two separate pathways: electrons involved in the Q cycle return back to QB via Cyt b_6. The main e^- pool is transferred to plastocyanin (PC) via iron sulfur proteins (FeS) and Cyt b_6, respectively. PC is a mobile e^- carrier which transfers e^- to PSI. The e^- from PSI reaction centers pass through A_0 and A_1 bound phylloquinone molecules, to FNR via Fx and FA/FB-bound iron sulfur proteins and ferredoxin (Fd). The ETC elements most sensitive to malfunctioning through elevated levels of TEs are indicated by arrows. For more details see section 20.4 in the text.

complexes and composed of Chl and proteins, i.e. the LHCs, core complexes containing RCs and other protein subunits. These components were reported to be targets for TE toxicity (Amunts et al., 2007; Faller et al., 2005; Qian et al., 2009; Umena et al., 2011; Sarvari, 2005).

Chlorophylls are highly sensitive to TEs and have been observed as precursors of ROS formation. In other words, Chls often act like unstable compounds where the energy cannot be transferred downstream because of the damage to components in the photosynthetic electron transfer system as a result of TE stress. The excited Chl also ends up producing ROS, which cause the functional loss of carbon fixation and overall plant performance rather than the quenching of harvested light energy (Fig. 3).

The analysis of *in vivo* Chl fluorescence kinetics has become one of the most potent and extensively used techniques available for plant physiological studies. It can serve as an efficient, non-destructive indicator of sublethal plant stress (Ekmekci et al., 2008; Vernay et al., 2008; Subrahmanyam, 2008). This method employs strong light for the excitation of Chls, which drive photosynthetic ETC and transient saturation of photosynthetic energy conversion (Fig. 3). The light energy absorbed by Chl molecules is reported to have three outcomes: i) it can be used in photochemistry (to drive photosynthesis), or ii) the excess energy can be dissipated as heat, or iii) it can be re-emitted as light (Chl fluorescence) (Maxwell and Johnson, 2000). Hence analyzing these processes can play a key role as an indicator of plant growth and function under TE stress.

20.4.1. Trace element effects on photosystem II

Measuring the Chl fluorescence *in vivo*, under different environmental stress conditions provides simple and reliable information on PSII activity, useful for the bioindication and biomonitoring of those stresses. The most important Chl fluorescence parameters related to PSII activity, such as the maximum quantum yield of PSII (F_v/F_m), photosynthetic electron transport rate (ETR), the quantum yield of PSII (Φ_{PSII}), non-photochemical quenching (NPQ) and photochemical quenching (qP) are listed in Table 3. The effects of TEs on Chl fluorescence and the above mentioned PSII activities have been well documented in different plant species during past decades (Table 4) (Maxwell and Johnson, 2000; Sigfridsson et al., 2004). The TE effect on these parameter values varies depending on the redox potential of the element, chemical reactivity and plant species. Relevant examples based on the recent literature are given in Table 4.

Cadmium has a negative impact on photosynthesis and on the activity of both PSII and PSI (Mobin and Khan, 2007; Shi and Cai, 2008; Shi et

Table 3

Selected PSII fluorescence parameters with abbreviations and formulae. F_o – minimum fluorescence yield of dark-adapted sample with PSII centers open; F_o' – minimum fluorescence yield of illuminated sample with PSII centers open; F_t – steady-state fluorescence yield; F_m – fluorescence yield of dark-adapted sample with PSII centers closed; F^o_m – F_m in the dark-adapted state; F_m' – maximum fluorescence yield of illuminated sample with PS II centers closed; F – fluorescence yield measured briefly before application of a saturation pulse; F_v – variable fluorescence ($F_m - F_o$) (Schreiber et al., 1986; Maxwell and Johnson, 2000; Klughamer and Schreiber, 2008)

Abbreviation	Photochemical parameters	Formula	References
F_v/F_m	Maximum quantum yield of PSII	$(F_m - F_o)/ F_m$	Maxwell and Johnson, 2000
Φ_{PSII}	Quantum yield of PSII photochemistry	$(F_m' - F_t)/F_m'$	Maxwell and Johnson, 2000
*q*P	Photochemical quenching of PSII	$(F_m' - F_t)/(F_m' - F_o')$	Maxwell and Johnson, 2000
NPQ	Non-photochemical quenching of PSII	$(F^o_m - F_m')/F_m'$	Maxwell and Johnson, 2000
Y(NPQ)	Quantum yield of light induced non-photochemical fluorescence quenching	$(F/F_m')-(F/F_m)$	Klughamer and Schreiber, 2008
Y(NO)	Quantum yield of non-light induced non-photochemical fluorescence quenching	F/F_m	Klughamer and Schreiber, 2008
ETR*	Relative electron transport rate	$[(F_m'-F)/F_m']\times$ PAR × 0.5 × 0.84	Schreiber et al., 2004

*PAR is the flux density of incident **p**hotosynthetically **a**ctive **r**adiation [μmol(quantum) m^{-2} s^{-1}]. Factor 0.5 is defined as the transport of one electron requiring the absorption of two quanta, as two photosystems are involved. Factor 0.84 – denotes that 84% of the incident quanta are absorbed by the leaf.

al., 2010). Photosystem II is more sensitive than PSI to Cd, so and it inhibits PSII function to a much greater extent than that of PSI (Chugh and Sawhney, 1999; Ekmekci et al., 2008). The examination of Cd toxicity on Chl fluorescence demonstrated that both the quantum yield of PSII (F_v/F_m) and effective quantum yield of PSII (Φ_{PSII}) were significantly reduced in *Ricinus communis* plants (Liu et al., 2011). Similar effects of different Cd treatments, have been also observed in other plants, namely in maize at 0.3, 0.6 and 0.9 mM Cd (Ekmekci et al., 2008), peanut at 10, 50 and 100 μM Cd (Shi and Cai, 2008), sunflower at 25, 50 and 100 mg Kg^{-1} Cd (Shi et al., 2010) and *Brassica juncea* at 25, 50 and 100 mg Kg^{-1} at (Mobin and Khan, 2007). Cadmium-treated leaves exhibited lower F_v/F_m and Φ_{PSII}, and the reductions were

Table 4

TEs effects on various chlorophyll fluorescence parameters

Plant species	TEs	Concentrations	Functions							Reference
			F_o	F_m	F_v/F_m	ETR	Φ_{PSII}	*qNP*	*qP*	
Ricinus communis	Cd	50 µM			↓		↓			Liu et al., 2011
Zea mays	Ni	0.1-0.2 µM	↓	↓	↓					Drążkiewicz and Baszyński, 2010
Zea mays	Cd	0.3-0.9 mM	↑	↓	↓	↓	↓	↓	↓	Ekmekçi et al., 2008
Spartina densiflora	Cu	9- 64 mmolL^{-1}	↑		↓		↓	↑↓		Mateos-Naranjo et al., 2008
Triticum aestivum	Cr	0.1-0.25 mM			↑	↓	↓	↑	↓	Sbubrahmanyam, 2008
Datura innoxia	Cr	0.05-2 mM	↑	↓	↓		↓		↓	Vernay et al., 2008
Hordeum vulgare	Cd	54 µM	↓	↓	↓	↓	↓	↑	↓	Vassilev and Manolov, 1999
Brassica napus	Cd	2-5 µM	↑↓	↓	↓					Larsson et al., 1998

approximately 5% for F_v/F_m and 47% for Φ_{PSII} in comparison to the control. The decrease in the F_v/F_m ratio may be explained in part by the negative effects of Cd on photochemical reactions, which affected the efficiency of PSII photochemistry and blocked the electron transport system. The decrease in Φ_{PSII} under Cd stress resulted in a reduced capacity of the carbon metabolism and in a low utilization of ATP and NADPH in the dark phase of photosynthesis (Liu et al., 2011).

In Cd-treated maize cultivars the F_v/F_m ratio decreased by approximately 16–24% in comparison to control plant (Ekmekci et al., 2008). As for the quantum yield of PSII, an examination showed that Φ_{PSII} diminished at 0.3–0.9 mM Cd concentrations in two maize cultivars. The Cd concentration of 0.9 mM caused the maximum decrease in these fluorescence parameters compared with those of the control. Along with F_v/F_m and Φ_{PSII} other activities like photochemical quenching qP and ETR (a decrease by 17–24%) were also found to decrease as the Cd concentration increased but no significant changes were observed in NPQ upon Cd treatment in maize cultivars (Ekmekci et al., 2008). The ETR values correspond to the light dependent rate of CO_2 fixation in control and Cd-treated plant. The F_o, F_v/F_m, Φ_{PSII} and qP were found to decline in metal-treated *Hordeum vulgare* (Vassilev and Manolov, 1999). The data on qP indicate an increased number of reduced (closed/inactive) RCs of PSII in Cd-treated plants, which implies that light absorption exceeds the capacity of ETR and CO_2 fixation and further increases the NPQ. However, Cd treated *Brassica napus* showed both increases and decreases in F_o values, depending on treatment periods. An increase in F_o suggests that non-functional PSII RCs, may also act as dissipative sink (Larsson et al., 1998).

As with other TEs, PSII is believed to be more sensitive for Pb than PSI and the inhibition site of Pb is also located on the electron donor side of PSII (Miles et al., 1972; Joshi and Mohanty, 2004). Studies on Pb treatment in barley showed a decline in Chl *a* fluorescence induction curves. It was suggested that Pb causes a down-regulation of PSII in order to avoid an over-reduction of primary electron acceptor Q_A and to reduce the load on the ETC (Kalaji and Loboda, 2007). A decreased rate of electron transfer was seen in Pb and other TE treated plants. The trace element-induced decrease in fluorescence yield is a result of inactivation of PSII-RCs (Vassilev and Manolov, 1999).

Cr ions alter the fluorescence parameters associated with PSII activity and activity. Also, Cr decreases the net photosynthetic rate by altering the chloroplast ultrastructure. A poorly developed lamellar system widely spaced thylakoids and a few grana were observed in Cr-treated plants. These structural changes were accompanied by alterations in the e^- transport from e^- donor side of PSI (Vernay et al., 2008; Subrahmanyam, 2008). Chromium reduced the net photosynthetic

rate, as well as the stomatal conductance and transpiration rate in wheat (Subrahmanyam, 2008) and *Datura innoxia* (Vernay et al., 2008). Subrahmanyam (2008) has reported that the F_v/F_m ratio, which represents the PSII photochemical efficiency in the dark-adapted state with fully open PSII, was not significantly affected by Cr treatment in *Triticum aestivum* (Subrahmanyam, 2008). A gradual decrease in the photochemical efficiency and quantum yield (Φ_{PSII}) of PSII was observed in *Datura innoxia* with increasing Cr concentration. The coefficient values of photochemical quenching (*q*P) and non-photochemical quenching (NPQ) under steady-state irradiation were significantly affected by Cr(VI) in wheat leaves. The *q*P was significantly reduced while the NPQ increased significantly under Cr(VI) treatment in both plants suggesting that the utilization of NADPH and ATP was inhibited under the reduced CO_2 assimilation caused by Cr(VI) stress. The increase in NPQ is due to an increased rate constant of the thermal dissipation of excitation energy, which down regulates the photosynthetic e^- transport mechanism (Vernay et al., 2008; Subrahmanyam, 2008). The lower F_m level in Cr-treated *Datura innoxia* that a structural alteration occurs in the pigment-protein complexes of PSII as well as change in the ultrastructure of the thylakoid membrane and damage to photosynthesis mostly localized in PSII (Vernay et al., 2008).

Nickel is an important micronutrient for plants and required for their metabolism. But excess Ni induces various toxic effects in PSII complexes, as indicated by alterations of chlorophyll fluorescence parameters (Drazkiewicz and Baszynski, 2010). In particular, Ni treatment at 0.2 μM caused a significant diminution in F_v values by about 63%. A similar tendency was also observed in the response to F_m values, which were lowered by 68% in maize plants under Ni stress (Drazkiewicz and Baszynski, 2010).

The toxic effect of As has been found to induce the decrease in ratios of Fv/Fo and Fv/Fm in maize (Stoeva et al., 2003). Nickel is also important micronutrient to plants and required for their metabolism. But excess Ni causes various toxic effects to the plant organisms including alterations of chlorophyll fluorescence parameters (Drazkiewicz and Baszynski, 2010). Particularly, Ni treatment at 0.2 μM caused a significant diminution in Fv values by about 63%. A similar tendency was also observed in the response to Fm values, which was declined by 68% in maize plants under Ni stress (Drazkiewicz and Baszynski, 2010). Recently, it has been reported that the Fv/Fm ratio was only slightly reduced in maize seedlings grown under Ni treatment, which further affect the ETS by inhibiting PSII donor and acceptor side (Drazkiewicz and Baszynski, 2010).

20.4.2. Trace element effects on photosystem I

The PSI pigment-protein complex in plants converts light energy into a trans-membrane charge separation, which finally leads to the reduction of carbon dioxide. In comparison to PSII, PSI activity has been found to be less sensitive to damage caused by TEs stress (Bazzaz and Govindjee, 1974; Atal et al., 1991; Sarvari, 2005; Mobin and Khan, 2007). However, Cd, Pb and Cr ions were reported to decrease the activity of electron transport in the PSI of isolated chloroplasts (Bazzaz and Govindjee, 1974; Wong and Govindjee, 1976; Shankar et al., 2004). Pb has been reported to affect the light harvesting complexes of PSI and PSII and the chloroplast coupling factor (Miles et al., 1972). Chromium is also found to decrease the net photosynthetic rate by altering the chloroplast ultrastructure and by inhibiting e^- transport processes by diverting the electrons from the donor side of PSI to Cr (VI) molecules (Shankar et al., 2004).

Studies on wheat leaves showed that, while Cd ions significantly decrease the activities of both PSII and PSI, the activity of PSII is much more sensitive than the activity of PSI. It has been explained that, contrary to PSI, Cd ions are able to modify the primary photochemistry and electron transport on the reducing or possibly oxidizing side of PSII, which results in an increase in the number of inactive reaction centers of PSII (Atal et al., 1991). Also, it is well known that TEs inhibit the site of water-oxidizing system of PSII which made it more sensitive to TEs (Mobin and Khan, 2007; Subrahmanyam, 2008). A study on the PSI activity in chloroplasts isolated from *Z. mays* seedlings showed that Cd decreased ferredoxin (Fd)-dependent $NADP^+$ photo-reduction, but did not affect the electron transport from 2,6-dichlorophenolindophenol to methylviologen. These results indicate that the metal interferes with electron transport at the reducing side of PSI (Siedlecka and Baszyvski, 1993).

Chugh and Sawhney (1999) reported that a 6-day treatment with 5 and 7.5 mM Cd inhibited PSII activity in pea chloroplasts by 21 and 36%, respectively, whereas the PSI activity was only slightly affected by a concentration of 10 mM. But on prolongation of the exposure of Cd (10 mM) to 12 days, the functioning of PSI also became significantly (40%) affected. Delayed effect of Cd on PSI activity points to a complexity of processes required to functions of PSI in stressed plants (Chugh and Sawhney, 1999)

Cu ions possibly affect photosynthetic activity by acting on prosthetic groups of enzymes or on polypeptides which are involved in electron transport chain (Maksymiec, 1997). Several plants have been used to study the inhibition of the rate of photosynthetic processes by Cu^{2+} (Maksymiec, 1997). The copper ion, as a constituent of plastocyanin, plays an important role in electron transport between PSII and PSI.

There were various contradictory reports about the site of action of the Cu^{2+} ion. Some authors proposed ferredoxin (Fd), i.e. the acceptor side of PSI, as a target of Cu stress. Further studies using electron paramagnetic resonance (EPR), indicated the D_1 and D_2 proteins on the donor side of PSII as a Cu target in the photosynthetic apparatus (Sersen et al., 1997).

Ni is different from other TEs, having a reverse effect on PSI. Ni treatment of isolated chloroplasts, at concentrations of 0.01 and 1.0 mM to chloroplast showed an increased activity of PSI by 15 and 6% over the control, while such treatment simultaneously lowered the PSII activity by 8 and 35%, respectively (Prasad et al., 2005). The overall photosynthetic ETR was significantly inhibited in Ni-treated chloroplasts. The greater sensitivity of PSII towards Ni might have resulted in interaction of this metal with the oxygen evolving complex, carriers of oxidizing as well as reducing side of PSII, and the reaction centre itself (Mohanty al., 1989, Prasad et al., 2005).

20.5. Summary and future perspective

Trace elements are toxic to plant organisms and impair their photosynthetic activity. TEs have their effect in interaction with other elements and alter the cellular ionic balance. TEs such as Cd, Pb, Hg, Ni, Cr, Cu and the metalloid (As) have been reported to decrease the accumulation of photosynthetic pigments in plant cells and tissues (Table 1). The inhibition of enzymes involved in photosynthetic pigment biosynthesis pathways is an important mechanism leading to the decrease of photosynthetic activity during stress induced by TEs (Table 2). On the other hand, due to their redox activity, TEs may induce significant alterations in the functioning of photosynthetic electron transfer. Thus, inhibition of photosynthesis during TE stress can be attributed both to damage to the photosynthetic apparatus and to a decrease in photosynthetic pigment accumulation.

The current scenarios in photosynthetic research have demonstrated that Chl fluorescence measurements offer a valuable alternative to plant bioassays for metal toxicity. *In vivo* analyses of chlorophyll fluorescence provide potential tools in the identification of TE action on plant photosynthetic parameters with very high accuracy and reproducibility. Short, it can be seen that Cd, Cr, Ni and Cu have a significant effect on the primary photochemistry of PSII (Table 4). In particular, the status of PSI can be a tool to find out the acute toxic effect of TEs. On the other hand, of TEs in plants is almost always associated with the high accumulation of inactivated PSII-RCs and with decreased requirements for production of NADPH.

The studies on TE toxicity reveal that the physiological responses of the same organism to a particular element may vary, depending on both physical and chemical conditions as well as on environmental factors. Studies on the action of TEs on pigment biosynthesis have been restricted to the major enzymes involved in the pathways and elucidating the toxicity from such multistep pathways will be a challenging area of research in future. Also, the elucidation of the fine structure and function of the photosystems and other ETC complexes are another area of interest which will help to find the sites of action on – and the special interactions of TEs with – the photosynthetic apparatus. Research into these components, bringing into play chlorophyll fluorescence analysis, help us to understand plant functions in the face of toxicity.

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Chapter 21. PHOTOSYNTHETIC PHYSIOLOGY AND PIGMENTS IN *LOBARIA PULMONARIA* LICHEN

Tamara Golovko, Olga Dymova, Iljia Zakhozhiy, Igor Dalke, and Ekaterina Kokovkina
Institute of Biology, Russian Academy of Sciences, Syktyvkar, Russia

Contents

21.1. Introduction

Lichens are resistant self-regulating association of fungus and algae. The presence of photosynthetic component turns fungus heterotrophic organism into autotrophic association. Green algae and/or cyanoprocariots are the most widely spread groups of the lichen photobionts. Due to close interaction of photo- and micobiont the lichens managed to occupy different habitats, frequently with extreme conditions, unfavorable to the other organisms.

Lobaria pulmonaria (L.) Hoffm (order Peltigerales W. Watson, family Labariaceae Chevoll.) is the folious epiphytic lichen with a broad area of habitat including Europe, Asia, Africa, North America and Australia (Yoschimura, 1971). As early as the last century *L. pulmonaria* was widely spread in the forest zone, however lately its occurrence decreased significantly. In Central Europe populations of *L. pulmonaria* suffered to a great extent due to air pollution and habitat destruction (Wert and Scheidegger, 2012). In the European North-East of Russia *L. pulmonaria* is frequently occurred in taiga forests, mainly in old aged aspen forests, valley fur woods and flood plain dendritic willows (Pystina and Semenova, 2009). Lichen is settled on the bark of deciduous and coniferous trees at the height of 2-3 metres, in separate cases up to 8-10 meters, it may be occurred even at arboreal litter.

L. pulmonaria is a classical example of symbiotic association which forms stable tripartite system. Lichen thallus of heteromeric type with clearly outlined layer of the photobiont. The primary photobiont of

L. pulmonaria is the green-alga *Dictyochloropsis reticulate* (Tschermak-Woess, 1995). Along with green alga cyanoprocariots, cyanobacteria of the class Nostoc, are involved in symbiosis. They are in special structures – cephalodias. Though cyanobacteria can photosynthesize, the key photobiont in tripartite lichen is green alga, and cyanobacteria carry out the process of biological nitrogen fixation.

Physical contacts of myco- and photobiont ensure exchange of the substances between the components of lichen association. The photobiont transfers carbohydrates derived from photosynthesis, and nitrogen compounds in case of nitrogen-fixing cyanobacteria such as *Nostoc,* to sustain heterotrophic metabolism of the mycobiont. The mycobiont supplies its photosynthetic partner with water and mineral nutrients (Honneger, 1991). Lichens are poikilohydric organisms. Their physiological activity depends on environmental conditions, the availability of moisture, light and favorable temperature. Light is vitally important factor for its autotrophic photobiont's partner. The quantity of light received by the photobiont during the period of thallus hydration may determine lichen growth (Palmqvist and Sundberg, 2000; Coxson and Stevenson, 2007). In summer when there is sufficient moisture and heat, photosynthetic activity of the lichen may be restricted by deficiency of light. In late autumn upon the tree leaf fall the lichens have more light, but low temperatures increase hazard of photoinhibition.

21.2. Seasonal changes of chlorophylls content in *Lobaria pulmonaria* thallus

Green pigments in lichen association are photobiont's markers. We have studied seasonal dynamics of the chlorophylls' content (Chl) in thalli, *L. pulmonaria* population, inhabiting in the mixed forest with prevalence of aspen trees. The sampling site was near the city Syktyvkar (61°34′ N, 50°33′ E). According to long-term data the average temperature of the coldest month, January, in this region was about −16 °C, the warmest one, July, +17 °C, and the frost free period was 90-105 days. The content of chlorophylls was determined spectrophotometrically in acetone extracts from freshly-sampled thalli.

The Chl (a+b) concentration per a unit of dry thallus mass varied from 0.9 up to 2.3 mg/g (Table 1), or averagely 150-180 mg per m^2. This was similar to the values given by S.C. Schofield et al. (2003) for such kind of the lichen inhabiting in the maple dominant deciduous forest of the South-East New Brunswick (Canada). Table 1 shows that to the end of the summer period the content of green pigments in thalluses increased, and to next year spring it decreased significantly. Minimal chlorophyll content in thallus was found in April, when the forest became lighter, and the average temperature was still low. We

Table 1

Content of the photosynthetic pigments in *Lobaria pulmonaria* thalli, mg/g DW

Date	Chlorophyll (*a*+*b*)	Chlorophyll *a* / Chlorophyll *b*	Chlorophyll in LHCII, %	Carotenoids	Chlorophyll / Carotenoids
18.04.12	1.41 ± 0.04	3.1 ± 0.1	53.0	0.40 ± 0.01	3.7 ± 0.2
14.06.12	1.52 ± 0.19	3.0 ± 0.4	56.4	0.40 ± 0.02	3.8 ± 0.3
02.08.12	2.00 ± 0.23	2.2 ± 0.2	70.4	0.44 ± 0.03	4.6 ± 0.3
13.11.12	1.30 ± 0.06	3.3 ± 0.1	50.8	0.41 ± 0.02	3.2 ± 0.1
13.12.12	1.27 ± 0.12	3.1 ± 0.1	53.7	0.38 ± 0.05	3.3 ± 0.1
15.01.13	1.64 ± 0.21	3.3 ± 0.1	51.5	0.48 ± 0.05	3.4 ± 0.7
03.04.13	1.06 ± 0.12	3.5 ± 0.3	49.8	0.38 ± 0.06	2.8 ± 0.2
24.04.13	0.86 ± 0.05	3.3 ± 0.1	51.2	0.28 ± 0.01	3.1 ± 0.1
24.06.13	1.28 ± 0.19	2.9 ± 0.2	56.7	0.39 ± 0.05	3.3 ± 0.1
29.07.13	1.89 ± 0.08	2.6 ± 0.3	61.7	0.43 ± 0.01	4.4 ± 0.3
01.08.13	1.48 ± 0.26	3.9 ± 0.2	46.1	0.43 ± 0.06	3.4 ± 0.1
11.09.13	2.30 ± 0.30	3.0 ± 0.1	54.5	0.70 ± 0.07	3.3 ± 0.1

Note: For the analysis of the samples are fixed in liquid nitrogen.

did not find significant change of the ratio Chl *a*/*b* within a year that might point to redistribution of the chlorophylls between the light-harvesting complexes and the reaction centers. The value of this index equaled, on the average, 3. The share of chlorophylls, belonging to the light-harvesting complex was made up 60-70% in summer, and it was 50-55% in winter. An increase of the number of the light-harvesting chlorophylls favors more efficient use of the light of low intensity by the lichen in summer, when forest cover becomes thicker, passing lower solar radiation.

21.3. Carotenoids and xanthophyll cycle conversion

Thalli *L. pulmonaria* were characterized by relatively high carotenoids concentration. The value of the ratio chlorophylls/carotenoids was made up averagely 3.5. Hence, approximately 25% of the whole pool of photosynthtetic pigments was for the share of carotenoids. Carotenoids' pool was presented mainly by xanthophylls; the content of β-carotene did not exceed 20% (Fig. 1a). Among xanthophylls lutein was made up a great part, 50% of carotenoids were for its share. Neoxathin (12-15%) and the xanthophyll cycle components (XC), violaxanthin (15-20%) and zeaxanthin (3-5%) were constantly present. The share of antheraxanthin, an intermediate of violaxanthin conversion into zeaxanthin, rised from 1 up to 5% of the carotenoids pool. The

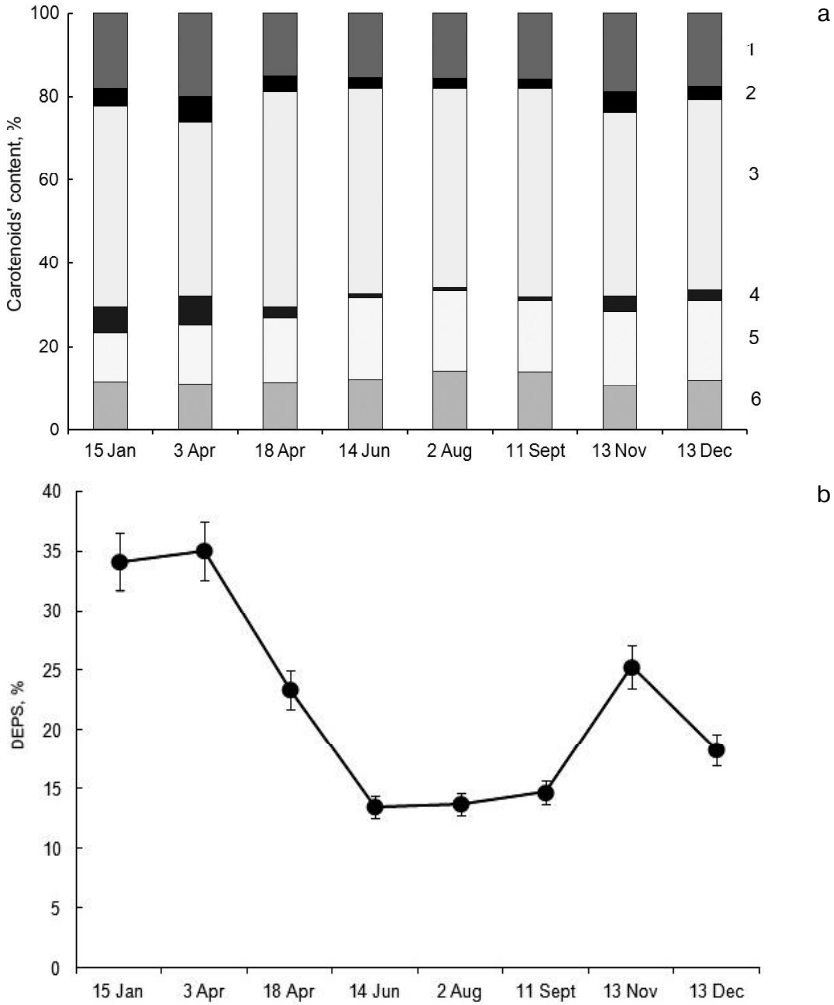

Fig. 1. Seasonal variation in the relative content of carotenoids (a) and deepoxidation (DEPS) of xantophylls cycle pigments (b) in *Lobaria pulmonaria* thalli. Pigments: 1 – β-carotene, 2 – zeaxanthin, 3 – lutein, 4 – antheraxanthin, 5 – violaxanthin, 6 – neoxanthin. DEPS = (zeaxanthin + 0.5antheraxanthin)/(violaxanthin + antheraxanthin + zeaxanthin).

level of conversion of the XC pigments (DEPS) changed strongly within a year: in summer-spring it was 1.5-2.0 times lower as compared to that one in winter and early spring (Fig. 1b).

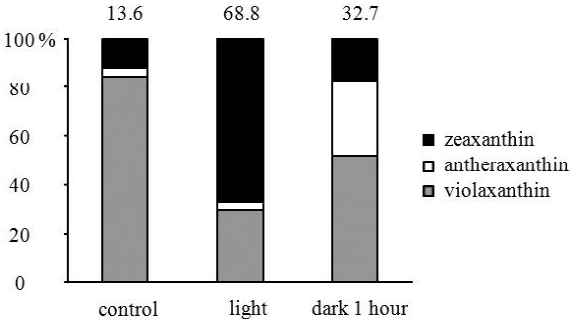

Fig. 2. Conversion state of xanthophylls cycle pigments (DEPS) in *Lobaria pulmonaria* thalli in summer.

In summer the activity of the cycle was 20% of its potential capability. This is supported by the data obtained in experiments on effect of thallus *L. pulmonaria* with intense light. Upon 1 hour of thallus exposition at photosynthetically active radiation (PAR) of 1000 μmol/m²s the DEPS increased by 5 times (Fig. 2). During exposition the amount of violaxanthin decreased approximately threefold, and of zeaxanthin increased more than 5 times. The reverse epoxidation occurred rather quickly. For 1 hour in the dark the share of antheraxanthin increased by an order, of violaxanthin increased by 40%, and of zeaxanthin decreased by 4 times. These results testify sensitivity of photosynthetic partner in the lichen association to high light.

21.4. Potential electron transport in PSII and CO_2-uptake

Photosynthetic light – response curves are means to quantitatively assessment of the light acclimation status of the photosynthetic organism by determining maximal rates and efficiencies of photosynthetic process. We studied light dependence of electron rate transport (ETR) in PSII and CO_2-exchange in *L. pulmonaria* thalli in various seasons.

It should be noted that in wet thallus the upper cortical layer created by fungous hyphae is elastic and transmits the light well to the alga layer that gives the lichen green color. While drying thallus is squeezed, its edges are curled, the thallus area is reduced of more than 30%, and it gets gray-brown color. This phenomenon is possible to see frequently in nature, when thalli are subjected to dessication. According to the some authors curling during dessication protects *Lobaria* thallus against photoinhibition (Bartak et al., 2006).

The value of potential photochemical activity of PSII (Fv/Fm) determined by induced fluorescence Chl *a* with a portable fluorimeter PAM-2100 was extremely low for air-dried thalli *L. pulmonaria* (Fig. 3). Thallus immersion into water for 10 s resulted in the increased mass of it of nearly by 65%, and keeping the thallus in water for 15 min increased its mass by 127%.

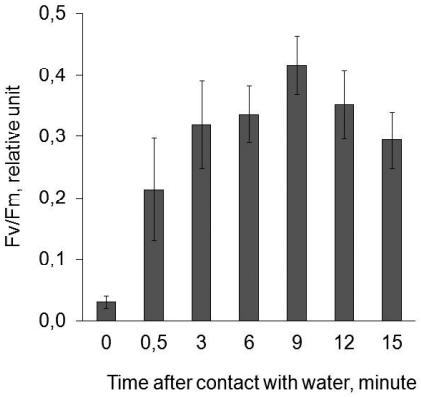

Fig. 3. Time recovery of the maximum photochemical efficiency of PSII (Fv/Fm) of the dry *Lobaria pulmonaria* thalli after short-term contact with water.

Thallus moistening was accompanied with changing of optical properties of the cortical layer and an increase of Fv/Fm up to 0.4-0.5 relative units. Nevertheless, water-retaining capacity of the wet thalli was rather low: they lost up to 50% of the absorbed water for 15 minutes at room conditions. As water is lost from the thallus, fluorescence decreases as well. Rehydration again increases fluorescence.

During dry and warm summer period we remarked that drought-stressed *L. pulmonaria* thalli were rehydrated with water vapour at night and re-establish at least partly the photosynthetic activity in the morning.

Hence, to obtain compatible data on potential photosynthetic activity of *L. pulmonaria* photobiont the freshly-sampled thalli were wetted and placed into the dampening chamber with further acclimation for 24 hrs at the room temperature at weak scattered light.

As seen from Fig. 4 the curves of the light dependence of ETR at various seasons were similar. Saturation of ETR with light was marked at PAR intensity of 450-550 μmol/m²s. Maximal ETR values in light saturation region were measured in June. In November-December the ETR value in freshly-sampled and acclimated under the room conditions thalli was lower than that in June only for 20-25%. If thalli sampled at the beginning of winter were acclimated for 24 hrs at 5 °C, then their ETR was averagely made up 50% from the values measured in thalli acclimated at 20 °C. These data show that the photobiont can rather quickly restore its photochemical activity in winter when the lichens were transfered into the favorable light-temperature conditions.

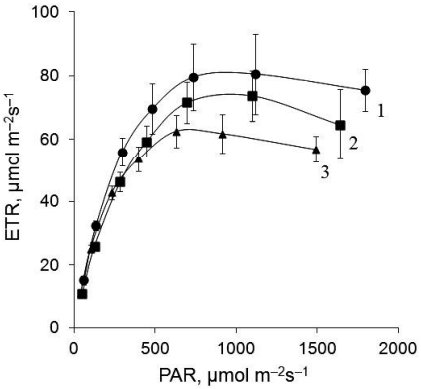

Fig. 4. Electron transport rate (ETR) light-curves of *Lobaria pulmonaria* thalli at various seasons: 1 – June, 2 – April, 3 – October.

In the *L. pulmonaria* habitats the PAR intensity within the vegetation period seldom exceeded 150-200 μmol/m²s. After a leaf fall and appearance of snow cover the PAR intensity could reach 400 μmol/m²s in sun days. Hence, in nature conditions PSII of the photobiont does not realize completely its capability to transport electrons due to the lack of the light and/or moisture in summer and low temperatures in winter.

Most likely in winter and early spring the XC plays a specified role in protection of the photobiont from photodestruction. Zeaxanthin, formed during deepoxidation of violaxanthin, is able to make safe heat dissipation of the energy excessively absorbed (Demming-Adams, 1990, 2003). Since enzymatic responses at low temperatures are inhibited, then the level of XC conversion increases in autumn, and the reverse epoxidation retards, allowing zeaxanthin to be accumulated. In autumn the lichens are usually moistened well, keeping high activity. According to our data (Fig. 1) the conversion of XC pigments in the *Lobaria* photobiont was 1.5-2.0 times higher in winter as compared to the summer.

There are data in the literature that poikilohydric photoautotrophs, mosses and lichens, have specific mechanisms of energy dissipation which protect desiccated organisms against photoinhibition (Heber et al., 2007; Heber, 2008; Gasulla et al., 2012). These mechanisms do not require a protonation reaction for activation of energy dissipation (Heber, 2008). In other words, they are not associated with the deepoxidation of violaxanthin. The main mechanism is based on dessication-induced conformational changes of the chlorophyll-protein complexes. Besides, the pigments and products of the second metabolism on the surface of hyphas of the upper cortical layer, can absorb and reflect the light. At squeezing the thallus and densing of the cortical layer these pigments shade alga layer, preventing the light entrance. Due to this effect we did not observe induction of the background Chl fluorescence (Fo) in the dessicated *Lobaria* thalli.

In wet thalli the value of the non-photochemical quenching of chlorophyll fluorescence (qN) increased significantly with the increasing of

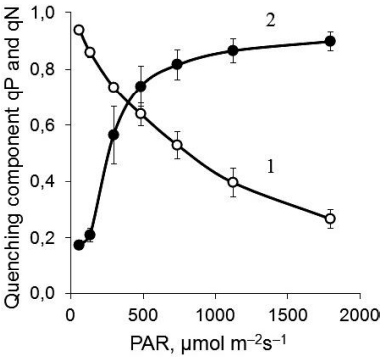

Fig. 5. Photochemical (1) and non-photochemical (2) quenching of chlorophyll fluorescence in *Lobaria pulmonaria* thalli under different light in June.

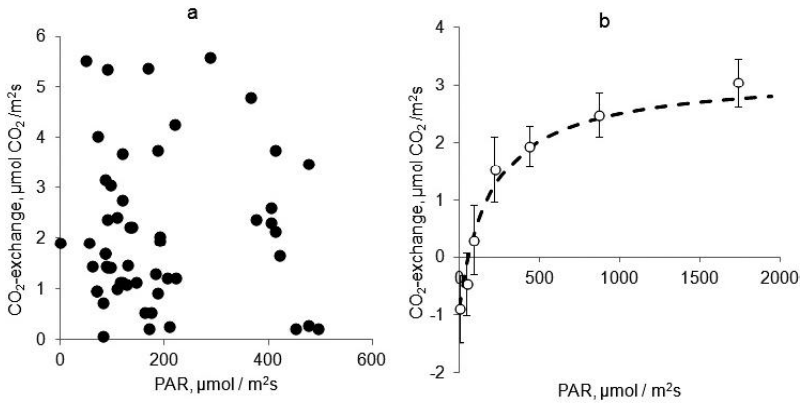

Fig. 6. *Lobaria pulmonaria* thalli rate of CO_2 net-absorption in habitats (a) and in controlled conditions (b) under different light.

PAR that pointed to dissipation of excessively absorbed energy (Fig. 5). Taking into account the high potential ability of XC to deepoxidation of violaxanthin into zeaxanthin in autotrophic symbiont *L. pulmonaria* (Fig. 2), it is possible to suggest that this mechanism contributes greatly into photoprotection of the wetted thalli.

In habitats the rate of CO_2-gas exchange in the thalli of *L. pulmonaria* varies greatly. Thus, in summer the rate of CO_2 net-uptake in some thalli reached 4-6 μmol/m²s (Fig. 6a). At the same time in other thalli was recorded CO_2 evolution at the light. This could be associated with their partial dessication. The assessments made in the laboratory conditions on the wetted thalli showed clear dependence of CO_2-gas exchange on light (Fig. 6b). Photosynthetic light-response curves obtained could be described with Michaelis-Menthen equation. Maximal rate of net-CO_2 uptake at light saturation was about 3 μmol/m²s. The CO_2 evolution rate in the dark was as low as three fold. On the basis of these data the rate of gross–CO_2 uptake in the thalli can reach 4 μmol/m²s at saturated light and the temperature of approximately 25 °C. The rate of gross-CO_2 uptake does not exceed 2 μmol CO_2/m²s at typical for *L. pulmonaria* light environment in nature. The same values were presented earlier by MacKenzie et al. (2001) for *L. pulmonaria* from deciduos forest in the South-West Canada.

21.5. Physiological responses of *Lobaria pulmonaria* to UV-radiation

UV-radiation is potentially hazard factor, which is able to influence on the living organisms and ecosystem processes (Qing et al., 2004).

In plant cell the chloroplasts, the centers of photosynthetic activity, are subjected to the damage under UV-radiation. Many authors marked the suppression of net photosynthesis, gene expression and synthesis of the key proteins of photosynthetic apparatus, enzymes of carbon metabolism, and photochemical reactions in the reaction centers of PSII (Fedina and Velitchkova, 2009; Kumari et al., 2014). The level of UV-radiation on the Earth surface depends on ozone layer, the thickness of which decreases greatly for the last time (Kerr and McElroy, 1993). This leads to an increse of the effluence of the UV-B (280-315 nm) and UV-A (315-400 nm) radiation. In the next decade an increase of intake of UV by 14% in the North hemisphere and by 40% in the South hemisphere is predicted.

Solhaug et al. (2003) showed that hydrated *L. pulmonaria* thalli exposed to natural UV-irradiance began to form melanins within a week; at the end of the experiment, thalli were dark brown. However, they could not detect protection of the photosynthetic apparatus by melanic pigments.

It has been studied by us the effects of the UV (A+B) radiation on CO_2-gas exchange and the antioxidative system of *L. pulmonaria* (Zakhozhiy et al., 2012). The thalli were collected at the beginning of August and placed into the chamber at the temperature of 18-20 °C with further constant moistening. The thalli were illuminated with lumenescence lamps: PAR 50 μmol/m²s, for 8 hours daily. Upon three days of acclimation, a part of the thalli were illuminated with UV-lamps daily (Camelion LH 26-3U Blacklight). The daily rate of UV (A+B) radiation was made up about 10 KJ/m² that was close to the natural rate of solar UV-B radiation entered on the open surface. 10 days later it has been found that control (−UV) and test (+UV) thalli differed by the xanthophylls content and the deepoxidation XC pigments. The DEPS value of (+UV)-thalli was 30% higher as compared to the (−UV) thalli. UV-radiation stimulated photosynthetic activity of the thalli (Fig. 7). At saturating irradiation the rate of CO_2 net-uptake in (+UV)-thalli was averagely by

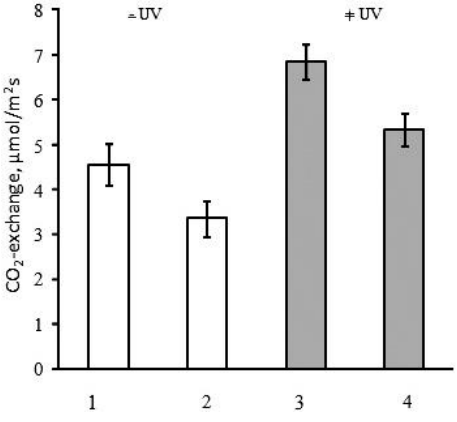

Fig. 7. Effects of UV (A+B) radiation on rate of CO_2 net-absorption in *Lobaria pulmonaria* thalli. 1, 3 – after night (16 h), 2, 4 – after light period (8 h).

35% higher relative to that one in (−UV) thalli. In (+UV) − thalli the respiration rate increased at night. The difference in respiration capacity of the control and test thalli was about 45%. Since fungus component consists of 80-90% biomass of lichens, we suggest that differences in respiration of the control and test thalli are caused by micobiont's response on UV-radiation greater than the reaction of photobiont.

It is known that UV-radiation effect on the pro/antioxidation balance of the living cells due to high formation of reactive oxygen species (ROS). The key producent of ROS in the cells are redox systems and, in particular, electron transport chains of the chloroplasts and mitochondrium. Changes in photosynthetic and respiration activity of the thalli of *L. pulmonaria* under UV-radiation served the basis to study the antioxidative enzymes activities and lipid peroxodation rates. No reliable differences were found between the test and control thalli on the activity of catalase and peroxidase. At the same time the activity of superoxidedismutase in (+UV)- thalli was higher by 40% relative to that one in (−UV)-thalli. The content of thiobarbituric acid-reactive products in (+UV)-thalli was lower by 30%. Physiological responses of the lichen *L. pulmonaria* to UV-radiation testify the ability of these organisms to protect from the affect of direct sun light. In natural conditions lichens may be subjected to these effects at falling of the sunlight flecks under the cover or at falling the shadow trees.

21.6. Conclusion

Populations of *L. pulmonaria* are abundant in old forests of middle taiga area at the European North-East Russia (Komi Republic). In warm months the low ambient light in the closed-canopy allows the lichen thalli to realize near 30% potential capability of net-CO_2 absorption. The character of the curves of light dependence of the rate of electron transport testifies high potential capacity of the PSII photobiont to electron transport in warm months and in winter after short adaptation of the thalli at the room temperature. It has been revealed a correlation between the rate of electron transport and the rate of net CO_2 uptake. In the region of light saturation of the rate of electron transport and net CO_2 absorption nonphotochemical quenching of the induced fluorescence of the chlorophyll *a* in PSII (qN) increases greatly. Zeaxanthin-dependent dissipation of the absorbed energy plays an important role in this process. The data obtained by us show that in summer the level of deepoxidation of XC pigments increased by 3-4 times upon exposition of the moisturized thalli to high light. In natural conditions the DEPS was much higher in winter and early spring than in summer. This favors to protection of photosynthetic apparatus from

photodestruction at low temperatures when illumination in the habitat of *L. pulmonaria* increases and the use of the light energy for the CO_2 assimilation is inhibited by low temperatures. It is also possible that heat energy dissipation favors to an increase of the temperature in thalli in cold time of the year.

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Part 5. HELPFUL ASPECTS OF PIGMENT STUDY

Chapter 22. REFLECTANCE-BASED NON-DESTRUCTIVE ASSAY OF LEAF CHLOROPHYLLS AND CAROTENOIDS

Alexei Solovchenko, Olga Chivkunova, and Mark Merzlyak*
M.V. Lomonosov Moscow State University, Moscow, Russia

Contents

22.1. Introduction

Chlorophylls (Chl) and carotenoids (Car) are essential pigments of higher plant assimilatory tissues (Lichtenthaler, 1987), responsible for absorption of light energy and for initial steps of its photochemical utilization; Car also fulfill a crucial function in protection of photosynthetic apparatus from photodestruction (Merzlyak and Solovchenko, 2002; Young and Lowe, 2001; Choudhury and Behera, 2001; Demmig-Adams et al., 1999). Different content and composition of Chl and Car determine the variations of higher plant color from dark-green to yellow. Other pigments are often involved in leaf and fruit coloration such as flavonoids (yellow) and anthocyanins (red) (the latter two groups are out of the scope of the present paper; for more detail see (Hughes, 2011; Steele et al., 2009; Merzlyak et al., 2008).

The absolute contents of the pigments, as well as their ratio, are important physiological characteristics, which could be recorded on

* Deceased.

the level of a single leaf, the whole plant, or even plant community. The content of Chl, the dominant pigment of green leaves, determines, to a great extent, the amount of Photosynthetic Active Radiation (PAR) absorbed by a leaf, the rate of photosynthesis, and plant productivity (Hallik et al., 2012; Hansson and Jensen, 2009; Merzlyak et al., 2009). Carotenoids augment Chl in light harvesting, stabilize pigment-protein complexes of photosynthetic apparatus (PSA) and prevent, via several mechanisms, damages to plants by the excess of visible radiation (Lichtenthaler, 1987; Young, 1991). The pigment content undergoes directional and specific changes in the course of plant growth and development during adaptation to unfavorable environmental conditions, as well as under various stresses and as a result of the damages (Biswal, 1995; Demmig-Adams and Adams, 2006; Gitelson et al., 2002; Merzlyak and Solovchenko, 2002; Merzlyak et al., 1999; Merzlyak et al., 1998).

Traditionally, pigment analysis in plant physiological and biochemical studies is carried out with spectrophotometry of organic solvent extracts. This method presumes destruction of the sample, hence it is time-consuming and coupled with artifacts due to pigment instability, incomplete extraction, the presence of light-absorbing impurities etc. (Merzlyak et al., 1996; Solovchenko et al., 2001). These circumstances make nondestructive estimation of pigment content with reflectance spectroscopy of intact tissues an attractive alternative to crude chemical methods. Indeed, both qualitative and quantitative changes in the pigment content of plant tissues should be inevitably apparent in tissue optical properties. Indeed, reflectance spectra of the leaves undergo remarkable changes as a result of mineral nutrition deficiency, pollutant intoxication, senescence and stresses, in particular during acclimation to strong solar irradiation, and in the course of senescence (Gitelson and Merzlyak, 1993; Merzlyak et al., 1998, 1999, 2008; Merzlyak and Solovchenko, 2002).

Nondestructive reflectance-based quantification of the pigments has a number of advantages such as rapid measurement of a large number of the samples. It is important to note that leaves remain intact after reflectance measurements, hence repeated measurements of the same sample turns to be feasible, making the reflectance-based pigment quantification highly suitable for monitoring of the plant objects.

Inexpensive portable reflectometers suitable for field measurements, providing reliable spectral data both from very small plant surface area and the whole plants were designed (Penuelas and Filella, 1998; Solovchenko et al., 2010; Richardson et al., 2002). Reflectance spectroscopy is widely used in global remote monitoring of agro- and phytocenoses. In recent years these approaches have also been implemented in "precision agriculture" technologies (Penuelas and Filella, 1998).

The basic theory of diffuse reflectance developed by Kubelka and Munk for a homogenous layer of "infinite thickness" yields a simple relationship between the intensity of the reflected light and the absorption and scattering coefficients of the medium (see (Kortum, 1969). A leaf, consisting of several structures with different refraction indices (cuticle, epidermis, and mesophyll) and containing high amounts of pigments, represents a complex optical system. Although detailed investigations of leaf optical properties have appeared in the literature (Fukshansky, 1981; Fukshansky et al., 1993; McClendon and Fukshansky, 1990), the most fruitful approaches for quantitative pigment analysis *in situ* were developed by considering the leaf as a "black box". A considerable body of researches was dedicated to the development of techniques for nondestructive analysis of plant pigments, and these issues attracted much attention during the last decade (Chappelle et al., 1992; Penuelas and Filella, 1998; Gilerson et al., 2010; Gitelson et al., 2003a, 2006, 2009). Foundation of the optical reflectance-based approach successfully applied by the author and his colleagues for *in situ* quantification of both photosynthetic and screening pigments has been laid in the works by Gitelson et al. (2009, 2006, 2003b, 2002, 1998). The present paper is a brief review of the techniques for quantitative estimation of Chl and Car in leaves with reflectance spectroscopy, including those developed in the laboratory of the authors.

22.2. Common features of plant reflectance spectra

Development of reflectance-based nondestructive techniques for plant pigments estimation requires deep understanding of their *in vivo* spectroscopy, localization, and the patterns of their changes during physiological processes in plants. Senescing leaves, featuring dramatic changes in Chl and Car, are suitable for demonstration of the influence of the pigments on leaf reflectance spectra (those of maple, as an example, are shown in Fig. 1). Leaves with low Chl content exhibit high reflectance (35-48%) at the wavelengths longer than 600 nm. The measurements performed on the leaves with extremely weak pigmentation showed that leaf tissues possessed high reflectance without any discernible spectral features in the near infrared (NIR), green and red parts of the spectrum. These investigations also revealed that apparent absorption of the light by leaves (amounting to 10-15%) in the range of 750-800 nm, described in the literature, is likely to result from incomplete collection of the transmitted light with an integrating sphere (Merzlyak et al., 2002, 2009). The presence of the pigments at very low amounts, which are difficult to quantify analytically (e.g., Chl content of 0.3-0.4 nmol/cm^2), manifests itself as distinct bands in leaf and fruit reflectance spectra (Fig. 1, see also Merzlyak et al.,

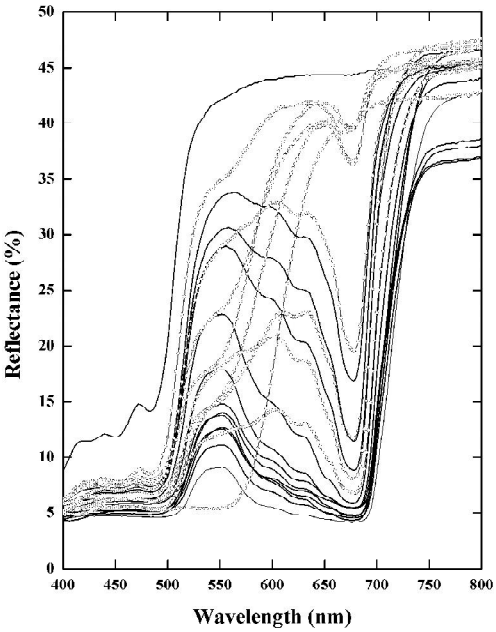

Fig. 1. Reflectance spectra of autumn maple (*Acer platanoides* L.) leaves. The chlorophyll content in leaves ranged from 0.1 to 60 nmol/cm^2 (see red-orange region in upper and lower spectra, respectively). Spectra of anthocyanin-free (<1 nmol/cm^2) leaves are shown by solid lines; broken lines correspond to leaves with anthocyanin content ranging from 2.1 to 40.8 nmol/cm^2 (upper and lower spectra, respectively).
Source: Merzlyak et al. 2003a.

2002, 2009). With increased pigment content up to 10-12 nmol/cm^2, the spectra contain pronounced features of Chl (pale-green leaves) and Car (yellow leaves) absorption. The reflectance spectra of green (Chl content ca. 30 nmol/cm^2) and especially dark-green leaves were poorly resolved.

The leaf reflectance in the main bands of Chl *a* absorption (near 440-450 and 670-680 nm) became saturated at Chl content of 10-15 nmol/cm^2. However, reflectance in these spectral regions did not drop below 4-5% even at very high concentrations of the pigment (Figure, see also (Gitelson et al., 2002; Merzlyak et al., 1999). This could be explained by reflection of the light by superficial leaf structures (cuticle and epidermis), containing very low amounts of the pigments (see spectra in (Solovchenko and Merzlyak, 2003; Pfiindel et al., 2006). Distinct bands attributable to Car absorption could be distinguished only in reflectance spectra of senescing (yellow) leaves on terminal stages of Chl degradation (see the uppermost curve in Figure and spectra in (Merzlyak et al., 1997, 1999).

It has been found that reciprocal reflectance, $1/R(\lambda)$, of the leaves (Gitelson et al., 2006; Gitelson et al., 2003b; Gitelson et al., 2009) at certain wavelength relates to pigment contents. This feature was used in the development of the models relating reflectance and pigment content. On the base of these models, the algorithms for estimation of chlorophyll and other pigments in leaves and fruits were developed (for review, see (Merzlyak et al., 2003a). Briefly, conceptual semi-analytical three-band model (Gitelson et al., 2006; Gitelson et al., 2003a) relating reflectance and content of the pigment of interest [P] was suggested in the form:

$$[P] \propto (R_{\lambda_1}^{-1} - R_{\lambda_2}^{-1}) \times R_{\lambda_3} \qquad \text{(Eq. 1)}$$

The model contains reflectances in three spectral bands (λ_1, λ_2, λ_3). Reflectance in the spectral band λ_1 is maximally sensitive to the pigment of interest; however, it is also affected by absorption by the other pigments contained in and scattering by plant tissue. To eliminate the effect of absorption by other pigments at reflectance R_{λ_1}, reflectance in spectral band R_{λ_2} has been used. R_{λ_2} is affected by absorption of the other pigments and is minimally affected by absorption of the pigment of interest. Thus, the difference ($R_{\lambda_1} - R_{\lambda_2}$) in Eq. 1 relating to the pigment of interest, however, is still affected by the scattering. To minimize this effect, reflectance in spectral band λ_3 should be governed mainly by scattering of the sample studied.

The following strategy (Gitelson et al., 2006) allows us to overcome some complications inherent in nondestructive analysis of plant pigments using reflectance spectra, as well as to employ the model (Eq. 1).

1) The detection of reflectance spectral bands governed predominantly by absorption of an individual pigment and sensitive to this pigment content.

2) The development of algorithms relating to reflectance at certain wavelengths with pigment content in the entire range of its variation.

3) Finding a way for elimination of chlorophyll contribution into reflectance required for other pigment analyses.

In the course of these studies, leaves of several plant species were analyzed at all stages of their development and in the wide range of their pigment content. The following criteria were used for validation of the approaches developed: (i) the algorithms should be sensitive only to the pigment of interest and insensitive to the other pigments or morphological-anatomical features of plants and (ii) they should be applicable to the data set obtained independently. For testing the second criterion we used leaves of different species which were collected in different years.

As a result of the analysis of reflectance spectra, the bands of *in situ* absorption of the leaves of pigments of different species were established (Gitelson et al., 2001, 2002, 2006; Merzlyak et al., 2003b; Gitelson and Merzlyak, 1996). The obtained results provided evidence that the conceptual model (Eq. 1) is applicable for an accurate nondestructive estimation of certain screening pigment content in leaves. The developed algorithms are (i) sensitive mainly to the pigment of interest and minimally sensitive to the contents of other pigments or morphological-anatomical features of the plant samples, and (ii) applicable to the data set obtained independently (Giannopolitis and Ries, 1977; Steele et al., 2009; Gitelson et al., 2009; Gitelson et al., 2003a; Gitelson et al., 2002).

22.3. Chlorophyll

In healthy anthocyanin-free leaves, Chl is the only pigment absorbing in green to far-red spectral range (Lichtenthaler, 1987; Merzlyak et al., 2002). In earlier investigations, reflectance minimum at 670-680 nm was employed for Chl analysis. Although the algorithms developed for these wavelengths showed a good sensitivity and linearity at low Chl content, they lost sensitivity to Chl over 10-15 nmol/cm^2 (Buschmann and Nagel, 1993; Gitelson and Merzlyak, 1994; Gitelson et al., 1996a).

Actually, spectral regions, where reflectance is sensitive to wide-range variations of Chl content (from 0 to 50-60 nmol/cm^2), were found aside from red maximum of Chl absorption: in the green (a broad band near 550-600 nm) and in the red (a narrow band near 700-705 nm) parts of the spectrum. In particular, these regions were revealed in the spectrum of standard deviation of reflectance calculated for leaves with wide variation of Chl content (Gitelson et al., 1996a; Gitelson et al., 2003b). It was found further that reflectances in these bands were hyperbolically related with Chl content (Fig. 2A). It should be noted that Chl absorption coefficients are very low in these bands. The linear relationship between inverse reflectance in certain spectral regions and pigment content is likely a fundamental feature of leaf reflectance spectra. It was used as a basis in the development of algorithms for estimation of Chl and other pigments.

One of the requirements for reliable algorithms of pigment analysis is their low sensitivity to morphological-anatomical traits of plant tissues. For leaves differing in pigment content, the lowest coefficient of variation of reflectance was found in the NIR region (Lichtenthaler et al., 1996; Gitelson and Merzlyak, 1994). Since leaf pigments possess no measurable absorption in the NIR, tissue reflectance in this region is thought to be determined by "internal" optical properties related to leaf thickness, water content, and light scattering. The scattering within plant tissues arises at interfacial boundaries separating phases with different refraction indices (Buschmann and Nagel, 1993; Merzlyak et al., 2002; Fukshansky, 1981).

Taking into account the above circumstances, the algorithms for estimation of Chl content were suggested in the form of simple ratios of reflectance coefficients at certain wavelengths: R_{NIR}/R_{550} and R_{NIR}/R_{700}. Note that R_{NIR} is insensitive and R_{700} and R_{550} are highly sensitive to Chl content (see e.g. Fig. 3). Both ratios were highly sensitive to Chl content in a wide range of its changes in leaves and fruits of diverse plant species and related linearly to the pigment content (Gitelson and Merzlyak, 1998; Lichtenthaler et al., 1996; Gitelson et al., 2003a). Furthermore, Chl determination could be performed in the broader spectral ranges: the algorithms in the form of $[1/R(\lambda) - 1/$

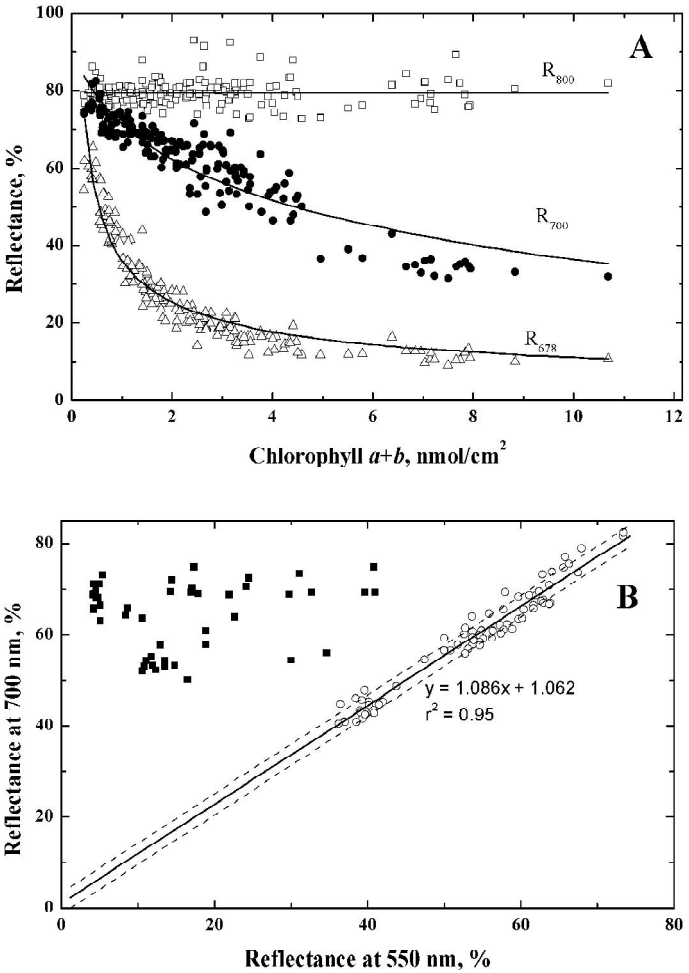

Fig. 2. Reflectances at 678, 700 and 800 nm versus chlorophyll content (A); reflectance at 700 nm vs. reflectance at 550 nm (B) in apple (*Malus* × *domestica* Borkh.) fruits. For green to green-yellow fruits, R_{550} vs. R_{700} is linear with determination coefficient higher than 0.95, whereas for anthocyanin-containing fruits, $R_{550} < R_{700}$ and fair relationship between them was disturbed. Solid lines represent the best-fit functions; dashed lines represent STD in B.

Source: Merzlyak et al. 2003b.

R_{NIR}] · R_{NIR} were shown to provide highly precise and linear estimates of leaf Chl content in the wavelength ranges of 530-580 and 695-735 nm (Gitelson et al., 2006).

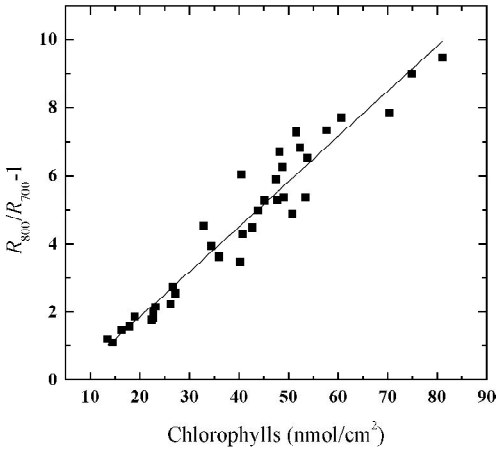

Fig. 3. Relationships between the index R_{800}/R_{700} −1 and chlorophyll content in apple leaves (r^2 > 0.98) (Solovchenko A., unpublished; in collaboration with Dr. L. Kozhina).

It is noteworthy that R_{NIR}/R_{550} and R_{NIR}/R_{700} ratios possessed similar sensitivity to the Chl content, which was due to high correlation between reflectance at 550 and 700 nm characteristic of healthy anthocyanin-free leaves. Furthermore, there is ground to believe that this correlation represents a universal feature of leaf reflectance spectra in the ranges predominantly or exclusively governed by Chl absorption (Gitelson et al., 2003a; Gitelson and Merzlyak, 1998). Thus, accumulation of anthocyanins (Fig. 1; see also (Gitelson et al., 2009; Gitelson et al., 2001)]) leads to a significant decrease of R_{550} relative to R_{700} (Fig. 2B). This greatly complicates the application of R_{NIR}/R_{550} index for Chl determination in the red leaves. At the same time, our studies showed that R_{NIR}/R_{700} index could be used for Chl analysis even at high anthocyanin content (Gitelson et al., 2001). Further details on the development of the indices and their characteristics are considered in (Gitelson and Merzlyak, 1996; Gitelson et al., 2003a).

Several other approaches, allowing efficient analysis of Chl in leaves, were developed using reflectance in the red region of the spectrum. Thus, the pigment content could be retrieved from the amplitude and position of the peak in the first derivative of a reflectance spectrum between 685 and 706 nm, the so-called “red edge” (Ding et al., 2009; Gitelson et al., 1996b).

22.4. Carotenoids

In green tissues the analysis of Car absorption in the blue region of the spectrum is problematic due to a strong overlapping absorption of Chl presented in high amounts in plant tissues (Merzlyak and Solovchenko, 2002; Gitelson et al., 2002; Demmig-Adams et al., 1996). Additional obstacles to the Car analysis in plants appear due to complex composition of these pigments undergoing transformation during fruit ontogeny and upon their adaptation to high-light conditions (Solov-

chenko et al., 2006). To estimate the effect of Car on reflection spectra it is necessary to remove a significant effect of Chl absorption. Normalization of reciprocal reflectance to reflectance at 678 nm (red Chl absorption band) removes in certain degree Chl effect: thus, the spectrum $[R_{800}/R(\lambda)]/R_{678}$ depends on the other factors but Chl (Merzlyak et al., 2003a; Gitelson et al., 2002). The quantitative Car estimation became feasible using the same 3-band model (Eq. 1) with λ_1 in the range of 510-530 nm (Merzlyak et al., 1998). To subtract the effect of Chl absorption on reflectance in spectral band λ_1, λ_2 was found to be optimal either in the green range (around 550 nm) or red edge range (700 nm). As for Chl and AnC retrieval, optimal λ_3 was in the NIR range beyond 750 nm. Two Carotenoid Reflectance Indexes (CRI) developed for leaves (Gitelson et al., 2002) were suggested as

$$CRI_1 = (R_{520}^{-1} - R_{700}^{-1}) \times R_{800}, \quad \text{(Eq. 2)}$$

or

$$CRI_2 = (R_{520}^{-1} - R_{550}^{-1}) \times R_{800}, \quad \text{(Eq. 3)}$$

where the first term in the parentheses is associated with combined absorption by Car and Chl, and the second one relates to Chl absorption. However, it should be mentioned that CRI is not applicable to anthocyanin-pigmented objects. In addition, flavonoids, when accumulated in high quantities, influenced considerably optical spectra and their absorption might have extended quite far into the visible spectrum. Therefore, one using reflectances for non-destructive determination of the higher plant pigments absorbing in the visible range should aware of the obstacles which could be caused by flavonoids when they are presented in high amounts (Merzlyak et al., 2005).

22.5. Chlorophyll-to-carotenoid ratio

The proportion between Car and Chl is an important characteristic of plant photosynthetic apparatus. The most dramatic changes in the content of these pigments occur at terminal stages of the leaf and fruit development in many plant species. Frequently, at these stages plant tissues retain certain amounts of Car or Car synthesis is induced on the background of Chl degradation. In chloroplasts of senescing plant tissues, plastoglobules rather than thylakoids become the predominant sites of Car localization (Merzlyak and Solovchenko, 2002; Merzlyak et al., 1999; Tevini and Steinmuller, 1985; Steinmuller and Tevini, 1985; Solovchenko et al., 2010). The analysis of green leaves with different pigment content revealed a strong correlation between reflectance in the red maximum of Chl absorption (near 678 nm) and in the spectral band near 500 nm governed by the combined absorption

of Chl and Car (Merzlyak et al., 1997; Merzlyak et al., 1999). In senescing coleus (*Coleus blumei* Benth.) leaves, characterized by a remarkably synchronous disappearance both of Chl and Car resulting in whitish leaf coloration, a high correlation of reflectances at these wavelengths was retained until advanced stages of Chl breakdown. By contrast, during chlorophyll degradation in yellowing leaves of deciduous trees (maple and chestnut) and in ripening fruits (e.g., apples and lemons) R_{678} increased significantly higher than R_{500}. As a result, a close correlation between reflectance at these wavelength characteristics of tissues with high chlorophyll content was broken (Merzlyak et al., 1997; Merzlyak et al., 1999).

For detection of relative changes in Chl and Car content, Plant Senescence Reflectance Index (PSRI) was suggested that used reflectance at 500 and 678 nm along with NIR reflectance: $(1/R_{678}-1/R_{500})/R_{NIR}$ (Gilerson et al., 2010). This index exhibited high correlation with the molar Car/Chl ratio in senescent maple leaves (Gilerson et al., 2010).

In leaves containing high amounts of Chl, R_{500} was somewhat higher than R_{678} that resulted in negative PSRI values. As mentioned earlier, the reflectance at 678 nm increased faster in the course of leaf senescence than that at 500 nm, which made PSRI positive. Therefore, the stage when PSRI turns to zero (i.e., $R_{678} = R_{500}$) could serve as a criterion for the onset of senescence in plants exhibiting Car retention. Our experiments showed that induction of Car synthesis occured at different stages of Chl degradation and the rates of relative changes in Chl and Car content varied between the plant species (Gilerson et al., 2010).

22.6. Conclusions and prospects

The results obtained during the last decade considerably extended possible applications of reflectance spectroscopy for estimation of pigment content and for assessment of physiological status of the plants. These achievements are really impressive, because some time ago reflectance spectroscopy was considered to be unable to provide useful information about plant organisms due to their low reflectance and poorly resolved spectra that seemed similar in different species (Gamon and Surfus, 1999). We consider these similarities as an evidence of common organization of photosynthetic apparatus and uniformity of its changes occurring during plant development and stress responses in higher plants.

The results presented in this review show that reflectance spectroscopy could be a useful and efficient tool for pigment analysis in plants. Remarkably, to retrieve nondestructively Chl and Car, the reflectance in only four spectral bands is sufficient. However, the possibilities of

application of this technique to leaves of other plant species need further verification. Another problem, which remains to be solved, is finding of the approaches for selective determination of Chl *a* and *b*. All this facilitates extensive application of reflectance spectroscopy for solving various issues of plant physiology on the level of individual leaves. The developed algorithms could be employed in remote sensing of vegetation status (Gitelson et al., 1997; Gitelson et al., 1996a). Fundamental spectral features of leaf reflectance, revealed in these studies, provide a basis for the development of this technology.

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Chapter 23. THE SPATIAL-TEMPORAL ESTIMATION OF VEGETATION CHLOROPHYLL INDEX: REMOTE-SENSING APPROACHES

Vladimir Elsakov
Institute of Biology, Russian Academy of Sciences, Syktyvkar, Russia

Contents

23.1. Introduction

Vegetation cover is the principal component of biocoenosis. It ensures carbon sink by means of photosynthetic accumulation and determines the productivity of ecosystems that can be measured by gasometrical, weight, and nowadays remote-sensing methods of study. The main optical features of phytocoenoses, influenced largely by total content of the pigments in above-ground phytomass of different plants, are actively used for isolating dominant profiles (for classification) of vegetation cover, quantitative assessment of plants seasonal development, revealing the succession stages or degradation of vegetation.

A series of studies conducted in phytocoenoses of different regions (Prince and Goward, 1995; Raynolds et al., 2006; Puma et al., 2007; Huemmrich et al., 2010, etc.) demonstrate the availability of close correlation between the volumes of energy vegetation cover absorbed/reflected in the ranges of red and near infrared radiation, absorbed photosynthetic active radiation (*APAR*) and productivity of the communities. This fact allows us to apply spectral indices for calculation of quantitative values of photosynthetically conditioned CO_2-flows and to treat these indices seasonal variability as a productivity indicator.

The chlorophyll index (ChI, kg/ha) is broadly used as a value that characterizes projective content of green pigments in phytocoenoses. For different geographical zones of Russia, positive correlation has

been found out between annual photosynthetic carbon sink, productivity of the communities and the projective content of chlorophyll (Voronin, 1995). Consequently, the analysis of spatial-time aspects of chlorophyll index distribution can help in revealing landscape, geographical, and seasonal volumes of photosynthetic carbon fixation by plant communities and their productivity.

Application of spectral vegetation indices for remote assessment of vegetation cover characteristics has numerous advantages because they are less dependent on changes in soils brightness, on illumination and observation geometric conditions, atmosphere transparency (Vinogradov, 1984). The spectral vegetation index can be treated as an integral character of above-ground vegetation photosynthetic activity due to positive correlation of the said above parameters:

$$\mathrm{VI} = f(ChI, APAR) \cdot \varepsilon, \quad (1)$$

where ChI – the projective content of chlorophyll in vegetation cover of the plot under study, $APAR$ –photosynthetic active radiation absorbed by vegetation cover, ε – a coefficient dependent on structural characteristics of vegetation communities, conditions of survey, and air condition. The most popular is the Normalized Difference Vegetation Index (NDVI). It is estimated as the normalized difference in brightness of image details (pixels) in visible red (0.6-0.7 μm, absorption maximum) and near infrared (0.7-1.0 μm, reflection maximum) ranges and is handled as an integral character of above-ground vegetation photosynthetic activity:

$$NDVI = (NIR\text{-}RED)/(NIR+RED), \quad (2)$$

where NIR – reflection in the near infrared spectral region, RED – reflection in the red spectral region. Despite chlorophyll plays a major role in formation of spectral characteristics of phytocoenoses, $ChlI$ distribution is seldom highly evaluated using remote-sensing data. The majority of investigations related with estimation of chlorophyll content by remote sensing data were done for water ecosystems (Kopelevich et al., 2006) and monospecific artificial agrocoenosis (Sid'ko and Shevyrnogov, 1998; Gitelson et al., 2012; Peng et al., 2011).

For this purpose, the leaf area index (LAI) is usually applied. High correlation between chlorophyll projective cover and the content of above-ground photosynthetic active phytomass (Tieszen and Johnson, 1968) makes it possible to use multispectral images also for identifying the content of above-ground phytomass:

$$ChI = \Sigma(P_{Ph}\ c)_n, \quad (3)$$

where P_{Ph} – autotrophic organs biomass of particular plants per an area unit, c – mean concentration of chlorophyll in the plants. However, identification should necessarily consider structural characteristics of phytocoenoses and all life forms in them. When plant communities have a tier structure and so high values of above-ground phytomass, correlation gets weaker (Golubyatnikov and Denisenko, 2006).

Correlation is the best in tundra zone due to a simple vertical structure of the communities, low total projective cover values, increased participation of mosses and lichens (Bliss and Matveyeva, 1992). For example, correlation between the NDVI and above-ground phytomass (g/m^2) values for the circumpolar area (by the AVHRR survey) has a form of exponential functions:

$y = 26.58e^{6.9357x}$, $R^2 = 0.8924$ (Raynolds et al., 2006);

$y = 24.907e^{7.1665x}$, $R^2 = 0.8941$ (Walker et al., 2003).

For tundra regions of Yamal, correlation between the NDVI values obtained in ground-based measurements and photosynthetic active biomass (g/m^2) (Epstein et al., 2009) can be represented as:

$y = 37.265e^{3.495x}$, $R^2 = 0.6176$.

For seaside meadows dominated by annual herbaceous psammophytes, correlation has a form of a linear function (by Landsat):

$y = 1612.5x - 131.6$, $R^2 = 0.83$ (Elsakov and Shchanov, 2005).

The present paper aims to study possible applications ways of the data obtained in spectrozonal surveys for distribution assessment and analysis of spatial-time changes of the *ChI* index within different phytocoenoses of Northern Eurasia.

23.2. Spectral vegetation indices and their importance in identifying distribution of chlorophyll projective content and observed productivity of phytocoenoses

Phytocoenoses of Northern Eurasia have a common trend in distribution of the *ChI* index: it decreases northwards from 35-40 kg/ha in the middle and south taiga, and in deciduous forests to 3-4 kg/ha in tundra (Voronin et al., 1995). Total projective chlorophyll concentration in spruce forests of the middle taiga subzone depends on the forest type, varying from 24.3 (haicap-moss spruce forest) up to 33.3 kg/ha (bilberry spruce forest) (Tuzhilkina and Bobkova, 2010). It makes 33.9 kg/ha in coniferous-deciduous phytocoenosis and 4.9-11.0 kg/ha (the lowest values among sphagnum-type forests) in pine forests (Tuzhilkina et al., 1998). For upland bogs, the index has a value of 5.7 kg/ha. Generally, its mean values for boreal botanic-geographical formations decrease from 40 in the middle and south taiga up to 20 kg/ha in the north taiga (Voronin et al., 2004). The *ChI* value in forest communities of the middle taiga is mainly made up by

autotrophic (up to 82% in mature spruce and 85% in coniferous-deciduous forests) part of the tree stand; northwards it gets lower (up to 30-40% in the north taiga) (Tuzhilkina and Bobkova, 2010).

Many authors indicate that a major part (from 70 to 90%) in total projective cover of chlorophyll in forest communities is composed of the first tree layer on the level of crown-making layer (Voronin, 1995). In this connection, application of remote-sensing methods is limited and advised for phytocoenoses with the *ChI* value less or equal to 24 kg/ha (Tsel'niker and Malkina, 1994).

For the tundra zone, the average projective chlorophyll content in phytocoenoses is 3.5 kg/ha (Voronin, 2006). For the tundra zone of Alaska, it varies from 3.2 up to 7.7 kg/ha (Tieszen and Johnson, 1968). In microgroups of the Khibini Mountains (tundra), the index varies within 3.3-9.7 kg/ha; it becomes less variable on transition to the level of communities and is 4.1-6.6 kg/ha (Shmakova and Kudryavtseva, 2002). The *ChI* value decreases according to the row: grasses (11.8 kg/ha) → shrubs/dwarf-birch (7.5-9.0) → sedge bogs (7.7) → black crowberry-dwarf-birch (5.8) → sparse willow forests (4.3) → cotton grass-mossy (4.2) → undershrub-mossy (3.6 kg/ha) phytocoenoses (Shmakova, 2006; Shmakova and Kudryavtseva, 2002; Tieszen and Johnson, 1968).

The materials of the *QuickBird* remote-sensing spectrozonal surveys of ultra-high resolution (resolution 2.44 m) made in the middle of vegetation period (6.7.2007) allowed us to analyze correlation between spectral indices and chlorophyll content values (literature data and own field measurements). As a simulating model plot we used tundra communities and sparse woods in the eastern part of the Bolshezemelskaya tundra (67°0′-67°6′ N, 54°46′-57°0′ E). After geometric correction and radiometric calibration (W/m^2 sr μm), the controlled classification with isolation dominant classes of phytocoenoses was done for the model plot's area; computation of the average for isolated classes values of the *NDVI* index was conducted.

The use of literature data and satellite data for adjacent communities estimated that linear statistically-important relation ($y = 24.0x - 5.1$; $n = 8$, $R^2 = 0.86$, $p = 0.01$) between total projective chlorophyll content values and the *NDVI* spectral vegetation index is available. The obtained data allowed us to extrapolate the *ChI* values to all image pixels and, hence, to get preliminary information on projective chlorophyll content for the plant communities previously unstudied (Fig. 1). The highest values were obtained for the class of willow forests and near-stream meadows (10.3 kg/ha on the average). The absence of closed canopy of vascular plants and availability of single mosses on the sites of the eroded peat lands and sandy areas were responsible for the lowest *ChI* values in the isolated classes (less than 0.4 kg/ha). The communities

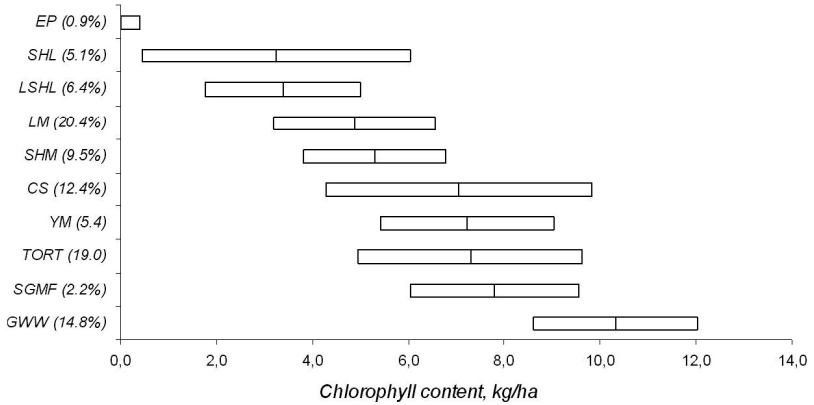

Fig. 1. Distribution of chlorophyll index in the isolated classes of vegetation cover by the simulating results. Keys: 1 – low(sub)shrub lichen tundra, LSHL, 2 – shrub lichen tundra, SHL, 3 – low(sub)shrub moss tundra, SHM, 4 – eroded peat, EP, 5 – grass (herbaceous) willow forests, GWW, 6 – sedge moss fen, SGMF, 7 – cotton grass (moss) swamp, CS, 8 – shrub mossy tundra, SHM, 9 – lichen-moss tundra, LM, 10 – yernik moss tundra, YM, 11 – sparse *B. tortuousa* wood, TORT. Mean values ± confidence interval (X±tSx, $p < 0.05$) are presented. Y-axis shows occurrence percents of the isolated community classes.

previously not considered of herbaceous-mossy open woods with *Betula tortuosa* had the *ChI* index of 7.3 kg/ha.

The region of the conducted field studies whose boundaries matched with the limits of image (9×10.9 km) was dominated by lichen-mossy tundra (LM) (20.4% of scene area), birch open woodlands (19%) and willow forests (14.8%). Totally, the above communities took 54.2% of the image area and, hence, played a key role in formation of mean values of the *ChI* index by image. For plant communities of the region (with exclusion for water objects, sites without vegetation cover, *ChI*>0) the index comprised 6.4 kg/ha. Water surfaces took 3.8% of the area.

A comparison of the data received by field gasometry with the LiCor-6200 (Nebraska, USA) infra-red gas analyzer and the *QuickBird* data which were simultaneously collected in adjacent phytocoenoses of simulating plot has shown that close positive correlations exist ($R^2 = 0.85$, $n = 18$, $p = 0.01$) between gross primary production (*GPP*), NDVI (Elsakov and Marushak, 2011) and the estimated values of chlorophyll index for the most particular areas (Fig. 2).

About 60% of the observed communities demonstrated an estimated linear dependence; the last communities had the observed CO_2 absorption intensity being lower (GWW community) or higher (LSHL, SHL and CS). These deviations might be caused both by the specificity of the reflected radiation registered characterizing a plot on the Earth surface

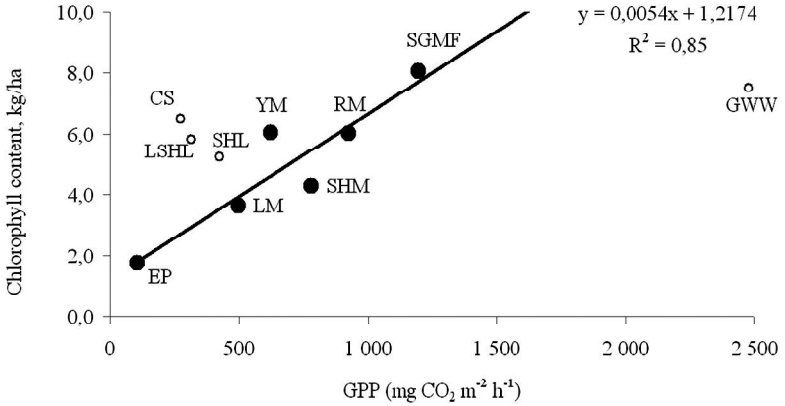

Fig. 2. Relationship between the *NDVI* and the gross primary production (*GPP*). Keys are the same as for Fig. 1.

and the specificity of species composition of the observed phytocoenoses, biological characteristics of the plants that make total phytomass value of the communities.

The use of identified dependence allowed us to extrapolate the results on chlorophyll index distribution and the observed gross primary production (GPP) to neighboring areas and to develop the thematic maps.

23.3. Interannual and seasonal changes in chlorophyll projective content by spectral data

Insufficient amount of different-season QuickBird images, high price and rare repetition are responsible for the need in other less expensive but also with less spatial resolution sensors for interannual and seasonal assessment of changes in quantitative phytocoenoses parameters. Landsat (Cohen and Goward, 2004) materials made in identical spectral ranges (0.63-0.69 μm and 0.76-0.90 μm), but with lower spatial resolution (30 m) are widely used nowadays. A comparison between the QuickBird and Landsat materials for one plot and one survey date (Fig. 3) has shown that images with less resolution have incorrect extreme values of the index as a result of spatial generalization. Variability of the Index drops that introduces an error into comparative analysis of quantitative characteristics at mosaic plots and small-sized phytocoenoses. Despite underestimate of the average values (for whole scene: $ChI_{\text{Landsat}} = 5.0$ kg/ha; $ChI_{\text{QuickBird}} = 6.1$ kg/ha; for vegetation cover pixels ($ChI>0$) $ChI_{\text{Landsat}} = 5.1$; $ChI_{\text{QuickBird}} = 6.4$ kg/ha), the application of Landsat scenes is rational.

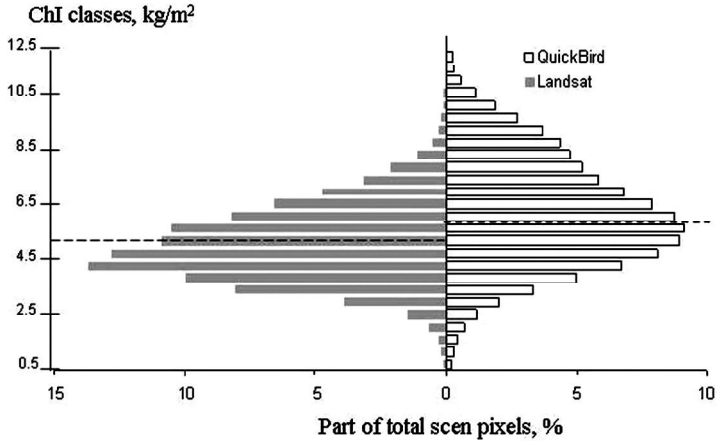

Fig. 3. Distribution of values of ChI classes for Landsat and QuickBird scenes with one survey date. Dotted line indicates mean values of general scene area ($ChI_{Landsat}$ = 5.0 ; $ChI_{QuickBird}$ = 6.1). The ordinate axe is classes of ChI groups.

The vegetation outlines obtained after classification of QuickBird scenes were used for isolation of composition-homogenous pixels at Landsat scene. A pixel was considered homogenous by class when 80% of its composition was represented by one Earth surface class of higher resolution. The analysis of seasonal changes in *Chl* index made for composition-homogenous classes demonstrated that index variability depends on seasonal specificity of vegetation period and climatic conditions of the years studied. The highest values of chlorophyll projective cover values do not match to different years and depend largely on the temperatures in the first part of vegetation period (Fig. 4).

For the class of herbaceous willow forests the chlorophyll projective cover maximums changed by 14-30 days from year to year. On condition of early vegetation period and stable warm period (2007) the index reached highest maximum values at early terms (towards July, 3). For single years, deviation of chlorophyll stock from mean value comprised 2.5-17%.

According to the results obtained, an assessment of quantitative values and trends in the *Chl* index for dominant communities using Landsat survey data is now complicated by various reasons. Firstly, a possibility of essential fluctuations in terms of phenological stages for years with different climatic conditions always exists. Seasonal dynamics in pigments' content in plants of cryolitozone is also a well-known fact (Petrov et al., 2010; Golovko et al., 2011). Consequently,

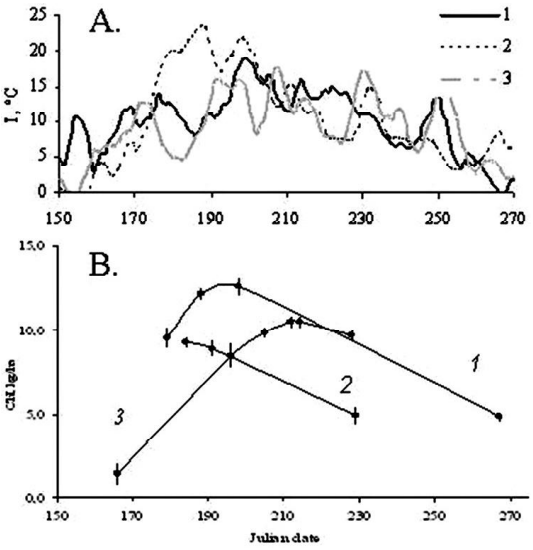

Fig. 4. Dynamics of average daily air temperatures leveled for 5 days in the study region (Vorkuta) (A.). Changes of the *ChI* index during vegetation period for herbaceous willow forests class by sensors data of different-season/different-year Landsat TM5 scenes. Keys: 2000 – 1, 2007 – 2, 2009 – 3 (B.).

same survey dates do not guarantee correct values. Secondly, quantitative comparison of phytocoenoses characteristics by remote-sensing data with different dates but the same phenological parameters does not exclude possible registration of the changes caused by seasonal positions of the Sun zenith angle (Elsakov and Teteryuk, 2011). These problems are able to be solved soon by putting into operation the new satellite observation systems Sentinel-2 within the program GMES (Global Monitoring for Environment and Security) that will allow frequent repeated surveys to be in 2-3 days.

23.4. Trends of the changes of ChI values in for estimation of climatically-conditioned transformation per 2000-2011 period

The analysis of convergence of NDVI volumes between MODIS and QuickBird scenes for the same date demonstrated that data of low spatial resolution can be comparable with generalized high resolution data (Fig. 5). The total pixels volume of QuickBird was spatially generalized to the MODIS pixel size calculated as an average volume of all the pixels included. Nevertheless, there is some shift of volumes related to the differences in sensors ($y = 0.997x - 0.293$, $r = 0.60$, $n = 1924$, $p = 0.001$). The obtained linear equations were used for recalibration of MODIS NDVI data ($NDVI_{MAX}$ for different years) for equatable with QuickBird NDVI volumes and for further calculation of ChI volumes on the basis of yearly established NDVI-ChI relations.

Variability of the chlorophyll projective cover index in climatically different years demonstrated the possibility to reveal and analyse the

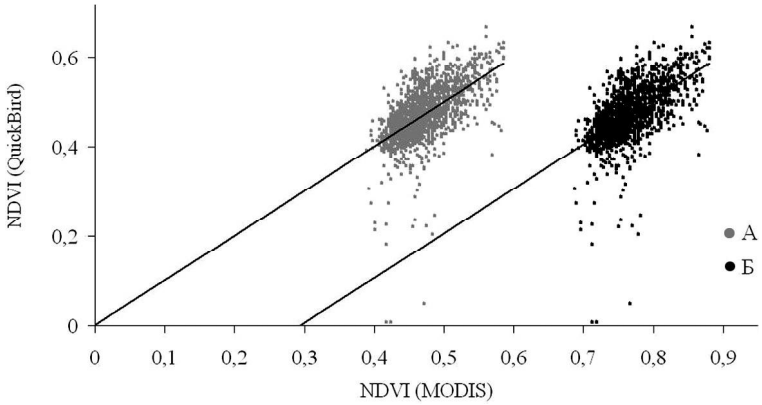

Fig. 5. The convergence of NDVI volumes between MODIS and QuickBird scenes for same date before (A) and after (B) correction procedure. The spatial resolution size of QuickBird images is recalculated to MODIS.

trends of climatic fluctuations trends on the area under study. Particularities in ChI distribution and changes within a separate geographical zone were observed at regional level by the Terra-MODIS (MOD13Q1.005) satellite survey data with spatial resolution of 0.25 km for the period of 2000-2010 (data source: modis.gsfc.nasa.gov). Despite the lower spatial resolution, the middle-resolution materials have advantages as no interference from micro- and mesorelief; frequent repetition of images (2 times a day) diminishes the impact of cloud canopy. The work included preparation of the NDVI index database using 16-day temporary composites (highest values of index for concerned period of time), selection of maximal index values for every year under study ($NDVI_{MAX}$). The highest index values were frequently registered for July-August (12-15 composites). Index correlation between the Terra-MODIS and Landsat data, and recalculation the $NDVI_{MAX}$ into ChI values based on the earlier models developed were made. According to the data obtained, the average index of maximal projective pigments content for the period of 2000-2010 (Fig. 6A) was estimated; the trends of index for the whole time interval were assessed (Fig. 6B).

Distribution of the *Chl* index undergoes the rules of vegetation cover distribution in Northern Eurasia and reflects current continental division into geographical zones influenced by climatic, geocryological and permafrost conditions of particular regions, by orographic features of the regions. History of location and degradation of permafrost rocks and possible changes of the south limits in modern conditions has been also demonstrated. Chlorophyll projective content is generally higher

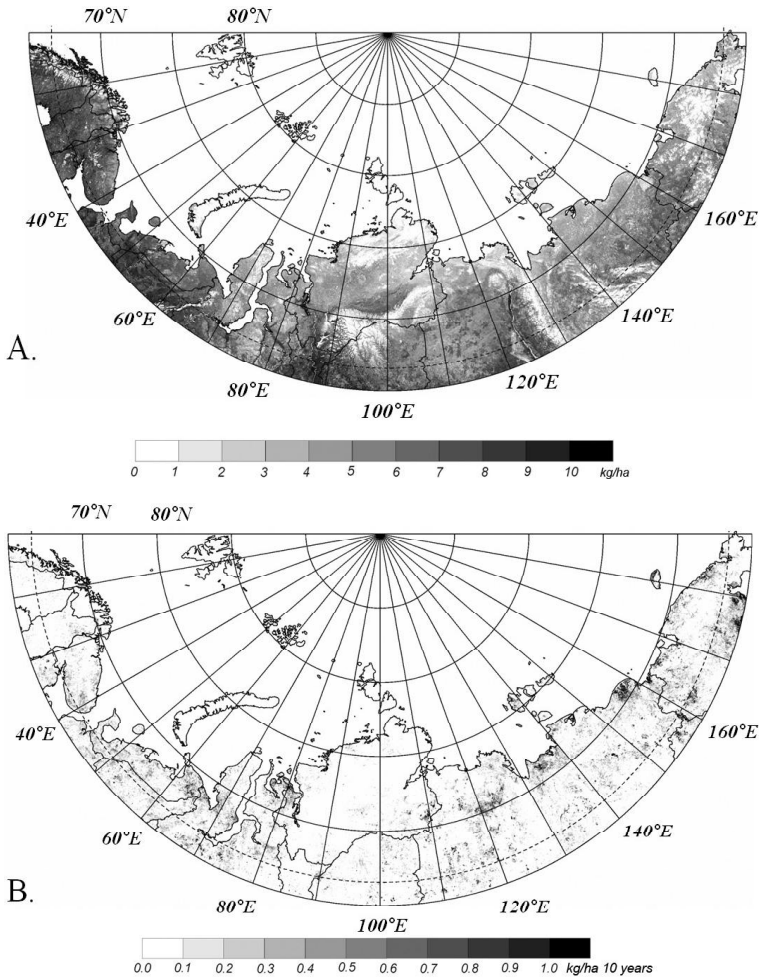

Fig. 6. Distribution of mean Chl values (kg/ha) on the territory of Northern Eurasia (A) and trend of index growth for the period of 2000-2011 (B). By the MODIS 2000-2011 data.

for the east-European tundra plots with "weak, warm" permafrost (Astakhov and Svensen, 2011) as compared to that one for the Trans-Urals (Zaural'e) and is presented in the value range of 5.7 ± 1.6 kg/ha (X±SD) for plots with the same vegetation types. For west-Siberian tundra plots situated at same latitude, the index value is considerably lower and makes up 3.2 ± 1.6 kg/ha. The current climate in the North-East of the Russian Plain is more ocean-oriented. Hence, climatic and

geocryological zones are less in length and are mainly represented by the south sub-Arctic tundra regions. Conservation of relict perennially frozen soils is also important in the east regions, consequently the European north-eastern regions are characterized by poor ice content in the mountain rocks (Baulin et al., 1981).

The average value of air carbon fixation by different phytocoenoses was estimated from 100 (Shmakova, 2006) to 314 kgC/kg Ch per year (Voronin, 2006). Recalculation of the ChI values allows the rules of photosynthetic carbon sink to be distributed within the region under study that can be counted into biomass characteristics with further active use during land management activities on reindeer grazing areas. However, analyzing coenotic, zonal, and landscape phytomass distribution parameters by remote-sensing materials should always consider the structure of phytocoenoses. For example, south tundra lands are largely presented by green vegetation, mainly by mosses and lichens (up to 40%) and woody above-ground parts of the shrubs and undershrubs (35-40%) (Bazilevich, 1993). According to the reservoir-and-flow-oriented model of production process (Voronin, 2006), all plants are divided into donor (phytotrophic) and acceptor (heterotrophic) parts linked by the conducting system. Consequently, plant communities also can be treated as a combination of autotrophic/heterotrophic parts. The data of spectrozonal survey highlight mainly the parameters of donor plant parts. For the majority of tundra communities, consisting usually of annual plants, the model is simplified. Net primary production (NPP) of photosynthesis is closely related to the photosynthetic carbon sink (Ph). Most tundra communities are described by the equation (Fig. 2).

A comparison of the values with the results obtained earlier in the region of the Vorkuta permafrost study station (Elsakov, 2003) has shown that intensity of the expected CO_2 absorption evaluated by above-ground phytomass stock exceeds the observed intensity in the communities dominated by the acceptor plants. For example, SGMF communities with not so dense *C. aquatilis* accumulate about 59% carbon in above-ground phytomass during the vegetation period. In addition, above-ground vascular plants accumulate 14.5% carbon (12.1% – in biomass of *C. aquatilis*) and mosses – 44.5%. The quantity of carbon assimilated in mossy layer increases along with increasing the thickness and density of mossy cover at model plots. For cotton-grass (CS) and mossy communities, this value makes up 74.2 and 87.2%, correspondingly. At the same time, a proportion of carbon accumulated in above-ground vascular plants phytomass (6.1 and 3.5%) and that of "not-considered" carbon stock (19.8 and 9.3%) decreases. In undershrub-mossy and undershrub-lichen-mossy communities, the green parts of vascular plants accumulate 5.7 and 17.5% carbon of NPP.

The analysis of interannual variability and ChI trends has indicated that index change within the area has spatially heterogeneous structure and depends largely on distribution of perennially frozen rocks. A response of the index to interannual climatic changes (fluctuations) is weak.

Severe climatic conditions of the Trans-Urals located in continental regions are less susceptible to climatic affects. The plots marked for stable changes seem to mark the areas with changing permafrost-climatic conditions. Visually (aerovisual observations) these changes are related to rapid willow forests growth on spatially neighboring plots in the regions of continuous perennially frozen rocks distribution (Fig. 6B). A comparison of the results obtained with the earlier observations on cryogenic landscapes time changes on the area (Elsakov and Marushchak, 2011) allows us to correlate the growth in phytocoenoses productivity at particular plots with thickening of the seasonally-thawing layer, temperature increase of the top perennially-frozen rocks fixed in the last tens years (Natural..., 2005).

The highest ChI values of 0.7-1.0 kg/ha corresponded to an increase of carbon accumulation in biomass with the intensity 7-30 kgC/ha annually for 10 years. The plots with high ChI values do not form a precise latitude belt; their location is usually dependent on the regions. These plots are sporadically situated and are found for the European North (eastern part of the Kola Peninsular, Malozemelskaya tunrda and Bolshezemelskaya tundra on unfrozen thaw pockets, Pai-Khoi), Western (southern part of the Yamal Peninsular and east of the Gydanskiy Peninsular), Middle (mean stream of the Olenek River) and Eastern Siberia (east of the Yano-Indigirskaya tundra, Kolymskaya tundra), some Arctic islands (north of the Wrangel Island and east of the Kotelnyi Island).

High ChI values, spatially belonging to the areas under serious stress conditions, are described in the literature. For example, reindeer grazing lands of the Turvaurgin community in the Nizhnekolymskiy district are found to be dramatically reduced and degraded (up to 30% of the grazing lands) due to rapid distribution of the shrubs (Shadrin, 2009). Coenotic activity and species abundance analysis of the latitude geographic species of vascular plants and lichens from different coenofloras have identified a moisture increase and a warmth decrease in dwarf-birch-undershrub-lichen-mossy and dryad tundra areas (south tundra subzone) of the Yamal Peninsular (Telyatnikov, 2002).

23.5. Summary and conclusions

The obtained results evidence that the materials of spectrozonal satellite surveys can be efficiently used for quantitative distribution

assessment of *Chl* index and for analysis of its seasonal and interannual changes in different phytocoenoses, vegetation types, and botanic-geographical zones. Different-year images can identify intensity and trend of phytocoenoses transformation in time (dynamic mapping), contribution of the landscape (facial), geomorphic and climatic conditions to carbon balance of the ecosystems; they also allow us to understand carbon balance formation in space on the level of particular regions. Sporadic field geo-botanic and ecologic studies on isolated parts of the area hinder in correct evaluation of the zone and longitude variations between phytocoenoses and hence require additional information.

The majority of the observed plots had relatively stable values of the *Chl* index during a year. However, the obtained results also identified some development trends of arctic phytocoenoses due to climate fluctuation; the index as an indirect indicator can help in assessing the trends of phytocoenoses productivity. The *Chl* index changes mark plots that response to global fluctuations and hence allow the areas that are largely transformed due to changing cryogenic conditions to be isolated. Consequently, the marked plots require further examination of the safety of engineering and transport structures on them, re-viewing the existing projects on land grazing lands for particular reindeer-breeding farms.

Acknowledgements

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Chapter 24. PLANT PIGMENTS AND HUMAN HEALTH

Olga Dymova[1], Ekaterina Lashmanova[2], and Tamara Golovko[1]
[1] Institute of Biology, Russian Academy of Sciences, Syktyvkar, Russia
[2] Syktyvkar State University, Syktyvkar, Russia

Contents

24.1. Introduction

Plants contain a variety of pigment molecules that absorb physiologically useful radiation. The functions of photosynthetic pigments were discussed in details in different chapters of this book. The question which will be considered in this chapter is nutraceutical value and possible beneficial role of consumption of natural plant pigments such as carotenoids, chlorophylls and anthocyanins. Anthocyanins are not photosynthetic pigments. They are colored flavonoids located mainly in vacuoles of the cells. This group of the pigments contributes to plants' protection from biotic and abiotic stress, including screening of photosynthetic apparatus from UV-radiation and light excess.

The contribution of individual plant pigments to human health maintenance is difficult to study and document (Lila, 2004). The natural pigments can be highly metabolized after ingestion. They are often able to show biological activity in combination with other phytochemicals in food.

24.2. Pigments as health supplements

24.2.1. Carotenoids

Carotenoids are the widest distributed group of the pigments. They have been identified in photosynthetic and nonphotosynthetic organisms: higher plants, algae, fungi, bacteria, and, at least, in one species of each form of animal life. Total production of carotenoids in nature is 10^8 ton/year. Since carotenoids are predominantly hydrocarbons, they are lipid soluble and found either in chloroplast membranes or in

specialized plastids called chromoplasts. The concentration of carotenoids in chromoplasts may reach very high levels. The carotenoid family of the pigments includes carotenes and xanthophylls (oxygenated carotenes). Carotenes are predominantly orange (β- and α-carotene) or red-orange (lycopene). β-carotene is the major carotenoid in higher plants and algae. Lycopene is the principal carotenoid in tomato fruit.

The most abundant xanthophylls in nature are fucoxanthin, lutein, violaxanthin and neoxanthin. Fucoxanthin can be found mainly in marine algae, whereas lutein, violaxanthin and neoxanthin are abundant in green leaves. The key biological functions of carotenoids are light capture, photosynthesis photoprotection, dissipation of excess light and quenching of singlet oxygen.

Carotenoids are bioactive substances in human diet. There are more than 600 carotenoids found in nature and about 50 carotenoids can be found regularly in human diet (Bendich, 1993). An interest to carotenoids and their possible role in maintaining human health appeared almost 100 years ago, when the connection between β-carotene and vitamin A (tocopherol) was established.

People cannot synthesize carotenoids and get them from food, mainly from fruits and vegetables (Grusak and DellaPenna, 1999). People from the developed countries receive about 70-90% of total carotenoid intake by eating fruits and vegetables. The primary carotenoids in fruits and vegetables consumed by different populations are lutein and β-carotene (O'Neill et al., 2001). The main sources of β-carotene are brocolli, carrot, kale, spinach etc. (Britton, 2009). Brocolli, green and yellow pepper, green leafy vegetables are rich in lutein (Table 1). However, 85% of lutein people receive from tomato fruit and tomato-based products (Bramley, 2000).

Berries are also relatively rich in carotenoids. We investigated the content of β-carotene and xanthophylls in four wild berries from taiga zone of the European North-East: cloudberry (*Rubus chamaemorus*), blueberry (*Vaccinium myrtillus*), cranberry (*Oxycoccus palustris*) and cowberry (*Vaccinium vitis-idaea*) (Lashmanova et al., 2012). The highest total carotenoid content among the berries

Table 1

Lycopene content of fruit and tomato products (summarized in Bramley, 2000)

Fruit/Tomato Lycopene Content	Product (μg/g fresh weight)
Fresh tomato	8.8±42.0
Watermelon	23.0±72.0
Pink guava	54.0
Pink grapefruit	33.6
Papaya	20.0±53.0
Tomato sauce	62.0
Tomato paste	54.0±1500.0
Tomato juice	50.0±116.0
Tomato ketchup	99.0±134.4
Pizza sauce	127.1

Source: Scott and Hart 1995; Tonucci et al. 1995; Rao and Agarwal 1999.

Table 2

Total carotenoid contents in berries, μg/100g DW

Carotenoids	*Rubus chamaemorus*	*Vaccinium myrtillus*	*Vaccinium vitis-idaea*	*Oxycoccus palustris*
Neoxanthin	20.1±1.1	9.6±1.5	0.99±0.02	27.1±1.5
Violaxanthin	12.9±2.7	12.1±2.4	1.53±0.17	11.3±1.8
Antheraxanthin	21.1±2.9	5.9±0.9	1.45±0.09	23.4±1.7
Lutein	50±15	183±24	12.28±1.09	48.1±4.8
Zeaxanthin	411±46	2.6±0.3	1.42±0.30	31.2±2.9
β-carotene	2320±200	43.9±4.8	4.42±0.25	56.6±2.1

Source: Lashmanova et al. 2012.

studied was found in cloudberry (2840 μg/100g dw) and blueberry (2140 μg/100 g) (Table 2). Cranberry and cowberry were characterized by significantly lower carotenoid content, 200 and 140 μg/100 g/DW, respectively. All berries contained β-carotene, but this pigment prevailed greatly in cloudberry (83% of total carotenoids). Lutein was the principal carotenoid in blueberry (71%) (Fig. 1). The major contributors to total carotenoid fund in cranberry were β-carotene (28%), lutein

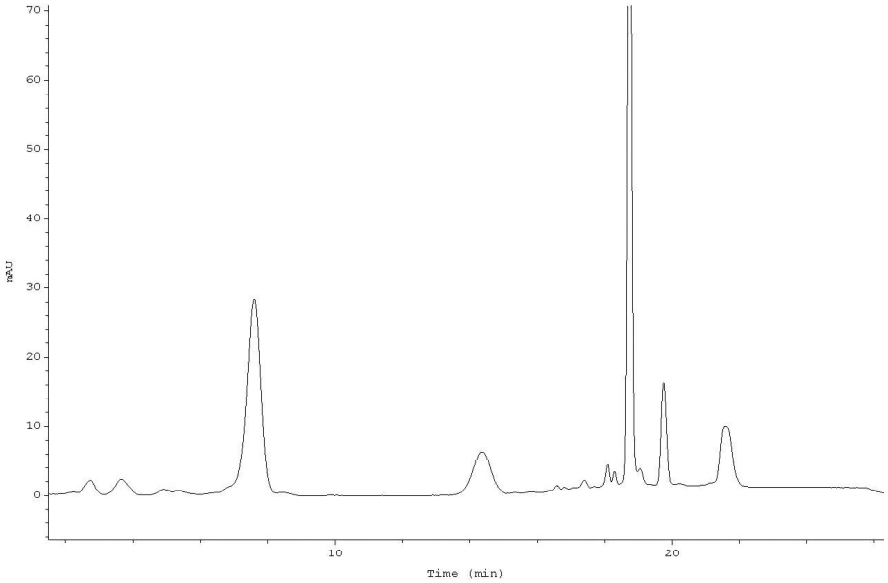

Fig. 1. HPLC chromatogram of the carotenoids in *Vaccinium myrtillus* berries. Pigments: 1 – neoxanthin; 2 – violaxanthin; 3 – antheraxanthin; 4 – lutein; 5 – zeaxanthin; 6 – β-carotene.

(23%) and neoxanthin (20%). With the exception of blueberry, only some amount of violaxanthin cycle pigments (violaxanthin, antheraxanthin and zeaxanthin) were revealed in the berries. There is not much data available on carotenoids concentrations in northern wild berries. Our data on the carotenoid content in blueberry are well correspondent with the data of Marinova and Ribarova (2007). However, the amount of β-carotene in our investigation was higher than that reported in Finnish cloudberry (Heinonen, 1989; Marinova and Ribarova, 2007).

The leafy vegetables, cultivated in greenhouse, are a valuable source of carotenoids in winter period. According to our experimental data the content of total carotenoids varied from 10 to 40 g/100 g FW depending on the varieties or sorts. Servings of 100 g of *Lactuca sativa* and *Eruca sativa* can fully satisfy the daily human requirements of carotenoids.

The concentration of carotenoids in fruits and vegetables can be changed by environmental and genetic factors (Sandmann, 2001). Bioavailabity of the carotenoids from different sources can differ depending on their intracellular location, physical state and food matrix (Britton, 2009).

Nowadays, food products and cosmetics may contain not only natural carotenoids, but also synthetic ones. Five major carotenoids (lycopene, β-carotene, canthaxanthin, zeaxanthin and astaxanthin) are being synthetized commercially (DelCampo et al., 2007). However, there is data that consumption of synthetic carotenoids in high amounts is less efficient (Southon, 2000) or can be even harmful (Van den Berg et al., 2000).

Carotenoids are bioactive substances in foods with powerful antioxidant bioactivity. An antioxidant is a substance that significantly reduces the adverse effects of reactive oxygen species in normal physiological conditions in human beings (Food and Nutrition Board, 2000). The physiological activity of carotenoids can be studied from two different positions (Vilchez et al., 2011). In one case, they can be studied as antioxidants (McNulty et al., 2007). For example, Ranga Rao et al. (2010) showed that consumption of microalgae increases the levels of antioxidant enzymes such as catalase, superoxide dismutase and peroxidase levels in plasma and liver of mice. In the second case, the activity of carotenoids can be studied from the position of their ability to effect cell differentiation and proliferation (Bertram, 1999). It was suggested that lycopene from tomatoes could prevent negative effects of oxidative stress on the skeletal system by increasing osteoblast's cell proliferation and the activity of differentiation marker alkaline phosphatase (Kim et al., 2003).

In the human eye macula lutein and zeaxanthin, which are usually called the macular pigment (MP), maintain the normal visual function

(Le et al., 2010). They reduce photooxidative effects of excessive blue light and protect the macula from adverse photochemical reactions (Mozaffarieh et al., 2003). Nowadays macular degeneration and cataracts are among the main causes of blindness. The formation of these diseases is associated with low concentrations of the MP (Olmedilla et al., 2001; Friedman et al., 2004). The concentrations of lutein and zeaxanthin in the macula can be increased by consuming supplements or natural sources such as spinach and maize (Landrum and Bohne, 2008). It was counted that the delay for cataract formation for 10 years will not only help people to have strong eyes longer, but will also save the USA economics for 5-6 billion USD (Taylor and Hobbs, 2001).

Carotenoids have been reported to have anti-inflammatory properties (Bolin et al., 2010). It was stated that due to carotenoid's ability to scavenge ROS, lycopene is capable to protect sperm from oxidative damage, which is associated with male infertility (Iwasaki and Gagnon 1999). Palan and Naz (1996) showed that men with antibody-mediated infertility had lower concentrations of lycopene in their sperm. The long-term consumption of lycopene can improve the lycopene levels in serum as well as sperm motility, sperm motility index, sperm morphology and the concentration of the functional sperm (Rao and Rao, 2007).

Dietary β-carotene can also provide immunomodulatory effects by reducing the UV-induced immunosuppression (Fuller, 1992) and increasing activity of natural killer cell (Santos, 1996). In recent decades many studies have also reported the ability of carotenoids to protect skin against UV light (Aust et al., 2005). The possibility of carotenoids to suppress the development of neurodegenerative diseases including Alzheimer's disease is being discussed (Rao and Balachandra, 2003). The inverse relationships between the level of the plasma carotenoids and the oxidative damage marker lymphocyte 8-OHdG were revealed by Haegele et al. (2000). The same inverse relationship between the level of lymphocyte DNA 8-OHdG and plasma levels of carotenoids in patients with Alzheimer disease was observed by Mecocci et al. (2002).

Carotenoids may be used as an inexpensive substance to prevent the development of certain cardiovascular diseases (Riccioni, 2009), which are the leading cause of death in the developed countries and the main health problem in the developing countries (Rosamond et al., 2008). However, different studies showed conflicting results regarding possible positive role of carotenoids in reducing the development of cardiovascular diseases (CVD). Numerous studies supported the hypothesis that the consumption of food rich in carotenoids reduced the cardiovascular disease mortality (Ito et al., 2006). Karrpi et al. (2012) stated that only the concentrations of β-carotene decreased CVD mortally, while the concentrations of lutein, α-carotene and the total

sum of carotenoids didn't significantly effect this index. At the same time another Finnish study showed that consumption of β-carotene by the smokers with previous myocardial infarction significantly increased a risk of fatal coronary event (Rapola et al., 1997). It was reported that high levels of another carotenoid lycopene in plasma and tissues reduced a risk of coronary heart disease (Rissanen et al., 2002), myocardial infarction (Ojha et al., 2013) and arteriosclerosis (Klipstein-Grobush, 2000).

In recent time, the possible beneficial role of carotenoid consumption in the development of different forms of cancer is actively discussed (Kucuk et al., 2002; Schwarz et al., 2008). β-carotene, α-carotene, lycopene, lutein, zeaxanthin, β-cryptoxanthin, fucoxanthin, canthaxanthin and astaxanthin showed chemopreventive activity, anti-carcinogenic activity in several tissues (Tanaka et al., 2012). At the same time high doses of β-carotene did not show such activity in clinical trials.

24.2.2. Chlorophylls

Chlorophylls are a group of green plant pigments involved in the process of photosynthesis. Chlorophylls are also used as a food additive. Chlorophyllin, a chlorophyll derivative, is a food-coloring agent, which has the index E141. The acceptable daily intake (ADI) for copper complexes of chlorophylls and chlorophyllins is 15 mg/kg body per day. The main sources of chlorophylls are sugar confectionery, desserts, sauces and condiments, cheese and soft drinks. A large amount of chlorophylls can also be found in green vegetables (Table 3).

Table 3

Concentration of major carotenoids and total chlorophylls in green vegetables (g/100 g vegetable sample)

Vegetables	Total chlorophylls	Total carotenoids
Beans	8400	2090
Broccoli	32 800	8230
Chive	63 400	12 200
Green bell pepper	8700	2180
Lettuce (curly)	37 700	6150
Lettuce (feld)	56 200	11 500
Lettuce (roquette)	57 500	11 500
Parsley	106 000	23 200
Peas	11 300	3160
Spinach	52 100	15 600

Source: Larsen and Christensen 2005.

Chlorophylls have many benefits for human health. It was stated that chlorophylls and its derivatives are able to stimulate immune system, benefit against sinusitis, fluid buildup and skin rashes, help to fight with anemia, eliminate molds in the body, remove toxins from the body, help to prevent cancer, clean the intestines, help to rejuvenate and energize organism, detoxify the liver, normalize blood pressure, and remove odors (Inanc, 2011). Chlorophylls can also prevent the formation of

calcium oxalate stones, better known as kidney stones, by inhibiting the growth of calcium oxalate dihydrate (Tawashi et al., 1982). Many studies have shown that chlorophylls and its derivatives act as antioxidants in the dark (Hoshina, 1998; Ferruzzi et al., 2002). In the light they act as pro-oxidants (Endo et al., 1985; Wanasundara and Shahidi, 1998).

24.2.3. Anthocyanins

Anthocyanins are colored flavonoids, which are responsible for red, pink, purple, and blue colors observed in plants. They are not photosynthetic pigments though they may participate in screen of photosynthetic apparatus from high light.

There are around 400 individual anthocyanins in the nature (Mazza and Miniati, 1993; Wrolstad, 2000). It was measured that the daily human consumption of anthocyanins is around 180-215 mg in the USA (Kuhnau, 1976). Berries and red wine are the main sources of anthocyanins. Servings of 100 g of berries can provide up to 500 mg of anthocyanins (Mazza and Miniati, 1993). In general, the concentration of anthocyanins in most of the fruits and vegetables is around 0.1-1.0% per DW (Swain and Bate-Smith, 1962).

Information on the possible positive effects of anthocyanins on human vision was discussed in the review by Ghosh and Konishi (2007). No effects of anthocyanins' consumption on night vision were found (Zadok et al., 1999). However, the recent studies (Ohguro et al., 2013) showed that oral consumption of blackcurrant anthocyanins may perform a beneficial role on vision by decreasing the intraocular pressure in healthy subjects and patients with glaucoma and increasing the ocular blood flow in patients with open-angle glaucoma.

Several epidemiological studies showed that coronary heart disease mortality can be decreased by moderate consumption of red wine (Graziano et al., 1993; Klatsky, 1994). This effect is partly a result of the presence of anthocyanins in red wine (Kanner et al., 1994).

Anthocyanins are also able to perform anti-inflammatory effects. The ability of anthocyanins to inhibit the activation of Nuclear Factor-κB, which controls expression of genes involved in the inflammatory response and secretion of pro-inflammatory chemokines and cytokines, in human monocytes was shown by Karlsen et al. (2007). Several studies showed the ability of anthocyanins to suppress secretion of pro-inflammatory chemokines and immunoregulatory cytokines (Herath et al., 2003; Cimino et al., 2006). Due to their anti-inflammatory abilities the anthocyane flavonoids in the natural juice from *Aronia melanocarpa* were revealed to reduce allergic reactions in rats (Borissova, 1994). Bertuglia et al. (1995) showed that *Vaccinium myrtillus* anthocyanosides were able to reduce microvascular impairments due to ischemia reperfusion injury.

Anthocynians are able to prevent the development of atherosclerosis by inhibiting the oxidation of low-density lipoproteins (LDL) (Pawlowicz, 2000) and protecting the integrity of the endothelial cells that line blood-vessel walls (Tsuda, 1998). In addition, anthocyanins relax blood vessels.

Anthocyanins had positive effects on type 2 diabetes by stimulating the insulin secretion and protecting β-cells from oxidative damage (Sancho and Pastore, 2012). However, different anthocyanins have different effects on the insulin secretion or no significant effect at all (Jayaprakasam et al., 2005).

There are other possible benefits of anthocyanins to human beings. Kang et al. (2003) showed that anthocyanins and cyaniding reduced the cell growth of human colon cancer cell lines HT 29 and HCT 116. In other studies it was revealed that anthocyanins, isolated from *Vitis coignetiae,* induced apoptosis in human colon cancer HCT-116 cells by suppressing Akt and activating p38-MAPK and in human hepatoma cells (Shin et al., 2009a; Shin et al., 2009b).

Numerous studies showed that consumption of the berries rich in anthocyanins retards and even reverses age-related deficits in motor and cognitive performance (Joseph et al., 1999; Bickford et al., 2000; Shukitt-Hale et al., 2009).

The anthocyanins profiles can also be used to check the authenticity of different food products like prune juice and black cherry jam (Boyles et al., 1993; Garcia-Viguera et al., 1997).

24.3. Conclusions

Plants are sources of different compounds that are used to maintain human health. Natural pigments – carotenoids, chlorophylls and anthocyanins, are bioactive compounds. They are able to perform antioxidant abilities, anti-inflamattory effects, stimulate immune system and etc. The results of numerous studies indicate that a well-balanced diet, which includes different plant pigments, plays an important role in prevention and therapy of many chronic human diseases. Further studies are required to determine the biological properties of natural plant pigments and their role in human health maintenance more comprehensively.

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