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Light-independent chlorophyll biosynthesis in gymnosperms

Disertační práce
Autoreferát

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ZHRNUTIE

Predložená práca sa zameriava na biosyntézu chlorofylu v nahosemenných rastlinách, ktorých typickou črtou je schopnosť tvorby chlorofylu nielen na svetle, ale aj v tme. Túto schopnosť zabezpečuje prítomnosť od svetla nezávislej protochlorofylid oxidoreduktázy (DPOR), ktorá je medzi inými prítomná v nahosemenných rastlinách, kde spolu s od svetla závislou protochlorofylid oxidoreduktázou zohrávajú dôležitú funkciu.

Svoju pozornosť sme zamerali predovšetkým na smrek obyčajný (*Picea abies* Karst.) a v menšej miere na rastliny smrekovca opadavého (*Larix decidua* Mill.). Obzvlášť veľkú pozornosť sme v práci venovali teoretickým základom danej problematiky, ktorá je aj nosným pilierom autoreferátu. Navyše, v samotnej dizertačnej práci dosiahnuté výsledky dokumentujeme prostredníctvom priložených publikácii.

Záverom možno konštatovať, že biosyntéza chlorofylu a jej regulácia v nahosemenných rastlinách závisí od: konkrétneho rastlinného druhu, vývinového štádia a faktorov okolitého prostredia, ako sú svetelné podmienky, teplota a zloženie okolitej atmosféry, atď. Teda je možné povedať, že syntéza chlorofylu podlieha prísnej regulácii na viacerých úrovniach. V našich záveroch sme taktiež zhrnuli výsledky postupnej, od svetla závislej aktivácie superkomplexov fotosystému II a vývoja kyslíka.

SUMMARY

This thesis is focused on chlorophyll biosynthesis in gymnosperm plants which are characterized by ability of chlorophyll formation also in dark. This is achieved by the presence of light-independent protochlorophyllide oxidoreductase (DPOR) which is typical *inter alia* for gymnosperms and plays an important role in chlorophyll biosynthetic pathway together with light-dependent protochlorophyllide oxidoreductase (LPOR). We aimed our attention especially on Norway spruce (*Picea abies* Karst.) and European larch (*Larix decidua* Mill.) plants. A special attention is paid to theoretical background of current state of knowledge in this field, especially in this review. Custom dissertation thesis consists of theoretical background followed by our results, documented by attached papers. Generally we can conclude that chlorophyll biosynthesis and its regulation in the gymnosperm plants depend on: plant species, developmental stage and environmental factors, such as light conditions, temperature, as well as composition of ambient atmosphere. Thus, chlorophyll formation may be regulated on various levels. We also summarized our results of gradual, light-dependent formation of PSII supercomplexes and oxygen evolution.

PUBLICATION LIST

I. **Stolárik T, Hedtke B, Šantrůček J, Ilík P, Grimm B, Pavlovič A** (2017) Transcriptional and post-translational control of chlorophyll biosynthesis by dark-operative protochlorophyllide oxidoreductase in Norway spruce. Photosynth Res 132: 165-179.

II. **Pavlovič A, Stolárik T, Nosek L, Kouřil R, Ilík P** (2016) Light-induced gradual activation of photosystem II in dark-grown Norway spruce seedlings. BBA-Bioenergetics 1857: 799-809.

III. **Stolárik T, Nožková V, Nosek L, Pavlovič A** (2018) The advantage of chlorophyll biogenesis in the dark: comparative study with seedlings of European larch (*Larix decidua*) and Norway spruce (*Picea abies*). Trees: *under review*

IV. **Stolárik T, Henselová M, Martinka M, Novák O, Zahoranová A, Černák M** (2015) Effect of low-temperature plasma on the structure of seeds, growth and metabolism of endogenous phytohormones in pea (*Pisum sativum* L.). Plasma Chem Plasma Process 35: 659-676.

CONTENT

1 INTRODUCTION

Photosynthesis is a very unique process on Earth which annually converts approximately 258 billion tons of carbon dioxide (Geider et al., 2001). Chlorophyll (Chl) is one of the most abundant organic compounds on Earth and as a part of the photosynthetic machinery, it absorbs light and is involved in energy transfer in the process of photosynthesis (von Wettstein et al., 1995; Chen, 2014). The term is derived from the Greek words χλωρός – *chloros* ("green") and φύλλον – *phyllon* ("leaf") and it was first isolated and named by French chemists J. B. Caventou and P. J. Pelletier (1817). The composition of the chlorophyll molecule (and also other photosynthetic pigments), its biosynthetic pathway and its regulation have been widely studied for decades, especially in angiosperm plants. However, chlorophyll biosynthesis in gymnosperms is still partially shrouded in mystery.

This doctoral thesis is focused on chlorophyll biosynthesis in gymnosperms, especially in two representatives of coniferous plants (also known as Pinophyta): Norway spruce (*Picea abies* Karst.) and European larch (*Larix decidua* Mill.). Besides a huge evolutionary role of conifers, as they have dominated forests for more than 200 million years, they also have ecological and economical importance (Nystedt et al., 2013). Gymnosperm plants (including conifers) also seemed to be an interesting target for research due to further peculiarities, e. g. the chlorophyll biosynthetic pathway. Already Morren (1858) noticed the ability of few photosynthetic organisms to synthesize chlorophyll in darkness and further investigations revealed the important role of protochlorophyllide (Pchlide) reduction for effective production of chlorophylls. In contrast to angiosperm plants, which use only light-dependent protochlorophyllide oxidoreductase (LPOR) for the reduction of Pchlide to chlorophyllide (Chlide) – precursors of Chl (Schoefs and Franck, 2003; Reinbothe et al., 2010), gymnosperms are also able to use a nitrogenase-like, light-independent protochlorophyllide oxidoreductase (DPOR) (Armstrong, 1998; Reinbothe et al., 2010).

In this study we focused on the effect of few regulatory and ecophysiological factors such as light, temperature, developmental stage, oxygen content in ambient air, etc. which are considered to affect chlorophyll biosynthesis. We focused our attention also on chlorophyll biosynthetic pathway, its regulation on transcriptional, translational and post-translational levels, structure of photosystems, formation of photosystem II supercomplexes, potential changes in plastid ultrastructure, and, the whole photosynthetic processes in general (see Chapter 8 – Publications). The first part of my doctoral thesis offers the complex insight into

the topic and summarizes recent scientific findings in this field. The second – supplementary part – is composed of my scientific papers.

2 ASSIMILATION PIGMENTS

Oxygenic photosynthesis requires chlorophyll (Chl) for absorption and transduction of light energy, and charge separation in reaction centres of photosystems I and II (PSI and PSII) to supply the electron-transport chain with electrons. Chlorophyll is bound to Chl-binding proteins which are assembled in the core complexes of PSI and PSII and their peripheral light-harvesting antenna complexes (Wang and Grimm, 2015), where they occur together with carotenoids. Besides their light-harvesting function, carotenoids have also a photoprotective function (DellaPenna and Pogson, 2006). Bacteriochlorophylls as well as bilins were also described in several photosynthetic organisms, e. g. photosynthetic bacteria, cyanobacteria, etc. (Blankenship, 2008).

2.1 Chlorophylls

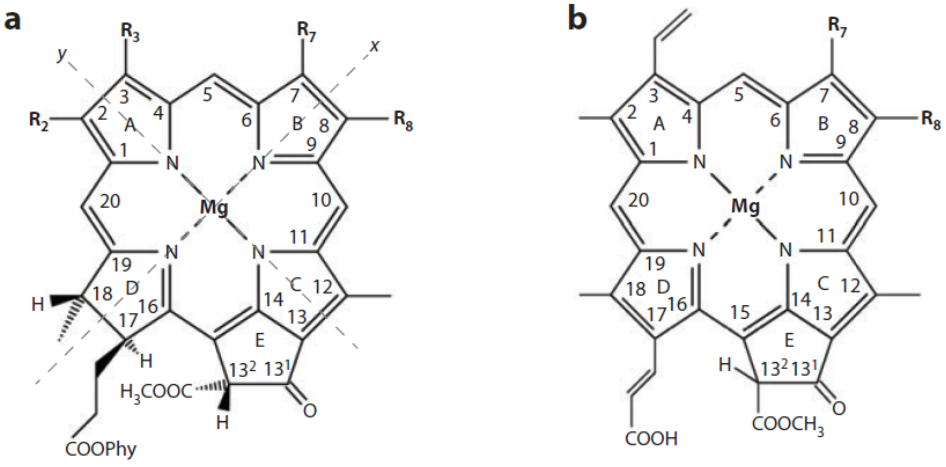
Chlorophylls are organic molecules, structurally based on tetrapyrroles; although chlorins would be a more concise term. This discrepancy is caused by the presence (tetrapyrrole molecule) or absence (chlorine molecule) of double bond between C-17 and C-18 carbon atoms in D-ring of the molecule (Fig. 1, Juselius and Sundholm, 2000). In the centre of the chlorine ring is magnesium (Mg) bound by coordinate covalent bond (Willstätter, 1906). All chlorophylls have a similar five-membered ring structure (pyrrole rings designated as A, B, C, D and E – isocyclic), with variations in side chains and/or reduction states (Chen, 2014). We know five types of chlorophyll molecule: Chl *a* (or 8-vinyl Chl *a*), Chl *b* (or 8-vinyl Chl *b*), Chl *c*, Chl *d* and Chl*f*. Each type of chlorophyll is characterized by different absorption spectra of the sunlight which enables the photosynthetic organisms to colonize a vast range of environments (Porra, 1989; Chen, 2014).

Chl *a* is the most abundant of all chlorophylls. The general structure of Chl *a* was elucidated by Hans Fischer in 1940 (Fischer and Orth, 1940). Chlorophyll *a* is present in the reaction centre (RC) and light-harvesting complexes (LHCs) of almost all oxygenic photosynthetic organisms including cyanobacteria, algae, and higher plants (Björn et al., 2009). The absorption spectrum of Chl *a* in 100% methanol is shown in Fig. 2. Various taxa of photosynthetic organisms contain different sets of light harvesting Chls. For example, Chl *a* occurs in glaucophytes, and in red algae together with Chl *d*. Chlorophylls *a*, *b*, *d* and [8-vinyl]-Chls *a* and *b* are present in cyanobacteria, Chls *a* and *b* in green algae and higher

plants, and Chl *a* and *c* in chromophytic algae (Govindjee and Satoh 1983; Murakami et al. 2004; Grimm et al. 2006; Scheer 2006). As mentioned above, functional groups (mainly formyl group – Fig. 1) bound in different positions cause different spectral properties, and the absorption maxima of chlorophylls are significantly shifted. This is documented in Fig. 2.

Chlorophyll *b* is characteristic for its formyl substitution in C-7 position and is considered as the second most abundant chlorophyll in oxygenic photosynthetic organisms (Chen, 2014). It functions as an accessory chlorophyll in the light-harvesting complexes, although it is not capable to act as so-called special pair in the reaction centre (role of Chl *a*) and primarily absorbs blue light (Fig. 2) (Chen, 2014). Interconversion between Chl *a* and Chl *b* (known as the chlorophyll cycle) may provide higher plants the ability to optimize light harvesting and thus, acclimation to varying light conditions, e. g. shade or a straight light irradiation (Kitajima and Hogan, 2003; Chen, 2014).

Chlorophyll *c* has not been found in vascular plants but it was described in (marine) algae including the photosynthetic Chromista (e. g. diatoms, brown algae) (Jeffrey and Vesk, 1997). Chlorophyll *c* has fully unsaturated porphyrin (Fig. 1b) which makes it different from all the other chlorophylls. It plays a role as light-harvesting pigment. There are several structural



Chlorin type

Chlorophylls	R_2	R_3	R_7	R_8	Q_y (nm)
Chl *a*	CH_3	$CH{=}CH_2$	CH_3	$CH_2{-}CH_3$	665*
8-vinyl Chl *a* (Chl a_2)	CH_3	$CH{=}CH_2$	CH_3	$CH{=}CH_2$	666#
Chl *b*	CH_3	$CH{=}CH_2$	CHO	$CH_2{-}CH_3$	652*
8-vinyl Chl *b* (Chl b_2)	CH_3	$CH{=}CH_2$	CHO	$CH{=}CH_2$	658#
Chl *d*	CH_3	CHO	CH_3	$CH_2{-}CH_3$	696*
Chl *f*	CHO	$CH{=}CH_2$	CH_3	$CH_2{-}CH_3$	707*

Porphyrin type

Chl *c*	R_7	R_8
Chl *c*1	CH_3	$CH_2{-}CH_3$
Chl *c*2	CH_3	$CH{=}CH_2$
Chl *c*3	$COOCH_3$	$CH{=}CH_2$

Fig. 1 Chemical structure of chlorophylls (Chls). **a:** Chemical structure of chlorophylls which have chlorine type macrocycles. **b:** Chemical structure of chlorophyll *c* family, the members of which are of the porphyrin type. Chlorin-type Chls are esterified by phytyl chain (Phy) of $C_{20}H_{39}$. **Chlorophyll *b***, **Chl *d*** and **Chl *f*** have **formyl group** substitutions at positions C-7, C-3 and C-2, respectively. The structural differences are red-coloured. $\mathbf{Q}_y$ means maximal red absorption (nm): asterisk-marked were recorded in 100% methanol; pound-marked in a mixture of methanol and acetone. (Modified according to Chen, 2014).

variations of Chl *c* (designated as c_1, c_2, c_3) which differ in substituents located at atoms C-7 and C-8 of B-ring (Fig. 1b). It has a blue-greenish colour and is an accessory pigment, particularly significant in its absorption of the light in blue wavelength region (approx. 447 – 452 nm) (Dougherty et al., 1970). This is also a reason why it occurs mainly in photosynthetic organisms living in great depths, with blue light penetrating water the furthest.

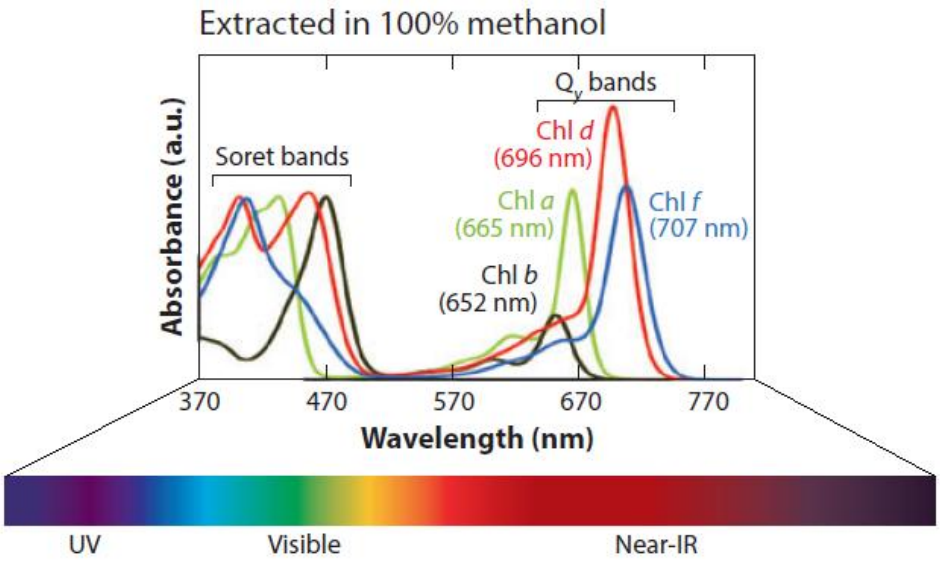


Fig. 2 Absorption spectra of chlorine-type chlorophylls in 100 % methanol (Chl *a*, Chl *b*, Chl *d* and Chl *f*). Soret-band maxima are arbitrarily scaled to a common height for comparison. (Modified according to Chen, 2014).

Chlorophyll *d* is a form of chlorophyll identified by Manning and Strain (1943). However, it was considered as an artificial product for more than 50 years until the discovery of a novel cyanobacterium *Acaryochloris marina* in 1996 (Miyasita et al., 1996). This confounded the traditional thinking about the irreplaceable function of Chl *a* in photosynthetic organisms, as >95% of all chlorophylls in this cyanobacterium was represented by Chl *d* (the content of Chl *a* was approx. 5%). The only structural difference between Chl *a* and Chl *d* is at C-3 position in A-ring, where vinyl group is replaced by formyl group (Fig. 1a) (Chen, 2014). Noticeably, the absorption maxima of Chl *d* are similar to bacteriochlorophyll *a* because it absorbs the light close to UV and IR spectral parts. It was also found that Chl *a* could be a precursor of Chl *d* (Schliep et al., 2010).

The most recently discovered type of chlorophyll is Chl *f* found in stromatolites (cyanobacterium *Halomicronema hongdechloris*) from Western Australia's Shark Bay (Chen et al., 2010). The molecule of Chl *f* is structurally related to Chl *a*, however in C-2 position of A-ring, a formyl group is present instead of a methyl group. The absorption maxima of Chl *f*

are significantly red-shifted (Fig. 2) and the functions of this chlorophyll type remain more or less unknown (Chen et al., 2012). The biosynthetic pathway of chlorophylls is described in detail in chapter 3.

2.2 Bacteriochlorophylls

Bacteriochlorophylls (Bchl/-s) differ from Chls by a substitution of vinyl group for acetyl group at C-3 of A-ring as well as a reduced double bond between C-7 and C-8 of B-ring. These substitutions offer the Bchls different spectral properties, mainly a shift of absorption maxima closer to UV and IR region (Grimm et al., 2006). These assimilation pigments occur in various phototrophic bacteria, and currently there are known bacteriochlorophyll *a, b, c, d, e* and *g*. Although Bchl *f* (20-desmethyl-Bchl *e*) was recently found only through mutation, we cannot exclude that it may exist naturally (Vogl et al., 2012). Authors prepared the mutant of green sulfur bacterium *Chlorobaculum limnaeum*, where *bchU* gene (bacteriochlorophyllide C-20 methyltransferase) was inactivated. The resulting *bchU* mutant synthesized Bchl f_F and no Bchl *e* was detected. The bacteria with Bchl*f* chlorosomes were much slower in growth, and the energy transfer from Bchl *f* aggregates to Bchl *a* was much less effective in comparison to WT. This may be a reason why Bchl *f* was not yet found in the nature in comparison with other bacteriochlorophylls of phototrophic bacteria.

Because my thesis is not focused on bacteriochlorophylls and anoxygenic photosynthesis, further information about Bchls is not provided here but can be found elsewhere (see Blankenship, 2008). However, it is possible to find Bchl-related partial information in the next chapters.

2.3 Bilins

Bilins are open tetrapyrrole molecules which occur in light-harvesting complexes (known as phycobilisomes) of cyanobacteria and red algae. Interestingly, they absorb the light between 550 – 650 nm, and the most important bilins are phycoerythrobilin and phycocyanobilin. They are synthesized through the Fe-branch of tetrapyrrole biosynthesis (Blakenship, 2008).

2.4 Carotenoids

Carotenoids are lipid-soluble compounds which play an important role in many photosynthetic processes, (e. g. light-harvesting, dissipation of excess energy and photoprotection through xanthophyll cycle).

Carotenoids are derivates of tetraterpenes, meaning that they are produced from 8 isoprene molecules and contain 40 carbon atoms (Armstrong and Hearst, 1996; DellaPenna and Pogson, 2006).

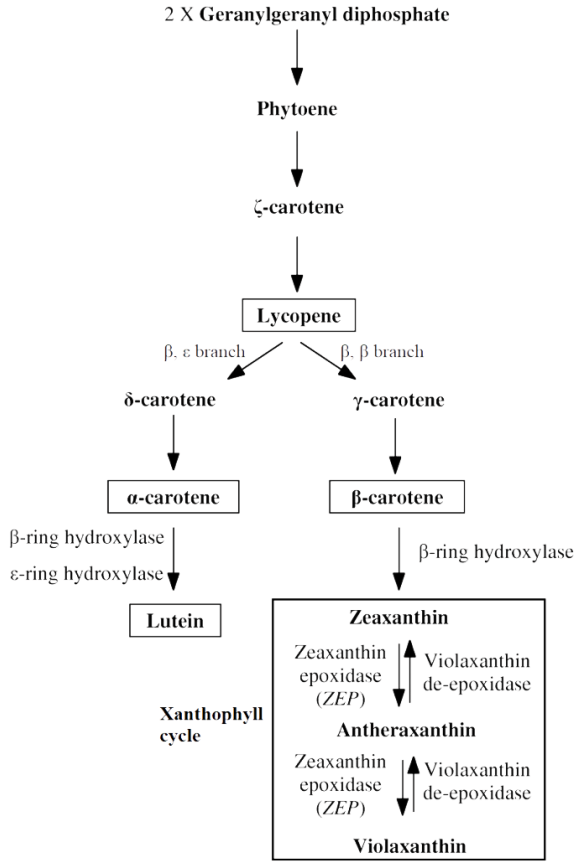


Fig. 3 Carotenoids biosynthetic pathway (modified according to Clotault et al., 2008). Inset shows xanthophyll cycle scheme in the right down part of the figure.

Carotenoids can be divided into two basic groups: carotenes and xanthophylls. Carotenes are pure hydrocarbons containing no oxygen (α-, β-carotene, lycopene, etc.), and on the other hand, xanthophylls contain oxygen in their molecules (lutein, violaxanthin, antheraxanthin, zeaxanthin, fukoxanthin, etc.).

In the biosynthetic pathway of carotenoids, the first committed step is the condensation of two molecules of geranylgeranyl diphosphate (GGDP) to produce phytoene (Fig. 3). Interestingly, GGDP can be used not only for phytoene synthesis, but also plays an

important role in the synthesis of chlorophylls, phylloquinone, gibberellins, and one branch of synthesis also leads to tocopherols (DellaPenna and Pogson, 2006). The carotenoid biosynthetic pathway continues through few intermediates to lycopene where the pathway divides into two branches characterized by different cyclic end-groups. Two beta rings lead to the β, β branch (β-carotene and its derivates: zeaxanthin, violaxanthin, antheraxanthin and neoxanthin), whilst one beta and one epsilon ring define the β, ε branch (α-carotene and its derivates). As briefly mentioned above, carotenoids perform various functions in plants. They are involved in photosystem assembly, light-harvesting and photoprotection, photomorphogenesis, non-photochemical quenching, lipid peroxidation and they also affect the function and size of light-harvesting antennae (Lokstein et al., 2002; Holt et al., 2005; DellaPenna and Pogson, 2006). Carotenoids are bound to proteins in the membranes and co-form LHC as accessory pigments. They absorb light energy between 400 – 500 nm and transmit it into the reaction centres. To avoid photooxidative damage of photosystems, xanthophylls provide very effective tool for excess energy dissipation, called xanthophyll cycle (Fig. 3). An epoxide group is introduced into both rings of zeaxanthin by zeaxanthin-epoxidase to form violaxanthin. Under high light stress which acidifies the lumen of thylakoids, violaxanthin deepoxidase is activated, resulting in increased levels of zeaxanthin (Niyogi, 1999). Finally, zeaxanthin dissipates excess of energy as heat (energy is not transferred to Chls).

Another important attribute of carotenoids is their ability to be synthesized also in the darkness and they are localized in tubular membranes around prolamellar bodies (PLB) as well as straightly in PLBs of etioplasts (DellaPenna and Pogson, 2006; Cuttriss et al., 2007).

3 CHLOROPHYLL FORMATION

The biosynthetic pathway of chlorophylls is a very complex process with several regulatory steps (Papenbrock and Grimm, 2001) and it has been well established in higher plants. However, some details of its compartmentalization and regulation remain obscure (Chen, 2014). The main steps of Chl biosynthesis are common for the majority of photosynthetic organisms, and the important differences and regulatory peculiarities between gymnosperm and angiosperm plants are described further.

3.1 General scheme of tetrapyrrole biosynthesis

The first steps of tetrapyrrole biosynthetic pathway are shared among chlorophylls, heme, siroheme and cobalamin (Chen, 2014). The pathway can be divided into four main parts: **1.** formation of 5-aminolevulinic acid (ALA); **2.** formation of protoporphyrin IX (Proto IX) from eight molecules of ALA; **3.** Mg-porphyrin branch leading to chlorophylls; **4.** heme-synthesizing branch (Papenbrock and Grimm, 2001). The scheme of this biosynthetic pathway is shown in Fig. 4.

At least two distinct pathways of ALA formation are known in the nature. Both of them were described in the phytoflagellate *Euglena gracilis* (Weinstein and Beale, 1983). One pathway is known as $\mathbf{C_4}$ pathway and occurs in animals, fungi and certain groups of bacteria, notably *Rhodobacter*, *Rhodospirillum* and *Rhizobium* (von Wettstein et al., 1995; Papenbrock and Grimm, 2001). This pathway utilizes a condensation reaction of glycine and **C4** moiety succinyl-CoA, catalyzed by pyroxidal phosphate-dependent enzyme – ALA-synthase (ALAS) (von Wettstein et al., 1995; Papenbrock and Grimm, 2001). The other, a three-step pathway is characteristic for the majority of bacteria, the Archaea, algae, and plants, and is called $\mathbf{C_5}$ pathway (Jordan, 1991; Papenbrock and Grimm, 2001), where ALA is derived from a **C5**-skeleton of glutamate. Glutamate is introduced by acetylation to $tRNA^{Glu}$ by glutamyl-tRNA synthetase (GluRS) and subsequently reduced by glutamyl-tRNA reductase (GluTR). Transamination is catalysed by glutamate-1-semialdehyde aminotransferase (GSAT) and it results in the formation of ALA (Fig. 4) (Friedmann et al., 1987; von Wettstein et al., 1995).

The part of the pathway between ALA and the tetrapyrrole Proto IX is highly conversed among all organisms. ALA-dehydratase (ALAD) condenses two molecules of ALA

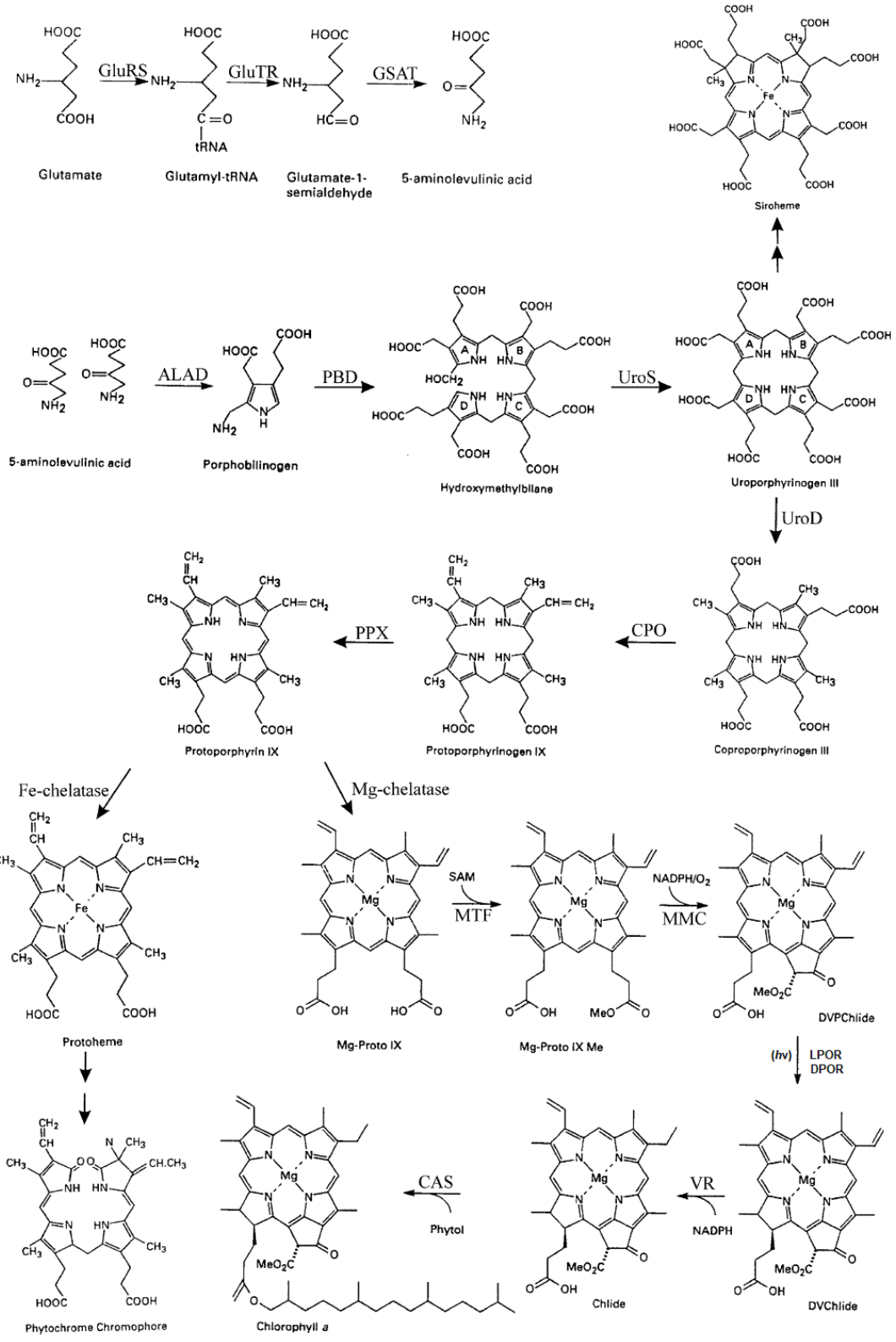


Fig. 4 Schematic diagram of the tetrapyrrole biosynthetic pathway. Eight units of 5-aminolevulinate form the tetrapyrrole skeleton. All intermediates from the glutamate to the plant tetrapyrrole end products are shown. The abbreviations of involved enzymes are explained within the text describing the biosynthetic pathway in Chapter 3. 1. (Modified according to Papenbrock and Grimm, 2001).

into monopyrrole porphobilinogen. Four molecules of porphobilinogen are polymerised to form, at first, a linear tetrapyrrole molecule – 1-hydroxymethylbilane using porphobilinogen deaminase (PBD). After an immediate isomerisation of this linear molecule, a ring molecule uroporphyrinogen III is arisen. The reaction is catalysed by uroporphyrinogen III synthase (UroS) (Papenbrock and Grimm, 2001). At this level, biosynthetic pathway branches for the first time. Methylation of uroporphyrinogen III leads to the synthesis of siroheme or (bacterial) cobalamin (vitamin B_{12}). On the other hand, biosynthesis may follow the way to Chl and heme, where the side chains of the porphyrin ring are further decarboxylated and oxidised to form protoporphyrin IX (Proto IX). That proceeds through a few intermediates: from uroporphyrinogen III to coproporphyrinogen III (catalyzed by uroporphyrinogen III decarboxylase – UroD), then it continues to protoporphyrinogen III (catalyzed by coproporphyrinogen oxidase – CPO) and finally to Proto IX, where the reaction is catalyzed by protoporphyrinogen oxidase (PPX) (Papenbrock and Grimm, 2001). Proto IX is the last common molecule in Chl and heme biosynthesis. At this step, the biosynthetic pathway branches, where metal chelation reactions of Proto IX catalyzed either by Fe-chelatase or Mg-chelatase divide the pathway into Fe-branch and Mg-branch, leading to heme and Chl formation, respectively (Papenbrock and Grimm, 2001; Tanaka and Tanaka, 2007). This part of the pathway is considered as an important regulatory point.

Fe-chelatase inserts Fe^{2+} into Proto IX in a reaction that does not require an input of energy, and is inhibited by the presence of ATP. On the contrary, the insertion of Mg^{2+} by Mg-protoporphyrin IX chelatase (Mg-chelatase) requires the presence of significant amount of energy, because the hydrolysis of ~15 ATP molecules per one metal ion insertion is required (Reid and Hunter, 2004). Recently, two isoforms of Fe-chelatase were described (FC1 or FeCh I and FC2 or FeCh II) and it was suggested that the individual isoforms contribute to heme biosynthesis in a different way. FC1 and FC2 most likely supply heme to different sets of heme-dependent proteins. FC1 may be in charge of heme synthesis for cytoplasmic, and more generally, extraplastidal heme-dependent proteins, whilst FC2 might be responsible for heme supply to the plastid-localized proteins requiring heme (Koch et al., 2004; Wang and Grimm, 2015). Moreover, FC2 possesses a light-harvesting Chl-binding (LHC) domain at the C-terminus, which may play a regulatory role in heme synthesis in photosynthetic plastids rather than in heme synthesis for other cellular heme-requiring proteins (Sobotka et al., 2011). Finally, the insertion of Fe^{2+} leads to the formation of protoheme and subsequently different types of heme, or to linear tetrapyrrole phytochromobilin (Papenbrock and Grimm, 2001).

Mg-chelatase is an enzyme which catalyzes the insertion of Mg^{2+} to Proto IX. This enzyme belongs to AAA^{+}-type chelatases and is composed of three subunits: H, I, and D. These subunits are conserved among species from cyanobacteria to higher plants (ChlH, ChlI, and ChlD); in bacteriochlorophyll biosynthesis they are commonly referred to as BchH, BchI, and BchD (Chew and Bryant, 2007). These three subunits are weakly associated with one another, and the three-subunit Mg-chelatase complex is relatively unstable (Reid and Hunter, 2004). The function of ChlD may be to provide a stable platform for ChlI subunits and form ChlI/ChlD polymeric complex. The binding of ATP (not hydrolysis) is important for the activation of the ChlI/ChlD complex (Jensen et al., 1999). This complex further interacts with the ChlH subunit and drives the ATP-dependent insertion of Mg^{2+} into Proto IX. ChlH is sensitive to the changing concentration of Mg^{2+}, which is reflected by the translocation of ChlH between chloroplast stroma and chloroplast envelope (Nakayama et al., 1998). Energy availability (besides other regulatory factors) is an important factor affecting the direction of the biosynthetic pathway – either to Chls or to hemes.

After the insertion of Mg^{2+}, Mg-protoporphyrin IX is created and immediately methylated by Mg-protoporphyrin IX methyltransferase (MTF) to Mg-protoporphyrin IX monomethylester. In a further step, Mg-protoporphyrin IX monomethyl ester cyclase (MMC) catalyzes the incorporation of atomic oxygen to Mg-protoporphyrin IX monomethylester (in case of oxygenic photosynthetic organisms) (Papenbrock and Grimm, 2001). This oxidative cyclization creates the E-ring, a distinctive isocyclic ring of all chlorophylls. Moreover, this ring structure is the unique characteristic of chlorophylls, in comparison to all other tetrapyrroles. Interestingly, in photosynthetic anoxygenic bacteria (e. g. green sulfur bacteria), a non-oxidizing cyclization mechanism unrelated to the oxidizing cyclization was also described (Ouchane et al., 2004). The anaerobic enzyme is a radical S-adenosyl-L-methionine (SAM) enzyme, and its crucial role in the regulatory mechanism is well described in Chapter 4. 1, part "Checkpoint at Proto IX level". The product of this reaction is divinyl protochlorophyllide (DV-Pchlide). There are known several models for the further fate of DV-Pchlide based on the reduction of 8-vinyl group on C-8 position of B-ring, since the activities of 8-vinyl reductase have been detected at five various levels: 1. Mg-proto IX monomethyl ester; 2. Mg-divinyl protochlorophyllide; 3. protochlorophyllide *a*; 4. chlorophyllide *a* and 5. chlorophyll *a* (Wang et al., 2013). (Note: Chlorophyllide *a* and chlorophyll *a* are final products of the biosynthetic pathway). However, the 8-vinyl reductases from different species show diverse and differing substrate preferences. There are more than five various 8-vinyl reductases with different reductive activities on the same or on different

substrates (Wang et al., 2013). Interestingly, marine *Prochlorococcus* species lack the reductase for vinyl group at C-8 position, hence they contain 8-vinyl Chl *a* and 8-vinyl-Chl *b* (Goericke and Repeta, 1992).

The next step of the pathway to chlorophylls is the reduction of protochlorophllide (Pchlide) to chlorophyllide (Chlide). According to the information mentioned above, DV-Pchlide as well as MV-Pchlide (monovinyl protochlorophyllide) may be reduced. This reduction is catalyzed by protochlorophyllide oxidoreductase (POR). Two different, unrelated forms of this enzyme have been described, one is dark-operative light-independent (DPOR), which does not require light for its function, and the other one is light-dependent NADPH protochlorophyllide oxidoreductase (LPOR). Both these enzymes reduce a double bond between C-17 and C-18 of D-ring (Fig. 5). [VR in the scheme (Fig. 4) means 8-vinyl reductase, if the vinyl group is reduced after the step of Pchlide conversion to Chlide] (Papenbrock and Grimm, 2001; Chen, 2014). Complex information regarding LPOR and DPOR distribution among organisms, structure, reduction mechanism etc. is stated in subchapters 3. 1. 1 and 3. 1. 2.

In the last step of chlorophyll biosynthetic pathway, Chlide *a* can be esterified with geranylgeranyl-pyrophosphate or phytyl-pyrophosphate by Chl synthase (Chl *a* synthase – CAS in this case). Then, it can be (partially) converted to Chl *b* by Chl *a* oxygenase (CAO) (Papenbrock and Grimm, 2001). CAO enzyme is a Rieske FeS centre-containing, non-heme-Fe monooxygenase that uses molecular oxygen and NADPH to perform two successive hydroxylations at the C-7 position of Chlide *a* (Tanaka et al., 1998). The gene encoding CAO is located in the nucleus and mature CAO protein can be found in thylakoid membranes. It is also possible that Chl *b* may be firstly generated *via* (P)Chlide *b* and it is esterified subsequently. Chl *a* and Chl *b* underlie the so-called chlorophyll cycle where Chl *b* can be reversibly converted to Chl *a*. Generally, Chl *a* (or Chlide *a*) is the precursor molecule for other types of chlorophylls – Chl *b*, Chl *d* and Chl *f*. However, the biosynthetic pathway leading to Chl *c* as the end product remains to be elucidated. Moreover, Larkum (2006) reported that Chl *c* may be the evolutionary precursor of chlorophylls. It also worth to mention that from Chlide *a*, bacteriochlorophyllide *a* – Bchlide *a* (the precursor of **bacteriochlorophyll *a*** – Bchl *a*) is formed by reduction of the double bond between C-7 and C-8 of B-ring; and this reduction is catalyzed by Chlide-oxidoreductase (COR; also known as chlorine-reductase). The reduction of the double bond in B-ring is coupled with hydration and oxidation of the 3-vinyl side group (Oster et al., 1997). COR is encoded by *bchX*, *bchY* and

bchZ genes which are similar to genes encoding the bacterial nitrogenase (which is also similar to DPOR enzyme) (Burke et al., 1993; Armstrong, 1998).

In the next two subchapters, we focus our interest on the crucial regulatory step in the chlorophyll biosynthetic pathway – protochlorophyllide reduction. This is realized by protochlorophyllide oxidoreductases: LPOR and/or DPOR.

3.1.1 Light-dependent protochlorophyllide oxidoreductase – LPOR

Light- and NADPH-dependent protochlorophyllide oxidoreductase (LPOR) (EC 1.3.33.1) is one of the very few enzymes that require light for their catalytic activity, thus it belongs to the group of photoenzymes. Another well-known photoenzyme is DNA photolyase (Begley, 1994; Reinbothe et al., 2010; Gabruk and Mysliwa-Kurdziel, 2015). LPOR is the only enzyme responsible for Pchlide reduction in angiosperm plants but it occurs together with the dark-operative POR (DPOR) in almost all evolutionary lower photosynthetic organisms, i. e. cyanobacteria, green algae and non-flowering plants: mosses, liverworts, hornworts, lycophyta, ferns, as well as gymnosperms (Armstrong, 1998; Fujita and Bauer, 2003; Yamamoto et al., 2011). Moreover, a gene encoding LPOR was also detected in anoxygenic photosynthetic bacteria and apparently introduced into their genome by horizontal gene transfer (Kaschner et al., 2014). Both of these enzymes catalyze the same stereospecific double bond reduction between C-17 and C-18 in D-ring of Pchlide (Fig. 5). LPOR is nuclear-encoded single-polypeptide enzyme that is post-translationally translocated to the plastids (Chen, 2014) and shows a high degree of similarity to the short-chain dehydrogenase-reductase (SDR) family. LPOR is present in high levels as a ternary complex with its substrate – photoactive Pchlide (emission at 655 nm), and with NADPH forming prolamellar bodies of etioplasts (Fujita, 1996; Schoefs and Franck, 2003; Reinbothe et al., 2010). Non-photoactive Pchlide has an emission maximum at 633 nm and serves as a precursor for photoactive Pchlide (Schoefs and Franck, 1998). Interestingly, although LPOR is not phylogenetically related to DPOR, they share a common sequence motif (a TFT motif) of unknown function (Gabruk and Mysliwa-Kurdziel, 2015). However, unlike genes encoding DPOR, several genes of LPOR isoforms may be present within one genome. This can be a result of horizontal gene transfer and/or genome duplication in dinoflagellates, achniophytes, and stramenopiles (Hunsperger et al., 2015). In land plants, the origin of multiple LPOR copies is unknown. In cucumber and pea, only one isoform of LPOR gene is present (Fusada et al., 2000), and its expression is positively photoregulated and remains unchanged during

greening and development. On the other hand, two LPOR isoforms are usually present in many land plants (e. g. *Hordeum vulgare*, *Pinus taeda*), known as LPORA (*por*A) and LPORB (*por*B) or additionally, also LPORC (*por*C) in *Arabidopsis thaliana* (Oosawa et al., 2000, Su et al., 2001). It is known, that expression level of LPOR isoforms differ in response to illumination: LPORA expression is strongly downregulated by light, however, LPORB is almost light-insensitive (Armstrong et al., 1995). LPORC is a dominant form in green mature tissues of *A. thaliana* and its expression is upregulated by high light irradiation (Oosawa et al., 2000, Masuda et al., 2003). Skinner and Timko (1998) reported that in contrast to angiosperms where LPOR is encoded by small nuclear gene family which contains two differentially expressed genes (*por*A and *por*B); for gymnosperm plants, a large multigene family is typical, being composed of two distinct subfamilies encoding *por*A and *por*B genes similar to those previously described in angiosperms. Surprisingly, for example in *Pinus taeda* these two *por* subfamilies are duplicated differently; *por*A family consist of two members, whilst *por*B contains at least 11 members (Skinner and Timko, 1998). LPORA and LPORB proteins show a size of 37 and 38 kDa, respectively (Skinner and Timko, 1998).

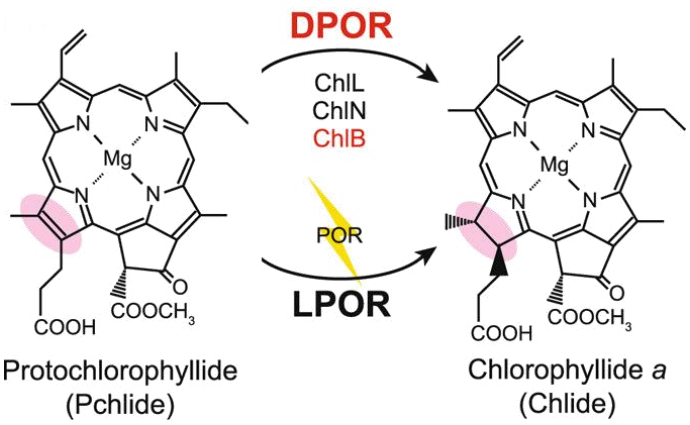


Fig. 5 The scheme shows a reduction of the double bond between C-17 and C-18 in D-ring of Pchlide (marked by purple oval); catalyzed by light-independent (DPOR) or light-dependent (LPOR) protochlorophyllide oxidoreductase. (Modified according Yamamoto et al., 2017).

As mentioned above, LPOR is a nucleus-encoded protein, post-translationally imported and localized in plastids (Fujita, 1996). Thus, the transport of the enzyme into plastids is guided by a transit peptide, located on its N-terminus (Gabruk and Mysliwa-Kurdziel, 2015). In barley which have two LPOR isoforms, it was investigated that in the structure of LPORA, there is a specific amino-acid motif of the transit peptide, crucial for the proper import of the enzyme into etioplasts. Thus, LPORA is predominantly imported into

etioplasts (with the presence of PLBs), and it was hypothesized that Pchlide binding by this transit peptide is required for the translocation of the enzyme. In contrast, the lack of this specific motif in the transit peptide of LPORB indicates, that LPORB does not require Pchlide for its transport into plastids – thus, may be located also in mature chloroplasts (Reinbothe et al., 2008).

The molecular mechanism of Pchlide reduction by LPOR remains to be elucidated, hence it is a target of intensive studies. However, it was shown that two photons are required for a single Pchlide molecule reaction, and the picosecond scale dynamics of the reaction was determined. It was also recently documented that Pchlide reduction consists of at least three major steps: **1.** light absorption; **2.** hydride transfer from the *pro*-S face of the nicotinamide ring of NADPH to C-17 in D-ring of Pchlide; **3.** proton transfer probably from Tyr280 to C-18 of Pchlide. Thus, light absorption promotes intramolecular charge transfer along the C-17 – C-18 bond, which allows subsequent events: the reduction of the mentioned double bond where NADPH serves as electron donor in the reaction (Wilks and Timko, 1995; Heyes et al., 2015). It was also investigated that LPOR may oligomerize which positively influences the effectiveness of the reaction (Gabruk et al., 2015).

3.1.2 Light-independent protochlorophyllide oxidoreductase – DPOR

The majority of gymnosperm plants, mosses, green algae, cyanobacteria, as well as anoxygenic photosynthetic bacteria are able to synthesize Chl also in the dark. This is ensured by light-independent Pchlide reduction, provided by dark-operative POR (or light-independent POR; DPOR) (Fujita, 1996). Phylogenetically, DPOR is the oldest enzyme that catalyzes Pchlide reduction. It is a plastid-encoded multisubunit complex which consists of three different polypeptides: ChlL, ChlN and ChlB, which form ChlL-dimer and ChlNB-heterotetramer. The average molecular weight of ChlL, ChlN and ChlB is 31 kDa, 61 kDa and 57 kDa, respectively. DPOR genes are extraordinarily conserved (Gabruk and Mysliwa-Kurdziel, 2015), and the three subunits of DPOR (L, N, B) remain almost unchanged from cyanobacteria to higher plants (Chen, 2014). The three subunits are responsible for the bacteriochlorophyll synthesis referred to as BchL, BchN and BchB which are orthologs of ChlL, ChlN and ChlB (Fujita, 1996; Reinbothe et al., 2010), whose amino acid sequences show significant similarities to *nif*H, *nif*D and *nif*K, respectively. These genes encode the subunits of bacterial nitrogenase (Raymond et al., 2004). ChlL protein and ChlNB proteins

are functionally equivalent to Fe protein and MoFe proteins, respectively, which are present in nitrogenase. Thus, ChlL (and Fe) proteins serve as an ATP-dependent electron donor for ChlNB (MoFe) proteins. ChlNB (as well as MoFe) represents the catalytic component, and provides an active site. In order to proceed with the electron transfer, ChlL (and Fe) uses a [4Fe-4S] cluster (Reinbothe et al., 2010). The reaction mechanism of DPOR is fundamentally different from that provided by LPOR. At first, a single electron transfer from the [4Fe-4S] cluster of L protein to the NB-cluster of NB protein is present. Further, electron transfer from the NB-cluster to the π electron cloud of Pchlide occurs and after several steps, the necessary stereospecificity of the reduction step occurring at C-17 = C-18 double bond is reached. Subsequently, a second electron transfer event takes place from ChlL to Pchlide through NB-cluster and completes the reaction, which leads to the formation of a single bond between C-17 and C-18. The oxidized L-protein is reduced back by ferredoxin (which serves as an electron donor for the reaction) for the next turnover. After that, another conformational change is achieved, which permits the release of Chlide, and the next reaction cycle may proceed (Nomata et al., 2005). Interestingly, the significant sensitivity of L-protein to oxygen belongs among the very important properties of DPOR (Yamamoto et al., 2009). (See chapter 4 for more).

4 REGULATION OF TETRAPYRROLE BIOSYNTHETIC PATHWAY

In this part, the regulation of chlorophyll biosynthesis is summarized. Besides transcriptional and translational regulation, post-translational level of chlorophyll biosynthesis regulation is extremely important, since it provides much greater extent of flexibility in a rapidly changing environment. Three main control points are discussed in detail, as well as the effect of various environmental factors. Finally, special attention is devoted to organisms having both Pchlide reductases: DPOR and LPOR.

4.1 General scheme of the pathway regulation

The control of tetrapyrrole biosynthesis predominantly optimizes the formation of adequate amounts of Chl and heme, and prevents the accumulation of metabolic intermediates. Due to their photochemical properties, the accumulation of free tetrapyrroles generates the highly reactive singlet oxygen upon illumination and may cause severe photooxidative damage. Tetrapyrrole biosynthesis is therefore expected to be tightly regulated at various levels by endogenous factors on one hand and environmental factors on the other hand (Chen, 2014). It is known that these regulatory steps are generally located **1.** at the beginning of the metabolic pathway for appropriate supply of the substrate into the pathway, and for defining the synthesis rate; **2.** at the branch points – to control the distribution of common intermediate molecules; **3.** at the step of the formation of end products to limit the metabolic flow by feedback control (Stitt, 1996).

Almost all enzymes of the pathway are nuclear-encoded (with exception of DPOR) and the metabolic pathway is located in various cellular compartments, where a tight regulation at various levels of gene expression is expected. (1) A rate-limiting step of the biosynthetic pathway is in its initial part – synthesis of ALA, which is crucial for the metabolic flow through the pathway. (2) Another significant regulatory point lies at the step of Proto IX, where the quantitative distribution of the intermediate is controlled in a direction of heme or Chl biosynthesis. (3) Pchlide reduction is the last regulatory step, and the level of Pchlide controls the inflow of ALA into the pathway by a feedback mechanism (Kannangara and Gough, 1979; Papenbrock and Grimm, 2001, Richter et al., 2010).

At first, the regulation of tetrapyrrole biosynthetic pathway was attributed to metabolic feedback control, where a crucial enzyme GluTR is involved, which is regulated by the concentration of heme (Vothknecht et al., 1998; Cornah et al., 2003). Because the activities of GluRS and GSAT were not affected by heme, GluTR was supposed to be the target molecule in ALA synthesis. This was also confirmed by *in vitro* experiments, where purified GluTR from barley was inhibited by heme (Vothknecht et al., 1996). The N-terminal 30 amino acid residues of mature GluTR were found to be required for heme inhibition and designated as the heme-binding domain (HBD; Vothknecht et al., 1998; Goslings et al., 2004). Analogically, from Mg-branch, there is a negative regulator as well – a FLU (FLUORESCENT) protein which acts on the same target enzyme (GluTR) (Meskauskiene and Apel, 2002). The deficiency of the negative regulator FLU is associated with an increasing content of Pchlide (Meskauskiene et al., 2001). FLU protein is active in the absence of light (in angiosperm plants), where the accumulation of Pchlide in high concentrations could cause severe photodamage if the plant was suddenly illuminated (Meskauskiene et al., 2001; Richter et al., 2010; Apitz et al., 2016). Thus, it is necessary to restrict the accumulation of Pchlide, and it is provided by the FLU-mediated signal pathway (Kauss et al., 2012). In a broader sense it means that FLU protects the plants during LPOR inactivation in the dark. In *A. thaliana*, FLU protein is bound in a FLU-containing membrane complex where four other enzymes are present besides FLU: CHL27, which is a subunit of Mg-protoporphyrin IX monomethylester oxidative cyclase, PORB and PORC isoforms of LPOR and geranylgeranyl reductase (Kauss et al., 2012).

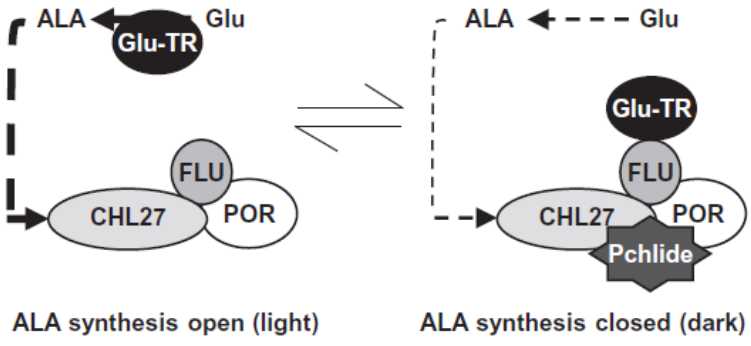


Fig. 6 The hypothetical mechanism of FLU action according to Kauss et al., 2012: FLU as a part of a membrane protein complex with (L)POR and CHL27 without Pchlide would not interact with GluTR, thus, ALA synthesis continues normally: ALA synthesis is open (light). However, when Pchlide is bound to the complex in dark conditions, FLU as a part of this complex should be able to interact with GluTR and inhibit it: ALA synthesis is closed (dark). This way ALA synthesis negatively correlates with Pchlide content.

In the presence of Pchlide, FLU inactivates ALA synthesis in the dark through the interaction with the C-terminus of GluTR (Goslings et al., 2004). A proposed mechanism of FLU action is shown in Fig. 6. Recently, a GluTR binding protein (GluTRBP) was described, and it forms a thylakoid-associated anchor for GluTR and contributes to the organization of ALA synthesis, and to separate the synthesis of ALA which is dedicated to heme. This mechanism ensures heme biosynthesis when other GluTR molecules are post-translationally inactivated by feedback inhibition of the chlorophyll branch by FLU (Czarnecki et al., 2011).

The post-translational regulation described above enables much greater flexibility in a rapidly changing environment (sunflecks, light/dark cycle) in comparison with transcriptional regulation, once sufficient amount of GluTR protein is synthesized. However, the synthesis of GluTR proteins is also under transcriptional control. The transcription of *HEMA* gene encoding GluTR is stimulated by light through phytochrome, and etiolated seedlings of angiosperms do not accumulate significant amount of GluTR in the dark (Gehring et al. 1977; Huang et al. 1989; Mohanty et al. 2006). GluTR regulation and ALA synthesis also depends on circadian rhythms, temperature, developmental stage, actual demand of Chl biosynthesis, as well as phytohormones (Kruse et al., 1997; Mohanty et al., 2006; Yaronskaya et al., 2006). Fine-tuned diurnal oscillations of the ALA-synthesizing capacity with a maximum in the first

half of the light period was determined in a few angiosperms, matching the oscillating levels of the light-harvesting genes transcripts (Kruse et al., 1997; Papenbrock et al., 1999) and suggesting a connection with the phytochrome-regulating system of *HEMA* gene encoding GluTR (Gehring et al., 1977; Mohanty et al., 2006).

A partially different situation must be present in photosynthetic organisms operating with both LPOR and DPOR enzymes. Because of the activity of DPOR in the dark, ALA-synthesizing capacity would not be inhibited to provide sufficient amount of metabolic precursors into Pchlide reduction for DPOR. This raises a question about the possible existence of a negative feedback regulator in such organisms. Initially, Falciatore et al. (2005) identified another related regulator of GluTR in *Chlamydomonas reinhardtii*, the "FLU-like protein" – FLP. FLPs can partially complement the *flu* mutation in *A. thaliana*. Demko et al. (2010) identified FLP in the Norway spruce seedlings despite the fact that the regulatory function of the light is alleviated in this species.When the spruce seedlings were cultivated in the dark, ALA synthesis was blocked only partially, which correlates with a high level of GluTR enzyme, also present in the dark. This indicates a phytochrome-independent regulation

of *HEMA* transcription, and relaxation of FLU-inhibitive activity on GluTR (Demko et al., 2009, 2010). Moreover, authors observed higher levels of GluTR protein in the early developmental stages in comparison with later stages of dark-grown *Larix decidua, Pinus mugo* and *P. sylvestris* seedlings (Demko et al., 2009; Breznenová et al., 2010). Their results indicate that for the regulation of ALA synthesis, the developmental stage may be more important than light. Our findings (Stolárik et al., 2017) suggest that GluTR is still positively regulated by light on transcriptional level in Norway spruce but to a lesser extent than in angiosperms in accordance with Demko et al. (2009, 2010). ALA synthesis is only partially a rate limiting step in Chl synthesis in the dark-grown gymnosperms, because the reduction of Pchlide to Chlide is more or less ineffective, as indicated higher Pchlide/Chl ratio after ALA-feeding in the dark and this point is a limiting step of Chl biosynthesis (Pavlovič et al., 2009).

Checkpoint at Proto IX level

The insertion of divalent Fe^{2+} or Mg^{2+} into Proto IX molecule decides about the further fate of the tetrapyrrole biosynthetic pathway. At this step, the chelating reaction is present and is secured by Fe-chelatase and Mg-chelatase, respectively (Papenbrock and Grimm, 2001; Tanaka and Tanaka, 2007). The subunit and isoenzyme compositions of these enzymes are described in Chapter 3. 1.

Generally, it was found that despite its energy requirement in the form of ATP (Walker and Willows, 1997; Jensen et al., 1999), Mg-chelatase has a higher affinity to Proto IX than Fe-chelatase (Walker et al., 1997, Guo et al., 1998). Besides of the energy supply requirements, it was also suggested that heme and Chl syntheses are spatially separated in different subcompartments of plastids (Roper and Smith, 1997). The substrate and product of Mg-chelatase, Proto IX and Mg-Proto IX, respectively, can be excited by light, forming triplet excited states, if they accumulate in excessive amounts in plants. The excited forms of chlorophyll intermediates immediately react with oxygen, which results in the formation of ROS and further inactivation of Mg-chelatase (Aarti et al., 2006). Therefore, light is the most important development regulator which significantly influences the effectiveness of heme or Chl formation through the control mechanisms affecting the Mg- and Fe- chelatases (Papenbrock and Grimm, 2001). The transcription of the genes encoding Mg-chelatase subunits is stimulated by light (Papenbrock et al., 1999; Yaronskaya et al., 2006). Mg-chelatase showed a higher activity 1 hour after transition from dark to light, which suggests its light-triggered post-translational modification. In contrast, a higher Fe-chelatase activity was determined in the very late phase of the illumination period. These findings show that the

expression and activities of both chelatases are coordinated and contribute to the appropriate allocation of Proto IX in the adaptive response to the daily usage of heme and Chl (Papenbrock and Grimm, 2001). Cultivation of plants in dark or in the light/dark periods followed this pattern, as suggest our findings in Norway spruce where the transcription of Fe-chelatase isoforms (especially FC1) was negatively photoregulated, but Mg-chelatase subunits showed a strong positive regulation by light (Stolárik et al., 2017).

Interestingly, the activity of Mg-chelatase can be sensed in early steps of the chlorophyll biosynthetic pathway, i. e. within ALA formation. Transgenic *Nicotiana tabacum* plants expressing antisense RNA for subunits CHLH or CHLI of Mg-chelatase showed chlorotic leaves as the result of decreased levels of Chl and heme. However, not only Mg-chelatase activity was decreased, but the accumulation of Proto IX was also lower, suggesting the existence of negative feedback mechanism in the Mg-branch of the pathway (Papenbrock et al., 2000). In this case, ALA-synthesizing capacity was also decreased (caused by the decreased transcripts of GluTR), as well as ALAD activity, which prevented over-accumulation of phototoxic products. In contrast, a reduced activity of Fe-chelatase in transgenic plants expressing Fe-chelatase antisense RNA caused Proto IX accumulation and thus, formation of leaf necrosis, but no decrease in ALA formation was observed. This indicates the absence of negative feedback loop in Fe-chelatase, in contrast to Mg-chelatase on ALA synthesis (Papenbrock et al., 2001).

Mg-chelatase is also under post-translational control through GENOMES UNCOUPLED 4 (GUN4) regulatory protein. This may represent another feedback control of Chl formation, because in cyanobacteria and higher plants, GUN4 binds to ChlH subunit of Mg-chelatase and forms a complex, binding with Proto IX and Mg-Proto IX, and activate Mg-chelatase (Larkin et al., 2003; Wilder et al., 2004). Shortly after the dark-light transition, metabolic activities increase (Papenbrock et al., 1999), leading to transient accumulation of Mg-Proto IX. Then, GUN4 acts as a shunt for excessive Mg-porphyrins. Upon binding these porphyrins, GUN4 assists in preventing photooxidative damage under the maintenance of high flux rates (Peter and Grimm, 2009).

Mg-chelatase forms a protein complex with another enzyme in chlorophyll biosynthetic pathway; Mg-protoporphyrin IX methyltransferase (MTF, Alawady et al., 2005). This enzyme catalyzes the transfer of methyl group to the 13-propionate side chain of Mg-Proto IX and produces Mg-Proto IX monomethyl ester (Fig. 4). Chlorophyll formation depends entirely on MTF protein, and *chlM*-null mutants of *A. thaliana* are unable of Chl synthesis. Thus, the inactivation of MTF inhibits the activity of Mg-chelatase and stimulates

the increased activity of Fe-chelatase. However, an enhanced activity of MTF leads to opposite profiles and Chl formation is stimulated (Alawady and Grimm, 2005; Pontier et al., 2007).

It was mentioned previously that the reduction of protochlorophyllide to chlorophyllide by light-independent or light-dependent manners represents one of the most important regulatory points in the whole biosynthetic pathway. Moreover, this step prominently differs among various species of photosynthetic organisms, reflecting the presence and potential coordination of LPOR/DPOR in response to various internal or environmental stimuli. Thus, the next sub-chapter offers a detailed description of this regulatory point.

4.2 Regulation of DPOR and LPOR activity by various stimuli among photosynthetic organisms

Regulation of light-dependent Chl synthesis in angiosperms has been described above, and gymnosperms together with lower plants share many points of this regulatory mechanism. However, several differences have been elucidated between angiosperm and gymnosperms, mainly caused by the presence of DPOR in conifers and lower plants. The presence of two biochemically and genetically distinct strategies for Chlide formation which have arisen during evolution, and coexist in many photosynthetic organisms, has a strong effect on their regulatory mechanisms (Armstrong, 1998). Demko et al. (2009) and Breznenová et al. (2010) reported that an additional reduction of Pchlide by DPOR enzyme enables the attenuation of the tight regulation of phytochrome-induced gene expression and LPOR-bound Pchlide repression of GluTR activity in cotyledons of gymnosperms. This light-independency is more prominent in early developmental stages, however, primary or secondary needles resemble the angiosperm plants more and the role of the light in Pchlide reduction seems to be irreplaceable (Skinner and Timko, 1999; Stolárik et al., 2017). Moreover, von Wettstein (1995) reported that DPOR is expressed only during the development of cotyledons, but DPOR expression during the development of primary and secondary needles was strongly alleviated, which was also confirmed by our results (Stolárik et al., 2017). These findings suggest a strong tissue and developmental specificity of DPOR expression. Interestingly, LPOR enzyme is also significantly influenced by developmental stage, as well as by light. The fast disappearance of LPOR protein was observed together with the decrease of its expression, despite the continuous greening of etiolated angiosperms after their illumination

(Holtorf et al., 1995). This contrasting result was resolved by the evidence of the presence of two forms of the enzyme with different expression patterns in several angiosperms. Whilst LPORA isoform was accumulated in the dark-grown seedlings and its mRNA together with the protein product rapidly declined after illumination due to the fast turnover of LPORA, LPORB remained more or less constant, independently of illumination. Not surprisingly, LPORA and LPORB were also described by Skinner and Timko (1998; 1999) in *Pinus taeda* and *P. nigra*, respectively. Also, Forreiter and Apel (1993) and Stabel et al. (1991) confirmed the presence of two LPOR isoforms in *Pinus mugo* and *Picea abies*, respectively. Moreover, LPORA is typical for PLB of etioplasts and etiochloroplasts of cotyledons where it is bound together with Pchlide and NADPH, and LPORB occurs mainly in mature tissues, e. g. secondary needles, where it is responsible for greening. However, the expression patterns in angiosperms and gymnosperms seem to be quite different; transcription of both LPOR isoforms was positively regulated by light, suggesting that the strong negative photoregulation described in angiosperms had not yet been established in gymnosperm plants (Skinner and Timko, 1999). These observations are consistent with our findings in Norway spruce (Stolárik et al., 2017).

Phytochrome regulation of LPOR expression was mentioned above. The potential regulation of DPOR by phytochrome was also investigated in *Marchantia paleacea* var. *diptera* (Suzuki et al., 2001). Authors claimed that phytochrome regulates the expression of *chlLNB* genes of DPOR. On the contrary, Shi and Shi (2006) suggest that larger amounts of *chlLNB* transcripts in the cells grown in light compared to the cells grown in dark could reflect either subtle differences in their transcription rates or in the stability of their transcripts under completely different growth conditions. Moreover, Eguchi et al. (2002) observed higher *chlL* transcript level in dark-grown *M. paleacea* var. *diptera* if compared with the light variant. The involvement of phytochrome in DPOR regulation in conifers has not yet been established, however negative photoregulation was documented in *Pinus thunbergii* and *Picea abies* cotyledons (Demko et al., 2010; Yamamoto et al., 2017; Stolárik et al., 2017).

Many authors provided the evidence that both Pchlide oxidoreductases contribute to the total Chl content in various photosynthetic organisms (Fujita and Bauer, 2003). An *in vitro* assay with crude cell extracts of *Pinus mugo* seedlings showed that LPOR is the major Pchlide reduction system and that DPOR only functions as an auxiliary system under the conditions, where light is hardly available (Forreiter and Apel, 1993). Thus, this may indicate that Chl formation is dependent on light intensity. The cells of green alga *Chlamydomonas reinhardtii* with any mutation in *chlL*, *N* or *B* genes, as well as particular nuclear loci (*y-1 – y-*

10) resulted in *yellow-in-the-dark* phenotype, if cultivated in conditions without the presence of light. However, if the same cells were cultivated under light conditions, they achieved wild-type phenotype and were able to synthesize enough Chl, thus LPOR can fully substitute the missing DPOR under light conditions (Cahoon and Timko, 2000). Similar results on algae, cyanobacteria and liverwortswere were obtained earlier by Fujita et al. (1992); Suzuki and Bauer (1992) and Suzuki et al. (1998). Recently, the studies on *Marchantia polymorpha* L. confirmed that DPOR is required for Chl biosynthesis under light limiting conditions (Ueda et al., 2014). The authors cultivated mutant plants in *chlB* gene of *M. polymorpha* under short and long day conditions and observed that the liverworts growing under short day conditions were significantly delayed in their development and accumulated much lower amount of Chl in comparison to wild type. Under long day conditions, mutant plants were indistinguishable from wild type indicating, that the role of DPOR in Chl biosynthesis decreases with a prolonged light period and that LPOR becomes essential for growth under high light conditions. Similar results were obtained by Fujita et al. (1998) who worked with a pair of *Plectonema boryanum* mutant, in which either LPOR or DPOR was inactivated. The results showed that the role of LPOR increases with increasing light intensity (>170 μmol m^{-2} s^{-1} PAR), however under low light conditions (10 – 25 μmol m^{-2} s^{-1} PAR) both PORs are required for sufficient Chl formation. Thus, in the situation with scarcity of photons, LPOR most likely cannot substitute the function of DPOR. These findings became a theoretical background to investigate the plasticity of Chl synthesis and photosystems formation in larch and spruce seedlings under deep shade conditions (Stolárik et al., 2018).

However, the formation of photosynthetic apparatus and Chl synthesis differs among gymnosperm species. For example, *Ginkgo biloba* does not express the genes for DPOR efficiently and is completely etiolated when growing in the dark (Pavlovič et al., 2010). On the other hand, *Picea abies* has the highest ability to synthesize Chl in the dark, whilst *Larix* and *Thuja* represent the conifers with strongly decreased ability in this way. This low ability is caused by two factors: non-synonymous mutations in DPOR genes in *Thuja* and insufficient *chlB* mRNA editing in *Larix* (Kusumi et al., 2006; Demko et al., 2009). Yamamoto et al. (2017) confirmed that *chlB* mRNA editing serves as an important regulatory system in *Pinus thunbergii*. The efficiency of *chlB* editing is decreased in the light and as a result, the unedited copy of ChlB protein is not able to interact with ChlL and thus to form a functional DPOR complex.

Besides light, temperature is one of the most important regulators of plant development. It was described that low temperature significantly inhibits Chl formation on

transcriptional level in angiosperms (Tewari and Tripathy, 1998; Mohanty et al., 2008). Our findings also confirmed that low temperature slows down the whole Chl biosynthetic pathway in Norway spruce as a representative of conifers (Stolárik et al., 2017) but the chlorophyll accumulation in the light was not inhibited so strongly in comparison with angiosperms. However, low temperature in combination with darkness inhibits Chl formation almost completely, indicating strong inhibition of DPOR activity under low temperature conditions (Muramatsu et al., 2001; Stolárik et al., 2017). However, transcription and translation of DPOR subunits were not negatively affected under such conditions. Thus, the significant decrease in Chl level seemed to be affected post-translationally. Moreover, Yamamoto et al. (2009; 2011) investigated that ChlL protein of DPOR is a primary target of oxygen, however this functional inactivation is not connected with a significant degradation of the protein(s) (Yamazaki et al., 2006). For example, LPOR deficient mutant of cyanobacterium *Leptolyngbya boryana* designated as YFP12 could grow photoautotrophically at maximally 3% oxygen concentration, which is an incomparably lower concentration to that found in ambient atmosphere (Yamazaki et al., 2006). Taking into account these information and if considering the increasing solubility of oxygen with a decreasing temperature (Henry, 1803), this provided a theoretical basis of our performed experiments, where we mimicked low-temperature conditions by the exposure of Norway spruce seedlings to higher oxygen content (Stolárik et al., 2017).

Chlorophyll biosynthesis and formation of pigment-protein complexes

In angiosperms, the accumulation of pigment-protein complexes can be controlled at multiple steps. The transcription of genes encoding chlorophyll-binding proteins is induced by light and regulated by phytochrome and blue light receptors. In addition, factors such as circadian regulation and the status of chloroplast influence the gene expression and further development (Tobin and Silverthorne, 1985). In addition, stable accumulation of chlorophyll-binding proteins has also been found to depend on the availability of chlorophylls (Chl). For example, mutants deficient in Chl*b* express normal level of the major LHCB polypeptides, but they were unstable in the absence of Chl*b* (Bellemare et al., 1982). Due to the absence of light, etiolated angiosperm plants do not accumulate significant amounts of chlorophyll-binding proteins in dark (Kanervo et al., 2008). In contrast, gymnosperms transcribe the genes encoding chlorophyll-binding proteins light-independently and the chlorophylls synthesized by DPOR stabilize proteins in thylakoid membranes (Yamamoto et al. 1991; Muramtasu et al., 2001). Thus, coniferous seedlings growing in the dark form relatively well developed etiochloroplasts instead of etioplasts (Walles and Hudák, 1975), with fully active

photosystem I (PSI) even during cultivation in full darkness (Oku et al., 1974; Kamachi et al., 1998). Photosystem II (PSII) is also formed under these conditions, however it remains in its latent form with an inactive oxygen-evolving complex (OEC) (Jansson et al., 1992; Shinohara et al., 1992; Pavlovič et al., 2016) until the chloroplasts are illuminated. Shinohara et al. (1992) elucidated that Mn integration into OEC is strictly light-dependent and indispensable for effective O_2 evolution. OEC is composed of three extrinsic proteins: PsbO, PsbP and PsbQ with relative molecular mass of 33 kDa, 23 kDa and 17 kDa, respectively. These proteins play a crucial role in Mn-cluster stabilization and the modulation of binding Ca^{2+} and Cl^- ions, which are irreplaceable during O_2 evolution (Yi et al., 2005). Not surprisingly, the chlorophyll *a*/*b* ratio is also higher in dark-developing seedlings, suggesting that the total size of light-harvesting complexes (LHC) is smaller than those in light-growing plants (Jansson et al., 1992; Stolárik et al., 2017). Yamamoto et al. (1991) reported that in dark-grown pine cotyledons sufficient amounts of total and translatable mRNA for LHCII are present, but the lower supply of Chl produced by DPOR is a limiting point in the dark. The imbalance between Chl*b* supply and the formation of LHC apoproteins lead to smaller antenna size in dark-grown cotyledons which are usually in monomeric conformation (Xue et al., 2017). In our study (Pavlovič et al., 2016), we have shown that the photoactivation of oxygen evolution in PSII is relatively fast and can be observed already in PSII that are not fully photochemically active. Continuously, the light-induced formation of fully active PSII centres is a gradual and long-term process in proper stoichiometric ratio requiring the synthesis and binding of LHCII to PSII, the assembly of OEC proteins and the final association of PSII into supercomplexes. Because DPOR provides only a limited supply of chlorophylls, we can conclude that light plays a crucial role not only in the assembly and activation of pigment-protein complexes involved in the process of photosynthesis in coniferous plants, but it also enhances Chl supply into this process.

5 SUMMARY

This doctoral thesis deals with chlorophyll (Chl) biosynthesis in gymnosperm plants, especially in Norway spruce (*Picea abies* Karst.) and partially in deciduous European larch (*Larix decidua* Mill.) – the important representatives of the Pinophyta division.

It is well-known that the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) is a crucial step in Chl biosynthetic pathway. This reaction may be catalyzed by light-dependent (LPOR) or light-independent (DPOR) protochlorophyllide oxidoreductase (POR). LPOR occurs separately, without the presence of DPOR, in angiosperm plants only, whilst they occur together among (almost) the rest of the plant kingdom including gymnosperms.

We have found that Chl biosynthesis in spruce depends not only on the presence or absence of light, but also on the developmental stage of the plant, as well as on the ambient temperature. Although the light's morphogenetic function is alleviated in cotyledons, in secondary needles it seems to be absolutely irreplaceable. This effect is well-documented on the absence of significant accumulation of Chl in secondary needles in comparison to cotyledons in the dark. DPOR activity is transcriptionally regulated by light and developmental stage, which was clearly shown mainly by analyses of gene expressions encoding the subunits of this enzyme. However, the ambient temperature regulates this enzyme post-translationally, which accounts for the etiolated phenotype of spruce seedlings cultivated at 7 °C in the dark, despite the normal levels of DPOR protein products. In this context, taking into account the increasing oxygen solubility proportionally to the decreasing temperature in the water, we have proposed that DPOR becomes a possible target of higher O_2 concentration. This hypothesis is based on the finding that ChlL subunit is oxygen-sensitive and DPOR enzyme is inactivated by this manner (Stolárik et al., 2017).

Following the experiments investigating the regulation mechanisms in Chl biosynthetic pathway we have dealt with the ecophysiological implications of having DPOR. Considering the pivotal role of DPOR under short day conditions where it significantly contributes to Chl formation, we have also decided to include larch (*Larix decidua*) seedlings into our experiments. This coniferous plant is typical for a decreased Chl concentration in the dark despite the presence of DPOR and this phenomenon was elucidated by insufficient *chlB* mRNA editing (which is a subunit of DPOR), thus, its' decreased activity. Based on several experiments, we have found that the larch seedlings, in contrast to spruce, showed a

remarkably decreased ability of Chl formation at very weak irradiance. This peculiarity can be possibly explained by the fact that under deep-shade conditions LPOR enzyme can not work properly due to its light-dependent character. The scarcity of photons for LPOR and dysfunctional DPOR in Europen larch are not able to satisfy the requirement for chlorophyll synthesis under these conditions. On the other hand, spruce had a significantly higher Chl amount despite growing in deep-shade, because of a functional DPOR presence. This causes the situation that spruce plants do not show an etiolated phenotype under such conditions, as well as the maximum quantum yield of PSII photochemistry values are comparable with the plants growing under optimal illumination, because even a low amount of photons was sufficient for the successful photoactivation of PSII. The presented data support the hypothesis about the mutual cooperation of both Pchlide-reducing enzymes under various light conditions (Stolárik et al., 2018).

Although dark-cultivated spruce seedlings possessed fully assembled and functional photosystem I (PSI), PSII was present only in its latent form with inactive oxygen-evolving complex (OEC). Using various laboratory techniques (fluorescence, thermoluminiscence, native and/or reducing gel electrophoresis, immunoblot, etc.) we have found out that after the illumination of plants, PSII is gradually activated. This includes fast Mn atoms incorporation into OEC and the whole assembly of this water-oxidizing complex, where PsbO, PsbQ and PsbP proteins play an important role. These proteins are localized inside the thylakoid membranes also in the dark, although they do not form a tight cluster with PSII. Interestingly, after 5 min. of illumination, a partial PSII activation was observable together with a detectable O_2 evolution. Even a 24-h illumination was not sufficient for full activation of PSII centres which include stable assembly of OEC and LHCII-PSII supercomplexes formation. This was observable only if the plants were cultivated under normal light/dark conditions (Pavlovič et al., 2016).

Finally, it is necessary to mention the “cost and benefit” of having DPOR. The reason why LPOR enzyme has appeared during evolution is quite simple: most likely it was a consequence of the transition from the anaerobic Archean Earth’s atmosphere to an aerobic atmosphere, similar to that existing in present times. Moreover, LPOR has at least three advantages over DPOR: primarily, it is insensitive to oxygen, as a photoenzyme it limits Pchlide photooxidative damage, and is energy-independent, i. e. does not require energy in the form of ATP. However, despite its ancient character and high energy requirements, the presence of DPOR enzyme in a functional state offers plants several advantages, mainly under specific light conditions. The presented doctoral thesis documents the regulation of DPOR on

transcriptional, translational and post-translational level; the ecophysiological implications of its presence in plants, and its importance in chlorophyll synthesis and PSII-supercomplexes formation in gymnosperm plants.

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Doctoral thesis

Light-independent chlorophyll biosynthesis in gymnosperms

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Annotation: This thesis is focused on chlorophyll biosynthesis in gymnosperm plants which are characterized by ability of chlorophyll formation also in dark. This is achieved by the presence of light-independent protochlorophyllide oxidoreductase (DPOR) which is typical *inter alia* for gymnosperms and plays an important role in chlorophyll biosynthetic pathway together with light-dependent protochlorophyllide oxidoreductase (LPOR). We aimed our attention especially on Norway spruce (*Picea abies* Karst.) and European larch (*Larix decidua* Mill.) plants. A special attention is paid to theoretical background of current state of knowledge in this field, followed by our results, documented by attached papers. Generally we can conclude that chlorophyll biosynthesis and its regulation in the gymnosperm plants depend on: plant species, developmental stage and environmental factors, such as light conditions, temperature, as well as composition of ambient atmosphere. Thus, chlorophyll formation may be regulated on various levels. We also summarized our results of gradual, light-dependent formation of PSII supercomplexes and oxygen evolution.

Content

Declaration

I hereby declare that this doctoral thesis is my original work, based on my own experiments. All other sources of information are acknowledged in section References.

Olomouc, 15th Dec. 2017 _____

Tibor Stolárik

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Abbreviations

ALA	5-aminolevulinic acid
Bchl	bacteriochlorophyll
cDNA	complementary DNA
Chl	chlorophyll
Chlide	chlorophyllide
DPOR	light-independent protochlorophyllide oxidoreductase
F_v/F_m	maximal quantum yield of photosystem II
GluTR	glutamyl-tRNA reductase
HPLC	high-pressure liquid chromatography
LHC	light-harvesting complex
LPOR	light-dependent NADPH protochlorophyllide oxidoreductase
OEC	oxygen-evolving complex
PAGE	polyacrylamide gel electrophoresis
PAR	photosynthetically active radiation
Pchlide	protochlorophyllide
PLB	prolamellar body
Proto IX	protoporphyrin IX
PSI	photosystem I
PSII	photosystem II
qPCR	quantitative real-time polymerase chain reaction
TEM	transmission electron microscopy
TL	thermoluminiscence

Note: Other abbreviations that are not listed here are explained directly in the text.

1 Introduction

Photosynthesis is a very unique process on Earth which annually converts approximately 258 billion tons of carbon dioxide (Geider et al., 2001). Chlorophyll (Chl) is one of the most abundant organic compounds on Earth and as a part of the photosynthetic machinery, it absorbs light and is involved in energy transfer in the process of photosynthesis (von Wettstein et al., 1995; Chen, 2014). The term is derived from the Greek words χλωρός – *chloros* ("green") and φύλλον – *phyllon* ("leaf") and it was first isolated and named by French chemists J. B. Caventou and P. J. Pelletier (1817). The composition of the chlorophyll molecule (and also other photosynthetic pigments), its biosynthetic pathway and its regulation have been widely studied for decades, especially in angiosperm plants. However, chlorophyll biosynthesis in gymnosperms is still partially shrouded in mystery.

This doctoral thesis is focused on chlorophyll biosynthesis in gymnosperms, especially in two representatives of coniferous plants (also known as Pinophyta): Norway spruce (*Picea abies* Karst.) and European larch (*Larix decidua* Mill.). Besides a huge evolutionary role of conifers, as they have dominated forests for more than 200 million years, they also have ecological and economical importance (Nystedt et al., 2013). Gymnosperm plants (including conifers) also seemed to be an interesting target for research due to further peculiarities, e. g. the chlorophyll biosynthetic pathway. Already Morren (1858) noticed the ability of few photosynthetic organisms to synthesize chlorophyll in darkness and further investigations revealed the important role of protochlorophyllide (Pchlide) reduction for effective production of chlorophylls. In contrast to angiosperm plants, which use only light-dependent protochlorophyllide oxidoreductase (LPOR) for the reduction of Pchlide to chlorophyllide (Chlide) – precursors of Chl (Schoefs and Franck, 2003; Reinbothe et al., 2010), gymnosperms are also able to use a nitrogenase-like, light-independent protochlorophyllide oxidoreductase (DPOR) (Armstrong, 1998; Reinbothe et al., 2010).

In this study we focused on the effect of few regulatory and ecophysiological factors such as light, temperature, developmental stage, oxygen content in ambient air, etc. which are considered to affect chlorophyll biosynthesis. We focused our attention also on chlorophyll biosynthetic pathway, its regulation on transcriptional, translational and post-translational levels, structure of photosystems, formation of photosystem II supercomplexes, potential changes in plastid ultrastructure, and, the whole photosynthetic processes in general (see Chapter 8 – Publications). The first part of my doctoral thesis offers the complex insight into

the topic and summarizes recent scientific findings in this field. The second – supplementary part – is composed of my scientific papers.

2 Assimilation pigments

Oxygenic photosynthesis requires chlorophyll (Chl) for absorption and transduction of light energy, and charge separation in reaction centres of photosystems I and II (PSI and PSII) to supply the electron-transport chain with electrons. Chlorophyll is bound to Chl-binding proteins which are assembled in the core complexes of PSI and PSII and their peripheral light-harvesting antenna complexes (Wang and Grimm, 2015), where they occur together with carotenoids. Besides their light-harvesting function, carotenoids have also a photoprotective function (DellaPenna and Pogson, 2006). Bacteriochlorophylls as well as bilins were also described in several photosynthetic organisms, e. g. photosynthetic bacteria, cyanobacteria, etc. (Blankenship, 2008).

2.1 Chlorophylls

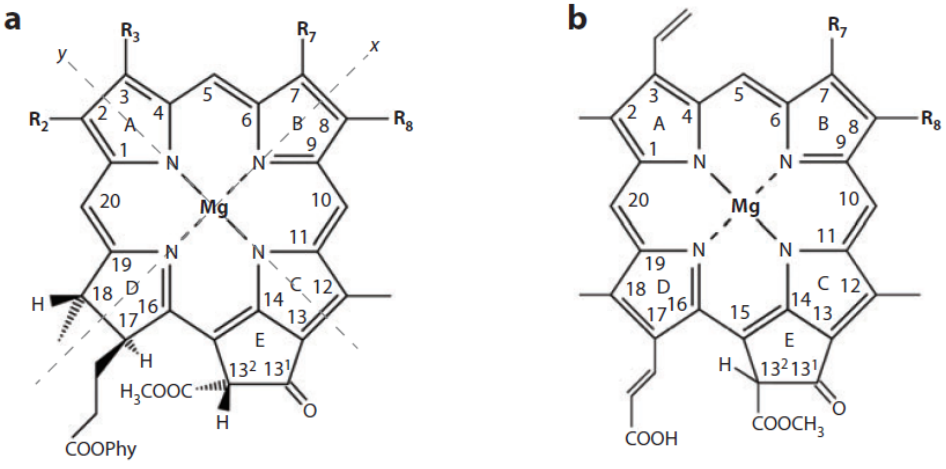
Chlorophylls are organic molecules, structurally based on tetrapyrroles; although chlorins would be a more concise term. This discrepancy is caused by the presence (tetrapyrrole molecule) or absence (chlorine molecule) of double bond between C-17 and C-18 carbon atoms in D-ring of the molecule (Fig. 1, Juselius and Sundholm, 2000). In the centre of the chlorine ring is magnesium (Mg) bound by coordinate covalent bond (Willstätter, 1906). All chlorophylls have a similar five-membered ring structure (pyrrole rings designated as A, B, C, D and E – isocyclic), with variations in side chains and/or reduction states (Chen, 2014). We know five types of chlorophyll molecule: Chl *a* (or 8-vinyl Chl *a*), Chl *b* (or 8-vinyl Chl *b*), Chl *c*, Chl *d* and Chl*f*. Each type of chlorophyll is characterized by different absorption spectra of the sunlight which enables the photosynthetic organisms to colonize a vast range of environments (Porra, 1989; Chen, 2014).

Chl *a* is the most abundant of all chlorophylls. The general structure of Chl *a* was elucidated by Hans Fischer in 1940 (Fischer and Orth, 1940). Chlorophyll *a* is present in the reaction centre (RC) and light-harvesting complexes (LHCs) of almost all oxygenic photosynthetic organisms including cyanobacteria, algae, and higher plants (Björn et al., 2009). The absorption spectrum of Chl *a* in 100% methanol is shown in Fig. 2. Various taxa of photosynthetic organisms contain different sets of light harvesting Chls. For example, Chl *a* occurs in glaucophytes, and in red algae together with Chl *d*. Chlorophylls *a*, *b*, *d* and [8-vinyl]-Chls *a* and *b* are present in cyanobacteria, Chls *a* and *b* in green algae and higher

plants, and Chl *a* and *c* in chromophytic algae (Govindjee and Satoh 1983; Murakami et al. 2004; Grimm et al. 2006; Scheer 2006). As mentioned above, functional groups (mainly formyl group – Fig. 1) bound in different positions cause different spectral properties, and the absorption maxima of chlorophylls are significantly shifted. This is documented in Fig. 2.

Chlorophyll *b* is characteristic for its formyl substitution in C-7 position and is considered as the second most abundant chlorophyll in oxygenic photosynthetic organisms (Chen, 2014). It functions as an accessory chlorophyll in the light-harvesting complexes, although it is not capable to act as so-called special pair in the reaction centre (role of Chl *a*) and primarily absorbs blue light (Fig. 2) (Chen, 2014). Interconversion between Chl *a* and Chl *b* (known as the chlorophyll cycle) may provide higher plants the ability to optimize light harvesting and thus, acclimation to varying light conditions, e. g. shade or a straight light irradiation (Kitajima and Hogan, 2003; Chen, 2014).

Chlorophyll *c* has not been found in vascular plants but it was described in (marine) algae including the photosynthetic Chromista (e. g. diatoms, brown algae) (Jeffrey and Vesk, 1997). Chlorophyll *c* has fully unsaturated porphyrin (Fig. 1b) which makes it different from all the other chlorophylls. It plays a role as light-harvesting pigment. There are several structural



Chlorin type

Chlorophylls	R_2	R_3	R_7	R_8	Q_y (nm)
Chl *a*	CH_3	$CH{=}CH_2$	CH_3	$CH_2{-}CH_3$	665*
8-vinyl Chl *a* (Chl a_2)	CH_3	$CH{=}CH_2$	CH_3	$CH{=}CH_2$	666#
Chl *b*	CH_3	$CH{=}CH_2$	CHO	$CH_2{-}CH_3$	652*
8-vinyl Chl *b* (Chl b_2)	CH_3	$CH{=}CH_2$	CHO	$CH{=}CH_2$	658#
Chl *d*	CH_3	CHO	CH_3	$CH_2{-}CH_3$	696*
Chl *f*	CHO	$CH{=}CH_2$	CH_3	$CH_2{-}CH_3$	707*

Porphyrin type

Chl *c*	R_7	R_8
Chl *c*1	CH_3	$CH_2{-}CH_3$
Chl *c*2	CH_3	$CH{=}CH_2$
Chl *c*3	$COOCH_3$	$CH{=}CH_2$

Fig. 1 Chemical structure of chlorophylls (Chls). **a:** Chemical structure of chlorophylls which have chlorine type macrocycles. **b:** Chemical structure of chlorophyll *c* family, the members of which are of the porphyrin type. Chlorin-type Chls are esterified by phytyl chain (Phy) of $C_{20}H_{39}$. **Chlorophyll *b*, Chl *d*** and **Chl *f*** have **formyl group** substitutions at positions C-7, C-3 and C-2, respectively. The structural differences are red-coloured. $\mathbf{Q}_y$ means maximal red absorption (nm): asterisk-marked were recorded in 100% methanol; pound-marked in a mixture of methanol and acetone. (Modified according to Chen, 2014).

variations of Chl *c* (designated as c_1, c_2, c_3) which differ in substituents located at atoms C-7 and C-8 of B-ring (Fig. 1b). It has a blue-greenish colour and is an accessory pigment, particularly significant in its absorption of the light in blue wavelength region (approx. 447 – 452 nm) (Dougherty et al., 1970). This is also a reason why it occurs mainly in photosynthetic organisms living in great depths, with blue light penetrating water the furthest.

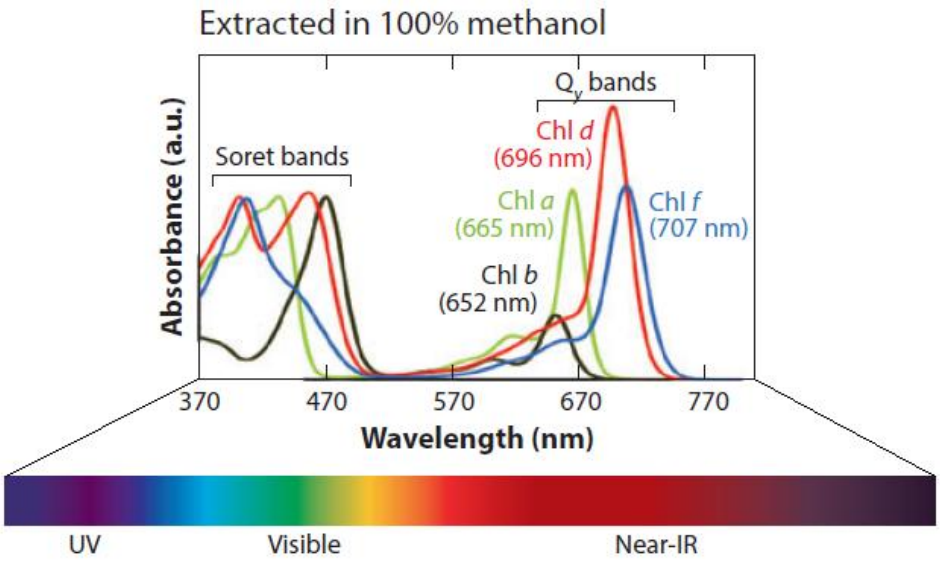


Fig. 2 Absorption spectra of chlorine-type chlorophylls in 100 % methanol (Chl *a*, Chl *b*, Chl *d* and Chl *f*). Soret-band maxima are arbitrarily scaled to a common height for comparison. (Modified according to Chen, 2014).

Chlorophyll *d* is a form of chlorophyll identified by Manning and Strain (1943). However, it was considered as an artificial product for more than 50 years until the discovery of a novel cyanobacterium *Acaryochloris marina* in 1996 (Miyasita et al., 1996). This confounded the traditional thinking about the irreplaceable function of Chl *a* in photosynthetic organisms, as >95% of all chlorophylls in this cyanobacterium was represented by Chl *d* (the content of Chl *a* was approx. 5%). The only structural difference between Chl *a* and Chl *d* is at C-3 position in A-ring, where vinyl group is replaced by formyl group (Fig. 1a) (Chen, 2014). Noticeably, the absorption maxima of Chl *d* are similar to bacteriochlorophyll *a* because it absorbs the light close to UV and IR spectral parts. It was also found that Chl *a* could be a precursor of Chl *d* (Schliep et al., 2010).

The most recently discovered type of chlorophyll is Chl *f* found in stromatolites (cyanobacterium *Halomicronema hongdechloris*) from Western Australia's Shark Bay (Chen et al., 2010). The molecule of Chl *f* is structurally related to Chl *a*, however in C-2 position of A-ring, a formyl group is present instead of a methyl group. The absorption maxima of Chl *f*

are significantly red-shifted (Fig. 2) and the functions of this chlorophyll type remain more or less unknown (Chen et al., 2012). The biosynthetic pathway of chlorophylls is described in detail in chapter 3.

2.2 Bacteriochlorophylls

Bacteriochlorophylls (Bchl/-s) differ from Chls by a substitution of vinyl group for acetyl group at C-3 of A-ring as well as a reduced double bond between C-7 and C-8 of B-ring. These substitutions offer the Bchls different spectral properties, mainly a shift of absorption maxima closer to UV and IR region (Grimm et al., 2006). These assimilation pigments occur in various phototrophic bacteria, and currently there are known bacteriochlorophyll *a*, *b*, *c*, *d*, *e* and *g*. Although Bchl *f* (20-desmethyl-Bchl *e*) was recently found only through mutation, we cannot exclude that it may exist naturally (Vogl et al., 2012). Authors prepared the mutant of green sulfur bacterium *Chlorobaculum limnaeum*, where *bchU* gene (bacteriochlorophyllide C-20 methyltransferase) was inactivated. The resulting *bchU* mutant synthesized Bchl f_F and no Bchl *e* was detected. The bacteria with Bchl*f* chlorosomes were much slower in growth, and the energy transfer from Bchl *f* aggregates to Bchl *a* was much less effective in comparison to WT. This may be a reason why Bchl *f* was not yet found in the nature in comparison with other bacteriochlorophylls of phototrophic bacteria.

Because my thesis is not focused on bacteriochlorophylls and anoxygenic photosynthesis, further information about Bchls is not provided here but can be found elsewhere (see Blankenship, 2008). However, it is possible to find Bchl-related partial information in the next chapters.

2.3 Bilins

Bilins are open tetrapyrrole molecules which occur in light-harvesting complexes (known as phycobilisomes) of cyanobacteria and red algae. Interestingly, they absorb the light between 550 – 650 nm, and the most important bilins are phycoerythrobilin and phycocyanobilin. They are synthesized through the Fe-branch of tetrapyrrole biosynthesis (Blakenship, 2008).

2.4 Carotenoids

Carotenoids are lipid-soluble compounds which play an important role in many photosynthetic processes, (e. g. light-harvesting, dissipation of excess energy and photoprotection through xanthophyll cycle).

Carotenoids are derivates of tetraterpenes, meaning that they are produced from 8 isoprene molecules and contain 40 carbon atoms (Armstrong and Hearst, 1996; DellaPenna and Pogson, 2006).

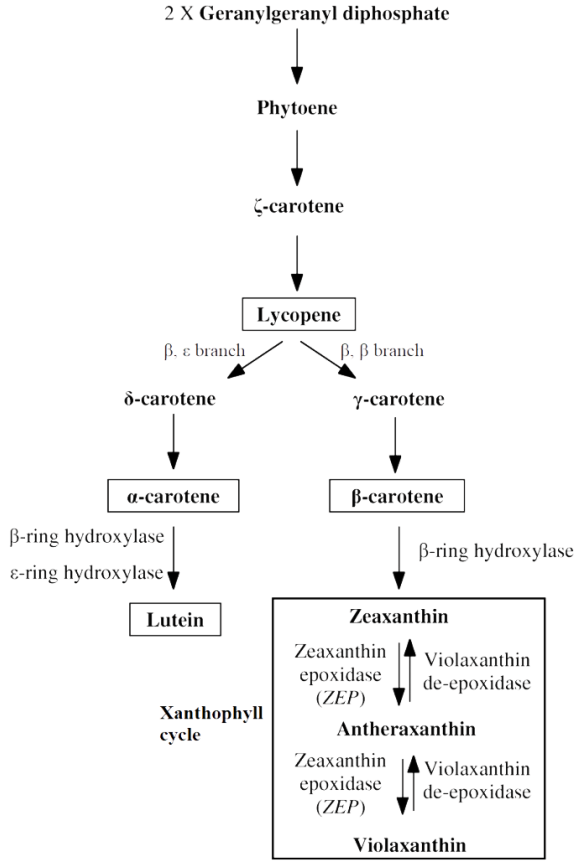


Fig. 3 Carotenoids biosynthetic pathway (modified according to Clotault et al., 2008). Inset shows xanthophyll cycle scheme in the right down part of the figure.

Carotenoids can be divided into two basic groups: carotenes and xanthophylls. Carotenes are pure hydrocarbons containing no oxygen (α-, β-carotene, lycopene, etc.), and on the other hand, xanthophylls contain oxygen in their molecules (lutein, violaxanthin, antheraxanthin, zeaxanthin, fukoxanthin, etc.).

In the biosynthetic pathway of carotenoids, the first committed step is the condensation of two molecules of geranylgeranyl diphosphate (GGDP) to produce phytoene (Fig. 3). Interestingly, GGDP can be used not only for phytoene synthesis, but also plays an

important role in the synthesis of chlorophylls, phylloquinone, gibberellins, and one branch of synthesis also leads to tocopherols (DellaPenna and Pogson, 2006). The carotenoid biosynthetic pathway continues through few intermediates to lycopene where the pathway divides into two branches characterized by different cyclic end-groups. Two beta rings lead to the β, β branch (β-carotene and its derivates: zeaxanthin, violaxanthin, antheraxanthin and neoxanthin), whilst one beta and one epsilon ring define the β, ε branch (α-carotene and its derivates). As briefly mentioned above, carotenoids perform various functions in plants. They are involved in photosystem assembly, light-harvesting and photoprotection, photomorphogenesis, non-photochemical quenching, lipid peroxidation and they also affect the function and size of light-harvesting antennae (Lokstein et al., 2002; Holt et al., 2005; DellaPenna and Pogson, 2006). Carotenoids are bound to proteins in the membranes and co-form LHC as accessory pigments. They absorb light energy between 400 – 500 nm and transmit it into the reaction centres. To avoid photooxidative damage of photosystems, xanthophylls provide very effective tool for excess energy dissipation, called xanthophyll cycle (Fig. 3). An epoxide group is introduced into both rings of zeaxanthin by zeaxanthin-epoxidase to form violaxanthin. Under high light stress which acidifies the lumen of thylakoids, violaxanthin deepoxidase is activated, resulting in increased levels of zeaxanthin (Niyogi, 1999). Finally, zeaxanthin dissipates excess of energy as heat (energy is not transferred to Chls).

Another important attribute of carotenoids is their ability to be synthesized also in the darkness and they are localized in tubular membranes around prolamellar bodies (PLB) as well as straightly in PLBs of etioplasts (DellaPenna and Pogson, 2006; Cuttriss et al., 2007).

3 Chlorophyll formation

The biosynthetic pathway of chlorophylls is a very complex process with several regulatory steps (Papenbrock and Grimm, 2001) and it has been well established in higher plants. However, some details of its compartmentalization and regulation remain obscure (Chen, 2014). The main steps of Chl biosynthesis are common for the majority of photosynthetic organisms, and the important differences and regulatory peculiarities between gymnosperm and angiosperm plants are described further.

3.1 General scheme of tetrapyrrole biosynthesis

The first steps of tetrapyrrole biosynthetic pathway are shared among chlorophylls, heme, siroheme and cobalamin (Chen, 2014). The pathway can be divided into four main parts: **1.** formation of 5-aminolevulinic acid (ALA); **2.** formation of protoporphyrin IX (Proto IX) from eight molecules of ALA; **3.** Mg-porphyrin branch leading to chlorophylls; **4.** heme-synthesizing branch (Papenbrock and Grimm, 2001). The scheme of this biosynthetic pathway is shown in Fig. 4.

At least two distinct pathways of ALA formation are known in the nature. Both of them were described in the phytoflagellate *Euglena gracilis* (Weinstein and Beale, 1983). One pathway is known as $\mathbf{C_4}$ pathway and occurs in animals, fungi and certain groups of bacteria, notably *Rhodobacter*, *Rhodospirillum* and *Rhizobium* (von Wettstein et al., 1995; Papenbrock and Grimm, 2001). This pathway utilizes a condensation reaction of glycine and **C4** moiety succinyl-CoA, catalyzed by pyroxidal phosphate-dependent enzyme – ALA-synthase (ALAS) (von Wettstein et al., 1995; Papenbrock and Grimm, 2001). The other, a three-step pathway is characteristic for the majority of bacteria, the Archaea, algae, and plants, and is called $\mathbf{C_5}$ pathway (Jordan, 1991; Papenbrock and Grimm, 2001), where ALA is derived from a **C5**-skeleton of glutamate. Glutamate is introduced by acetylation to $tRNA^{Glu}$ by glutamyl-tRNA synthetase (GluRS) and subsequently reduced by glutamyl-tRNA reductase (GluTR). Transamination is catalysed by glutamate-1-semialdehyde aminotransferase (GSAT) and it results in the formation of ALA (Fig. 4) (Friedmann et al., 1987; von Wettstein et al., 1995).

The part of the pathway between ALA and the tetrapyrrole Proto IX is highly conversed among all organisms. ALA-dehydratase (ALAD) condenses two molecules of ALA

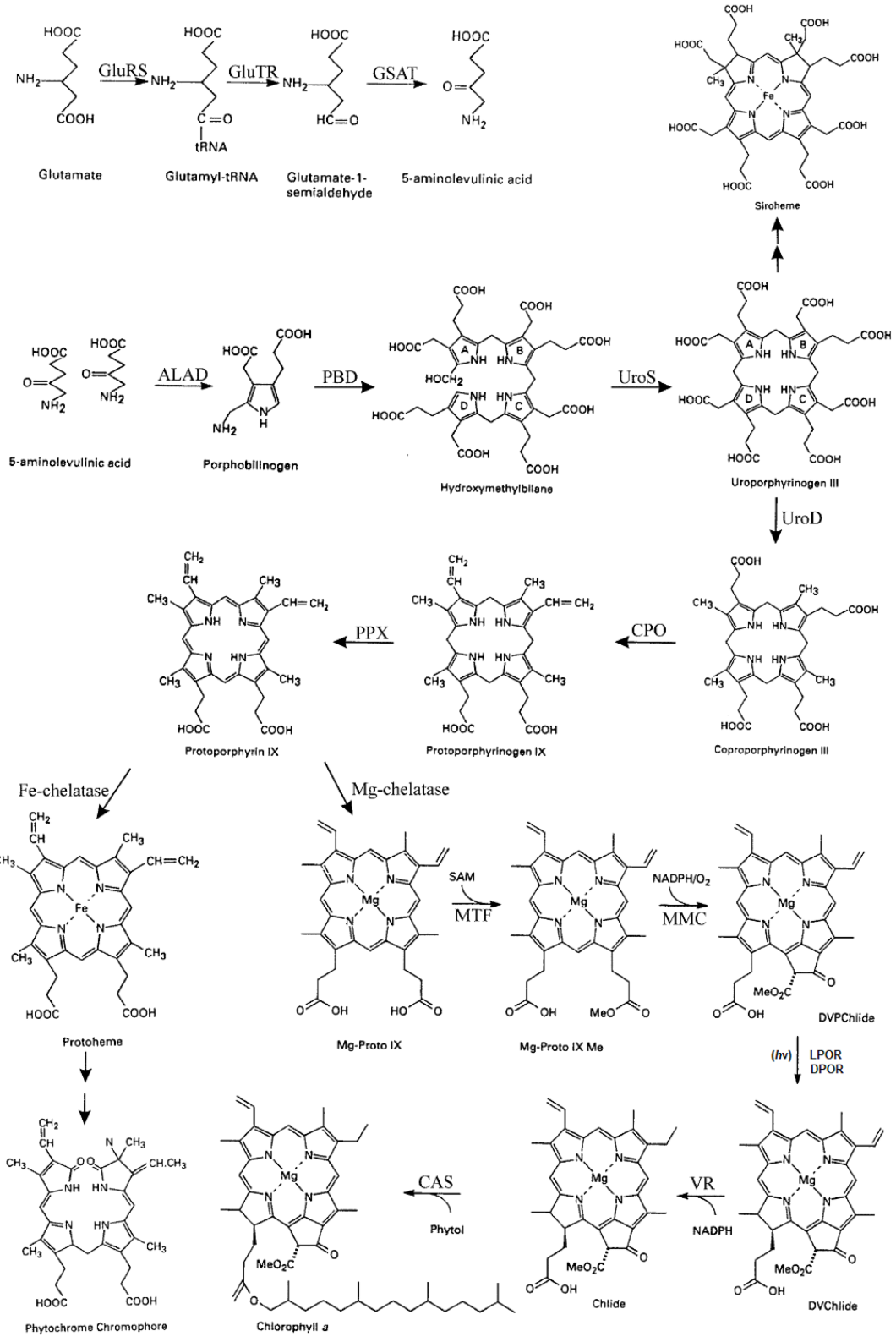


Fig. 4 Schematic diagram of the tetrapyrrole biosynthetic pathway. Eight units of 5-aminolevulinate form the tetrapyrrole skeleton. All intermediates from the glutamate to the plant tetrapyrrole end products are shown. The abbreviations of involved enzymes are explained within the text describing the biosynthetic pathway in Chapter 3. 1. (Modified according to Papenbrock and Grimm, 2001).

into monopyrrole porphobilinogen. Four molecules of porphobilinogen are polymerised to form, at first, a linear tetrapyrrole molecule – 1-hydroxymethylbilane using porphobilinogen deaminase (PBD). After an immediate isomerisation of this linear molecule, a ring molecule uroporphyrinogen III is arisen. The reaction is catalysed by uroporphyrinogen III synthase (UroS) (Papenbrock and Grimm, 2001). At this level, biosynthetic pathway branches for the first time. Methylation of uroporphyrinogen III leads to the synthesis of siroheme or (bacterial) cobalamin (vitamin B_{12}). On the other hand, biosynthesis may follow the way to Chl and heme, where the side chains of the porphyrin ring are further decarboxylated and oxidised to form protoporphyrin IX (Proto IX). That proceeds through a few intermediates: from uroporphyrinogen III to coproporphyrinogen III (catalyzed by uroporphyrinogen III decarboxylase – UroD), then it continues to protoporphyrinogen III (catalyzed by coproporphyrinogen oxidase – CPO) and finally to Proto IX, where the reaction is catalyzed by protoporphyrinogen oxidase (PPX) (Papenbrock and Grimm, 2001). Proto IX is the last common molecule in Chl and heme biosynthesis. At this step, the biosynthetic pathway branches, where metal chelation reactions of Proto IX catalyzed either by Fe-chelatase or Mg-chelatase divide the pathway into Fe-branch and Mg-branch, leading to heme and Chl formation, respectively (Papenbrock and Grimm, 2001; Tanaka and Tanaka, 2007). This part of the pathway is considered as an important regulatory point.

Fe-chelatase inserts Fe^{2+} into Proto IX in a reaction that does not require an input of energy, and is inhibited by the presence of ATP. On the contrary, the insertion of Mg^{2+} by Mg-protoporphyrin IX chelatase (Mg-chelatase) requires the presence of significant amount of energy, because the hydrolysis of ~15 ATP molecules per one metal ion insertion is required (Reid and Hunter, 2004). Recently, two isoforms of Fe-chelatase were described (FC1 or FeCh I and FC2 or FeCh II) and it was suggested that the individual isoforms contribute to heme biosynthesis in a different way. FC1 and FC2 most likely supply heme to different sets of heme-dependent proteins. FC1 may be in charge of heme synthesis for cytoplasmic, and more generally, extraplastidal heme-dependent proteins, whilst FC2 might be responsible for heme supply to the plastid-localized proteins requiring heme (Koch et al., 2004; Wang and Grimm, 2015). Moreover, FC2 possesses a light-harvesting Chl-binding (LHC) domain at the C-terminus, which may play a regulatory role in heme synthesis in photosynthetic plastids rather than in heme synthesis for other cellular heme-requiring proteins (Sobotka et al., 2011). Finally, the insertion of Fe^{2+} leads to the formation of protoheme and subsequently different types of heme, or to linear tetrapyrrole phytochromobilin (Papenbrock and Grimm, 2001).

Mg-chelatase is an enzyme which catalyzes the insertion of Mg^{2+} to Proto IX. This enzyme belongs to AAA^{+}-type chelatases and is composed of three subunits: H, I, and D. These subunits are conserved among species from cyanobacteria to higher plants (ChlH, ChlI, and ChlD); in bacteriochlorophyll biosynthesis they are commonly referred to as BchH, BchI, and BchD (Chew and Bryant, 2007). These three subunits are weakly associated with one another, and the three-subunit Mg-chelatase complex is relatively unstable (Reid and Hunter, 2004). The function of ChlD may be to provide a stable platform for ChlI subunits and form ChlI/ChlD polymeric complex. The binding of ATP (not hydrolysis) is important for the activation of the ChlI/ChlD complex (Jensen et al., 1999). This complex further interacts with the ChlH subunit and drives the ATP-dependent insertion of Mg^{2+} into Proto IX. ChlH is sensitive to the changing concentration of Mg^{2+}, which is reflected by the translocation of ChlH between chloroplast stroma and chloroplast envelope (Nakayama et al., 1998). Energy availability (besides other regulatory factors) is an important factor affecting the direction of the biosynthetic pathway – either to Chls or to hemes.

After the insertion of Mg^{2+}, Mg-protoporphyrin IX is created and immediately methylated by Mg-protoporphyrin IX methyltransferase (MTF) to Mg-protoporphyrin IX monomethylester. In a further step, Mg-protoporphyrin IX monomethyl ester cyclase (MMC) catalyzes the incorporation of atomic oxygen to Mg-protoporphyrin IX monomethylester (in case of oxygenic photosynthetic organisms) (Papenbrock and Grimm, 2001). This oxidative cyclization creates the E-ring, a distinctive isocyclic ring of all chlorophylls. Moreover, this ring structure is the unique characteristic of chlorophylls, in comparison to all other tetrapyrroles. Interestingly, in photosynthetic anoxygenic bacteria (e. g. green sulfur bacteria), a non-oxidizing cyclization mechanism unrelated to the oxidizing cyclization was also described (Ouchane et al., 2004). The anaerobic enzyme is a radical S-adenosyl-L-methionine (SAM) enzyme, and its crucial role in the regulatory mechanism is well described in Chapter 4. 1, part "Checkpoint at Proto IX level". The product of this reaction is divinyl protochlorophyllide (DV-Pchlide). There are known several models for the further fate of DV-Pchlide based on the reduction of 8-vinyl group on C-8 position of B-ring, since the activities of 8-vinyl reductase have been detected at five various levels: 1. Mg-proto IX monomethyl ester; 2. Mg-divinyl protochlorophyllide; 3. protochlorophyllide *a*; 4. chlorophyllide *a* and 5. chlorophyll *a* (Wang et al., 2013). (Note: Chlorophyllide *a* and chlorophyll *a* are final products of the biosynthetic pathway). However, the 8-vinyl reductases from different species show diverse and differing substrate preferences. There are more than five various 8-vinyl reductases with different reductive activities on the same or on different

substrates (Wang et al., 2013). Interestingly, marine *Prochlorococcus* species lack the reductase for vinyl group at C-8 position, hence they contain 8-vinyl Chl *a* and 8-vinyl-Chl *b* (Goericke and Repeta, 1992).

The next step of the pathway to chlorophylls is the reduction of protochlorophllide (Pchlide) to chlorophyllide (Chlide). According to the information mentioned above, DV-Pchlide as well as MV-Pchlide (monovinyl protochlorophyllide) may be reduced. This reduction is catalyzed by protochlorophyllide oxidoreductase (POR). Two different, unrelated forms of this enzyme have been described, one is dark-operative light-independent (DPOR), which does not require light for its function, and the other one is light-dependent NADPH protochlorophyllide oxidoreductase (LPOR). Both these enzymes reduce a double bond between C-17 and C-18 of D-ring (Fig. 5). [VR in the scheme (Fig. 4) means 8-vinyl reductase, if the vinyl group is reduced after the step of Pchlide conversion to Chlide] (Papenbrock and Grimm, 2001; Chen, 2014). Complex information regarding LPOR and DPOR distribution among organisms, structure, reduction mechanism etc. is stated in subchapters 3. 1. 1 and 3. 1. 2.

In the last step of chlorophyll biosynthetic pathway, Chlide *a* can be esterified with geranylgeranyl-pyrophosphate or phytyl-pyrophosphate by Chl synthase (Chl *a* synthase – CAS in this case). Then, it can be (partially) converted to Chl *b* by Chl *a* oxygenase (CAO) (Papenbrock and Grimm, 2001). CAO enzyme is a Rieske FeS centre-containing, non-heme-Fe monooxygenase that uses molecular oxygen and NADPH to perform two successive hydroxylations at the C-7 position of Chlide *a* (Tanaka et al., 1998). The gene encoding CAO is located in the nucleus and mature CAO protein can be found in thylakoid membranes. It is also possible that Chl *b* may be firstly generated *via* (P)Chlide *b* and it is esterified subsequently. Chl *a* and Chl *b* underlie the so-called chlorophyll cycle where Chl *b* can be reversibly converted to Chl *a*. Generally, Chl *a* (or Chlide *a*) is the precursor molecule for other types of chlorophylls – Chl *b*, Chl *d* and Chl *f*. However, the biosynthetic pathway leading to Chl *c* as the end product remains to be elucidated. Moreover, Larkum (2006) reported that Chl *c* may be the evolutionary precursor of chlorophylls. It also worth to mention that from Chlide *a*, bacteriochlorophyllide *a* – Bchlide *a* (the precursor of **bacteriochlorophyll *a*** – Bchl *a*) is formed by reduction of the double bond between C-7 and C-8 of B-ring; and this reduction is catalyzed by Chlide-oxidoreductase (COR; also known as chlorine-reductase). The reduction of the double bond in B-ring is coupled with hydration and oxidation of the 3-vinyl side group (Oster et al., 1997). COR is encoded by *bchX*, *bchY* and

bchZ genes which are similar to genes encoding the bacterial nitrogenase (which is also similar to DPOR enzyme) (Burke et al., 1993; Armstrong, 1998).

In the next two subchapters, we focus our interest on the crucial regulatory step in the chlorophyll biosynthetic pathway – protochlorophyllide reduction. This is realized by protochlorophyllide oxidoreductases: LPOR and/or DPOR.

3.1.1 Light-dependent protochlorophyllide oxidoreductase – LPOR

Light- and NADPH-dependent protochlorophyllide oxidoreductase (LPOR) (EC 1.3.33.1) is one of the very few enzymes that require light for their catalytic activity, thus it belongs to the group of photoenzymes. Another well-known photoenzyme is DNA photolyase (Begley, 1994; Reinbothe et al., 2010; Gabruk and Mysliwa-Kurdziel, 2015). LPOR is the only enzyme responsible for Pchlide reduction in angiosperm plants but it occurs together with the dark-operative POR (DPOR) in almost all evolutionary lower photosynthetic organisms, i. e. cyanobacteria, green algae and non-flowering plants: mosses, liverworts, hornworts, lycophyta, ferns, as well as gymnosperms (Armstrong, 1998; Fujita and Bauer, 2003; Yamamoto et al., 2011). Moreover, a gene encoding LPOR was also detected in anoxygenic photosynthetic bacteria and apparently introduced into their genome by horizontal gene transfer (Kaschner et al., 2014). Both of these enzymes catalyze the same stereospecific double bond reduction between C-17 and C-18 in D-ring of Pchlide (Fig. 5). LPOR is nuclear-encoded single-polypeptide enzyme that is post-translationally translocated to the plastids (Chen, 2014) and shows a high degree of similarity to the short-chain dehydrogenase-reductase (SDR) family. LPOR is present in high levels as a ternary complex with its substrate – photoactive Pchlide (emission at 655 nm), and with NADPH forming prolamellar bodies of etioplasts (Fujita, 1996; Schoefs and Franck, 2003; Reinbothe et al., 2010). Non-photoactive Pchlide has an emission maximum at 633 nm and serves as a precursor for photoactive Pchlide (Schoefs and Franck, 1998). Interestingly, although LPOR is not phylogenetically related to DPOR, they share a common sequence motif (a TFT motif) of unknown function (Gabruk and Mysliwa-Kurdziel, 2015). However, unlike genes encoding DPOR, several genes of LPOR isoforms may be present within one genome. This can be a result of horizontal gene transfer and/or genome duplication in dinoflagellates, achniophytes, and stramenopiles (Hunsperger et al., 2015). In land plants, the origin of multiple LPOR copies is unknown. In cucumber and pea, only one isoform of LPOR gene is present (Fusada

et al., 2000), and its expression is positively photoregulated and remains unchanged during greening and development. On the other hand, two LPOR isoforms are usually present in many land plants (e. g. *Hordeum vulgare*, *Pinus taeda*), known as LPORA (*por*A) and LPORB (*por*B) or additionally, also LPORC (*por*C) in *Arabidopsis thaliana* (Oosawa et al., 2000, Su et al., 2001). It is known, that expression level of LPOR isoforms differ in response to illumination: LPORA expression is strongly downregulated by light, however, LPORB is almost light-insensitive (Armstrong et al., 1995). LPORC is a dominant form in green mature tissues of *A. thaliana* and its expression is upregulated by high light irradiation (Oosawa et al., 2000, Masuda et al., 2003). Skinner and Timko (1998) reported that in contrast to angiosperms where LPOR is encoded by small nuclear gene family which contains two differentially expressed genes (*por*A and *por*B); for gymnosperm plants, a large multigene family is typical, being composed of two distinct subfamilies encoding *por*A and *por*B genes similar to those previously described in angiosperms. Surprisingly, for example in *Pinus taeda* these two *por* subfamilies are duplicated differently; *por*A family consist of two members, whilst *por*B contains at least 11 members (Skinner and Timko, 1998). LPORA and LPORB proteins show a size of 37 and 38 kDa, respectively (Skinner and Timko, 1998).

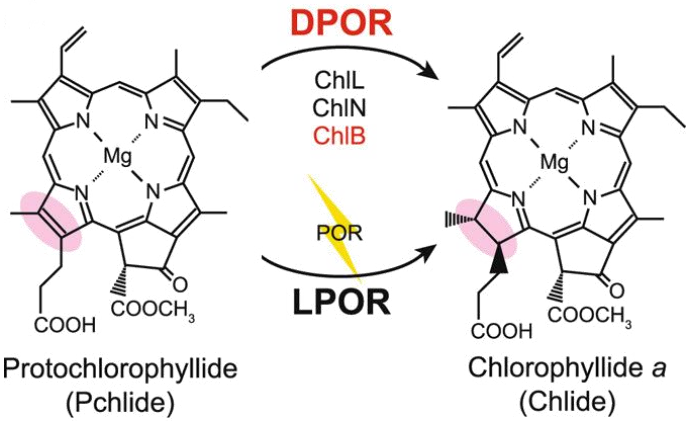


Fig. 5 The scheme shows a reduction of the double bond between C-17 and C-18 in D-ring of Pchlide (marked by purple oval); catalyzed by light-independent (DPOR) or light-dependent (LPOR) protochlorophyllide oxidoreductase. (Modified according Yamamoto et al., 2017).

As mentioned above, LPOR is a nucleus-encoded protein, post-translationally imported and localized in plastids (Fujita, 1996). Thus, the transport of the enzyme into plastids is guided by a transit peptide, located on its N-terminus (Gabruk and Mysliwa-Kurdziel, 2015). In barley which have two LPOR isoforms, it was investigated that in the structure of LPORA, there is a specific amino-acid motif of the transit peptide, crucial for the

proper import of the enzyme into etioplasts. Thus, LPORA is predominantly imported into etioplasts (with the presence of PLBs), and it was hypothesized that Pchlide binding by this transit peptide is required for the translocation of the enzyme. In contrast, the lack of this specific motif in the transit peptide of LPORB indicates, that LPORB does not require Pchlide for its transport into plastids – thus, may be located also in mature chloroplasts (Reinbothe et al., 2008).

The molecular mechanism of Pchlide reduction by LPOR remains to be elucidated, hence it is a target of intensive studies. However, it was shown that two photons are required for a single Pchlide molecule reaction, and the picosecond scale dynamics of the reaction was determined. It was also recently documented that Pchlide reduction consists of at least three major steps: **1.** light absorption; **2.** hydride transfer from the *pro*-S face of the nicotinamide ring of NADPH to C-17 in D-ring of Pchlide; **3.** proton transfer probably from Tyr280 to C-18 of Pchlide. Thus, light absorption promotes intramolecular charge transfer along the C-17 – C-18 bond, which allows subsequent events: the reduction of the mentioned double bond where NADPH serves as electron donor in the reaction (Wilks and Timko, 1995; Heyes et al., 2015). It was also investigated that LPOR may oligomerize which positively influences the effectiveness of the reaction (Gabruk et al., 2015).

3.1.2 Light-independent protochlorophyllide oxidoreductase – DPOR

The majority of gymnosperm plants, mosses, green algae, cyanobacteria, as well as anoxygenic photosynthetic bacteria are able to synthesize Chl also in the dark. This is ensured by light-independent Pchlide reduction, provided by dark-operative POR (or light-independent POR; DPOR) (Fujita, 1996). Phylogenetically, DPOR is the oldest enzyme that catalyzes Pchlide reduction. It is a plastid-encoded multisubunit complex which consists of three different polypeptides: ChlL, ChlN and ChlB, which form ChlL-dimer and ChlNB-heterotetramer. The average molecular weight of ChlL, ChlN and ChlB is 31 kDa, 61 kDa and 57 kDa, respectively. DPOR genes are extraordinarily conserved (Gabruk and Mysliwa-Kurdziel, 2015), and the three subunits of DPOR (L, N, B) remain almost unchanged from cyanobacteria to higher plants (Chen, 2014). The three subunits are responsible for the bacteriochlorophyll synthesis referred to as BchL, BchN and BchB which are orthologs of ChlL, ChlN and ChlB (Fujita, 1996; Reinbothe et al., 2010), whose amino acid sequences show significant similarities to *nif*H, *nif*D and *nif*K, respectively. These genes encode the

subunits of bacterial nitrogenase (Raymond et al., 2004). ChlL protein and ChlNB proteins are functionally equivalent to Fe protein and MoFe proteins, respectively, which are present in nitrogenase. Thus, ChlL (and Fe) proteins serve as an ATP-dependent electron donor for ChlNB (MoFe) proteins. ChlNB (as well as MoFe) represents the catalytic component, and provides an active site. In order to proceed with the electron transfer, ChlL (and Fe) uses a [4Fe-4S] cluster (Reinbothe et al., 2010). The reaction mechanism of DPOR is fundamentally different from that provided by LPOR. At first, a single electron transfer from the [4Fe-4S] cluster of L protein to the NB-cluster of NB protein is present. Further, electron transfer from the NB-cluster to the π electron cloud of Pchlide occurs and after several steps, the necessary stereospecificity of the reduction step occurring at C-17 = C-18 double bond is reached. Subsequently, a second electron transfer event takes place from ChlL to Pchlide through NB-cluster and completes the reaction, which leads to the formation of a single bond between C-17 and C-18. The oxidized L-protein is reduced back by ferredoxin (which serves as an electron donor for the reaction) for the next turnover. After that, another conformational change is achieved, which permits the release of Chlide, and the next reaction cycle may proceed (Nomata et al., 2005). Interestingly, the significant sensitivity of L-protein to oxygen belongs among the very important properties of DPOR (Yamamoto et al., 2009). (See chapter 4 for more).

4 Regulation of tetrapyrrole biosynthetic pathway

In this part, the regulation of chlorophyll biosynthesis is summarized. Besides transcriptional and translational regulation, post-translational level of chlorophyll biosynthesis regulation is extremely important, since it provides much greater extent of flexibility in a rapidly changing environment. Three main control points are discussed in detail, as well as the effect of various environmental factors. Finally, special attention is devoted to organisms having both Pchlide reductases: DPOR and LPOR.

4.1 General scheme of the pathway regulation

The control of tetrapyrrole biosynthesis predominantly optimizes the formation of adequate amounts of Chl and heme, and prevents the accumulation of metabolic intermediates. Due to their photochemical properties, the accumulation of free tetrapyrroles generates the highly reactive singlet oxygen upon illumination and may cause severe photooxidative damage. Tetrapyrrole biosynthesis is therefore expected to be tightly regulated at various levels by endogenous factors on one hand and environmental factors on the other hand (Chen, 2014). It is known that these regulatory steps are generally located **1.** at the beginning of the metabolic pathway for appropriate supply of the substrate into the pathway, and for defining the synthesis rate; **2.** at the branch points – to control the distribution of common intermediate molecules; **3.** at the step of the formation of end products to limit the metabolic flow by feedback control (Stitt, 1996).

Almost all enzymes of the pathway are nuclear-encoded (with exception of DPOR) and the metabolic pathway is located in various cellular compartments, where a tight regulation at various levels of gene expression is expected. (1) A rate-limiting step of the biosynthetic pathway is in its initial part – synthesis of ALA, which is crucial for the metabolic flow through the pathway. (2) Another significant regulatory point lies at the step of Proto IX, where the quantitative distribution of the intermediate is controlled in a direction of heme or Chl biosynthesis. (3) Pchlide reduction is the last regulatory step, and the level of Pchlide controls the inflow of ALA into the pathway by a feedback mechanism (Kannangara and Gough, 1979; Papenbrock and Grimm, 2001, Richter et al., 2010).

At first, the regulation of tetrapyrrole biosynthetic pathway was attributed to metabolic feedback control, where a crucial enzyme GluTR is involved, which is regulated by the concentration of heme (Vothknecht et al., 1998; Cornah et al., 2003). Because the activities of

GluRS and GSAT were not affected by heme, GluTR was supposed to be the target molecule in ALA synthesis. This was also confirmed by *in vitro* experiments, where purified GluTR from barley was inhibited by heme (Vothknecht et al., 1996). The N-terminal 30 amino acid residues of mature GluTR were found to be required for heme inhibition and designated as the heme-binding domain (HBD; Vothknecht et al., 1998; Goslings et al., 2004). Analogically, from Mg-branch, there is a negative regulator as well – a FLU (FLUORESCENT) protein which acts on the same target enzyme (GluTR) (Meskauskiene and Apel, 2002). The deficiency of the negative regulator FLU is associated with an increasing content of Pchlide (Meskauskiene et al., 2001). FLU protein is active in the absence of light (in angiosperm plants), where the accumulation of Pchlide in high concentrations could cause severe photodamage if the plant was suddenly illuminated (Meskauskiene et al., 2001; Richter et al., 2010; Apitz et al., 2016). Thus, it is necessary to restrict the accumulation of Pchlide, and it is provided by the FLU-mediated signal pathway (Kauss et al., 2012). In a broader sense it means that FLU protects the plants during LPOR inactivation in the dark. In *A. thaliana*, FLU protein is bound in a FLU-containing membrane complex where four other enzymes are present besides FLU: CHL27, which is a subunit of Mg-protoporphyrin IX monomethylester oxidative cyclase, PORB and PORC isoforms of LPOR and geranylgeranyl reductase (Kauss et al., 2012).

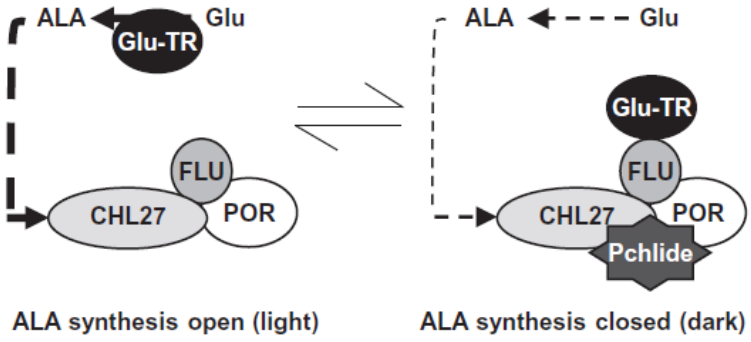


Fig. 6 The hypothetical mechanism of FLU action according to Kauss et al., 2012: FLU as a part of a membrane protein complex with (L)POR and CHL27 without Pchlide would not interact with GluTR, thus, ALA synthesis continues normally: ALA synthesis is open (light). However, when Pchlide is bound to the complex in dark conditions, FLU as a part of this complex should be able to interact with GluTR and inhibit it: ALA synthesis is closed (dark). This way ALA synthesis negatively correlates with Pchlide content.

In the presence of Pchlide, FLU inactivates ALA synthesis in the dark through the interaction with the C-terminus of GluTR (Goslings et al., 2004). A proposed mechanism of FLU action is shown in Fig. 6. Recently, a GluTR binding protein (GluTRBP) was described, and it forms a thylakoid-associated anchor for GluTR and contributes to the organization of

ALA synthesis, and to separate the synthesis of ALA which is dedicated to heme. This mechanism ensures heme biosynthesis when other GluTR molecules are post-translationally inactivated by feedback inhibition of the chlorophyll branch by FLU (Czarnecki et al., 2011).

The post-translational regulation described above enables much greater flexibility in a rapidly changing environment (sunflecks, light/dark cycle) in comparison with transcriptional regulation, once sufficient amount of GluTR protein is synthesized. However, the synthesis of GluTR proteins is also under transcriptional control. The transcription of *HEMA* gene encoding GluTR is stimulated by light through phytochrome, and etiolated seedlings of angiosperms do not accumulate significant amount of GluTR in the dark (Gehring et al. 1977; Huang et al. 1989; Mohanty et al. 2006). GluTR regulation and ALA synthesis also depends on circadian rhythms, temperature, developmental stage, actual demand of Chl biosynthesis, as well as phytohormones (Kruse et al., 1997; Mohanty et al., 2006; Yaronskaya et al., 2006). Fine-tuned diurnal oscillations of the ALA-synthesizing capacity with a maximum in the first half of the light period was determined in a few angiosperms, matching the oscillating levels of the light-harvesting genes transcripts (Kruse et al., 1997; Papenbrock et al., 1999) and suggesting a connection with the phytochrome-regulating system of *HEMA* gene encoding GluTR (Gehring et al., 1977; Mohanty et al., 2006).

A partially different situation must be present in photosynthetic organisms operating with both LPOR and DPOR enzymes. Because of the activity of DPOR in the dark, ALA-synthesizing capacity would not be inhibited to provide sufficient amount of metabolic precursors into Pchlide reduction for DPOR. This raises a question about the possible existence of a negative feedback regulator in such organisms. Initially, Falciatore et al. (2005) identified another related regulator of GluTR in *Chlamydomonas reinhardtii*, the "FLU-like protein" – FLP. FLPs can partially complement the *flu* mutation in *A. thaliana*. Demko et al. (2010) identified FLP in the Norway spruce seedlings despite the fact that the regulatory function of the light is alleviated in this species.When the spruce seedlings were cultivated in the dark, ALA synthesis was blocked only partially, which correlates with a high level of GluTR enzyme, also present in the dark. This indicates a phytochrome-independent regulation of *HEMA* transcription, and relaxation of FLU-inhibitive activity on GluTR (Demko et al., 2009, 2010). Moreover, authors observed higher levels of GluTR protein in the early developmental stages in comparison with later stages of dark-grown *Larix decidua*, *Pinus mugo* and *P. sylvestris* seedlings (Demko et al., 2009; Breznenová et al., 2010). Their results indicate that for the regulation of ALA synthesis, the developmental stage may be more important than light. Our findings (Stolárik et al., 2017) suggest that GluTR is still positively

regulated by light on transcriptional level in Norway spruce but to a lesser extent than in angiosperms in accordance with Demko et al. (2009, 2010). ALA synthesis is only partially a rate limiting step in Chl synthesis in the dark-grown gymnosperms, because the reduction of Pchlide to Chlide is more or less ineffective, as indicated higher Pchlide/Chl ratio after ALA-feeding in the dark and this point is a limiting step of Chl biosynthesis (Pavlovič et al., 2009).

Checkpoint at Proto IX level

The insertion of divalent Fe^{2+} or Mg^{2+} into Proto IX molecule decides about the further fate of the tetrapyrrole biosynthetic pathway. At this step, the chelating reaction is present and is secured by Fe-chelatase and Mg-chelatase, respectively (Papenbrock and Grimm, 2001; Tanaka and Tanaka, 2007). The subunit and isoenzyme compositions of these enzymes are described in Chapter 3. 1.

Generally, it was found that despite its energy requirement in the form of ATP (Walker and Willows, 1997; Jensen et al., 1999), Mg-chelatase has a higher affinity to Proto IX than Fe-chelatase (Walker et al., 1997, Guo et al., 1998). Besides of the energy supply requirements, it was also suggested that heme and Chl syntheses are spatially separated in different subcompartments of plastids (Roper and Smith, 1997). The substrate and product of Mg-chelatase, Proto IX and Mg-Proto IX, respectively, can be excited by light, forming triplet excited states, if they accumulate in excessive amounts in plants. The excited forms of chlorophyll intermediates immediately react with oxygen, which results in the formation of ROS and further inactivation of Mg-chelatase (Aarti et al., 2006). Therefore, light is the most important development regulator which significantly influences the effectiveness of heme or Chl formation through the control mechanisms affecting the Mg- and Fe- chelatases (Papenbrock and Grimm, 2001). The transcription of the genes encoding Mg-chelatase subunits is stimulated by light (Papenbrock et al., 1999; Yaronskaya et al., 2006). Mg-chelatase showed a higher activity 1 hour after transition from dark to light, which suggests its light-triggered post-translational modification. In contrast, a higher Fe-chelatase activity was determined in the very late phase of the illumination period. These findings show that the expression and activities of both chelatases are coordinated and contribute to the appropriate allocation of Proto IX in the adaptive response to the daily usage of heme and Chl (Papenbrock and Grimm, 2001). Cultivation of plants in dark or in the light/dark periods followed this pattern, as suggest our findings in Norway spruce where the transcription of Fe-chelatase isoforms (especially FC1) was negatively photoregulated, but Mg-chelatase subunits showed a strong positive regulation by light (Stolárik et al., 2017).

Interestingly, the activity of Mg-chelatase can be sensed in early steps of the chlorophyll biosynthetic pathway, i. e. within ALA formation. Transgenic *Nicotiana tabacum* plants expressing antisense RNA for subunits CHLH or CHLI of Mg-chelatase showed chlorotic leaves as the result of decreased levels of Chl and heme. However, not only Mg-chelatase activity was decreased, but the accumulation of Proto IX was also lower, suggesting the existence of negative feedback mechanism in the Mg-branch of the pathway (Papenbrock et al., 2000). In this case, ALA-synthesizing capacity was also decreased (caused by the decreased transcripts of GluTR), as well as ALAD activity, which prevented over-accumulation of phototoxic products. In contrast, a reduced activity of Fe-chelatase in transgenic plants expressing Fe-chelatase antisense RNA caused Proto IX accumulation and thus, formation of leaf necrosis, but no decrease in ALA formation was observed. This indicates the absence of negative feedback loop in Fe-chelatase, in contrast to Mg-chelatase on ALA synthesis (Papenbrock et al., 2001).

Mg-chelatase is also under post-translational control through GENOMES UNCOUPLED 4 (GUN4) regulatory protein. This may represent another feedback control of Chl formation, because in cyanobacteria and higher plants, GUN4 binds to ChlH subunit of Mg-chelatase and forms a complex, binding with Proto IX and Mg-Proto IX, and activate Mg-chelatase (Larkin et al., 2003; Wilder et al., 2004). Shortly after the dark-light transition, metabolic activities increase (Papenbrock et al., 1999), leading to transient accumulation of Mg-Proto IX. Then, GUN4 acts as a shunt for excessive Mg-porphyrins. Upon binding these porphyrins, GUN4 assists in preventing photooxidative damage under the maintenance of high flux rates (Peter and Grimm, 2009).

Mg-chelatase forms a protein complex with another enzyme in chlorophyll biosynthetic pathway; Mg-protoporphyrin IX methyltransferase (MTF, Alawady et al., 2005). This enzyme catalyzes the transfer of methyl group to the 13-propionate side chain of Mg-Proto IX and produces Mg-Proto IX monomethyl ester (Fig. 4). Chlorophyll formation depends entirely on MTF protein, and *chlM*-null mutants of *A. thaliana* are unable of Chl synthesis. Thus, the inactivation of MTF inhibits the activity of Mg-chelatase and stimulates the increased activity of Fe-chelatase. However, an enhanced activity of MTF leads to opposite profiles and Chl formation is stimulated (Alawady and Grimm, 2005; Pontier et al., 2007).

It was mentioned previously that the reduction of protochlorophyllide to chlorophyllide by light-independent or light-dependent manners represents one of the most important regulatory points in the whole biosynthetic pathway. Moreover, this step

prominently differs among various species of photosynthetic organisms, reflecting the presence and potential coordination of LPOR/DPOR in response to various internal or environmental stimuli. Thus, the next sub-chapter offers a detailed description of this regulatory point.

4.2 Regulation of DPOR and LPOR activity by various stimuli among photosynthetic organisms

Regulation of light-dependent Chl synthesis in angiosperms has been described above, and gymnosperms together with lower plants share many points of this regulatory mechanism. However, several differences have been elucidated between angiosperm and gymnosperms, mainly caused by the presence of DPOR in conifers and lower plants. The presence of two biochemically and genetically distinct strategies for Chlide formation which have arisen during evolution, and coexist in many photosynthetic organisms, has a strong effect on their regulatory mechanisms (Armstrong, 1998). Demko et al. (2009) and Breznenová et al. (2010) reported that an additional reduction of Pchlide by DPOR enzyme enables the attenuation of the tight regulation of phytochrome-induced gene expression and LPOR-bound Pchlide repression of GluTR activity in cotyledons of gymnosperms. This light-independency is more prominent in early developmental stages, however, primary or secondary needles resemble the angiosperm plants more and the role of the light in Pchlide reduction seems to be irreplaceable (Skinner and Timko, 1999; Stolárik et al., 2017). Moreover, von Wettstein (1995) reported that DPOR is expressed only during the development of cotyledons, but DPOR expression during the development of primary and secondary needles was strongly alleviated, which was also confirmed by our results (Stolárik et al., 2017). These findings suggest a strong tissue and developmental specificity of DPOR expression. Interestingly, LPOR enzyme is also significantly influenced by developmental stage, as well as by light. The fast disappearance of LPOR protein was observed together with the decrease of its expression, despite the continuous greening of etiolated angiosperms after their illumination (Holtorf et al., 1995). This contrasting result was resolved by the evidence of the presence of two forms of the enzyme with different expression patterns in several angiosperms. Whilst LPORA isoform was accumulated in the dark-grown seedlings and its mRNA together with the protein product rapidly declined after illumination due to the fast turnover of LPORA, LPORB remained more or less constant, independently of illumination. Not surprisingly, LPORA and LPORB were also described by Skinner and Timko (1998; 1999) in *Pinus taeda*

and *P. nigra*, respectively. Also, Forreiter and Apel (1993) and Stabel et al. (1991) confirmed the presence of two LPOR isoforms in *Pinus mugo* and *Picea abies*, respectively. Moreover, LPORA is typical for PLB of etioplasts and etiochloroplasts of cotyledons where it is bound together with Pchlide and NADPH, and LPORB occurs mainly in mature tissues, e. g. secondary needles, where it is responsible for greening. However, the expression patterns in angiosperms and gymnosperms seem to be quite different; transcription of both LPOR isoforms was positively regulated by light, suggesting that the strong negative photoregulation described in angiosperms had not yet been established in gymnosperm plants (Skinner and Timko, 1999). These observations are consistent with our findings in Norway spruce (Stolárik et al., 2017).

Phytochrome regulation of LPOR expression was mentioned above. The potential regulation of DPOR by phytochrome was also investigated in *Marchantia paleacea* var. *diptera* (Suzuki et al., 2001). Authors claimed that phytochrome regulates the expression of *chlLNB* genes of DPOR. On the contrary, Shi and Shi (2006) suggest that larger amounts of *chlLNB* transcripts in the cells grown in light compared to the cells grown in dark could reflect either subtle differences in their transcription rates or in the stability of their transcripts under completely different growth conditions. Moreover, Eguchi et al. (2002) observed higher *chlL* transcript level in dark-grown *M. paleacea* var. *diptera* if compared with the light variant. The involvement of phytochrome in DPOR regulation in conifers has not yet been established, however negative photoregulation was documented in *Pinus thunbergii* and *Picea abies* cotyledons (Demko et al., 2010; Yamamoto et al., 2017; Stolárik et al., 2017).

Many authors provided the evidence that both Pchlide oxidoreductases contribute to the total Chl content in various photosynthetic organisms (Fujita and Bauer, 2003). An *in vitro* assay with crude cell extracts of *Pinus mugo* seedlings showed that LPOR is the major Pchlide reduction system and that DPOR only functions as an auxiliary system under the conditions, where light is hardly available (Forreiter and Apel, 1993). Thus, this may indicate that Chl formation is dependent on light intensity. The cells of green alga *Chlamydomonas reinhardtii* with any mutation in *chlL*, *N* or *B* genes, as well as particular nuclear loci (*y-1 – y-10*) resulted in *yellow-in-the-dark* phenotype, if cultivated in conditions without the presence of light. However, if the same cells were cultivated under light conditions, they achieved wild-type phenotype and were able to synthesize enough Chl, thus LPOR can fully substitute the missing DPOR under light conditions (Cahoon and Timko, 2000). Similar results on algae, cyanobacteria and liverwortswere were obtained earlier by Fujita et al. (1992); Suzuki and Bauer (1992) and Suzuki et al. (1998). Recently, the studies on

Marchantia polymorpha L. confirmed that DPOR is required for Chl biosynthesis under light limiting conditions (Ueda et al., 2014). The authors cultivated mutant plants in *chlB* gene of *M. polymorpha* under short and long day conditions and observed that the liverworts growing under short day conditions were significantly delayed in their development and accumulated much lower amount of Chl in comparison to wild type. Under long day conditions, mutant plants were indistinguishable from wild type indicating, that the role of DPOR in Chl biosynthesis decreases with a prolonged light period and that LPOR becomes essential for growth under high light conditions. Similar results were obtained by Fujita et al. (1998) who worked with a pair of *Plectonema boryanum* mutant, in which either LPOR or DPOR was inactivated. The results showed that the role of LPOR increases with increasing light intensity (>170 μmol m^{-2} s^{-1} PAR), however under low light conditions (10 – 25 μmol m^{-2} s^{-1} PAR) both PORs are required for sufficient Chl formation. Thus, in the situation with scarcity of photons, LPOR most likely cannot substitute the function of DPOR. These findings became a theoretical background to investigate the plasticity of Chl synthesis and photosystems formation in larch and spruce seedlings under deep shade conditions (Stolárik et al., 2018).

However, the formation of photosynthetic apparatus and Chl synthesis differs among gymnosperm species. For example, *Ginkgo biloba* does not express the genes for DPOR efficiently and is completely etiolated when growing in the dark (Pavlovič et al., 2010). On the other hand, *Picea abies* has the highest ability to synthesize Chl in the dark, whilst *Larix* and *Thuja* represent the conifers with strongly decreased ability in this way. This low ability is caused by two factors: non-synonymous mutations in DPOR genes in *Thuja* and insufficient *chlB* mRNA editing in *Larix* (Kusumi et al., 2006; Demko et al., 2009). Yamamoto et al. (2017) confirmed that *chlB* mRNA editing serves as an important regulatory system in *Pinus thunbergii*. The efficiency of *chlB* editing is decreased in the light and as a result, the unedited copy of ChlB protein is not able to interact with ChlL and thus to form a functional DPOR complex.

Besides light, temperature is one of the most important regulators of plant development. It was described that low temperature significantly inhibits Chl formation on transcriptional level in angiosperms (Tewari and Tripathy, 1998; Mohanty et al., 2008). Our findings also confirmed that low temperature slows down the whole Chl biosynthetic pathway in Norway spruce as a representative of conifers (Stolárik et al., 2017) but the chlorophyll accumulation in the light was not inhibited so strongly in comparison with angiosperms. However, low temperature in combination with darkness inhibits Chl formation almost completely, indicating strong inhibition of DPOR activity under low temperature conditions

(Muramatsu et al., 2001; Stolárik et al., 2017). However, transcription and translation of DPOR subunits were not negatively affected under such conditions. Thus, the significant decrease in Chl level seemed to be affected post-translationally. Moreover, Yamamoto et al. (2009; 2011) investigated that ChlL protein of DPOR is a primary target of oxygen, however this functional inactivation is not connected with a significant degradation of the protein(s) (Yamazaki et al., 2006). For example, LPOR deficient mutant of cyanobacterium *Leptolyngbya boryana* designated as YFP12 could grow photoautotrophically at maximally 3% oxygen concentration, which is an incomparably lower concentration to that found in ambient atmosphere (Yamazaki et al., 2006). Taking into account these information and if considering the increasing solubility of oxygen with a decreasing temperature (Henry, 1803), this provided a theoretical basis of our performed experiments, where we mimicked low-temperature conditions by the exposure of Norway spruce seedlings to higher oxygen content (Stolárik et al., 2017).

Chlorophyll biosynthesis and formation of pigment-protein complexes

In angiosperms, the accumulation of pigment-protein complexes can be controlled at multiple steps. The transcription of genes encoding chlorophyll-binding proteins is induced by light and regulated by phytochrome and blue light receptors. In addition, factors such as circadian regulation and the status of chloroplast influence the gene expression and further development (Tobin and Silverthorne, 1985). In addition, stable accumulation of chlorophyll-binding proteins has also been found to depend on the availability of chlorophylls (Chl). For example, mutants deficient in Chl*b* express normal level of the major LHCB polypeptides, but they were unstable in the absence of Chl*b* (Bellemare et al., 1982). Due to the absence of light, etiolated angiosperm plants do not accumulate significant amounts of chlorophyll-binding proteins in dark (Kanervo et al., 2008). In contrast, gymnosperms transcribe the genes encoding chlorophyll-binding proteins light-independently and the chlorophylls synthesized by DPOR stabilize proteins in thylakoid membranes (Yamamoto et al. 1991; Muramtasu et al., 2001). Thus, coniferous seedlings growing in the dark form relatively well developed etiochloroplasts instead of etioplasts (Walles and Hudák, 1975), with fully active photosystem I (PSI) even during cultivation in full darkness (Oku et al., 1974; Kamachi et al., 1998). Photosystem II (PSII) is also formed under these conditions, however it remains in its latent form with an inactive oxygen-evolving complex (OEC) (Jansson et al., 1992; Shinohara et al., 1992; Pavlovič et al., 2016) until the chloroplasts are illuminated. Shinohara et al. (1992) elucidated that Mn integration into OEC is strictly light-dependent and indispensable for effective O_2 evolution. OEC is composed of three extrinsic proteins: PsbO, PsbP and PsbQ

with relative molecular mass of 33 kDa, 23 kDa and 17 kDa, respectively. These proteins play a crucial role in Mn-cluster stabilization and the modulation of binding Ca^{2+} and Cl^- ions, which are irreplaceable during O_2 evolution (Yi et al., 2005). Not surprisingly, the chlorophyll *a*/*b* ratio is also higher in dark-developing seedlings, suggesting that the total size of light-harvesting complexes (LHC) is smaller than those in light-growing plants (Jansson et al., 1992; Stolárik et al., 2017). Yamamoto et al. (1991) reported that in dark-grown pine cotyledons sufficient amounts of total and translatable mRNA for LHCII are present, but the lower supply of Chl produced by DPOR is a limiting point in the dark. The imbalance between Chl*b* supply and the formation of LHC apoproteins lead to smaller antenna size in dark-grown cotyledons which are usually in monomeric conformation (Xue et al., 2017). In our study (Pavlovič et al., 2016), we have shown that the photoactivation of oxygen evolution in PSII is relatively fast and can be observed already in PSII that are not fully photochemically active. Continuously, the light-induced formation of fully active PSII centres is a gradual and long-term process in proper stoichiometric ratio requiring the synthesis and binding of LHCII to PSII, the assembly of OEC proteins and the final association of PSII into supercomplexes. Because DPOR provides only a limited supply of chlorophylls, we can conclude that light plays a crucial role not only in the assembly and activation of pigment-protein complexes involved in the process of photosynthesis in coniferous plants, but it also enhances Chl supply into this process.

5 Summary

This doctoral thesis deals with chlorophyll (Chl) biosynthesis in gymnosperm plants, especially in Norway spruce (*Picea abies* Karst.) and partially in deciduous European larch (*Larix decidua* Mill.) – the important representatives of the Pinophyta division.

It is well-known that the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) is a crucial step in Chl biosynthetic pathway. This reaction may be catalyzed by light-dependent (LPOR) or light-independent (DPOR) protochlorophyllide oxidoreductase (POR). LPOR occurs separately, without the presence of DPOR, in angiosperm plants only, whilst they occur together among (almost) the rest of the plant kingdom including gymnosperms.

We have found that Chl biosynthesis in spruce depends not only on the presence or absence of light, but also on the developmental stage of the plant, as well as on the ambient temperature. Although the light's morphogenetic function is alleviated in cotyledons, in secondary needles it seems to be absolutely irreplaceable. This effect is well-documented on the absence of significant accumulation of Chl in secondary needles in comparison to cotyledons in the dark. DPOR activity is transcriptionally regulated by light and developmental stage, which was clearly shown mainly by analyses of gene expressions encoding the subunits of this enzyme. However, the ambient temperature regulates this enzyme post-translationally, which accounts for the etiolated phenotype of spruce seedlings cultivated at 7 °C in the dark, despite the normal levels of DPOR protein products. In this context, taking into account the increasing oxygen solubility proportionally to the decreasing temperature in the water, we have proposed that DPOR becomes a possible target of higher O_2 concentration. This hypothesis is based on the finding that ChlL subunit is oxygen-sensitive and DPOR enzyme is inactivated by this manner (Stolárik et al., 2017).

Following the experiments investigating the regulation mechanisms in Chl biosynthetic pathway we have dealt with the ecophysiological implications of having DPOR. Considering the pivotal role of DPOR under short day conditions where it significantly contributes to Chl formation, we have also decided to include larch (*Larix decidua*) seedlings into our experiments. This coniferous plant is typical for a decreased Chl concentration in the dark despite the presence of DPOR and this phenomenon was elucidated by insufficient *chlB* mRNA editing (which is a subunit of DPOR), thus, its' decreased activity. Based on several experiments, we have found that the larch seedlings, in contrast to spruce, showed a

remarkably decreased ability of Chl formation at very weak irradiance. This peculiarity can be possibly explained by the fact that under deep-shade conditions LPOR enzyme can not work properly due to its light-dependent character. The scarcity of photons for LPOR and dysfunctional DPOR in Europen larch are not able to satisfy the requirement for chlorophyll synthesis under these conditions. On the other hand, spruce had a significantly higher Chl amount despite growing in deep-shade, because of a functional DPOR presence. This causes the situation that spruce plants do not show an etiolated phenotype under such conditions, as well as the maximum quantum yield of PSII photochemistry values are comparable with the plants growing under optimal illumination, because even a low amount of photons was sufficient for the successful photoactivation of PSII. The presented data support the hypothesis about the mutual cooperation of both Pchlide-reducing enzymes under various light conditions (Stolárik et al., 2018).

Although dark-cultivated spruce seedlings possessed fully assembled and functional photosystem I (PSI), PSII was present only in its latent form with inactive oxygen-evolving complex (OEC). Using various laboratory techniques (fluorescence, thermoluminiscence, native and/or reducing gel electrophoresis, immunoblot, etc.) we have found out that after the illumination of plants, PSII is gradually activated. This includes fast Mn atoms incorporation into OEC and the whole assembly of this water-oxidizing complex, where PsbO, PsbQ and PsbP proteins play an important role. These proteins are localized inside the thylakoid membranes also in the dark, although they do not form a tight cluster with PSII. Interestingly, after 5 min. of illumination, a partial PSII activation was observable together with a detectable O_2 evolution. Even a 24-h illumination was not sufficient for full activation of PSII centres which include stable assembly of OEC and LHCII-PSII supercomplexes formation. This was observable only if the plants were cultivated under normal light/dark conditions (Pavlovič et al., 2016).

Finally, it is necessary to mention the “cost and benefit” of having DPOR. The reason why LPOR enzyme has appeared during evolution is quite simple: most likely it was a consequence of the transition from the anaerobic Archean Earth’s atmosphere to an aerobic atmosphere, similar to that existing in present times. Moreover, LPOR has at least three advantages over DPOR: primarily, it is insensitive to oxygen, as a photoenzyme it limits Pchlide photooxidative damage, and is energy-independent, i. e. does not require energy in the form of ATP. However, despite its ancient character and high energy requirements, the presence of DPOR enzyme in a functional state offers plants several advantages, mainly under specific light conditions. The presented doctoral thesis documents the regulation of DPOR on

transcriptional, translational and post-translational level; the ecophysiological implications of its presence in plants, and its importance in chlorophyll synthesis and PSII-supercomplexes formation in gymnosperm plants.

6 Experimental approach

This chapter is focused on plant material and experimental techniques, their principles and performance used in this doctoral thesis. However, specific details (e. g. solution concentrations) are shown in a corresponding article.

6.1 Plant material and culture conditions

The plants were cultivated under strictly controlled experimental conditions. We obtained the seeds of Norway spruce (Picea abies) and deciduous European larch (Larix decidua) from Semenoles, Liptovský Hrádok, Slovakia. The well-soaked seeds which had imbibed for at least 4 hours were then sown into well-moistened perlite substrate. The seedlings were cultivated under periodic light/dark cycle (12 h/12 h) or in complete darkness. The adult twigs of Norway spruce collected in Jeseníky, Czech Republic were cultivated under identical light and dark conditions. The period of seedlings cultivation was dependent on the ambient temperature; lower temperature (~7 °C) required longer cultivation. For more details (period of cultivation, temperature, light intensities during cultivation, composition of ambient atmosphere etc.) see attached articles in Chapter 8. After the cultivation, plant material was collected and frozen in liquid nitrogen and stored at -80 °C or immediately used for analyses. We have also cultivated etiolated barley seedling in the dark as a representative of angiosperms (without DPOR and Chl biosynthesis ability in the dark) to have a negative control for DPOR immunoblot analysis, as well as for Pchlide standard during its determination.

6.2 Assimilation pigments analysis

Already macroscopic observations revealed significant changes in chlorophyll concentration, which were confirmed by spectrophotometric measurements according to Lichtenthaler (1987). The crude tissues were ground in 80% (v/v) acetone under safe dim green light and Chl *a* and *b* concentration was determined at 663.2 and 646.8 nm, respectively, using double-beam spectrophotometer (Thermo Spectronic UV500, UV-Vis Spectro). If the chlorophyll concentration was very low and under the spectrophotometer detection limit, we used a HPLC method according to Papenbrock et al. (1999). The samples preparation for HPLC quantification is well described in our article – Stolárik et al. (2017), in section Materials and methods. The HPLC was also used for heme quantification, as described in Apitz et al. (2016).

We have also determined the amounts of intermediates leading to chlorophyll to describe the effect of various cultivation conditions on the whole Chl biosynthetic pathway. 5-aminolevulunic acid synthesizing capacity was measured according to Alawady and Grimm (2005). The exact steps of this procedure are described in our article (Stolárik et al., 2017). Protochlorophyllide determination in gymnosperm plants meets the problem due to small amount of Pchlide and a lower molar absorptivity of Pchlide in acetone compared to Chl caused by light-independent Chl biosynthesis. To avoid this problem, we separated Pchlide from all esterified tetrapyrroles as recommended by Selstam et al. (1987) by extraction with basic acetone and further washing with hexane. The amount of Pchlide was measured spectrofluorimetrically at λ_{ex} 438 and λ_{em} 633 nm in the hexane-washed acetone phase and quantified using pchlide standard from etiolated barley plants. For further details see Stolárik et al. (2017), section Materials and methods.

6.3 Chlorophyll fluorescence

Chlorophyll fluorescence is one of three possible fates of the absorbed light energy, besides heat and photochemistry. Thus, Chl fluorescence is the light re-emitted by chlorophyll molecules during the return from excited to basic states and it serves as a sensitive indicator of photosynthetic energy conversion *inter alia* in higher plants. The quantum yield of Chl *a* fluorescence from photosynthetic apparatus is 2–8%, reflecting open and closed reaction centres, respectively (Maxwell and Johnson, 2000). In my doctoral thesis we used various experimental techniques based on Chl fluorescence, and detailed information are presented below:

6.3.1 Low-temperature fluorescence measured at 77 K

The fluorescence emission spectra of photosynthetic organisms measured at the temperature of liquid nitrogen (77 K) provide important insights into the structural properties of photosynthetic apparatus, because the probability of vibrational energy states occurrence significantly decreases in comparison to room-temperature (Lamb et al., 2015). Moreover, 77 K temperature inhibits the electron transport within photosynthetic apparatus. Thus, emission bands recorded at 633 and 655 nm represent free Pchlide and Pchlide bound with PLBs, respectively. Chl *a* molecules bound to internal antennae of PSII – CP43 and CP47 give rise the fluorescence bands at 685 and 694 nm; and the light-harvesting complex of PSI at 733 nm. The presence of the mentioned bands changes according to the various light-

cultivating conditions, which was well-documented in Pavlovič et al. (2016) and Stolárik et al. (2017).

6.3.2 Very fast Chl fluorescence induction (FLI): the O-J-I-P curves

The measurement of very fast chlorophyll *a* fluorescence induction rise (FR) is a widely-used non-destructive method to observe the electron transport within the thylakoid membrane. We have measured FR with the Plant Efficiency Analyser (fluorometer) – (PEA, Hansatech, Norfolk, UK). The measurement was first introduced by Strasser and Govindjee (1991). A typical fluorescence induction curve of fully-developed dark-adapted plants is characterized by polyphasic rise called OJIP transient, where O, J, I and P mean the typical steps describing successive electron transport through the electron carriers. F_0 fluorescence (O-step) appears 40 – 50 μs after illumination and it originates from excited Chl molecules of light-harvesting antennae. The J, I and P steps appear after 2, 20 and 200 ms, respectively. It is widely accepted that J step reflects the Q_A^- accumulation, I is a representative of reduction Q_A^- and Q_B^- and J-step is typical for $Q_A^-Q_B^{2-}$ state hovewer several other explanations have been proposed (for review see Lazár, 2006). If the additional step designated as K appears about 400 μs after illumination, it means the inhibition on the donor side of PSII, i. e. the inhibition of OEC. The details are well described in Pavlovič et al. (2016). In specific situation the H and G step may appear, and H-step corresponds with the oxidation of P700/PC and subsequent G-step reflects a transient reduction of plastoquinone (PQ) pool, which is not followed by the redox change of P700/PC. The characterization of FR was described in Strasser (1997), Lazár (1999), Lazár and Schansker (2009), Stirbet and Govindjee (2012). For technical details and the performance of FR measurements, see Pavlovič at al. (2016) and Stolárik et al. (2017), sections Materials and Methods.

6.3.3 Quenching analysis

As the name suggests, the quenching analysis uses the fluorescence decay after a previous strong illumination of the sample. The measurement is initiated by switching on the measuring light, giving a measure of F_0 (min.) level of fluorescence in dark-adapted sample. Then, the saturation flash of the light is applied which causes closure of all reaction centres and allows the measurement of maximal fluorescence (F_m) in the dark-adapted state (F_m^0). Next, the actinic light is applied and at appropriate intervals, further saturating pulses are triggered and from each of these, F_m' (maximum fluorescence in the light) can be measured. F_t is a value of the steady-state fluorescence, measured immediately prior to (saturation) flash. After the flash, the actinic light may be switched off (preferably whilst simultaneously giving

a far-red light) and this allows to measure F_0' value (Maxwell and Johnson, 2000). The measured fluorescence quenching values compose the basis for important parameters calculation. In our articles we have used F_v/F_m parameter, which is the maximum quantum yield of PSII, and it was calculated as $(F_m - F_0)/F_m$. 1 – qP parameter was also used and informs about the ratio of "closed" reaction centres. Other commonly used fluorescence parameters may be found in Tab. 1 in Maxwell and Johnson (2000).

6.4 Oxygen evolution measurements

Besides the K-step of very fast fluorescence induction, which informs us about dysfunctional oxygen-evolving complex (OEC), there is also a straight technique suitable for O_2 evolution measurement – polarographically by the Clark's electrode. This electrode is composed of Ag anode and Pt cathode. The method is based on the changes of redox potential, which is measured as a difference of the voltage between the measuring and the reference electrode, which are under voltage and the electric current is proportional to the rate of oxygen reduction on the cathode surface (Clark et al., 1953). For our purposes we have measured oxygen evolution in thylakoid membranes suspension isolated according to Dau et al. (1995) and Hideg (1994). Details about thylakoid membranes isolation and oxygen evolution measurement performance are shownin Pavlovič et al. (2016).

6.5 Thermoluminiscence

Thermoluminiscence (TL) is a type of luminescence where the emitted light is measured after controlled warming of the sample. It is used *inter alia* for dating of ceramics, based on the imperfections in the lattice of crystals – hole and electron trapping levels. During the burning of the ceramics (of archeological artefacts) the trapping states of atom lattices – due to their imperfections – are formed and thus, not occupied by electrons. Over the centuries the ceramics are exposed to natural sources of radiation (cosmic rays, X-rays, etc.) which excite atoms in the lattice and electrons are trapped at trapping levels. Thus, proportionally to elapsed time, the lattice is more and more occupied by electrons. The next heating of the ceramics (during the TL measurement) causes the release of electrons from trapping state to exciting state and the energy is emitted in form of thermoluminiscence. Analogically, photosynthetic material after excitation – the charge separation in reaction centres at low temperature, the charged pairs are stabilized, similarly with formation of electron – hole pairs in ceramics (solids). The low temperature inhibits the movement of free electron carriers and

thus, the proper function of oxygen-evolving complex (Misra et al., 2001; Roncel and Ortega, 2005). TL in photosynthetic material was identified for the first time by Arnold and Sherwood (1957). The most of TL bands come from the recombination of charged pairs in PSII, which appear during linear, controlled heating of the photosynthetic sample and each band is characterized at specific temperature. The origin and characterization of TL bands may be found in Tab. 1 of Vass (2003) article, page 306. We have measured TL glow curves with a laboratory set-up based on cooled single-photon-counting detection system. The technical details of the measurement are presented in Materials and Methods section in Pavlovič et al. (2016).

6.6 Gas exchange measurements

Gasometric methods were used in our article (Stolárik et al., 2018), where all the technical details and measurement performance are provided. The method is based on measurements with an infra-red gas analyser LI-6400 portable photosynthesis (open) system (LI-COR Biosciences, Inc., Lincoln, NE, USA). The principle of the method is the measurement of gas exchange between leaf and its environment. It is secured by infra-red (IR) gas analyzer which functions on the absorption of IR light by heteroatomic molecules, e. g. CO_2, H_2O, NO, NH_3 at specific wavelengths. On the other hand, the isoatomic molecules like O_2, N_2, H_2 do not absorb IR light. Thus, commercial instruments for gas exchange function as absorptiometers, measuring in two wavelengths: 4.25 μm and 2.66 μm for CO_2 and H_2O, respectively. Modern gasometers allow the measurement of not only photosynthesis rate (A_N) and respiration (R_D), but also stomatal conductance (g_s) and transpiration (E) (Auble and Meyers, 1992).

6.7 Electrophoretic separation methods

Electrophoresis is a method used for the separation of compounds, based on the movement of charged particles (molecules) in an electric field. This electrokinetic phenomenon was observed for the first time in 1807 by Russian professors P. I. Strakhov and F. F. Reuss (Reuss, 1809). In our experiments, we have used three types of electrophoresis: clear native polyacrylamide gel electrophoresis (CN-PAGE), polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) for proteins and agarose-gel electrophoresis for the separation of nucleic acids.

The first two types of electrophoresis mentioned above are based on polyacrylamide (PolyAcrylamide Gel Electrophoresis – PAGE). The electrophoretic gel is prepared by the

mixture of monomeric (acrylamide) and dimeric (bisacrylamide) forms. Proportional amounts of these two compounds result in the final gel properties, characterized by size of pores in the gel, and thus the quality of separation (Rüchel et al., 1978). The composition of the gel is characterized by T and C values, where T means "Total" concentration of acrylamide and bisacrylamide and C indicates the percentual concentration of "Cross-linker", what is bisacrylamide. The polymerization of (complex) acrylamide solution is initiated by adding ammonium persulfate (APS), with the sulphate radicals serving as electron donors and the reaction is catalyzed by TEMED (N,N,N′,N′-tetramethylenediamine), which serves as an electron carrier.

We used SDS-PAGE (Sodium Dodecyl Sulphate – SDS) for the electrophoretic separation of proteins in denaturing conditions, where SDS binds the proteins, causes their denaturation and add them negative charge, which is used in their separation in an electric field. SDS together with some reducing agents (e. g. dithiotreitol, mercaptoethanol) causes the dissociation of quaternary and ternary protein structures, thus the protein complexes are dissociated into individual proteins. In SDS-PAGE technique, the proteins are separated according their molecular mass. In our experiments, we used Tricine system (Schägger – von Jagow system) (Schägger, 2006) which provides better separation of smaller molecules.

Gradient (4 – 8%) CN-PAGE gel (Clear-Native – CN) was used for the separation of pigment-protein supercomplexes. The separation of supercomplexes is well described in Kouřil et al. (2014). The principle of this separation method is based on non-reducing environment with mild, non-ionic detergents in low concentrations, which secure the solubilisation of protein complexes. In case of CN-PAGE, the proteins are negatively charged by a mild detergent – deoxycholate. The PSII-enriched membranes separated by CN-PAGE technique were prepared according to Berthold et al. (1981) with a few modifications (Caffarri et al., 2009). For further details, see Pavlovič et al. (2016), section Materials and Methods (2.7 Isolation and separation of PSII supercomplexes by clear native PAGE).

Agarose gel electrophoresis was used for nucleic acid separation to confirm the sizes of PCR amplification products in gene expression experiments. Agarose is a polysaccharide (linear polymer of D-galactose and 3,6-anhydro-L-galactose) obtained by agar purification, which is isolated from red algae. Agarose appears usually in the form of powder which is boiling in an appropriate buffer, most commonly 1 x TAE (Tris – acetate – EDTA) and the agarose solution solidifies after cooling. Analogically to polyacrylamide gels, agarose gels also have the network structure enabling nucleic acid separation according their molecular weight. The movement of nucleic acids in an electric field is secured by phosphate groups

which grant the negative charge to molecules (Serwer, 1983; Zimm and Levene, 1992). After electrophoretic separation, DNA is visualized by ethidium bromide. It is a compound intercalating among DNA and it fluoresces under UV radiation.

6.8 Real-time quantitative PCR

We used real-time quantitative polymerase chain reaction (qPCR) to elucidate the expression of the crucial genes whose products are involved in the chlorophyll biosynthetic pathway. qPCR is a method used in molecular biology and it is a variation of PCR where the amount of DNA products are spectrofluorometrically counted after each cycle of the amplification. Thus, it monitors the amplification of a targeted DNA molecule during the PCR, it means in real time, and not at its end as in conventional PCR. The input into the reaction consists of complementary DNA (cDNA) synthesized according to the RNA isolate (template); the reason is that the gene expression could be measured as the number of copies of an RNA transcript of the gene present in a sample, however RNA molecules are present in very small amounts and is essential to amplify it. This way, PCR is a method targeted on DNA, thus firstly it is necessary to reverse-transcribe RNA to cDNA and then qPCR can be performed (Bustin et al., 2009). The information about qPCR performance in our experiments is presented in Stolárik et al. (2017), section Materials and Methods and in Supplementary data.

6.9 Western blot

Western blot is an analytical semi-quantitative method widely used in several scientific sections, based on the immunodetection of the protein of interest (Corley, 2005). In our experiments, prior to Western blots, SDS-PAGE was performed for protein separation according their molecular weight. Subsequently, to make the proteins accessible for antibodies, they were transferred from the gel to a nitrocellulose membrane (commonly used is also polyvinylidene difluoride – PVDF membrane, usually for hydrophobic proteins). For the transfer we used a *semi-dry* electroblotting procedure, which uses the electric current to pull the proteins from the gel to the membrane. After the transfer, the effectiveness and integrity of proteins were checked by Ponceau-S staining. Before the incubation of the membrane in the antibody solution, the membrane must be stored in a blocking solution, which usually consists of 3 – 5% bovine-serum albumin, non-fat milk, etc. diluted in an appropriate buffer with a detergent (Tween 20), to prevent the non-specific bounds between

the antibody and the membrane. After blocking, the membranes were incubated in specific primary antibodies. The majority of antibodies were obtained from Agrisera (Vännäs, Sweden) and the antibodies against DPOR subunits were generously provided by prof. Yuichi Fujita (Nagoya, Japan). After washing the membranes of primary antibodies, the membranes were incubated in a secondary antibody (goat anti-rabbit IgG (H+L)-horseradish peroxidise conjugate) and subsequently visualised using Immobilon Western chemiluminiscent HRP substrate (Millipore, Billerica, MA, USA) on an Amersham Imager 600 (GE HealthCare Life Sciences, Japan). The chemiluminiscent visualisation is based on a reporter enzyme included in the secondary antibody (horseradish peroxidase), which converts the chemiluminiscent substrate (HRP substrate) to an unstable intermediate, which is stabilized by radiation of light-quantum – chemiluminiscence. The reference numbers of antibodies, as well as technical performance of western blot analyses are shown in our all articles, sections Materials and Methods.

6.10 Transmission electron microscopy (TEM)

For our purposes small sections of cotyledons were fixed in 5% glutaraldehyde and subsequently post-fixed in a solution of osmium tetroxide. Glutaraldehyde forms double bonds and perfectly stabilizes the sample. The samples were then gradually dehydrated in ethanol series with the final dehydratation by propylene oxide. In further steps, the samples were gradually satiated with an increasing concentration of the epoxide resin from Spurr Low-Viscosity Embedding Kit (Sigma-Aldrich, St. Louis, MO, USA). Finally, the samples were embedded in pure epoxide resin which polymerized and solidified at 70 °C overnight. Subsequently, the samples embedded in resin were cut to semithin sections (400 nm) using a glass knife. Semithin sections were stained by toluidine blue and examined by light microscope. Thereafter the samples were cut to ultrathin sections (90 nm) by a diamond knife, placed on copper grids and contrasted by uranyl acetate and lead citrate. Contrasting represents the substitution of the structures by huge atoms. Finally, the samples were observed by electron microscope Jeol JEM 2010 (Japan). For details, see Stolárik et al. (2018), section Materials and Methods.

The transmission electron microscopy is based on electrons instead of photons (used in optical microscopes) because the long wavelengths of visible light photons (380 – 760 nm) are one of the limiting factors for the image resolution. On the other hand, the wavelength of an electron depends on acceleration voltage inside the electron microscope. Electrons of

shorter wavelengths are the background for significantly higher resolution of an electron microscope, compared to an optical microscope. The electrons come from the electron source (electron gun) and aligned in an electron beam are interacting with the observed sample in one of several ways (transmission, scattering, reflection, secondary electron production, etc.). The subsequent passing of electrons through objective and projector lenses and the interaction with detector(s) ensure specific interaction-related visualization of sample structure (Reuss and Dykstra, 2003).

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8 Publications

8.1 Transcriptional and post-translational control of chlorophyll biosynthesis by dark-operative protochlorophyllide oxidoreductase in Norway spruce.

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ORIGINAL ARTICLE

Transcriptional and post-translational control of chlorophyll biosynthesis by dark-operative protochlorophyllide oxidoreductase in Norway spruce

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Abstract Unlike angiosperms, gymnosperms use two different enzymes for the reduction of protochlorophyllide to chlorophyllide: the light-dependent protochlorophyllide oxidoreductase (LPOR) and the dark-operative protochlorophyllide oxidoreductase (DPOR). In this study, we examined the specific role of both enzymes for chlorophyll synthesis in response to different light/dark and temperature conditions at different developmental stages (cotyledons and needles) of Norway spruce (*Picea abies* Karst.). The accumulation of chlorophyll and chlorophyll-binding proteins strongly decreased during dark growth in secondary needles at room temperature as well as in cotyledons at low temperature (7 °C) indicating suppression of DPOR activity. The levels of the three DPOR subunits ChlL, ChlN, and ChlB and the transcripts of their encoding genes were diminished in dark-grown secondary needles. The low temperature had minor effects on the transcription and translation of these genes in cotyledons, which is suggestive for post-translational control in chlorophyll biosynthesis. Taking into account the higher solubility of oxygen at low temperature and oxygen sensitivity of DPOR, we mimicked low-temperature condition by the exposure of seedlings to higher oxygen content (33%). The treatment resulted in an etiolated phenotype of dark-grown seedlings, confirming an oxygen-dependent control of DPOR activity in spruce cotyledons. Moreover, light-dependent suppression of mRNA and protein level of DPOR subunits indicates that more efficiently operating LPOR takes over the DPOR function under light conditions, especially in secondary needles.

Keywords Chill stress · Chlorophyll · DPOR · Low temperature · Protochlorophyllide · Norway spruce

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Introduction

Photosynthesis is a unique process on earth, which annually converts approximately 258 billion tons of carbon dioxide (Geider et al. 2001). The photosynthetic pigments, especially chlorophylls (Chls), are indispensable for light absorption, energy transduction as well as charge separation in all oxygenic photosynthetic organisms. Chls are end products of a multienzymatic and branched metabolic pathway. The first committed molecule of this tetrapyrrole biosynthetic pathway is 5-aminolevulinic acid (ALA) which is synthesized in plastids from glutamate by three enzymes: glutamyl-tRNA synthetase (GluRS), glutamyl-tRNA-reductase (GluTR), and glutamate-1-semialdehyde aminotransferase (GSAT). Eight molecules of ALA are combined to a first cyclic tetrapyrrole (uroporphyrinogen III) which is further converted by multiple enzymatic steps to protoporphyrin. Metal chelation reactions of protoporphyrin IX (Proto IX) catalysed by either Fe-chelatase or Mg-chelatase divide the

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pathway into the Fe-branch and the Mg-branch leading to heme and Chl formation, respectively (Papenbrock and Grimm 2001; Tanaka and Tanaka 2007).

One of the final steps of the Mg-branch is the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide). With the exception of anoxygenic photosynthetic bacteria and angiosperms, two different enzymes catalyse the Chlide formation in all photosynthetic organisms (Armstrong 1998; Schoefs and Franck 2003; Reinbothe et al. 2010; Gabruk and Mysliwa-Kurdziel 2015). The first enzyme is the light-dependent NADPH-protochlorophyllide oxidoreductase (LPOR or simply POR) encoded in the nuclear genome. The light-driven catalytic activity depends on light absorption of Pchlide resulting in a conformational change of LPOR. The second enzyme is a dark-operative protochlorophyllide oxidoreductase (DPOR), whose subunits are encoded in the plastome of plants or in the nucleoid of bacteria. DPOR is distributed not only among anoxygenic photosynthetic bacteria, but also among oxygenic phototrophs, i.e., cyanobacteria, green algae, and non-flowering plants (mosses, liverworts, hornworts, lycophyta, and ferns up to gymnosperms). However, it has never been found in angiosperms (Armstrong 1998; Fujita and Bauer 2003; Yamamoto et al. 2011). Interestingly, the two enzymes, DPOR and LPOR, coexist in most cyanobacteria, algae, and non-flowering plants (Armstrong 1998). Surprisingly, a gene encoding a LPOR variant was recently also detected in an anoxygenic photosynthetic bacterium and apparently introduced into the genome by horizontal gene transfer (Kaschner et al. 2014). Although both enzymes catalyse the reduction of Pchlide to Chlide by the same stereospecific reaction of the pyrrole ring D of Pchlide, they do not possess significant sequence similarity (Armstrong 1998; Reinbothe et al. 2010; Gabruk and Mysliwa-Kurdziel 2015), although TFT motif shares similar physicochemical properties in both types of the enzymes (Gabruk et al. 2012). LPOR is active as single polypeptide existing in higher plants in one (*Cucumis sativum*) or two isoforms (*Hordeum vulgare; Pinus taeda* designed as LPORA and LPORB) and belongs to the protein family of the short-chain dehydrogenases. LPORA is the predominant form of the enzyme present in etiolated tissue in prolamellar bodies (PLB). LPORB is responsible for Pchlide reduction during the later stages of greening and is present in minor amounts in thylakoid membranes. (Holtorf et al. 1995; Skinner and Timko 1999; Suzuki et al. 2001; Garrone et al. 2015; Gabruk and Mysliwa-Kurdziel 2015). In addition, a third isoform LPORC is known in *Arabidopsis thaliana* (Oosawa et al. 2000; Su et al. 2001). Completely different DPOR structurally resembles a protein complex of nitrogenase. It also consists of three subunits, which form two subcomplexes, the L (a ChlL dimer) and the NB (a ChlN–ChlB heterotetramer) proteins, respectively (Bröcker et al. 2010; Muraki et al. 2010).

It is known that the Chl and bacteriochlorophyll (Bchl) biosynthetic pathways underlie a strict regulation in angiosperms and photosynthetic bacteria, respectively, because accumulating Chl precursors can be highly phototoxic (Kruse et al. 1995; Bauer 2004; Zhong et al. 2009). In plants, light and the circadian clock are important regulatory factors for the transcriptional control of many genes involved in Chl biosynthesis and photosynthesis (Kruse et al. 1997). In particular, the regulation of ALA synthesis is considered to be rate-limiting for the metabolic flow into the pathway. The *HEMA* gene encoding GluTR is regulated inter alia by phytochrome (Gehring et al. 1977; Huang et al. 1989; Mohanty et al. 2006). Moreover, in the absence of light, transiently accumulating Pchlide mediates a feedback control on ALA synthesis by interaction of the negative regulator FLU with GluTR (Kauss et al. 2012). Thus, the plant is protected during LPOR inactivation in the dark against Pchlide accumulation and successive photooxidative damage (Meskauskiene et al. 2001; Goslings et al. 2004; Richter et al. 2010; Apitz et al. 2016).

Besides light, many other factors, e.g., temperature, stage of development or phytohormones, were described to adjust the Chl production in angiosperms (Mohanty et al. 2006; Yaronskaya et al. 2006). However, less is known about the regulation of Chl synthesis in organisms like gymnosperms, which have the two parallel enzymatic capacities for Pchlide synthesis. It has been described that the dominant regulatory role of light is alleviated in these organisms, as they express light-independently many photosynthesis- and Chl biosynthesis-related genes (Mukai et al. 1992; Skinner and Timko 1999; Muramatsu et al. 2001; Demko et al. 2009; Breznenová et al. 2010). Thus, instead of light, stage of development or temperature may play significant roles in the biogenesis of the photosynthetic apparatus under dark conditions (Ou and Adamson 1995; Skinner and Timko 1999; Muramatsu et al. 2001; Demko et al. 2009). However, the overall pattern of control mechanisms requires further investigation.

Molecular studies on conifers have a huge ecological and economical importance, because these trees dominate forests for more than 200 million years (Nystedt et al. 2013). We focused our study on the gymnosperm conifer species Norway spruce (*Picea abies*). Since it has been documented that Chl synthesis in dark-grown gymnosperm cotyledons is strongly suppressed at low temperature, and the needles are almost etiolated in the dark (Ou and Adamson 1995; Muramatsu et al. 2001), we examined the molecular mechanisms behind these observations. We cultivated and harvested cotyledons and secondary needles in dark and light as well as under room (23 °C) and low-temperature (7 °C) conditions. We

examined the expression of genes involved in Chl biosynthesis and the accumulation of the encoded proteins. The results showed that whereas in the light and in the secondary needles DPOR activity is regulated on transcriptional level, low temperature affects Chl biosynthesis post-translationally. The role of oxygen in post-translational control of DPOR activity is verified in land plants for the first time. Thus, these results reflect different regulations of LPOR and DPOR enzymes in conifers in response to different conditions.

Materials and methods

Plant material and culture condition

The seedlings of Norway spruce (*Picea abies* Karst.) were cultivated from well-soaked (4 h imbibed) seeds (Semenoles, Liptovský Hrádok, Slovakia) in well-moistened perlite at the temperature 23 ± 1 or 7 ± 1 °C in complete darkness (D23, D7 variants, respectively) or in the periodic light/dark cycle (12/12-h 100 µmol photons m^{-2} s^{-1} PAR, L23, L7 variants, respectively, referred as light/dark-grown thereinafter), for 17 days (at 23 °C) or 2 months (at 7 °C). Then, the cotyledons were harvested and frozen in liquid nitrogen and stored at −80 °C or immediately used for analyses. For further experiments, the twigs of Norway spruce were collected in Jeseníky (Czech Republic) in early spring stage, where the new secondary needles were hidden in the buds; twigs were transferred to dark (DN variant) and light/dark cycle (12/12-h 100 µmol photons m^{-2} s^{-1} PAR, LN variant, referred as light/dark-grown thereinafter) at 23 °C and acclimated for 2 months. The newly emerged secondary needles were immediately used or harvested and deep-frozen in liquid nitrogen.

For treatments with elevated oxygen concentration, the seeds were sown and the seedlings were cultivated in complete darkness for 17 days at 21 or 33% of oxygen in atmosphere at 23 °C. Two glass desiccators (volume 24 L, Kavalier, Czech Republic) with plants were ventilated in open flow regime with ambient or with oxygen-enriched air. The gas mixing system (GMS 150, PSI Brno, Czech Republic) and compressed N_2, O_2, and CO_2 (Messer, Germany) were used for artificial atmosphere. Flow rates (2 L min^{-1}), CO_2 concentration (400 ± 20 µmol mol^{-1}) as well as relative air humidity (80 ± 10%), controlled by two-channel due point generator and temperature controller (MGK-4, Walz, Germany), were equal for both desiccators.

Etiolated barley (*Hordeum vulgare*) was cultivated in complete darkness for extraction of Pchlide standard and western blot analyses (as negative control for DPOR enzyme).

Pigment analysis

For Chl $a+b$ determination, hundred mg of plant material was ground in a mortar and pestle with a small amount of sand and extracted with 80% (v/v) chilled acetone with $MgCO_3$ to avoid acidification and pheophytinization of the pigments. The samples were centrifuged at 10.000×*g* at 4 °C for 5 min. The concentration of Chl *a* and *b* was determined using a double beam spectrophotometer (Thermo Spectronic UV500, UV–Vis Spectro, MA, USA) at 663.2 and 646.8 nm according to Lichtenthaler (1987). In samples, where the Chl concentration was below detection limit of spectrophotometer (DN variant), a HPLC method was used (Papenbrock et al. 1999).

Characterization of Pchlide present in the conifers was not possible without phase separation of the pigments due to small amount of Pchlide and to the lower molar absorptivity of Pchlide in acetone compared to Chl. Therefore, we separated Pchlide from all esterified tetrapyrroles as recommended by Selstam et al. (1987). Pchlide was extracted from 100 mg of the plant material (fixed for 2 min in hot steam) in 3 mL acetone: 0.1 M NH_4OH (9:1, v/v). To separate Pchlide from the esterified tetrapyrroles, the extract was washed three times with an equal volume of hexane. The amount of Pchlide was measured spectrofluorometrically (Hitachi, F-4500, Japan) at λ_{ex} 438 and λ_{em} 633 nm in the hexane-washed acetone phase and quantified using a Pchlide standard. The Pchlide standard was prepared from etiolated barley plants according to Koski and Smith (1948) and spectrophotometrically quantified at 623 nm using the molar extinction coefficient in diethyl ether $\varepsilon = 3.56 \times 10^4$ M^{-1} cm^{-1} (Dawson et al. 1986). Using a dilution series of Pchlide standard in acetone:0.1 M NH_4OH (9:1, v/v), calibration curve was measured. All manipulations in the dark were performed under a dim green safelight.

ALA synthesizing capacity was measured according to Alawady and Grimm (2005). Hundred mg of cotyledons were incubated for 6 h with 20 mM K-phosphate buffer (pH 6.8) with 40 mM levulinic acid (inhibitor of ALA dehydratase) in the light (100 µmol photons m^{-2} s^{-1} PAR) or in the dark. Then, the plant material was slightly dried, quickly frozen in liquid nitrogen and ground using mortar and pestle. The powder was resuspended in 1 mL of 20 mM K-phosphate buffer pH 6.8 using vortex mixer and the samples were centrifuged for 20 min at 15.500×*g*. Then 400 µL of the supernatant was used and 400 µL of K-phosphate buffer was used as reference sample; 100 µL of ethyl acetoacetate was added to the supernatant as well as to the reference samples, and the mixture was subsequently boiled at 100 °C for exactly 10 min, cooled on ice for 5 min and mixed with an equal volume (500 µL) of modified Ehrlich´s reagent (with 2% (w/v) 4-dimethylaminobenzaldehyde).

The samples were centrifuged at room temperature (RT) for 25 min at 15.500×*g*, and absorbance of the chromophore was recorded at $\lambda = 525$, 553 nm and background at 650 nm.

HPLC quantification of tetrapyrroles

Fifty mg of frozen plant material was homogenized using a Retsch mill (under dim light conditions). Then 250 µL of cold acetone:0.2 N NH_4OH (9:1) was added, and the mixture was stored for 20 min at −20 °C, then well homogenized and centrifuged for 10 min at 4 °C with maximal speed. The supernatant was transferred to a new reaction tube and 100 µL of this was re-centrifuged for 30 min and used for Proto IX determination but no significant amounts were detectable. The pellet was resuspended in 300 µL acetone and the extraction repeated. The new supernatant was added to the first and the extraction was repeated, if necessary. The combined supernatants were centrifuged at 4 °C for 30 min at 14.000 rpm. 100 µL of the supernatant was used for determination of Chl content in DN variant only, where it was below the detection limit of the spectrophotometer used. For heme determination, the pellet was mixed with 100 µL of acetone/HCl/dimethyl sulfoxide (10:0.5:2) and incubated for 20 min at RT with occasional vortexing. The mixture was centrifuged for 30 min at RT at 16.000×*g*. The supernatant was used for heme quantification as described in Apitz et al. (2016).

Low-temperature fluorescence emission spectra (77 K)

For organization of pigment-protein complexes, fluorescence emission spectra of the cotyledons and secondary needles were measured at low temperature using a fluorescence spectrophotometer Hitachi F-4500 (Tokyo, Japan) with the spectral band widths of 10 and 2.5 nm for excitation and emission monochromator, respectively. The plant material was immersed in liquid nitrogen (77 K) in an optical Dewar flask and measured. The excitation wavelength was set to 440 nm.

Real-time quantitative PCR (qPCR)

Fifty mg of plant tissue was frozen by liquid nitrogen and homogenized using steel beads in a Retsch mill at 30 s^{-1} for 1 min. Then 1100 µL of Lysis Solution RP (InviTrap® Spin Plant RNA Mini Kit, Stratec, Germany) was added and extraction was performed according to the supplier's instructions. In addition, a supplemental protocol for DNA digestion was followed by using a RTA Spin filter. For isolation of RNA from secondary needles, the volume of Lysis Solution RP was ten-times increased.

The quality of isolated RNA (500 ng per sample) was examined by agarose gel electrophoresis in 0.5 × TBE buffer. To remove DNA, 2 µg of RNA was mixed with 1 µL of 10 × reaction buffer, 0.5 µL RiboLock®, and 1 µL 10 x DNase I to give a final volume of 10 µL. Following an incubation at 37 °C for 30 min, reactions were stopped by adding 1 µL 50 mM EDTA and incubation at 65 °C for 10 min.

For the reverse transcription, 11 µL RNA (DNase-treated), 0.5 µL ddH_2O, 4 µL RT buffer, 0.5 µL RiboLock® RNAse inhibitor, 2 µL 10 mM dNTPs, 1 µL RevertAid® reverse transcriptase, and 1 µL random hexamer primers were used. As a control, all components except RevertAid® reverse transcriptase were applied. Tubes were incubated at 25 °C for 10 min, following 60 min at 42 °C and reaction was finished at 70 °C for 10 min. Finally, the cDNA was diluted using 80 µL of ddH_2O. The cDNA was amplified with SensiMix™ SYBR® (Bioline, UK) on CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). The qPCR reaction mix contained 1 µL of cDNA, 3 µL Sensi-Mix™, 1 µL ddH_2O, and 1 µL of specific primers (Supplemental Table 1) and was performed as follows: (1) initialization (10 min at 95 °C), (2) denaturation (15 s at 95 °C), (3) annealing (15 s at 61 °C), (4) elongation (25 s at 72 °C) and 44 cycles of steps 2, 3, and 4. The melting curve was determined from 65 to 95 °C (increment 0.5 °C for 5 s). Expression rates were calculated relative to TUB (tubulin), according to the $2^{-\Delta C(t)}$ method (Livak and Schmittgen 2001; Schmittgen and Livak 2008). The specific primers for qPCR were designed according to BLAST analyses of the protein sequences and alignments with sequences of *Arabidopsis thaliana, Pinus taeda*, and *Picea abies*. Sizes of PCR amplification products were confirmed by agarose gel electrophoresis.

SDS–PAGE and western blots

Total proteins from the spruce cotyledons and secondary needles were isolated using extraction buffer containing 28 mM DTT, 28 mM Na_2CO_3, 175 mM sucrose, 5% SDS, 10 mM EDTA and protease inhibitors (Set VI, Calbiochem, Darmstadt, Germany). The samples were heated for 30 min at 70 °C. The concentration of total soluble proteins in the samples was determined using the Bicinchoninic Acid Kit for Protein Determination (Sigma-Aldrich, St. Louis, MO, USA), and absorbance was measured at 562 nm (Thermo Spectronic UV500, UV–Vis Spectro, MA, USA). The same amount of proteins was separated in a 10% (v/v) SDS–polyacrylamide gel (Schägger 2006) followed by transfer to a nitrocellulose membrane (Bio-Rad, Germany) by Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). To check the correct protein transfer, the membranes were stained by Ponceau-S. After blocking

in TBS-T containing 5% BSA overnight at 4 °C, the membranes were incubated with the primary antibody at room temperature (or at 8 °C, if overnight) with soft agitation. Antibodies against protein D1 (AS05 084), PsbS (AS09 533), PsbO (AS06 142-33), PsbP (AS06 167), PsbQ (AS06 142-16), Lhcb 1–6 (AS01 004, AS01 003, AS01 002, AS04 045, AS01 009, AS01 010), Lhca 1–4 (AS01 005, AS01 006, AS01 007, AS01 008), LPOR (AS05 067), GluTR (AS10 689), and RbcL were purchased from Agrisera (Vännäs, Sweden). Antibodies against DPOR subunits (ChlL, N, and B) were generously provided by Prof. Yuichi Fujita (Nagoya, Japan). After washing, the membrane was incubated 1 h in the secondary antibody (goat anti-rabbit IgG (H+L)-horseradish peroxidase conjugate) with dilution 1:10,000 (Bio-Rad, Hercules, CA, USA). Signals were visualized and quantified using Immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) on an Amersham Imager 600 (GE HealthCare Life Sciences, Japan).

Samples after incubation at elevated oxygen levels were subjected to oxyblots for the detection of oxidized proteins. The measurement of protein CO groups after their derivatization with 2,4-dinitrophenylhydrazine (DNPH), which led to the formation of a stable dinitrophenyl (DNP) hydrazone product, was performed according to the protocol of Dalle-Donne et al. (2003). After blotting, nitrocellulose membranes were washed 10 min in 1× TBS and then incubated for 30 min with DNPH; the stock solution of DNPH (49.5 mg in 5 mL 50% H_2SO_4) was diluted in 250 mL of 1× TBS. The membranes were washed for 5 min in 1× TBS, blocked overnight in 5% BSA in 1× TBS-T at 4 °C, washed again by 1× TBS for 10 min and followed by incubation in primary anti-DNPH antibody (dilution 1:500 to 5% BSA in 1× TBS) for 1 h. After incubation with the primary antibody, the membrane was washed with 1× TBS-T; 1× for 15 min, 2× for 5 min and 1× TBS for 5 min. The secondary antibody (dilution 1:10,000 to 5% BSA in 1× TBS, Bio-Rad, Hercules, CA, USA) was added, followed by 5× washing in 1×TBS-T for 10 min, and finally by 1× TBS for 10 min. The signals were visualized by Amersham Imager 600 (GE HealthCare Life Sciences, Japan).

Statistical analysis

To evaluate the significance of the data, one-way analysis of variance (ANOVA) followed by Tukey's test (Origin 8.5.1., Northampton, USA) or Student's *t* test (Excel, Microsoft Office) were used.

Results

Accumulation of tetrapyrroles and their precursors

Dark-grown secondary needles (DN) and cotyledons cultivated at low temperature (D7) were entirely etiolated (Fig. 1). The dark-grown cotyledons cultivated at room temperature (D23) showed weaker green pigmentation than L23 (Fig. 1). Spectrophotometric measurements confirmed the macroscopic phenotype in chl $a+b$ concentration (Fig. 2a). Only traces of Chl were detected in dark cultivated secondary needles (DN) and HPLC confirmed the

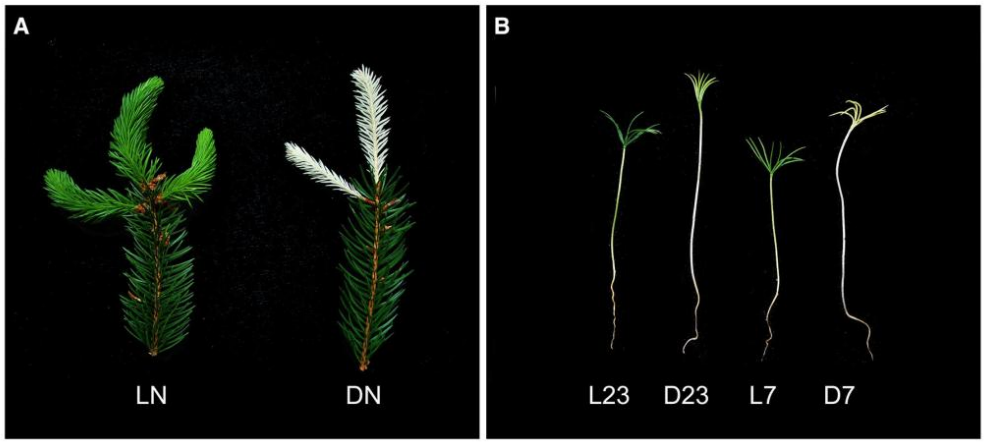

Fig. 1 Morphological observation and comparison of chlorophyll content in needles (**a**) and cotyledons (**b**) of *P. abies* Branches of Norway spruce with secondary needles were cultivated in light/dark cycle (12/12-h, 100 µmol m^{-2} s^{-1} PAR) at 23 °C (LN) or in complete darkness at the same temperature (DN). 17-day-old seedlings were cultivated in light/dark cycle at 23 °C (L23) and 7 °C (L7) and in complete darkness at 23 °C (D23) and 7 °C (D7)

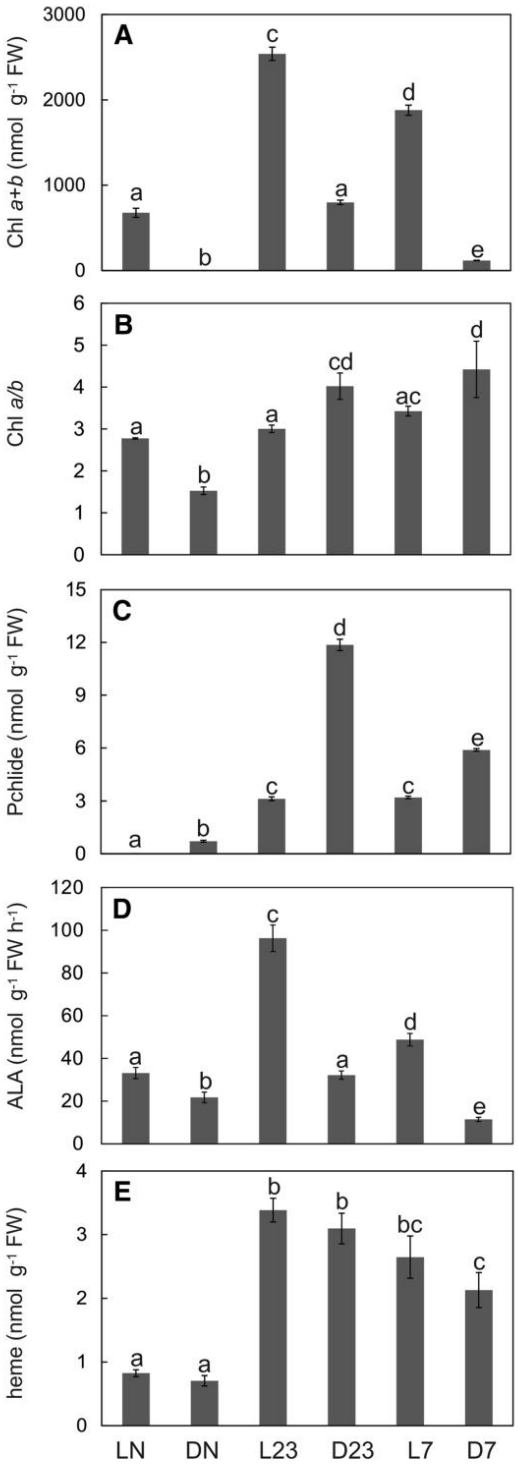

Fig. 2 Content of assimilation pigments, heme and its precursors. ▶ Chl $a+b$ content (**a**), Chl a/b ratio (**b**), protochlorophyllide (Pchlide) content (**c**), ALA synthesizing capacity (**d**) and heme content (**e**). Branches of Norway spruce with secondary needles were cultivated in light/dark cycle (12/12-h, 100 µmol m^{-2} s^{-1} PAR) at 23 °C (LN) or in complete darkness at the same temperature (DN). 17-day-old seedlings were cultivated in light/dark cycle at 23 °C (L23) and 7 °C (L7) and in complete darkness at 23 °C (D23) and 7 °C (D7). Means ± SD, $n=4$. *Different letters* denote significant differences at $P<0.05$ (ANOVA, Tukey's test)

presence of Chl *a* and *b* in only picomolar concentrations (chl $a=620$ pmol g^{-1} FW; chl $b=410$ pmol g^{-1} FW). In general, the Chl content in cotyledons was lower in dark-grown seedlings in comparison to those grown in light/dark cycle at the same temperature. However, decreased temperature caused a strong reduction in Chl accumulation. When cotyledons were incubated at 7 °C in the dark (D7), they contained only 15% of the Chl content of dark-grown seedlings at 23 °C (D23). Light/dark-grown cotyledons at 7 °C (L7) contained 75% of the Chl content compared to the samples cultivated at 23 °C (L23). The Chl level of the D7 seedlings did not increase after transfer to 23 °C during a 1-week-incubation in the dark, but increased after transfer to light (Fig. S1). Higher Chl *a*/*b* ratios were detected in dark-grown cotyledons irrespective of the temperature conditions as well as in light/dark-grown secondary needles (Fig. 2b).

The Pchlide content was strongly decreased in light due to the activity of LPOR (Fig. 2c). In the dark, Pchlide accumulated to lower amounts in the secondary needles (DN) compared to cotyledons (D23). Low temperature (D7) resulted in decreased contents of Pchlide in dark-grown cotyledons (Fig. 2c).

The ALA synthesizing capacity determines the synthesis rate of substrate for the tetrapyrrole biosynthetic pathway and was significantly decreased in all dark-grown in comparison to light/dark-grown samples. Moreover, the ALA synthesis rate was more inhibited at low temperature (Fig. 2d). Application of exogenous 20 mM ALA did not increase Chl content in the D7 variant (Fig. S1).

The heme content reflects the activity in the Fe-branch of tetrapyrrole biosynthesis. The lowest heme content was detected in secondary needles cultivated both at light/dark and dark conditions (LN and DN). Almost four times higher concentration was found in light/dark-grown cotyledons and its content slightly decreased at lower temperature under dark conditions (D7) (Fig. 2e).

Low-temperature fluorescence spectra (77 K)

It is expected that the pigment molecules are bound to proteins in PSI and PSII complexes. This can be confirmed by the measurement of the low-temperature

fluorescence emission spectrum of leaf samples (77 K, Fig. 3). In the light/dark-grown plants (LN, L23, L7), the emission peaks at 685 and 695 nm document the presence of the core antennae of PSII, CP43, and CP47, respectively (Nakatani et al. 1984). Emission at 735 nm corresponds to PSI (Fig. 3a, c, e). These emission maxima were also present in dark-grown cotyledons, with the slight shift of PSI emission to shorter wavelengths (D7, D23, Fig. 3d, f). Dark-grown needles (DN) seemed not to form significant amounts of pigment-protein complexes of PSII and PSI (Fig. 3b), coinciding with a very low Chl accumulation (Fig. 2a). In addition, the fluorescence maxima observed in the spectra of dark-grown cotyledons and needles (DN, D7, D23) indicate the presence of free Pchlide (633 nm) and Pchlide bound to LPOR and $NADPH^+$ in PLB (655 nm) (Selstam et al. 1987; Fig. 3b, d, f). Relatively more free Pchlide molecules were present in dark-grown needles (DN) in comparison to cotyledons (D7, D23), which contain Pchlide bound mainly to PLB (compare F_{633} and F_{655}; Fig. 3b, d, f).

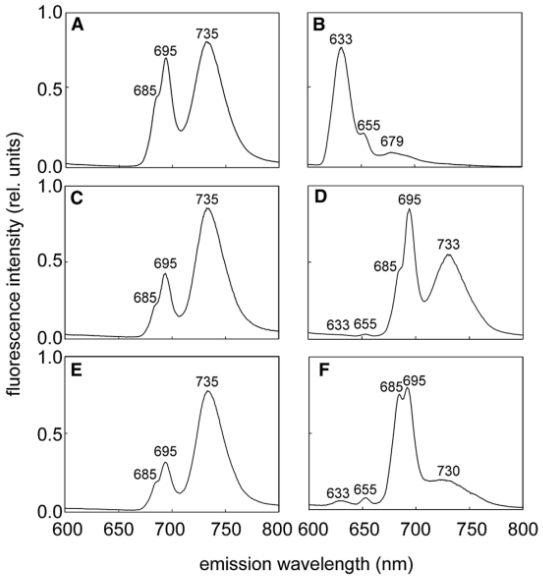

Fig. 3 Low-temperature fluorescence emission measured at 77-K. Secondary needles cultivated under the light/dark cycle (12/12-h, 100 µmol m^{-2} s^{-1} PAR) at 23 °C (**a**), secondary needles cultivated in complete darkness at 23 °C (**b**), cotyledons cultivated under the light/dark cycle at 23 °C (**c**), cotyledons cultivated in complete darkness at 23 °C (**d**), cotyledons cultivated under the light/dark cycle at 7 °C (**e**), cotyledons cultivated in complete darkness at 7 °C (**f**). The results shown are representative of four measurements

Expression of genes involved in tetrapyrrole biosynthesis

It is expected that the different Chl contents under the various cultivation conditions and developmental stages of our seedlings are due to differences in the expression of important genes involved in Chl biosynthesis and protein assembly into pigment-protein complexes. We found that the expression of many genes underlies either positive or negative light-dependent regulation (Fig. 4). Positive photoregulation was observed for *RBCL* (large subunit of Rubisco), *HEMA* (encoding GluTR), *CHLH* and *CHLD* (genes encoding subunits of Mg-chelatase), total *LPOR* (encoding both *LPORA* and *LPORB*), and *CAO* (encoding Chl *a* oxidase) in cotyledons and needles. Low-temperature treatment (D7, L7) also inhibited transcription of many of these genes, most noticeable *LPOR* and *HEMA*. On the other hand, genes for DPOR subunits (*CHLN, CHLL, CHLB*) as well as the *FC1* isoform of Fe-chelatase were negatively regulated by light exhibiting the highest transcript accumulations under dark conditions. Transcription of genes for DPOR subunits was not affected by temperature, but was developmentally regulated. Secondary needles had much lower transcript levels of DPOR subunits in comparison to cotyledons, particularly under dark conditions. Transcript levels of LPOR were similar between needles and cotyledons (Figs. 4, 6).

Immunoblot analysis

Total protein extracts were subjected to SDS-PAGE followed by immunoblotting to semi-quantify protein levels (Fig. 5). First, we analysed Chl-binding proteins of PSII (D1, Lhcb1–6), PsbS, and PSI (Lhca1–4) as well as proteins PsbO, PsbP, PsbQ of the oxygen-evolving complex (OEC) of PSII. Levels of Chl-binding proteins coincided with Chl accumulation in spruce (Fig. 2a), i.e., low amount Chl-binding proteins were found in DN and D7 variants. All the Chl-binding proteins except Lhcb3 and Lhcb6 were detected under D23 conditions in contrast to etiolated barley grown under the same conditions, what is consistent with inability to synthesize Chl in the dark in angiosperms. As expected, the PsbS protein, which is an important component for dissipating excess light energy via regulation of non-photochemical quenching, accumulated to highest level in L7 variant. The large subunit of Rubisco (RbcL) was immunodetected in all samples with slightly higher content under light conditions. This is in accordance with the transcription of the *RBCL* gene (Fig. 4).

Regarding proteins involved in Chl biosynthesis, the amount of GluTR correlated with Chl accumulation (Fig. 2a), ALA synthesizing capacity (Fig. 2d) and *HEMA* transcription (Fig. 4). The highest GluTR content

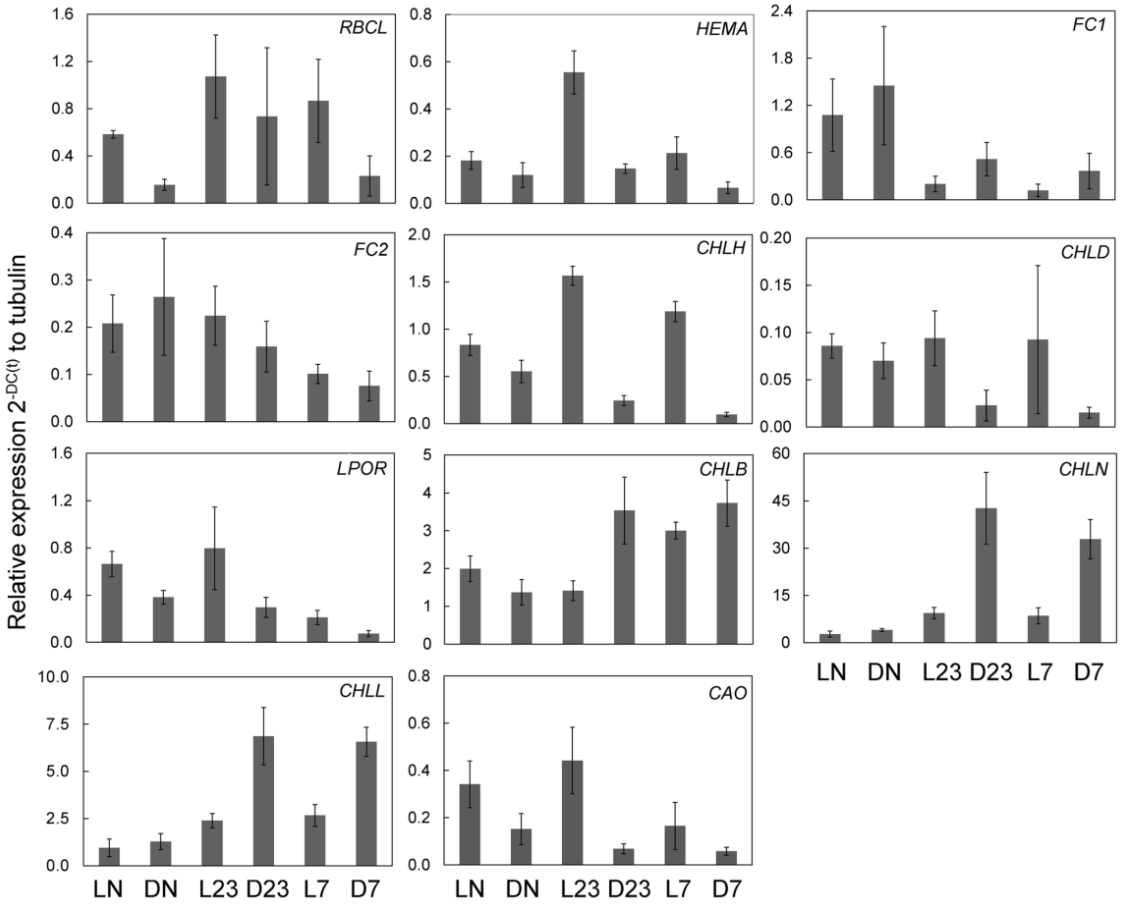

Fig. 4 Quantification of gene expression relative to tubulin by real-time PCR. *RBCL* (large subunit of Rubisco); *HEMA* (glutamyl-tRNA-reductase); *FC1, FC2* (isoforms of Fe-chelatase), *CHLH, CHLD* (subunits of Mg-chelatase); total *LPOR* (*light*-dependent protochlorophyllide oxidoreductase, LPOR), *CHLB, CHLN, CHLL* (subunits of *light*-independent protochlorophyllide oxidoreductase, DPOR) and *CAO* (chlorophyll *a* oxygenase). Branches of Norway spruce with the secondary needles were cultivated under light/dark cycle (12/12-h, 100 µmol m^{-2} s^{-1} PAR) at 23 °C (LN) or in complete darkness at the same temperature (DN), and 17-day-old seedlings were cultivated under the light/dark cycle at 23 °C (L23) and 7 °C (L7) and in complete darkness at 23 °C (D23) and 7 °C (D7). The results are the means ± SD from three independent experiments

was immunodetected in the light (Fig. 5), while cold and darkness resulted in significant decrease of mRNA (Fig. 4) and protein levels (Figs. 5, 6). On the contrary, both LPOR and DPOR were negatively light-regulated with the highest protein content found in the dark. Given the known sequenced genome of Norway spruce and based on homology search of protein and cDNA from *Pinus mugo* (Forreiter and Apel 1993), we identified two candidate genes in Norway spruce as *LPORA*: joined gene model MA_10433385g0030 and MA_10433385g0020 and *LPORB*: gene model MA_91782g0010, indicating that Norway spruce has also two isoforms of LPOR. Although our antibody is immunoreactive against both isoforms of LPOR, we were not able to immunodetect double band in our Western blots. Low temperature decreased total amount of LPOR protein, but the amount of DPOR subunits (ChlN, ChlB) was only slightly affected if at all by low temperature. The signal intensity for these proteins was weak in both variants of secondary needles (LN, DN). Unfortunately, we were not able to detect ChlL subunit in spruce. We used barley as a negative control for immunodetection of DPOR subunits and no signal was detected.

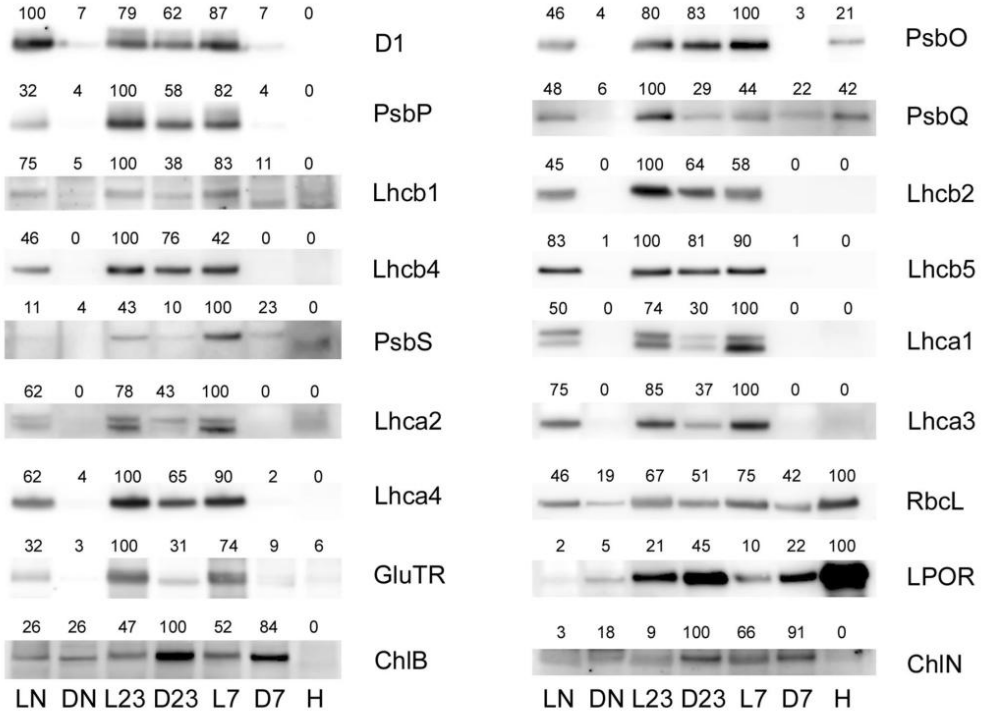

Fig. 5 Immunoblotting analyses showing the changes in relative amounts of proteins. The same amount of total proteins (20 µg) was electrophoresed in 10% (v/v) SDS–polyacrylamide gel and subjected to Western blot analysis. Branches of Norway spruce with the secondary needles were cultivated under the light/dark cycle (12/12-h, 100 µmol m^{-2} s^{-1} PAR) (LN) or in complete darkness at the same temperature (DN), and 17-day-old seedlings were cultivated under the light/dark cycle at 23 °C (L23) and 7 °C (L7) and in complete darkness at 23 °C (D23) and 7 °C (D7). As a negative control, dark-grown etiolated angiosperm *Hordeum vulgare* (H) was used. Protein content in the bands was quantified by chemiluminescence. The blots are representatives of three independent experiments

Fig. 6 Correlation of gene expression involved in chlorophyll biosynthesis in response to light, low temperature and development. Increase up to 50% (0), 50–100% (↑), 100–200% (↑↑), >200% (↑↑↑) and decrease up to 50% (0), 50–100% (↓), 100–200% (↓↓), >200% (↓↓↓). *ND* not determined (antibodies used did not give a specific signal)

	mRNA level			protein level		
	light	low temperature	development	light	low temperature	development
GluTR (*HEMA*)	↑↑↑	↓↓	↓↓	↑↑↑	↓↓	↓↓
Mg-chelatase	↑↑↑	0	0	ND	ND	ND
LPOR	↑↑	↓↓↓	0	↓↓	↓↓	↓↓↓
DPOR	↓↓↓	0	↓↓↓	↓	0	↓↓↓
CAO	↑↑↑	↓	0	ND	ND	ND

Impact of high oxygen concentration on Chl biosynthesis in the dark

Intriguingly, despite the very low Chl content in cotyledons of seedlings growing under dark and cold conditions (D7), the mRNA as well as the protein levels of DPOR subunits are comparable to D23 (Figs. 4,5,6), indicating a post-translational regulation of DPOR activity. Because DPOR activity is ATP-dependent and oxygen-sensitive, we investigated the effect of these factors. First, we intended to examine the seedlings of D7 variant supplied with 3% sucrose for 2 months whether Chl synthesis is limited

by substrate availability for ATP synthesis. We did not observe any effect of this treatment on Chl accumulation under cold conditions in darkness (Fig. S2). Second, taking into account that solubility of gases in aqueous solutions depends on temperature (Henry 1803), the solubility of oxygen increases from 8.58 mg L^{-1} at 23 °C to 12.14 mg L^{-1} at 7 °C. We increased the oxygen concentration from atmospheric 21 to 33% to investigate possible O_2 effects independently from the decrease in temperature. At this increased partial pressure, the concentration of oxygen in distilled water is around 13.95 mg L^{-1}. Seedlings cultivated in the dark at room temperature at elevated oxygen level for 17 days contained only one-third of the Chl found at atmospheric concentration of O_2 (Fig. 7a, b), indicating that a higher oxygen partial pressure inhibits Chl synthesis, most likely at the level of DPOR activity. Under elevated oxygen conditions, the content of RbcL, LPOR, and ChlN subunit of DPOR did not change, while the levels of ChlB and GluTR were significantly decreased (Fig. 7c). In order to exclude increased oxidative stress under elevated O_2 concentration, we performed an OxyBlot test using DNPH. The levels of oxidized proteins remained the same when both experimental variants were compared (Fig. S3).

Discussion

Unlike angiosperms, gymnosperms have two mechanisms for Chl formation: one that requires light for the reduction of Pchlide to Chlide, and a second mechanism that can carry out Pchlide reduction in the absence of light (Armstrong 1998; Reinbothe et al. 2010). It has been assumed that the light-dependence of Pchlide reduction and consequently Chl formation is an evolutionary adaptation resulting in a bioenergetically more conservative lifestyle due to tight regulation of gene expression (Skinner and Timko 1999) and replaced costly light-independent mechanisms which are ATP dependent. Furthermore, oxygen sensitivity of DPOR is an unfavorable property in oxygenic photosynthetic organisms (Yamazaki et al. 2006; Nomata et al. 2016). Our study showed that the role of light for Chl synthesis is alleviated relative to angiosperms but light is still involved in control of Chl biosynthesis in coniferous Norway spruce in addition to tightly temperature and development-dependent control.

Regulatory role of light is alleviated but still present in gymnosperm cotyledons

Light is the most important modulator of plant morphogenesis and physiology, as it could also be seen for Chl biosynthesis in angiosperms. Chl synthesis is initially regulated at the level of ALA synthesis, which is considered

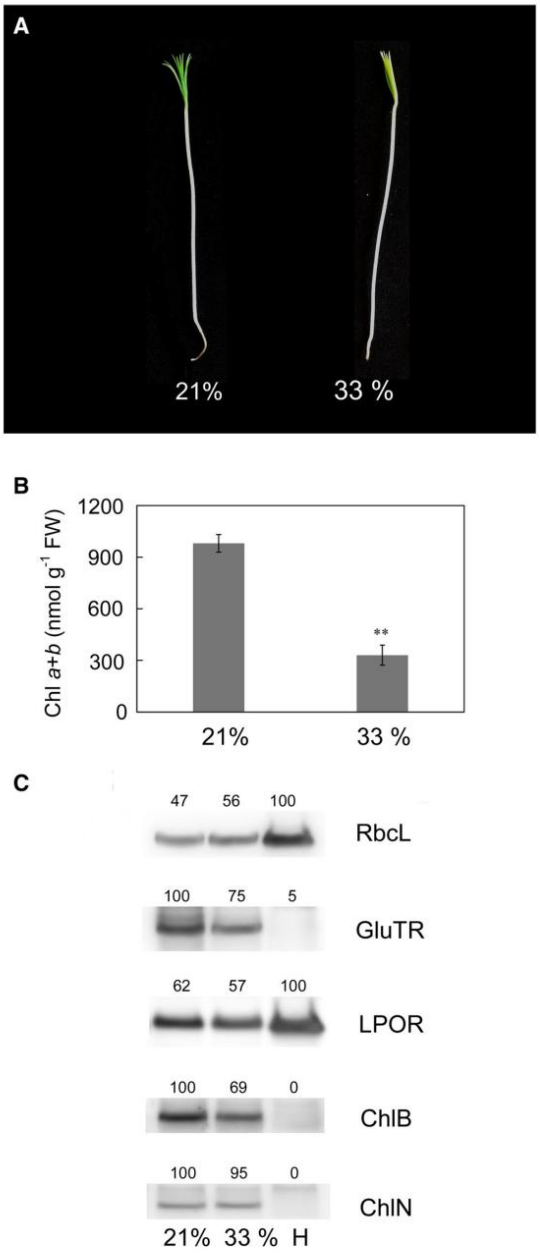

Fig. 7 Effect of increased atmospheric oxygen concentration (33%) on chlorophyll biosynthesis compared to control (21%) in dark-grown spruce seedlings cultivated at 23 °C. Morphological observation (**a**), chl $a+b$ content, means ± SD, $n=4$, **significant differences at $P<0.01$ (Student's t test) (**b**), and immunoblot analyses of selected proteins (**c**), the blots are representatives of three independent experiments. Atmospheric oxygen concentration (21%), elevated oxygen concentration (33%) and *H. vulgare* as negative control (H)

the rate-limiting step. In etiolated angiosperms, light stimulates the transcription of the key enzyme in ALA synthesis, glutamyl-tRNA-reductase (GluTR, encoded by *HEMA*) through phytochrome (Gehring et al. 1977; Huang et al. 1989; Mohanty et al. 2006). In contrast, ALA synthesis as well as GluTR expression are relatively high in dark-grown conifers in comparison to light/dark-grown (Figs. 2d, 5). This is in accordance with previous studies (Dražič and Bogdanovič 2000; Demko et al. 2009, 2010; Breznenová et al. 2010). Nevertheless, ALA synthesis is the rate-limiting step, as exogenous application of ALA increases Pchlide and Chl formation in spruce cultivated in the dark (Pavlovič et al. 2009). However, in light/dark-grown angiosperms, ALA formation declines immediately after transition from light to dark despite the presence of identical amounts of GluTR. This hints at a post-translational regulatory mechanism, presumably based on interaction of the negative regulator FLU with GluTR (Meskauskiene et al. 2001; Goslings et al. 2004; Richter et al. 2010). Recently, it has been suggested that FLU is part of a LPOR-CHL27 complex bound to the thylakoid membranes (Kauss et al. 2012). When accumulating Pchlide is bound to LPOR in the dark, FLU was hypothesized to interact with GluTR, resulting in inhibition of ALA synthesis (Kauss et al. 2012; Apitz et al. 2016). However, in cotyledons of gymnosperms, ALA is still synthesized at a significant rate despite the putative repression of ALA synthesis by the potentially inhibiting protein complex of LPOR-bound Pchlide and FLU-like proteins (Demko et al. 2010). In conifers, Pchlide is metabolized also by DPOR activity. This favors the attenuation of the tight regulation of phytochrome-induced gene expression and LPOR-bound Pchlide repression of GluTR activity in cotyledons of gymnosperms. This light-independency is more pronounced in early developmental stages of cotyledons and is diminished during ontogeny of gymnosperms (Demko et al. 2009; Breznenová et al. 2010). It can be suggested that two separate ALA pools exist, which provide independently appropriate substrate amounts for LPOR and DPOR. Such a mechanism would provide adequate amounts of the substrate for DPOR in the dark, while a GluTR fraction providing a putative second area of ALA synthesis for metabolite supply of LPOR is inhibited.

In contrast to GluTR, LPOR gene expression and protein abundance dramatically decreased after illumination of etiolated angiosperms (Holtorf et al. 1995). Rapid disappearance of LPOR protein was enigmatic, since it was inconsistent with the observed continued formation of chlorophyll after extended periods of illumination. This apparent contradiction was resolved by the demonstration that two forms of the enzyme are present in most angiosperms. Whereas *LPORA* accumulated in dark-grown angiosperms and its mRNA as well as protein rapidly disappeared after illumination, *LPORB* remained at an approximately constant level. Although our qPCR did not recognize *LPORA* and *LPORB*, our results are consistent with findings of Skinner and Timko (1998, 1999) in *Pinus taeda* and *P. nigra* seedlings, where on the contrary to angiosperms, transcription of both isoforms as well as total *LPOR* pool were positively regulated by light. Thus, the regulation of *LPOR* mRNA level in gymnosperms appears to be different from that observed in angiosperms, suggesting that the strong negative regulation of *LPOR* transcription in response to light had not yet been established in the gymnosperms (Skinner and Timko 1999). However, rapid decline of LPORA protein due its rapid turnover after illumination is present in both angiosperms (Armstrong et al. 1995; Holtorf et al. 1995) and gymnosperms (Stabel et al. 1991; Forreiter and Apel 1993; Skinner and Timko 1999). The high level of LPORA protein in dark-grown cotyledons masks the ability to easily detect the presence of the LPORB isoform making quantification impossible, but it seems that light reduced level of both isoforms in pine (Skinner and Timko 1998, 1999).

The mRNA and protein accumulations of DPOR subunits were repressed by light in accordance with our previous results (Demko et al. 2010), indicating a negative transcriptional control of DPOR in response to light. Thus, because of improved bioenergetically more efficiently operating LPOR, LPOR takes over the DPOR function under light conditions. This was documented by the fact that illumination of dark-grown spruce and pine had stimulatory effect on chlorophyll accumulation (Mariani et al. 1990; Demko et al. 2009; Breznenová et al. 2010; Pavlovič et al. 2016).

Secondary needles depend on light for Chl synthesis

In contrast to cotyledons, dark-grown secondary needles of spruce were etiolated and contained ten times less GluTR in comparison to LN and D23, indicating a stronger light-dependent regulation of Chl synthesis at this developmental stage (Fig. 5). The transcription of LPOR genes followed the pattern described in cotyledons but with overall much lower accumulation of LPOR protein (Figs. 4, 5). Reduced level of LPOR protein in secondary needles is consistent with reduced overall Pchlide level (Fig. 2c) and Pchlide bound in PLB in dark-grown needles (Fig. 3b). Moreover, Skinner and Timko (1999) did not detect any LPORA protein despite transcription of both isoforms in secondary needles of pine, but they did not use etiolated but only re-darkened needles for three days. Consistent with studies on pine (Skinner and Timko 1999), the mRNA and protein levels of DPOR subunits were strongly repressed in secondary needles resulting in only picomolar concentrations of Chl and a strong reduction of pigment-protein complexes of PSI and PSII in the dark, indicating transcriptional control of

Chl biosynthesis (Figs. 2a, 4, 5). Thus, chlorophyll metabolism of the secondary needles of gymnosperms resembles more than that of angiosperm tissue, although strong negative regulation of *LPOR* mRNA in response to light has not yet been established. We propose that already in secondary needles, the Chl synthesis is mainly controlled by light, including the use of LPOR.

Transcriptional and post-translational regulation of LPOR and DPOR activity, respectively, in response to low temperature

Temperature plays a prominent role in determining plant productivity worldwide. Whereas low temperature (7 °C) inhibited Chl accumulation in angiosperm maize, cucumber and wheat by 90% (van Huystee and Hodgins 1989; Tewari and Tripathy 1998; Mohanty et al. 2006) in light/dark-grown spruce, it was inhibited only by 25% (Fig. 2a). In our study, the expression of several genes was downregulated in response to chilling stress in the light in accordance with the above-mentioned studies. Reduced ALA synthesis (Fig. 2d) correlated with reduced transcript (*HEMA*, Fig. 4) and protein content (GluTR, Fig. 5) for key-regulatory step. The transcription of ferrochelatase (mainly *FC2*) and substrate-binding *ChlH* (subunit of Mg-chelatase) was partially reduced (Fig. 4). The expression of LPOR was also inhibited by low temperature (Figs. 4, 5), but the mRNA and protein contents of DPOR subunits were not negatively affected in light, indicating a differential regulation of LPOR and DPOR by low temperature (Figs. 4, 5).

But low temperature inhibited Chl biosynthesis almost completely in dark-grown spruce seedlings (Figs. 1, 2a), confirming the results on *Pinus thunbergii* seedlings (Muramatsu et al. 2001). Chill stress in the dark had almost the same effect on transcription of genes involved in Chl biosynthesis as cold treatment under circadian light/dark conditions, as it was determined for candidate genes and their encoded proteins in ALA synthesis (*HEMA* and GluTR), Mg-chelatase (*ChlH*), Fe-chelatase (*FC2*), LPOR (*LPOR*) (Figs. 4, 5). However, similar to light conditions, low temperature did not reduce transcription of DPOR subunits (*ChlN, ChlB, ChlL*) and the protein content was only reduced by 10–20% in comparison to D23. This is in contrast to strong inhibition of Chl accumulation in the D7 variant. This result would favor a post-translational regulation of DPOR activity. In contrast to LPOR, which requires light as a source of energy for substrate conversion, DPOR enzyme requires at least four ATP molecules for reduction of Pchlide to Chlide (Nomata et al. 2016). Thus, decreased levels of ATP should result in reduced DPOR activity at low temperature. However, feeding with sucrose as substrate for respiration did not increase Chl accumulation (Fig. S2). Another peculiar property of DPOR is oxygen-sensitivity (Yamazaki et al. 2006; Yamamoto et al. 2009, 2011) and could play a significant role in inhibition of DPOR activity under low temperature. Since oxygen solubility increases at low temperature (Henry 1803), we attempted to mimic the effect of low temperature by increasing oxygen partial concentration at room temperature. This resulted in an inhibition of Chl synthesis by 67% (Fig. 7a, b). The maximum oxygen level under which the LPOR deficient mutant YFP12 of cyanobacterium *Leptolyngbya boryana* could grow photoautotrophically was only 3% oxygen; a much lower concentration than found in terrestrial ecosystems (21%). It has been assumed that oxygen tolerance increased during evolution, since gymnosperms are able to synthesize Chl in the dark under ambient O_2 concentration. Yamamoto et al. (2009) found the ChlL protein of DPOR to be the primary target of oxygen. Its sensitivity to oxygen differs among organisms, being lowest in the above-mentioned prokaryotic cyanobacteria. However, the ChlL protein of the moss *Physcomitrella patens* revealed an oxygen sensitivity comparable to the anoxygenic prokaryote *Rhodobacter capsulatus* with both organisms being more sensitive to oxygen than prokaryotic cyanobacteria (Yamamoto et al. 2011). These results suggest that DPOR from oxygenic photosynthetic organisms did not acquire oxygen tolerance during evolution, but might have evolved to have very efficient scavenging systems such as the water–water cycle to remove oxygen from chloroplast (Yamamoto et al. 2009, 2011). DPOR is inactivated by oxygen without significant degradation of its subunits (Yamazaki et al. 2006). These results are consistent with our results, showing components of DPOR to be reduced only slightly under elevated oxygen and to the same extent as in response to low temperature in the dark (Figs. 5, 7 c). Decreased Chl synthesis and DPOR activity under elevated oxygen levels resulted in a decreased content of GluTR. Our ALA feeding experiments showed that ALA synthesis was not exclusively the rate-limiting step of Chl biosynthesis in the dark at low temperature and that DPOR cannot be reversibly activated after transferring the seedlings to room temperature (Fig. S1). In contrast, in *Pinus thunbergii* seedlings, it was demonstrated that DPOR was reversibly inactivated at low temperature and Chl synthesis was restored after transfer to 23 °C for one week in the dark (Muramatsu et al. 2001). The reason for this interspecific discrepancy remains unknown.

Organization of pigment-protein complexes under different conditions

Chl biosynthesis strongly correlates with accumulation of pigment-protein complexes of PSI and PSII (Figs. 2a, 3, 5), since Chl-binding proteins are unstable in the absence of bound pigment molecules (Fig. 5, Hobber

and Argyroudi-Akoyunoglou 2004). The stable assembly of pigment-protein complexes in the dark requires the light-independent synthesis not only of Chl but also of its binding-proteins. Transcripts of *cab (lhc)* and *psbA* genes and their encoded proteins, LHCP and D1, respectively, accumulate light-independently in gymnosperm cotyledons (Figs. 3, 5, 6; Muramatsu et al. 2001). The absence of Lhcb3 and Lhcb6 proteins irrespective of light and temperature conditions is in accordance with recent findings of Kouřil et al. (2016) who reported an evolutionary loss of their respective genes in the genera *Pinus* and *Picea* and a resulting different organization of LHCII–PSII supercomplexes. The dark-grown spruce cotyledons contain considerably less LHCII in comparison to light/dark-grown cotyledons (Fig. 5, Jansson et al. 1992; Pavlovič et al. 2016). This is consistent with an increased ratio of Chl*a*/*b* (Fig. 2b) and decreased transcription of *CAO* (Fig. 4) in dark-grown cotyledons. Moreover, the majority of LHCII proteins are in free monomeric form with poor connection to PSII core in thylakoid membranes of conifers developed in the dark (Pavlovič et al. 2009, 2016; Xue et al. 2017). Despite the presence of all components of photosynthetic apparatus in the dark, PSII remains in latent form until illumination. The light-induced incorporation of Mn^{2+} ions, PsbP and PsbQ proteins into OEC, plays an important function in photoactivation, formation, and stabilization of LHCII–PSII supercomplexes in granal membranes and is essential for effective photochemistry in conifers (Shinohara et al. 1992; Pavlovič et al. 2016).

Conclusion

This study shows that Chl biosynthesis in the dark in Norway spruce strongly depends on developmental stage, temperature, and light. Although the regulatory role of light is alleviated, it is still present in cotyledons, while secondary needles, in contrast, reveal a strong light-dependency of Chl synthesis. DPOR activity is developmentally and light-dependently regulated at the transcriptional level, whereas temperature partially regulates DPOR activity post-translationally. This is in contrast to the transcriptional regulation observed for LPOR in response to low temperature. We suggest that post-translational inhibition of DPOR activity by low temperature in the dark is partially caused by sensitivity of DPOR protein to increased oxygen levels. The inhibition of DPOR activity by different environmental factors is sensed for a feedback control of ALA synthesis and the whole biosynthetic pathway is down-regulated at the transcriptional level (e.g., reduced transcription of *HEMA*) to prevent accumulation of phototoxic tetrapyrrole metabolites. The down-regulation of Chl biosynthesis catalysed by DPOR in the light and the low temperature may represent a possible regulatory mechanism known as photostasis during chilling stress or frost hardening in evergreen conifers (Öquist and Huner 2003). To attain photostasis during winter with reduced carbon assimilation and termination of growth, conifers must reduce its antenna size and Chl content and increase capacity for non-photochemical quenching (through increased PsbS protein content) of absorbed energy to protect PSII against photoinhibition (Öquist and Huner 2003; Savitch et al. 2010). On the other hand, DPOR is most likely suitable for Chl synthesis in cotyledons under short-day conditions (Ueda et al. 2014). Ecophysiological implications of the presence of DPOR in conifers are currently under investigation in our laboratory.

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8.2 Light-induced gradual activation of photosystem II in dark-grown Norway spruce seedlings.

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Light-induced gradual activation of photosystem II in dark-grown Norway spruce seedlings

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ABSTRACT

Gymnosperms, unlike angiosperms, are able to synthesize chlorophyll and form photosystems in complete darkness. Photosystem I (PSI) formed under such conditions is fully active, but photosystem II (PSII) is present in its latent form with inactive oxygen evolving complex (OEC). In this work we have studied light-induced gradual changes in PSII function in dark-grown cotyledons of Norway spruce (*Picea abies*) via the measurement of chlorophyll *a* fluorescence rise, absorption changes at 830 nm, thermoluminescence glow curves (TL) and protein analysis. The results indicate that in dark-grown cotyledons, alternative reductants were able to act as electron donors to PSII with inactive OEC. Illumination of cotyledons for 5 min led to partial activation of PSII, which was accompanied by detectable oxygen evolution, but still a substantial number of PSII centers remained in the so called PSII-Q_B-non-reducing form. Interestingly, even 24 h long illumination was not sufficient for the full activation of PSII centers. This was evidenced by a weak attachment of PsbP protein and the absence of PsbQ protein in PSII particles, the absence of PSII supercomplexes, the suboptimal maximum yield of PSII photochemistry, the presence of C band in TL curve and also the presence of up-shifted Q band in TL in DCMU-treated cotyledons. This slow light-induced activation of PSII in dark-grown cotyledons could contribute to the prevention of PSII overexcitation before the light-induced increase in PSI/PSII ratio allows effective operation of linear electron flow.

1. Introduction

A key step in chlorophyll biosynthesis in plants is the reduction of protochlorophyllide (Pchlide) to chlorophyllide catalyzed by Pchlide oxidoreductase (POR). While in angiosperms this reduction is strictly light-dependent and catalyzed by so called light-dependent POR (LPOR, e.g. [1,2]), in gymnosperms this reaction can be catalyzed also by light-independent dark-operative POR (DPOR, e.g. [2,3]. Since the transcription of genes encoding thylakoid proteins and their synthesis in gymnosperms is also light-independent, chlorophylls can be assembled into protein complexes in the dark, forming both photosystem II (PSII) and photosystem I (PSI) [4–11]. While PSI developed in the dark is fully active, PSII remains in its latent form with inactive oxygen evolving complex (OEC) until the chloroplasts are illuminated [12–16]. Photoactivation of PSII does not require synthesis of new proteins and is connected with ligation of four Mn atoms in OEC [14,16–18].

The OEC of higher plants is composed of three extrinsic proteins: PsbO (33 kDa), PsbP (23 kDa) and PsbQ (17 kDa). While PsbO protein plays a central role in the stabilization of the Mn-cluster, PsbP and PsbQ proteins appear to modulate the binding of Ca^{2+} and Cl^- ions, which are required for efficient oxygen evolution [19]. Recent experiments with transgenic plants revealed that PsbO and PsbP proteins are indispensable for PSII function and oxygen evolution in higher plants [20–22]. Büchel et al. [23] have shown that the minimal protein core needed for the photoactivation of PSII, i.e. for the initiation of OEC function, is composed of four PSII reaction center subunits (D1, D2, α and β subunits of cytochrome b_{559}), the inner antenna CP47 and several smaller hydrophobic subunits. The lumenal loops of CP47 and D2 proteins enable the docking of PsbO protein [24], which then provides a docking site for PsbP protein. Both PsbP and PsbO proteins are needed for the binding of PsbQ, which stabilizes their association [25,26]. In addition, the PsbP together with PsbQ proteins is necessary for the formation PSII supercomplexes (PSII dimer bound to light harvesting complexes of PSII, LHCII) [27,28] which are known to be the most efficient oxygen-evolving PSII structure [29].

When exposed to light, PSII formed in dark-grown gymnosperms can be readily photoactivated. This process involves the ligation of Mn

Abbreviations: DPOR, dark-operative protochlorophyllide oxidoreductase; F_m, maximal fluorescence in the dark-adapted state; F_v, variable fluorescence; F_0, minimal fluorescence in the dark-adapted state; FNR, ferredoxin-$NADP^+$ oxidoreductase; LPOR, light-dependent protochlorophyllide oxidoreductase; OEC, oxygen evolving complex; PAR, photosynthetically active radiation; PSI, photosystem I; PSII, photosystem II; Q_A, primary stable quinone acceptor of PSII; Q_B, secondary stable quinone acceptor of PSII; PQ, plastoquinone.

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atoms and the assembly of OEC proteins into functional OEC, but not the assembly of LHCII [13,14,16]. In this work we have shown that although the photoactivation of PSII in dark-grown Norway spruce cotyledons takes less than 5 min, PSII is not fully functional even after 24 h of illumination. The most important difference between PSII from light-grown plants and photoactivated PSII originating from dark-grown plants is the absence of PSII supercomplexes in the latter. The formation of PSII supercomplexes is probably crucial for the coordination of PSII activity at the donor and acceptor side. Our results support the suggestion that PsbP and PsbQ proteins are important for the formation of PSII supercomplexes and efficient operation of PSII photochemistry.

2. Materials and methods

2.1. Plant material and culture conditions

Seeds of *Picea abies* (Semenoles, Liptovský Hrádok, Slovakia) were first imbibed 4 h in the water and then germinated and cultivated in the moist perlite in complete darkness. When indicated, the dark-grown seedlings were placed in a growth chamber at temperature 23 ± 1 °C and illuminated with continuous white light (80 μmol photons m^{-2} s^{-1}, 1, PAR) for up to 24 h. Light-grown plants were cultivated in a growth chamber at temperature 23 ± 1 °C with periodic 12/12-h day/night cycle (80 μmol photons m^{-2} s^{-1}, PAR). The measurements were performed in cotyledons of 18-day-old seedlings. All manipulations with the dark-grown seedlings were performed under dim green safety light.

2.2. Low temperature fluorescence emission spectra (77 K)

Fluorescence emission spectra of cotyledons were measured at low temperature using a fluorescence spectrophotometer Hitachi F-4500 (Tokyo, Japan) with the spectral bandwidths of 10 and 2.5 nm for excitation and emission monochromator, respectively. The cotyledons were immersed in liquid nitrogen in an optical Dewar flask and measured at 77 K. The excitation wavelength was set to 440 nm.

2.3. Measurements of chlorophyll a fluorescence and absorption changes at 830 nm

Prior to measurements, illuminated seedlings (illuminated for 5, 20, 60 min and 2, 4, 8, 24 h at 80 μmol photons m^{-2} s^{-1}, PAR) were transferred to the dark for 30 min dark-adaptation. The fast chlorophyll *a* fluorescence induction was measured at room temperature using a PEA-fluorometer (Hansatech, King's Lynn, Norfolk, UK) over a time span of 20 μs to 20 s with a data acquisition rate of 10 μs for the first 2 ms, 1 ms between 2 ms and 1 s, and 100 ms thereafter. In the case of dark-grown spruce seedlings, various excitation light intensities were used (1000, 3000, 5000, 7000, 9000 μmol m^{-2} s^{-1} PAR, 650 nm) to document light-dependency of the K-step appearance. Otherwise 7000 μmol m^{-2} s^{-1} PAR of excitation light intensity was used for measurements of fast chlorophyll fluorescence rise curve during illumination. Maximum quantum yield of PSII (F_v/F_m) was calculated as ($F_m - F_0$) / F_m (see e.g. [30]).

For the estimation of the redox state of plastoquinone (PQ) pool, the excitation pressure on PSII (1 − qP parameter) was measured using saturation pulse method. The spruce cotyledons were dark-adapted for 30 min and then minimal fluorescence (F_0) was measured using the excitation intensity of 0.1 μmol m^{-2} s^{-1} (PAR) (Fluorcam FM700, Photon System Instruments, Brno, Czech Republic). Thereafter, the maximum fluorescence (F_m) was then measured using a saturation pulse (3000 μmol m^{-2} s^{-1}, PAR, duration: 1200 ms). Fluorescence induction was measured for 10 min using actinic light (80 μmol m^{-2} s^{-1}, PAR). For the determination of 1 − qP parameter ten saturation pulses were triggered during the fluorescence induction. The actinic light was turned off and F_0' value was recorded. The 1 − qP parameter was calculated using the measured F_m level in the last pulse (F_m') according to Maxwell and Johnson [30].

For the simultaneous monitoring of PSII and PSI activities, the fast chlorophyll *a* fluorescence induction was measured together with absorption changes at 830 nm using Dual-PAM-100 (Walz GmbH, Germany). We used 6 s long actinic light pulse (635 nm, 2000 μmol m^{-2} s^{-1} PAR) with data acquisition rate 600 μs. For the anaerobic measurements, spruce seedlings (dark-grown and 5 min illuminated) were placed into polypropylene bag that was continually (30 min in the dark) flushed with argon gas and chlorophyll *a* fluorescence and P700 changes were monitored simultaneously as described above.

2.4. Thermoluminescence measurements

Thermoluminescence glow curves were measured with a laboratory set-up based on a cooled single-photon-counting detection system Hamamatsu (photon counter CS410, photomultiplier R639, cooler C2761, Japan) (for details see [31]). The dark-grown, 5 min, 24 h illuminated and light-grown seedlings were dark adapted for 30 min. Then cotyledons were cut from the seedlings, attached to the holder and cooled from room temperature to −60 °C using liquid nitrogen. During the cooling the cotyledons were illuminated with dim green light (0.5 μmol photons m^{-2} s^{-1} PAR). This low intensity green light was used to prevent rapid photoactivation of dark-grown seedlings during sample cooling. When indicated, the cotyledons were incubated in 50 μM DCMU during the dark adaptation, cooled down in the dark and at −55 °C one single turnover flash was triggered (XST 103 xenon flash lamp, Walz, Germany). The TL glow curves were recorded during warming to 80 °C at a heating rate of 0.5 °C s^{-1}.

2.5. Measurements of photosynthetic pigment concentration

Dark-grown, light-grown, and dark-grown spruce cotyledons illuminated for 5 min and 24 h were ground with a small amount of sand and extracted with 80% (v/v) chilled acetone with $MgCO_3$ to avoid acidification and pheophytinization of the pigments. The samples were centrifuged at 10,000 ×*g* at 4 °C for 5 min. The concentration of chlorophyll *a*, *b* and sum of carotenoids in the supernatant was determined spectrophotometrically (Jenway 6705 UV/Vis, Bibby Scientific, Essex, UK) according to Lichtenthaler [32].

2.6. Isolation of thylakoid membranes and measurements of oxygen evolution

Thylakoid membranes were prepared from dark-grown, light-grown and dark-grown spruce cotyledons illuminated for 5 min and 24 h according to Dau et al. [33] and Hideg [34]. Spruce cotyledons were homogenized in a buffer containing 400 mM sucrose, 35 mM Hepes, 400 mM NaCl, 4 mM $MgCl_2 \cdot 6H_2O$, 5 mM sodium ascorbate and 0.2% BSA (pH = 7.2). Homogenate was filtered through 2 layers of nylon (40 μm mesh). The filtrate was centrifuged 6 min at 5000 ×*g* (4 °C). Pellet was resuspended in cold buffer containing 25 mM Hepes, 150 mM NaCl, 8 mM $MgCl_2 \cdot 6H_2O$ and 1 mM EDTA-Na (pH = 7.5) and centrifuged 10 min at 5000 ×*g* (4 °C). Pellet was resuspended in cold resuspension buffer containing 400 mM sucrose, 50 mM Hepes, 15 mM NaCl and 5 mM $MgCl_2 \cdot 6H_2O$ (pH = 7.2) and centrifuged 5 min at 5000 ×*g* (4 °C). Finally, pellet was resuspended in small volume of cold resuspension buffer, chlorophyll concentration was measured according to Lichtenthaler [32] and the samples were stored at −80 °C.

Oxygen evolution was measured polarographically with a Clark-type electrode (Hansatech, King's Lynn, Norfolk, UK) in 915 μl resuspension buffer containing 400 mM sucrose, 40 mM MES, 15 mM NaCl, and 5 mM $MgCl_2$, pH = 6.5). 10 μl of thylakoid membrane suspension (1–2 mg chl *a* + *b* ml^{-1}) and 20 μl 70 mM electron acceptor, 2-phenyl-*p*-benzochinone (PPBQ), were added to resuspension

buffer. Actinic light was provided by white LED diodes (2000 μmol m^{-2} s^{-1} PAR, Photon Systems Instruments, Brno, Czech Republic). The activity was recalculated on the same amount of chlorophyll.

2.7. Isolation and separation of PSII supercomplexes by clear native PAGE

PSII-enriched membranes were prepared from dark-grown, light-grown, and dark-grown spruce cotyledons illuminated for 5 min and 24. Membranes were prepared according to Berthold et al. [35] with a few modifications [36]. Spruce cotyledons were homogenized in a solution containing 20 mM Tricine KOH (pH = 7.8), 400 mM NaCl, 2 mM $MgCl_2$ and protease inhibitors 0.2 mM benzamidine and 1 mM ε-aminocaproic acid. Homogenate was filtered through 2 layers of nylon (40 μm mesh). Solution was centrifuged for 10 min at 1400 ×*g* and the pellet resuspended in a solution containing 20 mM Tricine KOH (pH = 7.8), 150 mM NaCl, 5 mM $MgCl_2$ and protease inhibitors as before. This solution was centrifuged for 10 min at 4000 ×*g*, pellet resuspended in 20 mM Hepes (pH = 7.5), 15 mM NaCl, and 5 mM $MgCl_2$ (buffer R) and centrifuged again for 10 min at 6000 ×*g*. The pellet was finally resuspended in a small volume of R. Chlorophyll concentration was adjusted to 2.5 mg/ml and then PSII membranes were prepared by solubilizing stacked thylakoids at 2.1 mg/ml final concentration with 3/16 volumes of 20% Triton X100 (*w*/*v*), 15 mM NaCl, and 5 mM $MgCl_2$ for 20 min on ice by soft agitation. Non-solubilized material was removed by 5 min centrifugation at 3500 ×*g*, supernatant was then centrifuged for 30 min at 40,000 ×*g*, pellet washed once with solution R to remove excess detergent and then centrifuged as before. Finally, PSII membranes were resuspended in a small volume of 20 mM Hepes (pH = 7.5), 400 mM sorbitol, 15 mM NaCl, and 5 mM $MgCl_2$. PSII enriched membranes were frozen in liquid nitrogen and stored at −80 °C. The entire preparation was performed in the cold under dim green light. For the PSII supercomplex preparation, thylakoid and PSII enriched membranes (10 μg of chlorophylls) were solubilized by n-dodecyl-β-D-maltoside at a detergent:chlorophyll mass ratio 12 and supplemented by sample buffer (50 mM Hepes, 400 mM sucrose, 5 mM $MgCl_2$, 15 mM NaCl, 10% glycerol, pH = 7.2) to the final volume of 30 μl. Nonsolubilized membranes were removed by centrifugation at 22,000 ×*g*, 4 °C and supernatant was directly loaded onto gel. Clear-native polyacrylamide gel electrophoresis (CN-PAGE) was performed using the 4–8% gradient resolving gel optimized for separation of large supercomplexes as described in Kouřil et al. [37]. The same amount of chlorophylls was electrophoresed.

2.8. SDS-PAGE and Western blots

The proteins from thylakoid and PSII enriched membranes were isolated using extraction buffer containing 28 mM DTT, 28 mM Na_2CO_3, 175 mM sucrose, 5% SDS and 10 mM EDTA and protease inhibitors (Set VI, Calbiochem, Darmstadt, Germany). The samples were heated for 30 min at 70 °C. Concentration of total soluble proteins in samples was determined using the Bicinchoninic Acid Kit for Protein Determination (Sigma-Aldrich, St. Louis, MO, USA) and absorbance was measured at 562 nm (Jenway 6705 UV/Vis, Bibby Scientific, Essex, UK). The same amount of proteins was electrophoresed in Tricine-10% (v/v) SDS-polyacrylamide gel according to Schägger [38] followed by transfer to the nitrocellulose membrane (Biorad, Germany) by Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Biorad, Hercules, CA, USA). To check the correct protein transfer, the membranes were stained with Ponceau Red. After blocking in TBS-T containing 5% BSA overnight at 4 °C, the membranes were incubated with the primary antibody for 1 h at room temperature with soft agitation. Antibodies against proteins of photosystem II (D1), PsbS, OEC (PsbP, PsbQ, PsbO), photosystem I (PsaA), cytochrome *f* and light harvesting antennae (Lhcb1, 2, 4, 5, Lhca1–4), were purchased from Agrisera (Vännäs, Sweden). After washing, the membrane was incubated for 1 h with the secondary antibody in dilution 1:10,000; the goat antirabbit IgG (H + L)-horseradish peroxidase conjugate (Bio-Rad, Hercules, CA, USA). Blots were visualized using Immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, MA, USA). After washing, the blots were visualized and quantified by a gel scanner Amersham Imager 600 (GE HealthCare Life Sciences, Japan).

2.9. Statistical analysis

Data from chlorophyll fluorescence, P700 absorbance changes and thermoluminescence measurements are representatives of a total of four–six independent measurements. To evaluate the significance of the data (oxygen evolution, chlorophyll pigment content and F_v/F_m, 1 − qP and F_0 parameters) analysis of variance (ANOVA followed by Tukey's test, Origin 8.5.1., Northampton, USA) was used.

3. Results and discussion

3.1. Assembly of PSI and PSII antennae

Dark-grown spruce seedlings are able to synthesize significant amount of chlorophyll in the dark due to the presence of DPOR (Table 1). The chlorophyll molecules do not remain free, but they are bound to various proteins in PSI and PSII complexes, which can be documented by the measurement of the low temperature (77 K) fluorescence emission spectrum (Fig. 1). The bands observed in the spectrum of dark-grown spruce cotyledons indicate the presence of free Pchlide (633 nm), Pchlide bound to prolamellar bodies (655 nm), Chl *a* molecules bound to internal antennae of PSII (CP43, 685 nm and CP47, 694 nm) and to the light-harvesting complex of PSI (LHCI, 733 nm). After 5 min illumination of the seedlings, the Pchlide molecules were not detected in the emission spectrum (Fig. 1, inset), which can be explained by the catalytic activity of LPOR [1,2].

The shape of the fluorescence emission spectrum of PSII (i.e. 685 and 694 nm bands) did not change during 24 h illumination and was identical with that of light-grown cotyledons, which implies that the antennae CP43 and CP47 are properly assembled in PSII even in the dark. The PSI emission increased relatively to PSII emission during prolonged illumination, but it did not reach the level of the light-

Table 1
Oxygen evolution rate in thylakoid membranes isolated from cotyledons of Norway spruce, pigment content and chlorophyll fluorescence parameters measured in intact cotyledons (minimal fluorescence, F_0, excitation pressure, 1 − qP). Values with different letters in the rows denote statistically significant differences at $P < 0.05$ (ANOVA, Tukey's test). Means ± s.d., n = 5, ND — not detectable, N/A — not applicable.

	Dark-grown	5 min illuminated	24 h illuminated	Light-grown
O_2 evolution (nmol O_2 mg^{-1} chl $a + b$ s^{-1})	ND a	3.1 ± 0.4 b	46.5 ± 5.0 c	32.8 ± 3.1 d
chl $a + b$ (μg g^{-1} FW)	887 ± 25 a	860 ± 30 a	1057 ± 21 b	2264 ± 36 c
Chl a/b	3.55 ± 0.04 a	3.51 ± 0.03 a	3.59 ± 0.05 a	3.07 ± 0.03 b
car + xan (μg g^{-1} FW)	214.8 ± 5.5 a	205.8 ± 4.5 a	251.8 ± 5.5 b	469.4 ± 9.5 c
(Chl $a + b$)/(car + xan)	4.13 ± 0.07a	4.18 ± 0.05 a	4.20 ± 0.02 a	4.83 ± 0.02 b
1 − qP	N/A	N/A	0.19 ± 0.03 a	0.22 ± 0.05 a
F_0 (a.u.)	697 ± 17 a	710 ± 93 a	472 ± 31 b	496 ± 48 b

grown seedlings (Fig. 1). This finding can be explained by lower amount of PSI in dark-grown and dark-grown illuminated cotyledons or by higher reabsorption of PSII emission in light-grown cotyledons [39], which is associated with increasing concentration of chlorophylls (Table 1). However, this reabsorption would lead to a preferential decrease of the intensity of the emission band at 685 to that at 694 nm, which was not observed (Fig. 1). Thus, the higher PSI to PSII ratio in light-grown cotyledons is probably responsible for the observed change in PSI emission intensity. This was also confirmed by Western blot analysis of PsaA protein (reaction center of PSI), Lhca1–4 proteins (PSI antennae) and D1 protein (reaction center of PSII) (Fig. 7A). In dark-grown and dark-grown illuminated plants, the relative amount of PSI (PsaA) compared to PSII (D1) is lower than in light-grown plants. The relatively low PSI/PSII ratio was also documented in dark-grown *Pinus palustris* seedlings by 2D SDS-PAGE [6].

3.2. PSII electron transport in dark-grown cotyledons

The functionality of photosynthetic electron transport chain in dark-grown spruce cotyledons was studied by the measurement of fast chlorophyll *a* fluorescence rise (FR) and the saturation pulse method. The measurement of FR is the frequently used non-destructive method to probe electron transport in thylakoid membranes. In fully developed dark-adapted plants, in response to illumination of high intensity, the

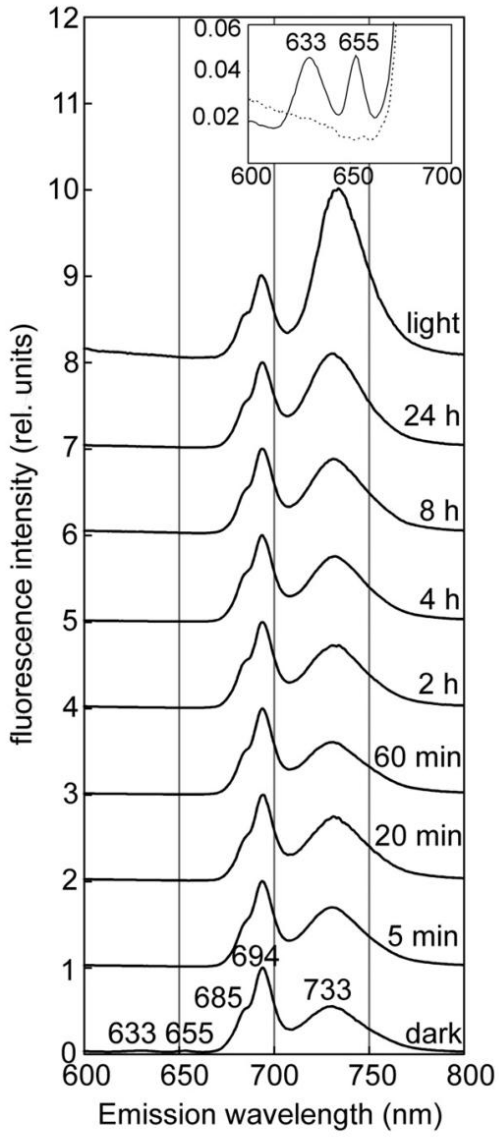

Fig. 1. Low-temperature (77 K) fluorescence emission spectra in spruce cotyledons. Cotyledons were either light-grown (light) or dark-grown and illuminated for 0 (dark), 5, 20, and 60 min and 2, 4, 8 and 24 h. Excitation wavelength was 440 nm. The spectra are normalized to the fluorescence intensity at 694 nm and are vertically shifted. The inset shows the detailed spectra of both forms of Pchlide present in dark-grown seedlings (solid line) and their photoreduction after 5 min of illumination (dashed line). Presented data are representatives of a total of four measurements.

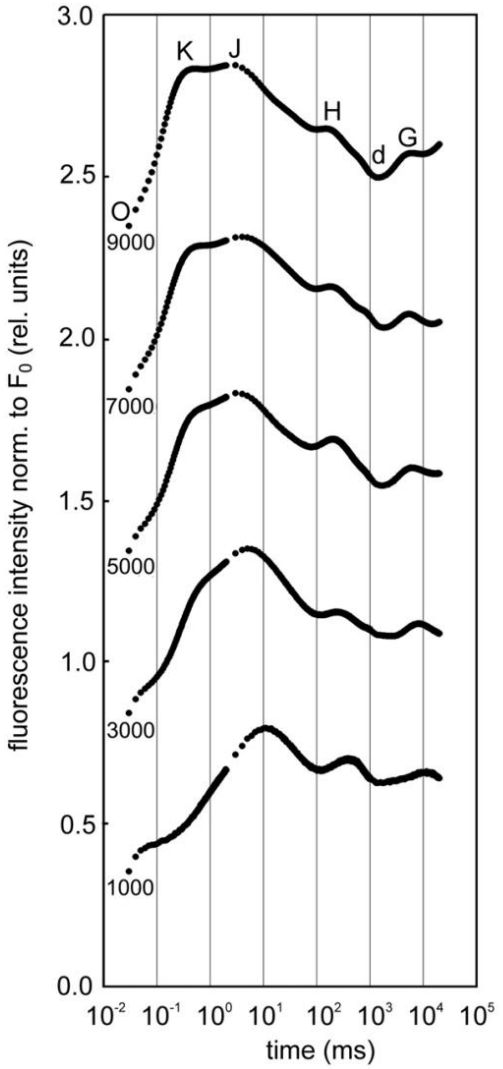

Fig. 2. Chlorophyll *a* fluorescence rise in dark-grown spruce cotyledons measured in time span of 30 μs–20 s at different intensities of exciting light (1000–9000 μmol photons m^{-2} s^{-1}, 650 nm, indicated). Curves are normalized to the F_0 level, vertically shifted and are plotted on a logarithmic time scale. Presented data are representatives of a total of four measurements.

fluorescence intensity (yield) usually shows a polyphasic rise called OJIP transient. The steps J, I and P in FR represent temporary accumulation of reduced electron carriers involved in linear electron transport in thylakoid membranes (for recent reviews see e.g. Lazár and Schansker [40], Stirbet and Govidjee [41,42]).

Fig. 2 shows the FRs measured in dark-grown cotyledons at different intensities of exciting light. A clear step appeared at about 400 μs in FRs measured with high intensity of exciting light. The time position of this step and its disappearance at lower exciting light indicate that this step can be assigned as step K. This step appears in FR whenever the rate of electron transport from P680 to the acceptor side of PSII exceeds the rate of electron transport from the donor side of PSII to P680 [43]. While the fluorescence rise to the K peak reflects a transient reduction of Q_A after the first charge separation in PSII (i.e. a formation of the $Tyr_Z^+ Q_A^-$ form, Tyr_Z — secondary electron donor of PSII), the fluorescence decrease after the K peak is explained by the forward electron transport to Q_B (i.e. formation of the $Tyr_Z^+ Q_A Q_B^-$ form, [44,45]). The appearance of this step in FR is well documented in leaves exposed to heat, which results in the damage of OEC [43,46–48]. In plants exposed to heat, ascorbate serves as a slow alternative electron donor to PSII [45]. This electron donation explains why other steps are often observed in FR after the K step [45,49].

Interestingly, an additional step at about 2 ms also appeared together with the K step in FR of dark-grown cotyledons under higher excitation intensities (Fig. 2). The time position of this step and its gradual shift to longer times for lower exciting light shows that this is a typical J step, representing the accumulation of Q_A^- with a weak reduction of acceptors beyond Q_A in unstressed leaves [50]. Tóth et al. [45] have shown that the J step can appear together with the K step in FR when the heated leaves with damaged OEC are preilluminated by high light for several seconds. The authors have shown that this phenomenon corresponds with the reduction of PQ pool by the electrons coming from PSII during the preillumination. Sometimes the K and J steps appear in FRs of heat-stressed plants without the preillumination [44,46], which could be explained by intensive heat-induced dark reduction of PQ pool by stromal reductants [51]. Taking into account these findings we can conclude that in our dark-grown spruce cotyledons there is a functionless OEC, but when illuminated, PSII can take electrons from an alternative source (e.g. ascorbate) and some chloroplast reductants are probably able to reduce PQ pool in the dark. Traces of additional steps after the K and J steps in FR of dark-grown cotyledons (the H and G steps, see hereinafter) support the idea of functional electron transport chain in thylakoid membranes without OEC (Fig. 2).

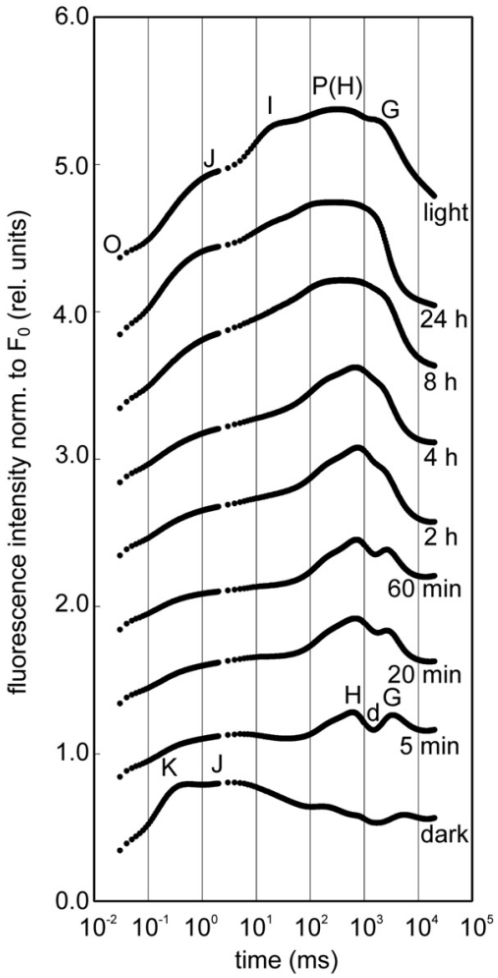

Fig. 3. Chlorophyll *a* fluorescence rise measured in time span of 30 μs–20 s at excitation light intensity of 7000 μmol photons $m^{-2} s^{-1}$ (650 nm) in light-grown and dark-grown spruce cotyledons that were illuminated (80 μmol photons $m^{-2} s^{-1}$, PAR) for different time periods (0–24 h, indicated). Seedlings were dark-adapted for 30 min before the fluorescence measurement. Curves are normalized to F_0 level, vertically shifted and plotted on a logarithmic time scale. Presented data are representatives of a total of 4–6 measurements.

3.3. Electron transport in thylakoid membranes of illuminated dark-grown cotyledons

Illumination of dark-grown cotyledons for 5 min led to dramatic changes in FR (Fig. 3). The K step disappeared and distinct peaks at about 700 ms and 3.5 s became dominant in FR. The time position of these peaks allows us to identify them as H and G steps, respectively (for nomenclature see Lazár [52]). They have been observed in foraminifers, diatoms, zooxanthellae, lichens, gymnosperms and angiosperms growing under intermittent light [40,53–60]. Using the simultaneous measurements of FR and absorption changes at 820 nm it has been found that the descending part of the H step corresponds with the oxidation of P700/PC (the electron donor of PSI/plastocyanin), while the subsequent G step was attributed to a transient reduction of PQ pool which is, however, not followed by the redox change of P700/PC [57]. Ongoing illumination of dark-grown spruce cotyledons for up to 24 h led to a gradual disappearance of the G step and to a better resolution

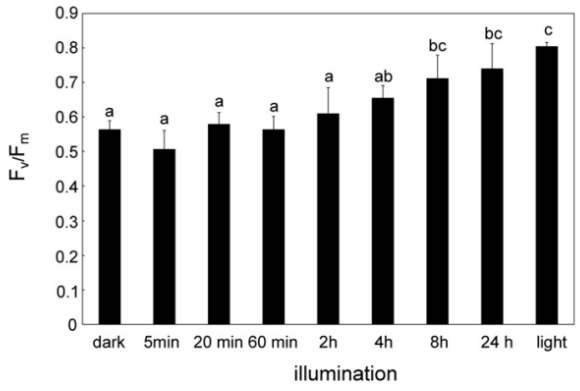

Fig. 4. Maximum quantum yield of photosystem II photochemistry (F_V/F_M) in dark-grown cotyledons in response to illumination (80 μmol photons $m^{-2} s^{-1}$, PAR) for different time periods (0–24 h, indicated) and in light-grown cotyledons. The F_V/F_M ratios were calculated from the chlorophyll *a* fluorescence curves (Fig. 3). Means ± s.d., n = 4–6. Different letters denote significant differences at P = 0.05 (ANOVA, Tukey's test).

of the J and I steps in FR (Fig. 3). The F_v/F_m ratio gradually increased from about 0.55 to 0.75 during the 24 h illumination. This ratio was about 0.8 for light-grown cotyledons, representing the value of fully functional PSII (Fig. 4).

In order to find out whether the H and G steps in FR of spruce cotyledons are attributed correctly, we have measured FRs simultaneously with the absorption changes of P700/PC (Fig. 5). In the time-course of P700/PC redox changes, three different phases can be distinguished, similar to those described in *Trebouxia*-possesing lichens [57] or in diatoms *Thalassiosira pseudonana*, *Phaeodactylum tricornutum* and *Cyclotella meneghiniana* [59]. These are: (i) the initial oxidation of P700/PC during the first 10–20 ms due to electron flow within PSI, (ii) the transient reduction of P700/PC between 20 and 200 ms by electrons from PSII and (iii) the final oxidation of P700/PC after approximately 200 ms. Due to technical limitations, the simultaneous measurement of FR and P700/PC redox changes was performed using lower intensity of exciting light (2000 μmol photons m^{-2} s^{-1}, PAR) and lower recording rate than the FRs shown in Figs. 2 and 3. Under such conditions, the K step in the FR of dark-grown cotyledons is not distinguishable, but the H and G steps appeared in FR after 5 min and longer illumination as in Figs. 2 and 3. As expected, the fluorescence decline after the H peak was accompanied by the oxidation of P700/PC and the fluorescence rise to the G step was not connected with reduction of P700/PC [57]. It is clearly visible that in dark-grown cotyledons, the final oxidation of P700/PC was much faster (reached the saturation level at about 1.3 s) than in cotyledons illuminated for 5 min and longer time (saturation at about 3 s and more). In dark-grown cotyledons the electron donation from PSII to PSI is very limited (see above), thus the final oxidation of P700/PC was relatively fast, limited only by the activation of the outflow of electrons at the acceptor side of PSI [57].

The relatively fast final oxidation of P700/PC during several seconds of intensive illumination of dark-adapted plants is typical for phylogenetically older plants. Shirao et al. [61] have compared this final oxidation phase in 101 plant species and have concluded that it is much faster in gymnosperms than in angiosperms. The fast final oxidation of P700/PC was observed also in lichens [57,60], diatoms [59,62] or green algae [63]. On the basis of the experiments with various chemical inhibitors, Ilík et al. [57] have suggested that the final oxidation of P700/PC could reflect a fast activation of FNR (ferredoxin-$NADP^+$ oxidoreductase) or Mehler reaction at the acceptor side of PSI and is not connected with the activity of PTOX (plastid terminal oxidase). Later it has been shown that this final oxidation is slowed down or eliminated (in the used time frame) under anaerobic conditions [59,62,63], which indicates that the Mehler-peroxidase reaction is responsible for the final oxidation of P700/PC. We favor this interpretation as in spruce cotyledons we have also observed that this final oxidation is oxygen dependent (Fig. S1) and not affected by propylgallate, a well-known inhibitor of PTOX (data not shown).

3.4. Photoactivation of PSII detected by thermoluminescence

In order to clarify the mechanism of the gradual completion of PSII function in dark-grown seedlings exposed to light, we have measured the thermoluminescence glow curves (TL), which represent a useful tool for the study of the photoactivation of OEC in PSII. The formation of functional OEC is associated with the appearance of characteristic TL bands that reflect a recombination of the reduced PSII quinone acceptors (Q_A^- and Q_B^-) with the forming S states of OEC. In order to maximize the number of TL bands characterizing the formation of active PSII, we have excited the cotyledons continuously during the cooling down to

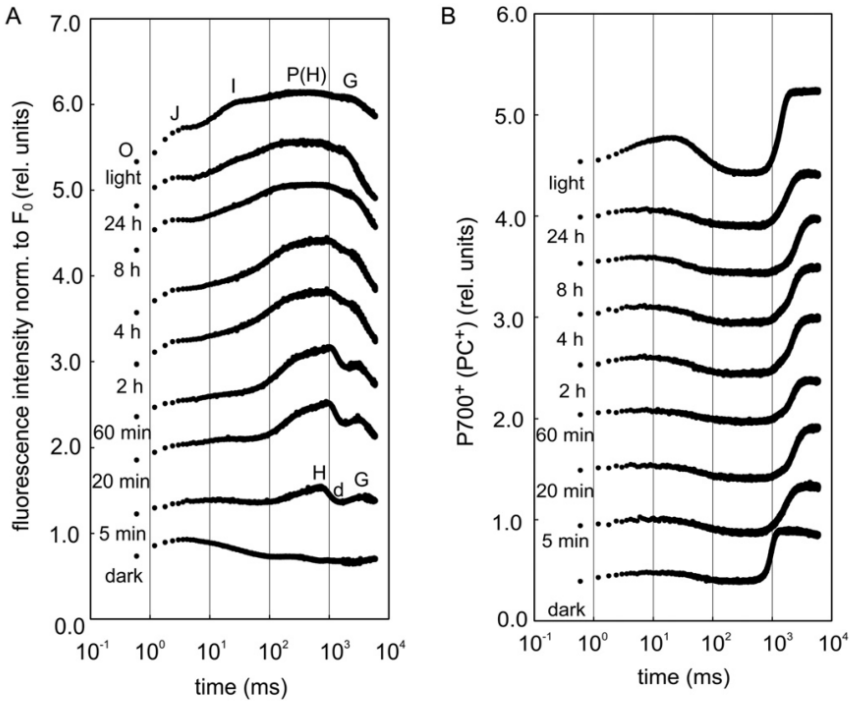

Fig. 5. Simultaneous measurement of chlorophyll *a* fluorescence rise (A) and P700/PC absorbance changes at 830 nm (B) in dark-grown spruce cotyledons that were illuminated (80 μmol photons m^{-2} s^{-1}, PAR) for different time periods (0–24 h, indicated) and in light-grown cotyledons. Seedlings were dark-adapted for 30 min before the measurement. Light intensity of exciting light was 2000 μmol photons m^{-2} s^{-1} (635 nm). Curves are vertically shifted (without any normalization) and are plotted on a logarithmic time scale. Presented data are representatives of a total of four measurements.

−60 °C. For the detection of so called Q band in the TL curve (see below), we have used cotyledons treated with DCMU (an inhibitor of the electron transport between Q_A and Q_B) that were excited by single turnover flash.

We used four distinguished stages of developing spruce cotyledons (dark-grown, dark-grown illuminated for 5 min and 24 h, light-grown) for thermoluminescence measurements. In dark-grown cotyledons excited during cooling we have observed clear TL bands peaking at about −25 °C and 55 °C (Fig. 6A). Both these bands have been already observed in samples with Mn-depleted PSII [64,65], implying that OEC does not operate in dark-grown spruce cotyledons due to the absence of Mn atoms [16]. The high-temperature TL band was termed C band and attributed to the recombination Q_A^- Tyr_D^+ (Tyr_D — tyrosine of D2 protein at the donor side of PSII) (see Johnson et al. [66]). The low temperature band could represent the Z_V band originating in $P680^+ Q_A^-$ recombination [67] and/or the A_T band, which originates in recombination of $Tyr_Z^+ Q_A^-$ [68].

A relatively short, 5 min illumination of dark-grown cotyledons was sufficient for the appearance of a typical B band (at about 35 °C) in the TL glow curve (Fig. 6A, Table S2). As B band originates in $S_{2(3)}^+ Q_B^-$ recombination, this result indicates that OEC is already functional in PSII. In cotyledons illuminated for 5 min and subsequently treated with DCMU, the C band became dominant and additional bands at about 5 °C and 20 °C were observed in the TL curve. While the 5 °C band represents a typical Q band coming from $S_2^+ Q_A^-$ recombination in DCMU treated samples [69] and its presence is expectable with respect to the intensive B band measured without DCMU, the origin of the 20 °C band is not clear. The 20 °C band probably represents the up-shifted Q band, which was observed in DCMU-treated stromal thylakoids [70] or DCMU-treated leaves of transgenic tobacco with severely down-regulated PsbP protein of OEC [27]. The absence of PsbP in PSII destabilizes grana stacking [27], thus the 20 °C band could really originate in PSII from non-appressed thylakoids. In fact, non-appressed thylakoids are very abundant in dark-grown spruce seedlings [71].

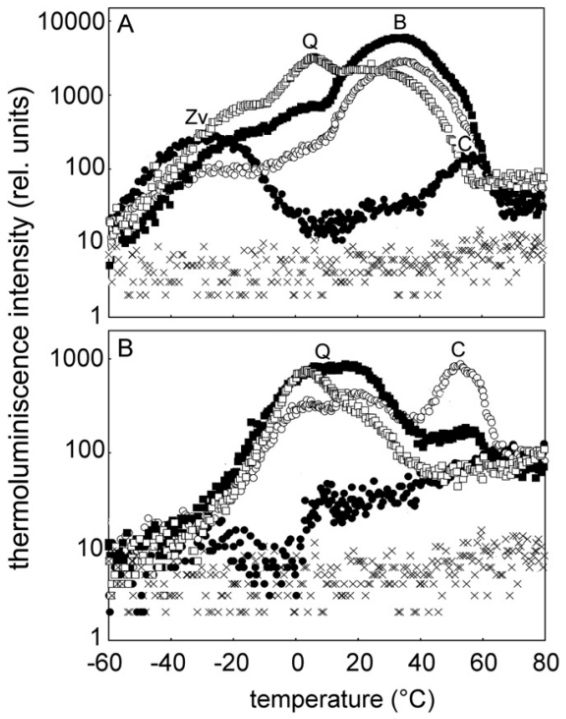

Fig. 6. Typical thermoluminescence glow curves with dark-grown (●), dark-grown illuminated for 5 min (○) and 24 h (■) and light-grown (□) spruce cotyledons untreated (A) and treated with 50 μM DCMU (B). The cotyledons were illuminated during cooling (from 25 to −60 °C) by dim green light (0.5 μmol photons m^{-2} s^{-1}) (A) or by a single turnover flash at −55 °C (B). The background signal (×) was detected with non-illuminated dark-grown seedlings. The y-axis is plotted on logarithmic time scale. Presented data are representatives of a total of 4–5 measurements.

TL glow curves of cotyledons after 24 h illumination showed higher B band intensity, however a shoulder of the C band remained visible as well as after 5 min illumination (Fig. 6A). Compared to TL curves of cotyledons illuminated for 5 min, a dramatic decrease of the C band intensity accompanied by an increase of both Q bands was observed in DCMU-treated cotyledons (Fig. 6B). These results agree with the well-known phenomenon that the gradual decrease of the C/B band or C/Q band intensity ratio in untreated or DCMU-treated samples, respectively, reflects the formation of active PSII centers (so called PSIIα or PSII-Q_B-reducing) at the expense of inactive PSII (so called PSIIβ or PSII-Q_B-nonreducing, [72,73]). The formation of active PSII centers is accompanied with a decrease in F_0 values despite increasing chlorophyll content (Table 1).

TL glow curves of light-grown cotyledons showed the absence of the C band in both DCMU-treated and untreated samples and the suppression of the 20 °C Q band in DCMU-treated cotyledons, which both reflect the presence of a high portion of active PSII centers. Interestingly, a dominant band at about 5 °C (probably the Q band) instead of the B band appeared in untreated light-grown cotyledons. This finding could be associated with a reduced accumulation of Q_B^- in active PSII during weak continuous excitation upon cooling to −60 °C.

3.5. Accumulation of photosynthetic pigments and development of oxygen evolution

For the four distinguished stages of developing spruce cotyledons (dark-grown, dark-grown illuminated for 5 min and 24 h, light-grown) we have also determined the concentrations of photosynthetic pigments and measured the rate of oxygen evolution in thylakoid membranes separated from the seedlings (Table 1). As expected, the dark-grown and 5 min illuminated cotyledons contained the lowest amount of chlorophylls and carotenoids. After 24 h illumination the pigment concentration significantly increased, but it was still about a half of that observed in light-grown seedlings. The lowest chlorophyll *a*/*b* ratio and the highest ratio of chlorophylls to carotenoids were observed in light-grown seedlings (Table 1). A relatively high chlorophyll *a*/*b* ratio in dark-grown gymnosperms is common and is attributed to the low amount of LHCII (Fig. 7) [5,6,8,13,74].

The measurement of oxygen evolution in thylakoid membranes isolated from dark-grown plants confirmed the absence of detectable oxygen evolution, which corresponds with the appearance of the K step in FR (Figs. 2 and 3) as well as with TL data (Fig. 6). After 5 min illumination of dark-grown spruce seedlings, oxygen evolution was detectable, which again agrees with the disappearance of the K step in FR (Figs. 2 and 3) and with the appearance of B band in TL (Fig. 6A). After 24 h illumination of the seedlings the rate of oxygen evolution was high, even higher than in the membranes isolated from light-grown seedlings (Table 1). This finding can be explained by the fact that the rate of oxygen evolution is related to chlorophylls ($a + b$) and that the light-grown seedlings have relatively higher extent of LHCII (as indicated by the lower chlorophyll *a*/*b* ratio, Table 1) and PSI than dark-grown seedlings illuminated for 24 h. Indeed, the relative amount of PSII reaction centers (per chlorophyll) estimated from the amount of the D1 protein using immunoblotting was lower in thylakoid membranes isolated from the light-grown seedlings than from dark-grown seedlings illuminated for 24 h (Fig. S2). Thus, in the measurement of oxygen evolution, the effect of the slightly higher PSII photochemical activity in light-grown seedlings compared to dark-grown seedlings illuminated for 24 h (as deduced e.g. from the F_V/F_M ratio, see Fig. 4) was shielded by the increased chlorophyll/PSII ratio in light-grown seedlings.

3.6. Organization of PSII complexes

The results described above indicate that the light-induced development of fully active PSII centers in dark-grown spruce seedlings is a long-term process that involves also the assembly of LHCII with PSII. In order to reveal how the development of PSII photochemical activity is connected with the assembly of PSII complexes during 24 h of illumination, we have performed analysis of selected PSII proteins in isolated thylakoid membranes using SDS-PAGE and Western blots (Fig. 7). We have focused mainly on the level of PSII reaction center protein (D1), OEC subunits (PsbO, PsbP, PsbQ), and light harvesting antennae of PSII (Lhcbs) and PsbS protein. The levels of cytochrome *f* and PsaA, the PSI reaction center protein, and the light harvesting antennae of PSI (Lhca1–Lhca4), were also determined. The results showed that all the selected components of photosynthetic apparatus are present in thylakoid membranes of dark-grown cotyledons and their content was unchanged or increased with prolonged illumination, which corresponds with studies published by Shinohara et al. [5] and Canovas et al. [6] using dark-grown pine seedlings. As expected, the highest amounts of these proteins were detected in the light-grown plants. We failed to detect Lhcb3 and Lhcb6 proteins. The antibodies against *Arabidopsis* Lhcb3 and Lhcb6 proteins are probably not specific for spruce (the epitopes share less than 50% similarity, communication with the company Agrisera AB), which questions the presence of these proteins in spruce at all.

In order to investigate the association of OEC proteins to PSII, we have isolated also the proteins from PSII particles. The protein analyses revealed a gradual increase in the amount of PsbO bound to PSII with prolonged illumination, low amount of PsbP bound to PSII in comparison to light-grown plants and a complete absence of PsbQ in PSII particles during the 24 h illumination (Fig. 7B). Comparing these results with that obtained with thylakoid membranes (Fig. 7A) indicates that PsbP and PsbQ proteins are loosely associated with PSII in dark-grown and dark-grown illuminated seedlings (Fig. 7B). The loosely bound PsbP, and consequently PsbQ, in PSII during 24 h illumination explain why the maximum yield of PSII photochemistry remained slightly below optimal values (Fig. 4, [20]). It has been reported that the absence of PsbP in OEC causes a release of Ca^{2+} ions from the Mn-cluster [22], which is important for the formation of photochemically active PSII [75]. When Ca^{2+} ions are missing at the donor side of PSII, the PSII remains in the

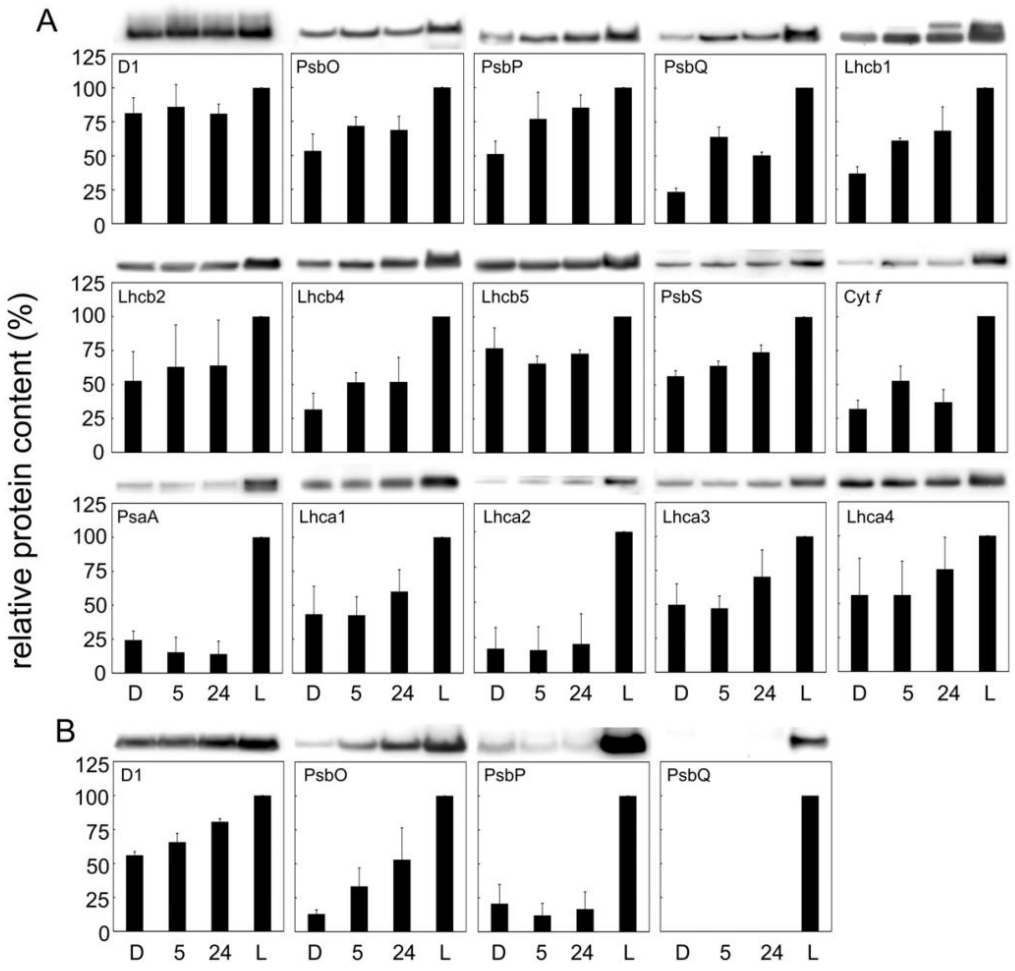

Fig. 7. Western blots of isolated thylakoid (A) and PSII membranes (B). Samples were separated in 10% (v/v) SDS-polyacrylamide gel and subjected to Western blot analysis. D — dark-grown plants; 5, 24 — dark-grown plants subsequently illuminated for 5 min or 24 h, respectively; L — light-grown plants. The presented blots are representatives of three independent experiments. Protein content (relative to L) in the bands was quantified by chemiluminescence. The statistical evaluation of the protein content is based on three independent experiments (means ± s.d.).

form of so called PSII-Q_B-nonreducing centers [76], where the electron transfer from Q_A to Q_B is slowed down due to the up-shifted redox potential of Q_A [77]. The importance of PsbP for the formation of photochemically active PSII was also evidenced in PsbP RNAi suppressed *Arabidopsis* plants [78]. The absence of PsbQ in PSII complex of spruce seedlings during 24 h illumination may not be crucial for the functioning of OEC in PSII [20].

Actually, it has been reported that the PsbP together with PsbQ proteins is necessary for the formation of PSII–LHCII supercomplexes [27,28,36,79], which have the highest rate of oxygen evolution among PSII containing structures [29]. Therefore, we have performed clear-native electrophoresis of protein complexes from thylakoid membranes and PSII particles isolated from dark-grown, dark-grown illuminated and light-grown cotyledons. Because we have had problems with complete solubilization of thylakoid membranes from dark-grown and dark-grown illuminated seedlings using n-dodecyl-β-D-maltoside (Fig. S3), we used Triton X treatment for the isolation of PSII particles with subsequent solubilization with n-dodecyl-β-D-maltoside (Fig. 8). Using both approaches we obtained the same results PSII–LHCII supercomplexes were present only in thylakoid membranes and PSII particles from light-grown seedlings (Fig. 8, Fig. S3). The absence of the supercomplexes in the dark-grown and dark-grown illuminated spruce seedlings corresponds with the increased content of PSII monomers and dimers (Fig. 8), which are connected with lower rate of O_2 evolution [29]. The absence of PSII–LHCII supercomplexes together with lower relative content of PSI (Fig. 7A) may prevent overexcitation of PSII and may maintain electron balance between both photosystems (see the same 1 − qP values in Table 1). Indeed, transgenic plants with lower amount of PsbP protein and thus decreased relative amount of PSII–LHCII supercomplexes have reduced level of PSI [27].

4. Conclusion

In this study we have shown that photoactivation of oxygen evolution in PSII is relatively fast and can be observed already in PSII that are not fully photochemically active. At the same time we present data indicating that the light-induced formation of fully photochemically active PSII centers is a gradual and long-term process that includes the synthesis and binding of LHCII to PSII, the assembly of OEC proteins PsbP and PsbQ into PSII and finally the association of PSII into supercomplexes. The slow activation of PSII function seems to have physiological importance as in the dark-grown seedlings the PSI/PSII stoichiometry is rather low (Figs. 1 and 7A, [6]) and faster PSII activation (the assembly of PSII into supercomplexes) could lead to over-reduction of electron carriers in linear electron flow upon illumination. The PSI/PSII stoichiometry is changing due to coordinated expression of photosynthetic genes, which are under light-dependent redox control [80]. The photoactivated PSII centers that are not fully photochemically active could be identical to PSII-Q_B-nonreducing centers, which are localized in stromal thylakoids [81]. The stromal thylakoids, indeed, dominate in the early stage of light-induced greening of dark-grown spruce seedlings [71]. The appearance of PSII-Q_B-nonreducing centers after the photoactivation is supported by the appearance of the C band in TL glow curves (see Andrée et al., [72]). This type of PSII centers can be important as they are able to cope with eventual excessive light by effective recombination process within PSII [75]. However, we have also observed the up-shifted Q band in TL after the 24 h illumination of spruce seedlings, which was more intensive than the C band. This unusual TL band probably represents a fraction of not fully active PSII with missing PsbP (see Ido et al. [27]). The loosely bound PsbP, and consequently PsbQ, in PSII prevents the formation of PSII–LHCII supercomplexes and grana stacking [21,27,82] and thus over-excitation of PSII, indicating the important role of these proteins in keeping the balance between PSII and PSI.

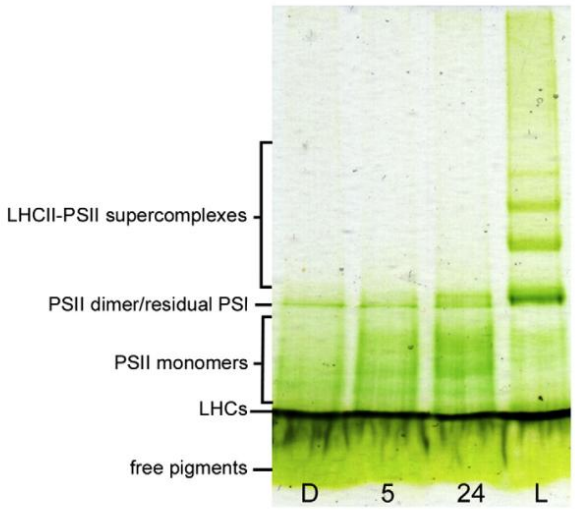

Fig. 8. Separation of pigment–protein complexes by clear native PAGE. PSII enriched thylakoid membranes isolated from spruce cotyledons were solubilized by n-dodecyl-β-D-maltoside. D — dark-grown plants; 5, 24 — dark-grown plants subsequently illuminated for 5 min and 24 h, respectively; L — light-grown plants.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2016.02.009.

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8.3 The advantage of chlorophyll biogenesis in the dark: comparative study with seedlings of European larch (*Larix decidua*) and Norway spruce (*Picea abies*).

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The advantage of chlorophyll biosynthesis in the dark: comparative study with seedlings of European larch (*Larix decidua*) and Norway spruce (*Picea abies*)

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Key message

The ability to green in the dark represents mechanism for shade tolerance in seedlings of Norway spruce

Conflict of interest

The authors declare that they have no conflict of interest.

Author Contribution:

Conceived and designed the experiments: TS and AP

Conducted the experiments: TS, VN, LN and AP

Analyzed the data: TS, VN and AP

Contributed reagents/materials/analysis tools: AP

Wrote the manuscript: TS, AP

Abstract

In contrast to angiosperm plants, gymnosperms possess two different enzymes for reduction of protochlorophyllide to chlorophyllide: dark-operative, light-independent protochlorophyllide oxidoreductase (DPOR), consisting of three subunits: ChlL, ChlN and ChlB, and the light-dependent protochlorophyllide oxidoreductase (LPOR). European larch seedlings (*Larix decidua* Mill.), in contrast to Norway spruce (*Picea abies* Karst.), accumulate only very low amount of chlorophylls in the dark due to the inactive DPOR enzyme. In this study we used these two species to investigate the advantage of co-existence of two protochlorophyllide oxidoreductases on chlorophyll synthesis under different cultivation light conditions. We found that under the deep shade conditions, the larch seedlings are partially etiolated with low quantum yield of photosystem II photochemistry caused by inefficient LPOR function under these subliminal values of irradiance and by the inactive DPOR enzyme. In contrast, the spruce accumulated a significant amount of chlorophylls under the deep shade conditions due to the co-existence of active DPOR and LPOR enzymes. Moreover, although PSII developed in the dark had an inactive oxygen evolving complex, even very low irradiance is sufficient for photoactivation of PSII proved by the high values of quantum yield of photosystem II (F_v/F_m) and disappearance of K-step in chlorophyll *a* fluorescence induction under deep shade conditions in spruce. We did not find any advantage of having DPOR enzyme under the high light conditions, what is consistent with decreasing abundance of DPOR subunits with increasing light intensities. Thus, presence of active DPOR enzyme may represent molecular basis for shade tolerance in conifer seedlings.

Key words

chlorophyll, larch, light acclimation, shade, spruce

Introduction

Chlorophyll (Chl) biosynthesis and its regulation have been widely studied for decades. Reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) seems to be the most crucial step in Chl biosynthetic pathway and the process of plant greening. In this reaction, pyrrole ring D of Pchlide is reduced and chlorins are formed (Skinner and Timko 1999, Gabruk and Mysliwa-Kurdziel 2015). In nature, two Pchlide reductases can catalyse this reaction. One of them is light-dependent NADPH-Pchlide oxidoreductase (LPOR) and the other one is the light-independent Pchlide reductase (DPOR, Armstrong 1998, Gabruk and Mysliwa-Kurdziel 2015).

The ability of light-independent chlorophyll biosynthesis (i.e. in the dark) is closely correlated with the presence of the genes for DPOR in DNA. The enzyme occurs in oxygenic phototrophs, i.e. cyanobacteria, green algae and non-flowering plants (mosses, liverworts, hornworts, lycophyta, ferns, gymnosperms) and anoxygenic bacteria (Armstrong 1998, Fujita and Bauer 2003, Yamamoto et al. 2011). DPOR is ATP-dependent and oxygen sensitive reductase consisting of three plastid-encoded subunits, which form two sub-complexes, the L (ChlL dimer) and the NB (ChlN–ChlB heterotetramer) resembling bacterial nitrogenase (Bröcker et al. 2010, Muraki et al. 2010). DPOR has never been found in angiosperms (Armstrong 1998).

In angiosperms, the same stereospecific reduction of the pyrrole ring D of Pchlide is catalysed exclusively by LPOR (Armstrong 1998, Reinbothe et al. 2010, Gabruk and Mysliwa-Kurdziel 2015). Instead of four molecules of ATP used by DPOR for single Pchlide reduction (Nomata et al., 2016), two photons of light are required for a single-molecule reduction by LPOR (Sytina et al., 2008). In contrast to DPOR, LPOR is a nuclear-encoded, single-polypeptide which occurs in one (*Cucumis sativa*), two (*Hordeum vulgare*) or three isoforms (*Arabidopsis thaliana*), designated as LPORA, LPORB and LPORC. LPORA form is a predominant form of the enzyme present especially in prolamellar bodies (PLBs) of etiolated plants. LPORB is located in PLB or in the thylakoid membranes in the lower amounts and is responsible for Pchlide reduction during the later stages of greening (Holtorf et al. 1995, Skinner and Timko 1999, Suzuki et al. 2001, Garrone et al. 2015, Gabruk and Mysliwa-Kurdziel 2015). So far, *Arabidopsis thaliana* is the only identified organism with third isoform LPORC (Oosawa et al. 2000, Su et al. 2001; Masuda et al., 2003; Gabruk and Mysliwa-Kurdziel 2015). The presence of LPOR enzyme together with DPOR was demonstrated within all photosynthetic organisms with the exception of anoxygenic

photosynthetic bacteria and angiosperms (Forreiter and Apel 1993, Armstrong 1998, Schoefs and Franck 2003, Reinbothe et al. 2010, Gabruk and Mysliwa-Kurdziel 2015).

Gymnosperms belong to the group of plants which have two Pchlide reductases. The ability to green in the dark is the highest in cotyledons and appears to decrease in more developed tissue (primary and secondary needles of conifers, Schoefs and Franck, 1998; Stolárik et al., 2017). But many authors have noticed that the efficiency of chlorophyll biosynthesis and formation of photosynthetic apparatus in the dark are highly variable among conifers and within the whole gymnosperm group (Armstrong 1998). For example, the conifers, *Thuja occidentalis* and *Larix decidua* seedlings show a low ability to synthesize Chl in the dark despite presence of the genes encoding subunits of DPOR, whilst spruce (*Picea abies*) accumulates the highest amount of Chl of all the Pinaceae when cultivated in darkness (Mariani et al. 1990, Fujita and Bauer 2003, Kusumi et al. 2006, Demko et al. 2009). Demko et al. (2009) and Kusumi et al. (2006) claimed that this phenomenon is caused by insufficient *chl*B mRNA editing in DPOR in the case of *Larix* or non-synonymous mutations of some of the DPOR genes in *Thuja*. RNA-editing of *chlB* transcripts is required for restoration of codons for conserved leucine and tryptophane in both: *P. abies* and *L. decidua*. Whereas the efficiency of mRNA editing in *P. abies* is high, in *L. decidua* is rather low (Demko et al., 2009). Recently, Yamamoto et al. (2017) showed causal relationship between RNA editing of *chlB* mRNA and DPOR activity. ChlB protein translated from unedited copy of *chlb* RNA is not able to form stable complex with ChlN subunit and thus formation of active DPOR complex. Among gymnosperms, also *Ginkgo biloba* showed a deficiency in Chl-formation in the dark, despite the presence of the corresponding genes, which are probably not expressed in significant amount (Chinn and Silverthorne 1993, Pavlovič et al. 2009).

Chlorophylls do not occur as free pigment molecules. Instead, they are bound in proteins of photosystem I (PSI) and photosystem II (PSII). Since the expression of the genes encoding thylakoid proteins is also light-independent in conifers, chlorophylls can be assembled into pigment-protein complexes also in the dark, forming both photosystem II (PSII) and photosystem I (PSI) but in lower abundance (Yamamoto et al. 1991, Shinohara et al., 1992a; Muramatsu et al. 2001, Pavlovič et al. 2016, Stolárik et al. 2017). While PSI developed in the dark is fully active, PSII remains in its latent form with poorly connected light harvesting antennae (LHCII) and with inactive oxygen evolving complex (OEC) until the illumination of chloroplasts. Photoactivation of PSII does not require synthesis of any new proteins and is connected with ligation of four Mn atoms in OEC and stable assembly of its

proteins (PsbO, PsbP and PsbQ) (Shinohara et al. 1992b, Kamachi et al. 1994, Pavlovič et al. 2016, Xue et al. 2017).

But what is an advantage of having two Pchlide reductases? As shown in cyanobacteria and green algae, mutants lacking DPOR are able to synthesize Chl and grow photoautotrophically in the light as well as wild type (Fujita et al. 1992, Suzuki and Bauer 1992). This fact strongly suggests that the enzymatic activity of LPOR is high enough to totally substitute that of DPOR in the light. However, there should be some uncharacterized conditions where DPOR activity is critical to survive, otherwise it is difficult to explain why both LPOR and DPOR are retained among such a broad range of oxygenic phototrophic organisms. Fujita and Bauer (2003) suggested that co-existence of two different Pchlide reduction systems contributes to balance in synthesis of appropriate amount of Chl responding to alternations in light conditions in cyanobacterial cells. A pair of *Plectonema boryanum* mutant in which either LPOR or DPOR was inactivated by gene-targeted mutagenesis has shown that the role of LPOR in Chl biosynthesis increases with increasing light intensity (> 170 μmol m^{-2} s^{-1} PAR) and both LPOR and DPOR are themselves sufficient for growth under low light conditions (10 – 25 μmol m^{-2} s^{-1} PAR) (Fujita et al. 1998). Recent studies confirmed that DPOR is particularly required for Chl synthesis under short-day conditions in liverwort *Marchantia polymorpha* (Ueda et al. 2014). They cultivated the *chl*B gene mutants of *M. polymorpha* under long (16-h L/8-h D) and short (8-h L/16-h D) day conditions and observed a different development of *M. polymorpha* according the length of light period. If cultivated under the short-day conditions, the mutant plants were delayed in their development and accumulated significantly lower amount of Chl in comparison to wild type.

During development of conifer seedlings in natural light conditions, both Pchlide reductases certainly contribute to Chl accumulation (Fujita and Bauer, 2003). However, because conifers are non-model plant systems and reliable genetic system has not been established, it is challenging to quantify the contribution of DPOR and LPOR to net Chl production in conifers. An *in-vitro* assay with crude cell extracts of *Pinus mugo* seedlings showed that LPOR is the major Pchlide reduction system and that DPOR only functions as an auxiliary system under the conditions, where the light is available (Forreiter and Apel, 1993). On the other hand, Fujita and Bauer (2003) suggested that under the light-limiting conditions, where the activity of LPOR is reduced, DPOR could become the dominant system in conifers however the evidence is lacking. To elucidate the co-existence of DPOR and LPOR to the Chl synthesis under different light conditions in conifers, we used two species (*L. decidua* and *P. abies*), which strongly differ in their ability to synthesize Chl in the dark. Because of

unavailability of gymnosperm mutant plants, the improper ChlB editing in *L. decidua* cotyledons regardless of light conditions (Demko et al. 2009), makes it a good experimental candidate as a species with disrupt DPOR function in contrast to *P. abies* seedlings which synthesize and accumulate the highest amount of chlorophyll in the dark among Pinaceae (Fujita and Bauer, 2003). Using this experimental approach, our study showed that co-existence of functional DPOR and LPOR enzyme may provide the molecular mechanism for sustaining high quantum yield and effective photochemistry of PSII under the light limiting conditions in *P. abies* seedlings and thus adaptations to shade conditions.

Materials and methods

Plant material and culture conditions

The seedlings of the European larch (*Larix decidua* Mill.) and Norway spruce (*Picea abies* Karst.) were cultivated from well-soaked (4 hours imbibed) seeds (Semenoles, Liptovský Hrádok, Slovakia) in well-moistened perlite at the temperature 23 ± 1 °C, under five different intensities of illumination: total darkness, 0.5, 20, 100 and 1400 μmol photons m^{-2} s^{-1} PAR) with periodic light/dark cycle (12/12-h), for 17 days. Then, the cotyledons were collected, frozen in liquid nitrogen and stored at – 80 °C or immediately used for analyses.

Pigment analysis

For Chl *a* + *b* determination, 100 mg of cotyledons was ground in a mortar with a pestle, using a small amount of sand and extracted with 80 % (v/v) chilled acetone with $MgCO_3$ to avoid acidification and pheophytinization of the pigments. The samples were centrifuged at 10.000g at 4 °C for 10 min. The concentration of Chl *a* and *b* was determined using a double beam spectrophotometer (Thermo Spectronic UV500, UV-Visible Spectro, MA, USA) at 663.2 nm and 646.8 nm and calculated according to Lichtenthaler (1987).

Characterization of Pchlide present in the conifers was not possible without phase separation of the pigments due to small amount of Pchlide and to the lower molar absorptivity of Pchlide in acetone compared to Chl. Therefore, we separated Pchlide from all esterified tetrapyrroles as recommended by Selstam et al. (1987). Pchlide was extracted from 100 mg of cotyledons (fixed for 2 min in hot steam) in 3 mL acetone: 0.1 M NH_4OH (9:1, v/v). To separate Pchlide from the esterified tetrapyrroles, the extract was washed three times with an equal volume of hexane. The amount of Pchlide was measured spectrofluorometrically (Hitachi, F-4500, Japan) at λ_{ex} 438 and λ_{em} 633 nm in the hexane-washed acetone phase and quantified using a Pchlide standard. The Pchlide standard was prepared from etiolated barley

plants according to Koski and Smith (1948) and spectrophotometrically quantified at 623 nm using the molar extinction coefficient in diethyl ether $\varepsilon = 3.56 \times 10^4\ M^{-1}\ cm^{-1}$ (Dawson et al. 1986). Using a dilution series of Pchlide standard in acetone:0.1 M NH_4OH (9:1, v/v), calibration curve was measured. All manipulations in the dark were performed under a dim green safelight.

Low temperature fluorescence emission spectra (77 K)

For organization of pigment-protein complexes, fluorescence emission spectra of the cotyledons were measured at low temperature using a fluorescence spectrophotometer Hitachi F-4500 (Tokyo, Japan) with the spectral band widths of 10 and 5 nm for excitation and emission monochromator, respectively. The plant material was immersed in liquid nitrogen (77 K) in an optical Dewar flask and measured. The excitation wavelength was set to 440 nm.

SDS-PAGE and Western blots

Total proteins from the spruce cotyledons were isolated using extraction buffer containing 28 mM DTT, 28 mM Na_2CO_3, 175 mM sucrose, 5 % SDS and 10 mM EDTA and protease inhibitors (Set VI, Calbiochem, Darmstadt, Germany). The samples were heated for 30 min at 70 °C. The concentration of total proteins in the samples was determined using the Bicinchoninic Acid Kit for Protein Determination (Sigma-Aldrich, St. Louis, MO, USA) and absorbance was measured at 562 nm (Thermo Spectronic UV500, UV-Visible Spectro, MA, USA). The same amount of proteins (25 μg) was separated in a 10 % (v/v) SDS-polyacrylamide gel (Schägger, 2006) followed by transfer to a nitrocellulose membrane (Bio-Rad, Germany) by Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). To check the correct protein transfer, the membranes were stained by Ponceau-S. After blocking in TBS-T containing 5 % BSA overnight at 4 °C, the membranes were incubated with the primary antibody at room temperature (or at 4 °C, if overnight in the case DPOR antibodies) with soft agitation. Antibodies against proteins PsbA (D1) (AS05 084), PsbB (CP47) (AS04 038), PsbC (CP43) (AS11 1787), PsbD (D2) (AS06 146), Lhcb 2, 4 and 5 (AS01 003, AS04 045, AS01 009), PsbS (AS09 533), PsbO (AS06 142-33), PsbP (AS06 167), PsbQ (AS06 142-16), PsaB (**AS10 695)**, Lhca 1-4 (AS01 005, AS01 006, AS01 007, AS01 008), Cyt*f* (AS08 306), RbcL (AS03 037), GluTR (AS10 689) and LPOR (AS05 067), were purchased from Agrisera (Vännäs, Sweden). Antibodies against DPOR subunits (ChlN and ChlB) were generously provided by Prof. Yuichi Fujita (Nagoya, Japan). After washing, the membrane was incubated 1 h in the secondary antibody (goat anti-rabbit

IgG (H+L)-horseradish peroxidase conjugate) with dilution 1:10000 (Bio-Rad, Hercules, CA, USA). Signals were developed using Immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) and visualized and quantified on an Amersham Imager 600 (GE HealthCare Life Sciences, Japan).

Chlorophyll a fluorescence measurements

We measured fast chlorophyll induction curves in the larch and spruce seedlings growing at the different light intensities after 30 minutes of dark adaptations using PEA-fluorometer (Hansatech, King's Lynn, Norfolk, UK). Dark-adapted cotyledons were gently fixed in the clip and suddenly illuminated by a flash of intensity 7000 μmol photons m^{-2} s^{-1} PAR (λ = 650 nm) over a time span of 20 μs to 20 s with a data acquisition rate of 10 μs for the first 2 ms, 1 ms between 2 ms and 1 s, and 100 ms thereafter. The F_0 level was measured as the fluorescence at 40 μs. The F_m is maximum recorded fluorescence reached either at K (dark-grown and samples illuminated with low light intensity) or P-steps. Maximum quantum yield of PSII (F_v/F_m) was calculated as: $(F_m - F_0)/F_m$ (Maxwell and Johnson 2000).

For photoactivation experiments, the seedlings of the larch and spruce were cultivated in the dark and illuminated for 1, 5 min and 1 hour with light intensities used for cultivation (i.e. 0.5, 20, 100 and 1400 μmol photons m^{-2} s^{-1} PAR). Because of slow photoactivation at 0.5 μmol photons m^{-2} s^{-1} PAR, the illumination in this variant of experiment continued for the next 2, 3 and 6 hours. The fast chlorophyll *a* fluorescence induction was measured in dark-grown and illuminated cotyledons (but 30 min dark-adapted before measurements) at room temperature using a PEA-fluorometer (Hansatech, King's Lynn, Norfolk, UK) as described above.

Gas exchange

Measurement of photosynthetic parameters was performed in a laboratory at ambient temperature 25 °C (t) and 35% relative air humidity (RH). Light response curves were measured with a LI-6400 portable photosynthesis system (LI-COR Biosciences, Inc., Lincoln, NE, USA) using a LED-based light source accessory (6400-40 LCF) providing red (635 nm) and blue (470 nm) light. Cotyledons of the light-adapted conifer seedlings were non-invasively inserted into leaf chamber with stable experimental conditions (t_{leaf} = 23 °C, RH ~ 75 %, CO_2 concentration = 380 ppm, PAR = 2000 μmol photons m^{-2} s^{-1}). After equilibration of adjusted chamber conditions (about 15 minutes) the light response curves were logged during following 50 minutes. Desired lamp setting were 2000, 1500, 1000, 750, 500, 300,

100, 50, 0 μmol photons m^{-2} s^{-1} PAR with 20 % of blue light. Biomass enclosed in the leaf chamber was dried at 70 °C for 3 days and used for computation of the rate of CO_2 assimilation (nmol g^{-1} DW s^{-1}) for each sample.

Electron microscopy

Cotyledons were cut into 1.5 mm long sections by a razor and immediately fixed in 5 % (v/v) glutaraldehyde and postfixed in 1 % osmium tetroxide at room temperature. Fixed samples were gradually dehydrated in ethanol series and the dehydratation was finished by pure propylene oxide. The samples were embedded in Spurr Low-Viscosity Embedding Kit epoxide resin (Sigma-Aldrich, St. Louis, MO, USA). Semithin sections were stained by 0.5 % (w/v) toluidine blue and examined using light-microscope. Ultrathin sections were contrasted by 2 % (w/v) uranyl acetate and 2 % (w/v) lead (II) citrate and observed by electron microscope (Jeol JEM 2010, Japan).

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's test (Origin 8.5.1., Northampton, USA) or *t*-test (Excel, Microsoft Office) were used for evaluation of the significance of the data.

Results

Pigment accumulation

Larch seedlings cultivated in complete darkness and under the weak light conditions (0.5 μmol photons m^{-2} s^{-1} PAR) are characterized by etiolated or very pale green phenotype (Fig. 1A). This is in contrast to Norway spruce seedlings which are significantly greener, if cultivated under the same conditions (Fig. 1B). The macroscopic observations were confirmed also by the spectrophotometric measurements of chl *a*+*b* content (Fig. 2A). In general, higher chlorophyll content was found in the cotyledons of spruce seedlings in comparison to larch, especially in the dark and under the weak light cultivation conditions (0.5 μmol photons m^{-2} s^{-1} PAR). The chlorophyll content increased with increasing light intensity in both species, but at the highest irradiations (1400 μmol photons m^{-2} s^{-1} PAR) decreased. The chlorophyll *a*/*b* ratio (Chl *a*/*b*) slightly increased with increasing light intensities in the range between 20 – 1400 μmol photons m^{-2} s^{-1} PAR in both species, indicating the reduction of light-harvesting antenna system relative to PSII core with increasing light intensities (Lichtenthaler and Babani, 2004). The higher Chl *a*/*b* ratio was found in dark-grown spruce seedlings indicating

reduced amount of light-harvesting antennae relative to PSII core in spruce under such conditions (Fig. 2B). The Chl fluorescence emissions at 685 and 695 nm at temperature 77K document the presence of the core antennae of PSII, CP43, and CP47, respectively, in both species even in the dark (Fig. 3A,B; Nakatani et al. 1984). Emission at ~ 733 nm corresponds to PSI, which was very flat in the case of dark-grown larch (Fig. 3A). The peaks became dominant in seedlings cultivated in the light (Fig. 3C-J).

Dark-grown cotyledons accumulated the highest level of Pchlide (Fig. 2C). The fluorescence maxima at 77K observed in the spectra of dark-grown cotyledons indicate the presence of free Pchlide (F_{633nm}) as well as Pchlide bound to LPOR and NADPH in PLBs (F_{655nm}, Fig. 3A,B). The elevated level of Pchlide was also found under the deep shade conditions (0.5 μmol photons m^{-2} s^{-1} PAR) in both species (Fig. 2C) which was predominantly bound in PLBs (F_{655nm}, Fig. 3C,D).

Immunoblot analysis

The whole tissue protein extracts from cotyledons were subjected to SDS-PAGE followed by immunoblotting to semi-quantify protein levels (Fig. 4). First, we analysed Chl-binding proteins of PSII (D1, D2, CP43, CP47, Lhcb2, 4 and 5), protein responsible for energy-dependent non-photochemical quenching (PsbS), as well as the proteins of oxygen-evolving complex of PSII (PsbO, PsbP, PsbQ), followed by chlorophyll-binding proteins of PSI (PsaB, Lhca1-4) in both conifer species. Because the chlorophyll-binding proteins are unstable in the absence of chlorophylls, the levels of Chl-binding proteins correlate very well with the chlorophyll accumulation in both studied species (Fig. 2A). The level of chlorophyll-binding proteins increased with increasing light intensities, it culminated at 20 – 100 μmol photons m^{-2} s^{-1} PAR and usually decreased at the highest light intensity (1400 μmol photons m^{-2} s^{-1} PAR). Interspecific differences are also conspicuous, especially in the plant cultivated in total darkness and weak light conditions (0.5 μmol photons m^{-2} s^{-1} PAR). Under such conditions, larch cotyledons accumulated very low amount of chlorophyll-binding proteins if at all, while all these proteins were easily immunodetected in spruce cotyledons. The proteins of OEC (PsbO, PsbP, PsbQ) and Cyt *f* from Cyt b_6f complex usually followed the same pattern. The level of PsbS protein gradually increased with increasing irradiance in both species of conifers confirming its photoprotective role in thermal dissipation of excess absorbed light energy in plants, measured as non-photochemical quenching of chlorophyll fluorescence (Niyogi et al. 2004). The level of large subunit of Rubisco (RbcL) also increased with increasing light

intensities in larch, but culminated at moderate light intensities in spruce (20 – 100 μmol photons m^{-2} s^{-1} PAR).

Glutamyl-tRNA reductase (GluTR) is the target enzyme for regulating aminolevulinic acid and tetrapyrrole biosynthesis in response to environmental and endogenous signals (Fang et al., 2016). Its level correlates well with Chl accumulation (Fig. 2A), with the lowest levels in D and 0.5 variants of cultivation, increasing with light intensity and then decreasing at the highest light intensity (Fig. 3). On the other hand, LPORA was negatively light-regulated. with the highest protein amounts detected in the spruce and larch seedlings cultivated in darkness and deep shade conditions (0.5 μmol photons m^{-2} s^{-1} PAR). The similar pattern as in the case of LPOR was followed also by the subunits of DPOR (ChlB and ChlN). The signal from ChlN in the larch was not specific.

Quantum yield of photosystem II photochemistry

One of the most intriguing results offered the measurement of maximum quantum yield of PSII (F_V/F_M), especially in the seedlings cultivated under deep shade conditions (0.5 μmol photons m^{-2} s^{-1} PAR), where the larch seedlings showed only subliminal values of this parameter, in contrast to the spruce seedlings reflecting standard F_V/F_M values (Fig. 5). The values of F_V/F_M reflect the potential quantum efficiency of PSII and are used as a sensitive indicator of plant photosynthetic performance, with optimal values of around 0.83 measured for most plant species (Maxwell and Johnson 2000). This indicates that LPOR in the larch with almost inactive DPOR, cannot provide sufficient amounts of chlorophylls to PSII under the deep shade conditions, however low light intensity is sufficient for photoactivation of PSII in spruce which is built up by chlorophylls synthesized almost exclusively by DPOR.

To confirm this hypothesis, we performed illumination experiments of dark-grown spruce and larch seedlings using measurements of fast chlorophyll fluorescence induction (FR). In the dark, both species showed a prominent K-step, indicating donor side inhibition of PSII (Strasser 1997). After illumination with 0.5 μmol photons m^{-2} s^{-1} PAR the K-step gradually disappeared and new steps (J, I, P) appeared indicating successful photoactivation. This process was speeded up after illumination of dark-grown seedlings with higher light intensities, especially in Norway spruce (20, 100 μmol photons m^{-2} s^{-1} PAR, Fig. S1, S2), but was photoinhibitory at the highest light intensity in both species (1400 μmol photons m^{-2} s^{-1} PAR, Fig. S3). The K-step persisted much longer in larch seedlings (Fig. 6, S1, S2) supported by low amount of OEC and chlorophyll-bindings proteins in dark-grown seedlings and necessity to synthesize them *de novo* after illumination (Fig. 4).

Rate of photosynthesis

In general, rate of net photosynthesis (A_N) was higher in the larch in comparison to the spruce seedlings. Only the spruce seedlings growing at 0.5 μmol photons m^{-2} s^{-1} PAR overcome A_N in the larch at low light intensities; the light compensation point was significantly lower in spruce than in the larch cotyledons (41 ± 30 vs. 143 ± 31 μmol photons m^{-2} s^{-1} PAR), indicating an effective utilization of limited number of photons for CO_2 assimilation in accordance to high F_V/F_M values at 0.5 μmol photons m^{-2} s^{-1} PAR in spruce (Fig. 5). However, even the low chlorophyll content in the larch growing at 0.5 μmol photons m^{-2} s^{-1} PAR (Fig. 2A) was sufficient for net CO_2 uptake at higher illumination which exceeds that in the spruce (Fig. 7) – a typical difference between sun and shade adapted plants. Increasing cultivation irradiance had no effect on A_N in spruce, in the larch the maximum A_N increased up to 100 μmol photons m^{-2} s^{-1} and then declined in plant growing at 1400 μmol photons m^{-2} s^{-1} PAR (Fig. 7). Also the light compensation points were comparable between species growing in the range 20 – 1400 μmol photons m^{-2} s^{-1} PAR (data not shown).

Chloroplasts ultrastructure

The chloroplasts (or rather etiochloroplasts) from complete darkness (Fig.8A,F) are characterized by the presence of the prolamellar bodies (PLB), consistent with accumulation of LPOR protein (Fig. 4) and Pchlide which are bound in them (Fig. 2C,3). The thylakoid system is poorly developed in the larch. The grana are minute and consist of only one or two discs (thylakoids, Fig. 8A inset). Larch etiochloroplasts contain prominent plastoglobuli assembled into groups. The better developed thylakoid system appears in the spruce containing up to five aggregated thylakoids of large diameter (Fig. 8F inset). The presence of the large starch grains is also typical for both species (Fig. 8A, F). Chloroplasts from plants cultivated under the deep shade conditions (0.5 μmol photons m^{-2} s^{-1} PAR) still contain PLB which are smaller than those from complete darkness. The thylakoid system is better developed in both species (Fig 8B, G insets) and aggregated plastoglobuli observed in the dark-grown larch seedlings are more dispersed (Fig. 8B). Massive granal formation (up to 20 thylakoids) is typical for the chloroplasts in seedlings of both species cultivated under 20 μmol photons m^{-2} s^{-1} PAR, as well as a complete disappearance of the starch grains (Fig. 8C, H and insets). Very similar situation is observable in the cotyledons cultivated at 100 μmol photons m^{-2} s^{-1} PAR, however some smaller starch grains are present (Fig. 8D, I and insets). In general, it seems that the grana are more stacked in spruce chloroplasts than in the larch at the given light intensity. In the plants cultivated under the highest light intensity (1400 μmol

photons m^{-2} s^{-1} PAR), the chloroplasts with more prominent starch grains are typical (Fig. 8E, J).

Discussion

Our study confirmed reduced ability to synthesize chlorophyll in the dark in *L. decidua* (Fig. 1, 2A), as was found previously, caused by ineffective *chlB* (DPOR subunit) mRNA editing (Karpinska et al. 1997, Demko et al. 2009). Recently, Yamamoto et al. (2017) showed causal relationship between RNA editing of *chlB* mRNA and DPOR activity. Since *P. abies* has the highest ability to synthesize chlorophyll in the dark among conifers (Fujita and Bauer 2003), these two species are suitable models in non-model group of plants for investigation of contribution both Pchlide reductases to chlorophyll accumulation. Although, chlorophyll accumulation depends not only on synthesis but also on its stabilization in pigment-protein complexes embedded in thylakoid membranes, it has been postulated that total translatable mRNA for pigment-protein complexes is sufficient and not limiting (they are expressed light-independently) but the assembly of PSII is limited by the supply of Chl by DPOR in the dark (Yamamoto et al. 1991, Mukai et al. 1992, Shinohara et al. 1992, Muramatsu et al. 2001).

Our study clearly showed that under the shade conditions (0.5 - 20 μmol photons m^{-2} s^{-1} PAR) larch cotyledons had lower maximum quantum yield of PSII photochemistry (F_V/F_M) in comparison to spruce (Fig. 5). Thus, it is evident that mainly under 0.5 μmol photons m^{-2} s^{-1} PAR, LPOR enzyme cannot provide sufficient amount of chlorophylls for effective photochemistry in the larch, where DPOR enzyme is almost inactive. On the other hand, spruce cotyledons had the highest F_V/F_M values under these conditions due to the presence of active DPOR which together with LPOR (activity of which is probably minimal at such low photon flux rate) provide sufficient amount of chlorophylls as building blocks for PSII assembly. The inefficiency of LPOR enzyme at such low light intensity is also documented by the presence of Pchlide (Fig. 2C) bound in PLB (Fig. 3C,D, 8B,G) in both investigated species. The lower content of Pchlide in the larch seedlings despite non-functional DPOR is consistent with our previous results and can be account for down-regulation of whole biosynthetic pathway (Demko et al., 2009). Indeed, the presence of active DPOR has not significant impact on Pchlide levels, as both dark-grown angiosperms and gymnosperms contain comparable content of Pchlide (Selstam et al. 1987).

Although DPOR enzyme can synthesize significant amount of chlorophylls which are bound to pigment-protein complexes in the dark and low irradiance (Fig. 2A, 3, 4), the PSII is inactive as F_V/F_M values and presence of K-step in chlorophyll *a* fluorescence induction in

dark-grown seedlings indicate (Fig. 5,6). Thus PSII needs short period of illumination for photoactivation. This phenomenon is well known in gymnosperm seedlings (Jansson et al. 1992, Shinohara et al. 1992b, Kamachi et al. 1994, Pavlovič et al. 2016). Photosystem II membranes isolated from dark-grown conifer seedlings had negligible Mn content and O_2 evolution from OEC. All intrinsic proteins are present in PSII membranes but with reduced amount of PsbO and PsbP. PsbQ protein is completely missing from OEC. Also the amount of LHCII is substantially less in dark-grown conifers with high portion of free monomeric form of LHCII caused by limited supply of Chl *a* and Chl *b* (Jannson et al. 1992, Pavlovič et al. 2016, Xue et al. 2017). The process of photoactivation involves integration of Mn ions, PsbP and PsbQ proteins from thylakoid lumen into functional OEC (Shinohara et al. 1992b, Kamachi et al. 1994, Pavlovič et al. 2016) and increased the overall amount and proportion of LHCII within the assembled LHCII-PSII supercomplexes (Pavlovič et al. 2016, Xue et al. 2017) resulting in more stacked grana in light-grown plants (Fig. 8). In our study, the inhibition of OEC is clearly seen as K-step in measurements of FR (Fig. 6). This step appears in FR whenever the rate of electron transport from P680 to the acceptor side of PSII exceeds the rate of electron transport from the donor side of PSII to P680 (Strasser 1997). While the fluorescence rise to the K-peak reflects a transient reduction of plastoquinone A (Q_A) after the first charge separation in PSII (i.e. a formation of the $Tyr_Z^+Q_A^-$ form, Tyr_Z - secondary electron donor of PSII), the fluorescence decrease after the K-peak is explained by the forward electron transport to plastoquinone B (Q_B, i.e. formation of the $Tyr_Z^+Q_AQ_B^-$ form (Srivastava et al. 1997, Tóth et al. 2007). The appearance of this step in FR is well documented in leaves exposed to heat, which results in the damage of OEC (Strasser 1997, Lazár and Ilík 1997, Lazár 1999, Brestič et al., 2012). On the other hand, the steps J, I and P in FR represent temporary accumulation of reduced electron carriers, mainly Q_A and Q_B involved in linear electron transport in thylakoid membranes, i.e. functionality of PSII (for review see Lazár 2006). Thus, using this non-destructive method, disappearance of K-step and appearance of J, I and P steps inform us about the photoactivation of PSII.

In our experiments, it is clearly seen that the photoactivation is dependent on light intensity, it is much faster at higher light intensities in the spruce. However, the highest light intensity (1400 μmol photons m^{-2} s^{-1} PAR) had negative effect on photoactivation in both species (Fig. S3). It is well known that PSII, with an incomplete donor side, is very sensitive to photoinhibition (Jansson et al. 1992). On the other hand, even such low light intensity as 0.5 μmol photons m^{-2} s^{-1} PAR is sufficient for photoactivation, what explain the high values of F_V/F_M in spruce cultivated 17 days under this light intensity (Fig. 5). The photoactivation

of larch cotyledons is delayed (Fig. 6, S1 S2) most probably caused by necessity to synthesize sufficient amount of PSII proteins *de novo* as is known in angiosperms which do not possess DPOR enzyme (Fig. 4; Kanervo et al. 2008). This delayed photoactivation in larch in comparison to spruce seedlings has been well described previously (Mariani et al. 1990, Demko et al. 2009). From these experiments it is clearly seen that despite the high abundance of LPOR under the deep shade conditions (Fig. 4), its catalytic activity is insufficient for Pchlide reduction under deep shade conditions due to the limited supply of photons (Fig. 2A). However the same conditions provide sufficient amount of light for photoactivation of PSII built up from chlorophylls synthesized mainly by DPOR (Fig. 5). Because spruce is known as a shade plants while larch belong to the group of sun trees, the ability to green in the dark is probably a part of molecular basis for shade tolerance of some gymnosperms (Walles and Hudák 1975, Fujita and Bauer 2003). The shade adaptations of spruce is obvious also from light response curve of photosynthesis with lower compensation point, early light saturation and overall lower A_N (Fig. 7) and from pigment analyses (higher Chl content and lower Chl*a*/*b* ratio in the spruce, Fig. 2A, B) – a typical adaptations or acclimation response of shade plants (Lichtenthaler and Babani 2004).

Our study is in agreement with the study of Ueda et al. (2014) and Fujita et al. (1998), who showed that DPOR is required under conditions of light limitation (short day conditions) in liverwort (*M. polymorpha*) and LPOR becomes essential for growth under high light conditions in cyanobacteria (*P. boryanum*). This is further supported by the fact that the protein level of two subunits of DPOR (ChlB and ChlN) is decreasing with increasing light intensity (Fig. 4). In the spruce, the level of DPOR subunits is regulated on transcription level and the expression of *ChlB*, *ChlN* and *ChlL* genes is downregulated by light (Stolárik et al. 2017). Negative photoregulation of DPOR subunits was also described by Breznenová et al. (2010) and Yamamoto et al. (2017) in three species of pines and also in algae *Chlamydomonas reinhardtii* by Cahoon and Timko (2000), but it is not general in other organisms having both Pchlide reductases (Fujita and Bauer, 2003). Besides transcriptional regulation, decreased activity of DPOR in the light may be caused by increased oxygen evolution from OEC and DPOR sensitivity to oxygen (Nomata et al. 2006; Stolárik et al., 2017). On the other hand, the decreased level of LPOR protein with increasing irradiance (Fig. 4) is inconsistent with chlorophyll accumulation in the light (Fig.1, 2A). This apparent paradox is well known in angiosperms (Armstrong et al. 1995, Holtorf et al. 1995) and gymnosperms (Stabel et al. 1991, Forreiter and Apel 1993, Skinner and Timko 1999). For a long time, rapid disappearance of LPOR protein in the light was enigmatic, since it was

inconsistent with the observed continued formation of chlorophyll after extended periods of illumination. Although it has been suggested that even minute amounts of LPOR, still detectable in green plants, may be sufficient to support continuous synthesis and accumulation of Chl in the light (Griffiths 1985; Holtorf et al., 1995), this apparent contradiction was resolved by the demonstration that two forms of the enzyme are present in most angiosperms and gymnosperms. Whereas LPORA accumulated in dark-grown angiosperms and gymnosperms and it rapidly disappeared after illumination, LPORB remained at an approximately constant level continuing in chlorophyll formation (Armstrong et al. 1995; Holtorf et al. 1995, Skinner and Timko, 1999). Our antibody bound probably only to LPOR A, because double band has never been observed in our Western blots.

In conclusion, our study showed that co-existence of DPOR and LPOR enzymes provide advantage for plants growing under the deep shade conditions, where limited number of photons are insufficient for LPOR enzyme, but are sufficient for photoactivation of the latent PSII built up almost exclusively by DPOR. Under the high light conditions, the level of DPOR enzyme decreased and it is tempting to assume that its contribution to chlorophyll formation is minor and LPOR takes over its functions. This is not surprising, because chlorophyll synthesis by DPOR is very costly (4 ATP per one formed molecule of Chlide, Nomata et al. 2016), while LPOR enzyme uses “cheap” two available photons of light for its enzymatic catalysis (Sytina et al., 2008). We have not found any advantage of having DPOR under the high light conditions, confirming the distribution of DPOR enzyme mainly in the shade adapted plants (mosses, ferns, liverworts etc.). Thus, the ability to green in the dark is probably a part of molecular basis for shade tolerance in some non-flowering plants

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Figure legends:

Fig. 1 Seedlings of *L. decidua* (A) and *P. abies* (B) growing under different light intensities.

Fig. 2 Chlorophyll *a* + *b* content (A), chlorophyll *a*/*b* ratio (B) and protochlorophyllide content (C) in *L. decidua* (white bars) and *P. abies* (black bars) cotyledons. Different letters (lower case for *L. decidua* and upper case for *P. abies*) denote significant differences among different light intensities. One way analysis of variance (ANOVA) followed by Tukey's test, $P < 0.05$, Asterisks denote significant difference between species at given light intensity (**, $P < 0.01$, *, $P < 0.05$, Student's *t*-test), means ± s.d., n = 4-5.

Fig. 3 Low-temperature fluorescence emission measured at 77-K from cotyledons. Dark-grown larch (A), dark-grown spruce (B), larch growing at 0.5 μmol m^{-2} s^{-1} PAR (C), spruce growing at 0.5 μmol m^{-2} s^{-1} PAR (D), larch growing at 20 μmol m^{-2} s^{-1} PAR (E), spruce growing at 20 μmol m^{-2} s^{-1} PAR (F), larch growing at 100 μmol m^{-2} s^{-1} PAR (G), spruce growing at 100 μmol m-2 s-1 PAR (H), larch growing at 1400 μmol m^{-2} s^{-1} PAR (I), spruce growing at 1400 μmol m^{-2} s^{-1} PAR. The results shown are representative of four measurements.

Fig. 4 Western blot analysis of photosynthesis- and chlorophyll biosynthesis-related proteins in *L. decidua* (A) and *P. abies* (B) seedlings. The same amount of total proteins (25 μg) was electrophoresed in 10% (v/v) SDS–polyacrylamide gel and subjected to Western blot analysis. Protein content in the bands was quantified by chemiluminescence.

Fig. 5 Maximum quantum yield of photosystem II (F_v/F_m) in *L. decidua* (white bars) and *P. abies* (black bars) cotyledons after 17 days of growing at different light intensities. Different letters (lower case for *L. decidua* and upper case for *P. abies*) denote significant differences among different light intensities at $P < 0.05$. One way analysis of variance (ANOVA) followed by Tukey's test. Asterisks denote significant difference between species at the given light intensity at $P < 0.01$ (Student's t-test). Means ± s.d., n = 4-5.

Fig. 6 Photoactivation experiments at the light intensity 0.5 μmol m^{-2} s^{-1} PAR in *L. decidua* (A) and *P. abies* (B). Chlorophyll *a* fluorescence rise measured at excitation light intensity of 7000 μmol photons m^{-2} s^{-1} PAR (650 nm) in dark-grown spruce cotyledons that were illuminated (0.5 μmol photons m^{-2} s^{-1}, PAR) for different time periods (0, 1, 5 min, 1, 2, 3, 6 h as indicated). Seedlings were dark-adapted for 30 min before the fluorescence measurement. Curves are normalized to F_0 level, vertically shifted and plotted on a logarithmic time scale. The vertical lines indicate the position of K-step. Presented data are representatives of a total of 4–5 measurements. The values below each curve indicate average F_v/F_m, means ± s.d., n = 4-5.

Fig. 7 Rate of net photosynthesis (A_N) in *L. decidua* (A) and *P. abies* (B) cotyledons. Plants growing at 0.5 μmol m^{-2} s^{-1} PAR (closed circles), 20 μmol m^{-2} s^{-1} PAR (open circles), 100 μmol m^{-2} s^{-1} PAR (closed squares) and 1400 μmol m^{-2} s^{-1} PAR (open squares). Means ± s.d., n = 3-4.

Fig. 8 Plastid ultrastructure of *L. decidua* (A-E) and *P. abies* (F-J) cotyledons. Dark-grown plants (A,F), growing at 0.5 μmol m^{-2} s^{-1} PAR (B,G), 20 μmol m^{-2} s^{-1} PAR (C,H), 100 μmol m^{-2} s^{-1} PAR (D,I), 1400 μmol m^{-2} s^{-1} PAR (E,J). * - prolamellar bodies, S - starch grains, arrows denote aggregated plastoglobuli, Bars = 600 nm. The inserted panels show thylakoid membranes of corresponding plastids in detail. The length of inset long side corresponds to 1μm.

Supplemental figures

Fig. S1 Photoactivation experiments at the light intensity 20 μmol m^{-2} s^{-1} PAR in *L. decidua* (A) and *P. abies* (B). Chlorophyll *a* fluorescence rise measured at excitation light intensity of 7000 μmol photons m^{-2} s^{-1} PAR (650 nm) in dark-grown spruce cotyledons that were illuminated (20 μmol photons m^{-2} s^{-1}, PAR) for different time periods (0, 1, 5 min and 1 h as indicated). Seedlings were dark-adapted for 30 min before the fluorescence measurement. Curves are normalized to F_0 level, vertically shifted and plotted on a logarithmic time scale. The vertical lines indicate the position of K-step. Presented data are representatives of a total of 4–5 measurements. The values below each curve indicate average F_V/F_M, means ± s.d., n = 4-5.

Fig. S2 Photoactivation experiments at the light intensity 100 μmol m^{-2} s^{-1} PAR in *L. decidua* (A) and *P. abies* (B). Chlorophyll a fluorescence rise measured at excitation light intensity of 7000 μmol photons m^{-2} s^{-1} PAR (650 nm) in dark-grown spruce cotyledons that were illuminated (100 μmol photons m^{-2} s^{-1}, PAR) for different time periods (0, 1, 5 min and 1 h as indicated). Seedlings were dark-adapted for 30 min before the fluorescence measurement. Curves are normalized to F_0 level, vertically shifted and plotted on a logarithmic time scale. The vertical lines indicate the position of K-step. Presented data are representatives of a total of 4–5 measurements. The values below each curve indicate average F_V/F_M, means ± s.d., n = 4-5.

Fig. S3 Photoactivation experiments at the light intensity 1400 μmol m^{-2} s^{-1} PAR in *L. decidua* (A) and *P. abies* (B). Chlorophyll a fluorescence rise measured at excitation light intensity of 7000 μmol photons m^{-2} s^{-1} PAR (650 nm) in dark-grown spruce cotyledons that were illuminated (1400 μmol photons m^{-2} s^{-1}, PAR) for different time periods (0, 1, 5 min and 1 h as indicated). Seedlings were dark-adapted for 30 min before the fluorescence measurement. Curves are normalized to F_0 level, vertically shifted and plotted on a logarithmic time scale. The vertical lines indicate the position of K-step. Presented data are representatives of a total of 4–5 measurements. The values below each curve indicate average F_V/F_M, means ± s.d., n = 4-5.

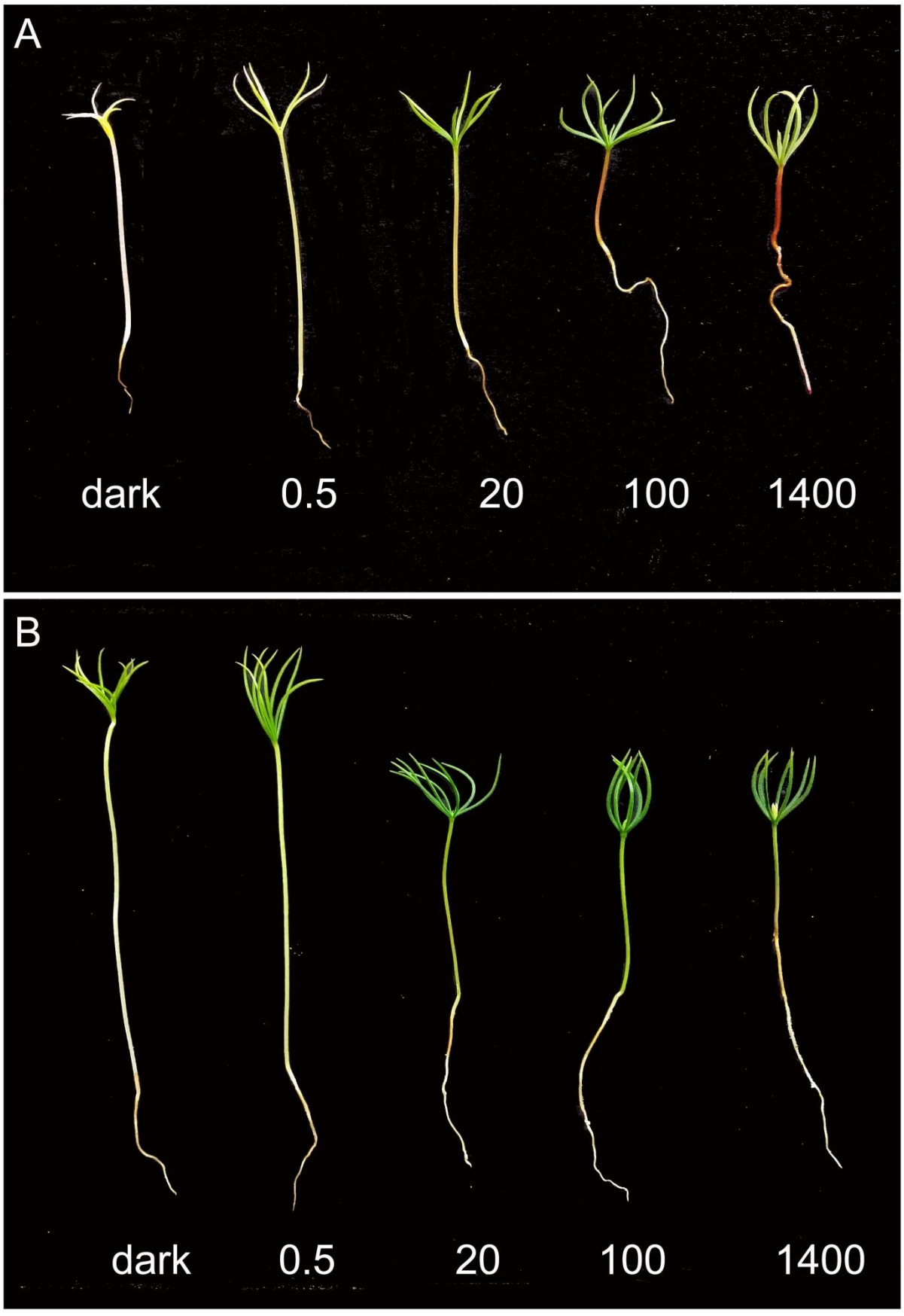

Fig. 1

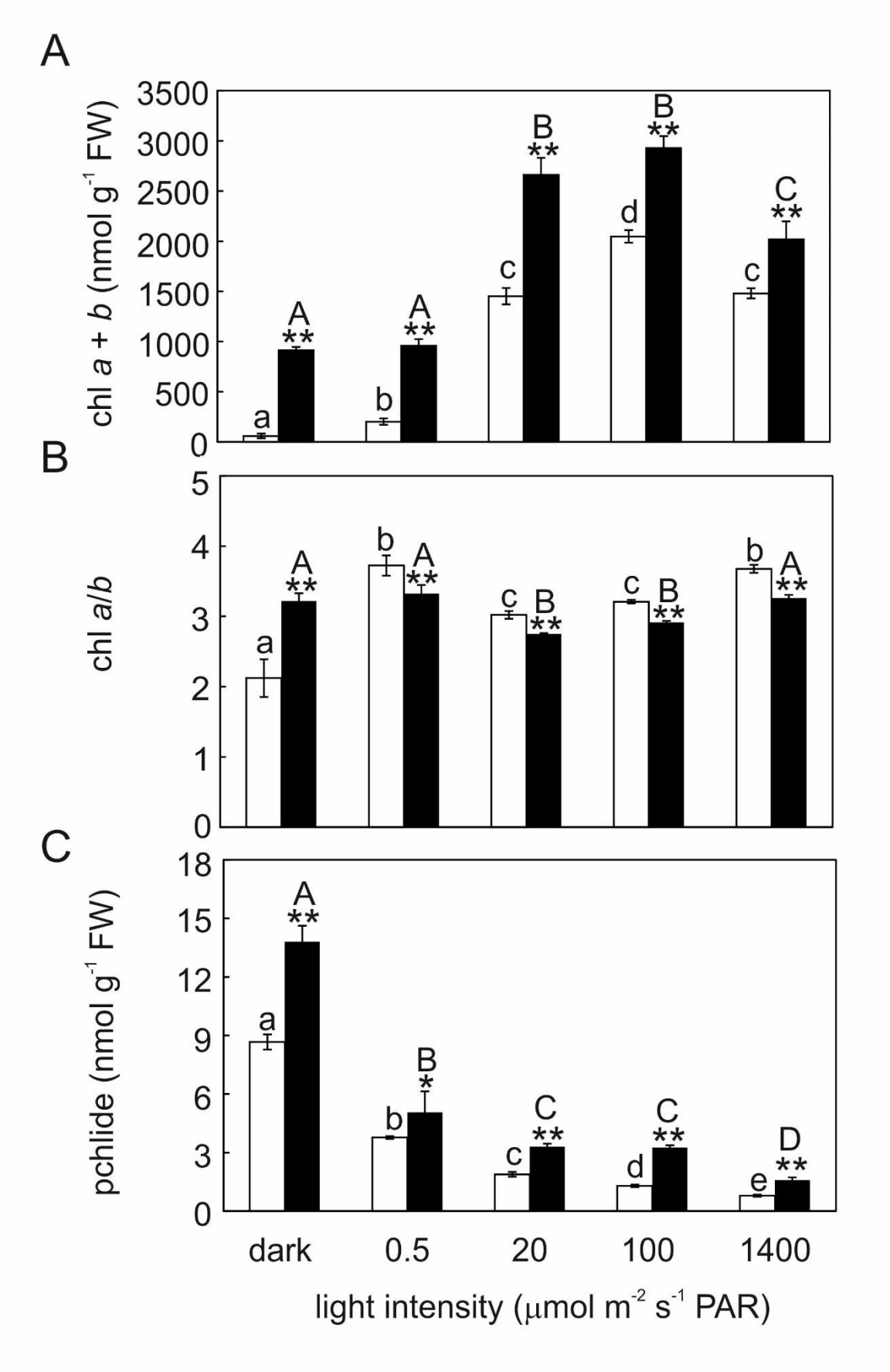
A
chl a + b (nmol g-1 FW)
3500
3000
2500
2000
1500
1000
500
0
B
chl a/b
5
4
3
2
1
0
C
pchlide (nmol g-1 FW)
18
15
12
9
6
3
0
dark
0.5
20
100
1400
light intensity (μmol m-2 s-1 PAR)

Fig. 2

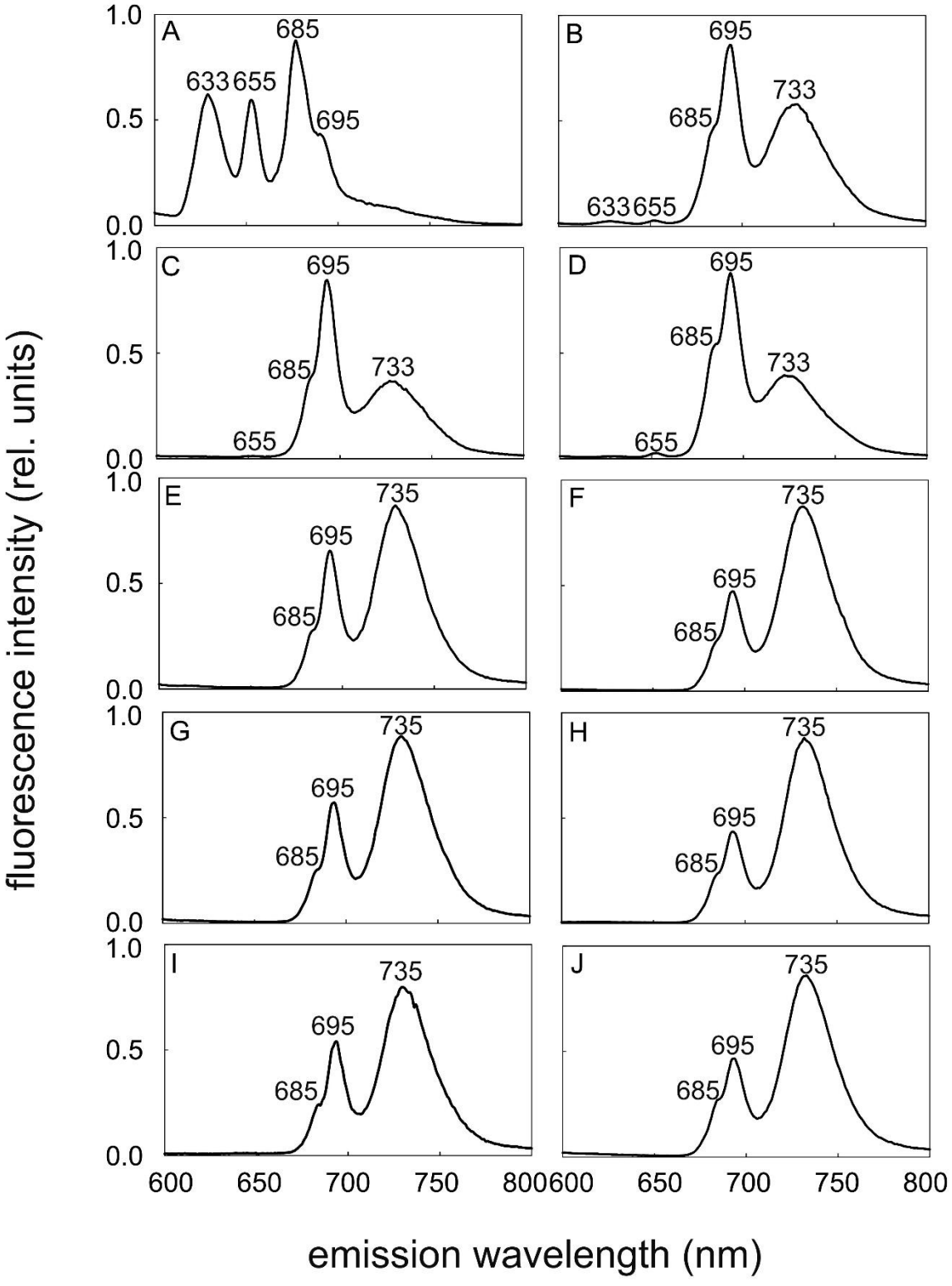
A
685
633
655
695
B
695
733
685
633
655
C
695
685
733
655
D
695
685
733
655
E
735
695
685
F
735
695
685
G
735
695
685
H
735
695
685
I
735
695
685
J
735
695
685
1.0
0.5
0.0
600
650
700
750
800
fluorescence intensity (rel. units)
emission wavelength (nm)

Fig. 3

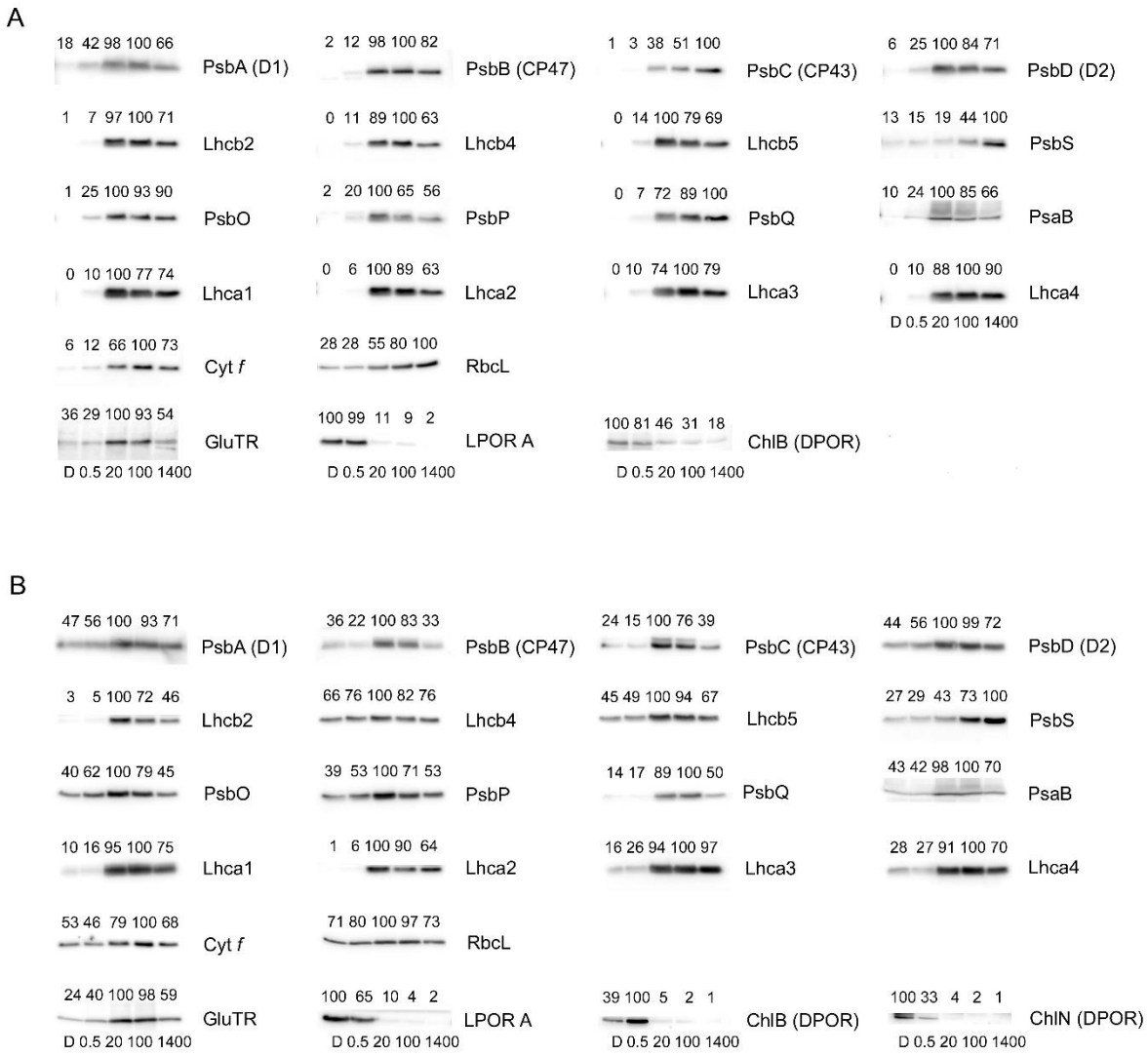
A
18 42 98 100 66
PsbA (D1)
2 12 98 100 82
PsbB (CP47)
1 3 38 51 100
PsbC (CP43)
6 25 100 84 71
PsbD (D2)
1 7 97 100 71
Lhcb2
0 11 89 100 63
Lhcb4
0 14 100 79 69
Lhcb5
13 15 19 44 100
PsbS
1 25 100 93 90
PsbO
2 20 100 65 56
PsbP
0 7 72 89 100
PsbQ
10 24 100 85 66
PsaB
0 10 100 77 74
Lhca1
0 6 100 89 63
Lhca2
0 10 74 100 79
Lhca3
0 10 88 100 90
Lhca4
D 0.5 20 100 1400
6 12 66 100 73
Cyt f
28 28 55 80 100
RbcL
36 29 100 93 54
GluTR
100 99 11 9 2
LPOR A
100 81 46 31 18
ChlB (DPOR)
D 0.5 20 100 1400
D 0.5 20 100 1400
D 0.5 20 100 1400
B
47 56 100 93 71
PsbA (D1)
36 22 100 83 33
PsbB (CP47)
24 15 100 76 39
PsbC (CP43)
44 56 100 99 72
PsbD (D2)
3 5 100 72 46
Lhcb2
66 76 100 82 76
Lhcb4
45 49 100 94 67
Lhcb5
27 29 43 73 100
PsbS
40 62 100 79 45
PsbO
39 53 100 71 53
PsbP
14 17 89 100 50
PsbQ
43 42 98 100 70
PsaB
10 16 95 100 75
Lhca1
1 6 100 90 64
Lhca2
16 26 94 100 97
Lhca3
28 27 91 100 70
Lhca4
53 46 79 100 68
Cyt f
71 80 100 97 73
RbcL
24 40 100 98 59
GluTR
100 65 10 4 2
LPOR A
39 100 5 2 1
ChlB (DPOR)
100 33 4 2 1
ChlN (DPOR)
D 0.5 20 100 1400
D 0.5 20 100 1400
D 0.5 20 100 1400
D 0.5 20 100 1400

Fig. 4

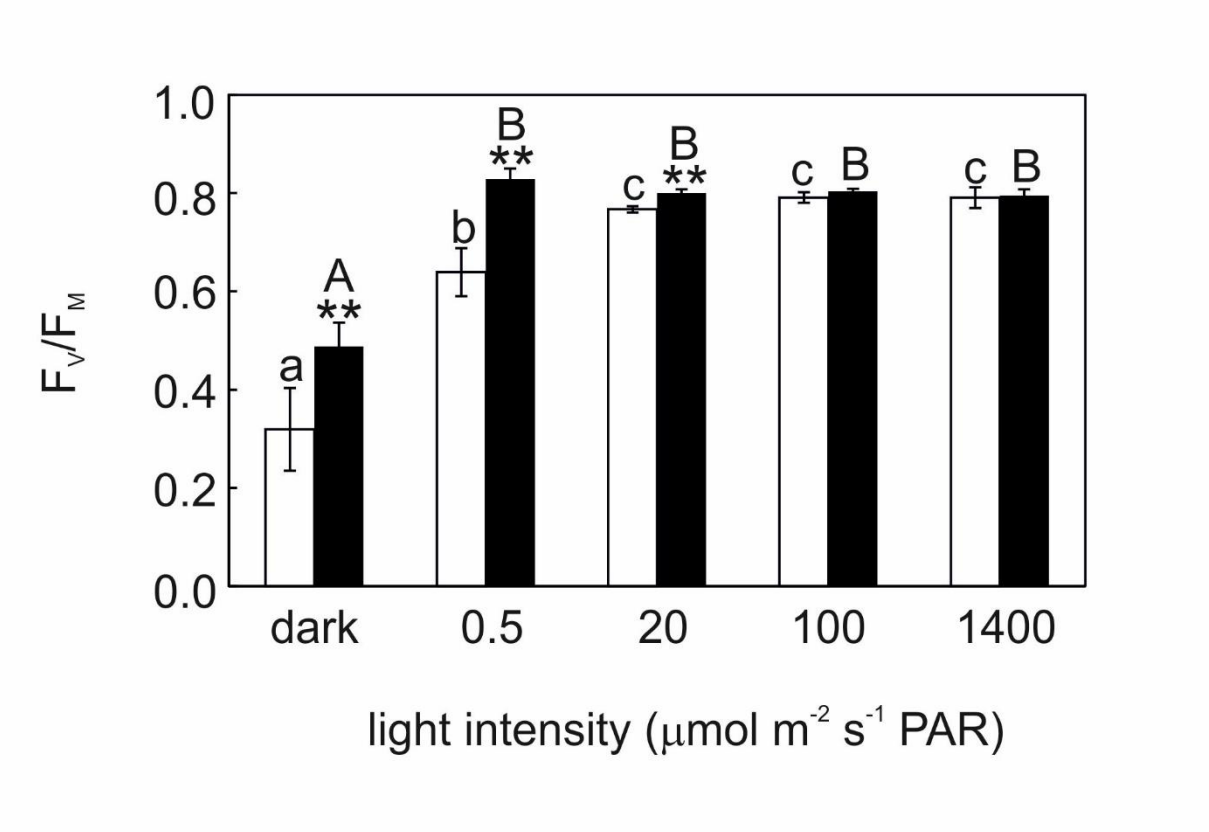
1.0
0.8
0.6
0.4
0.2
0.0
F_V/F_M
a
A
**
b
B
**
c
B
**
c
B
c
B
dark
0.5
20
100
1400
light intensity (μmol m^{-2} s^{-1} PAR)

Fig. 5

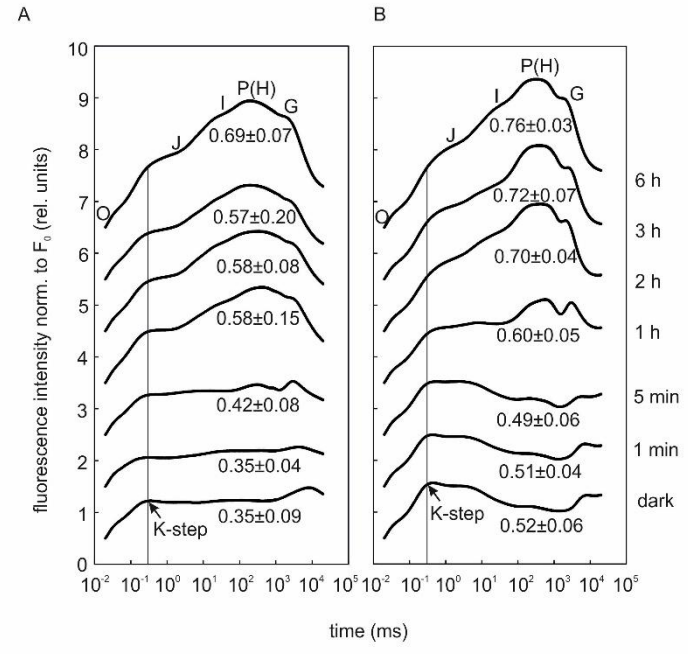
A
B
fluorescence intensity norm. to F_0 (rel. units)
O
J
I
P(H)
G
0.69±0.07
0.57±0.20
0.58±0.08
0.58±0.15
0.42±0.08
0.35±0.04
0.35±0.09
K-step
0.76±0.03
0.72±0.07
0.70±0.04
0.60±0.05
0.49±0.06
0.51±0.04
0.52±0.06
6 h
3 h
2 h
1 h
5 min
1 min
dark
time (ms)

Fig. 6

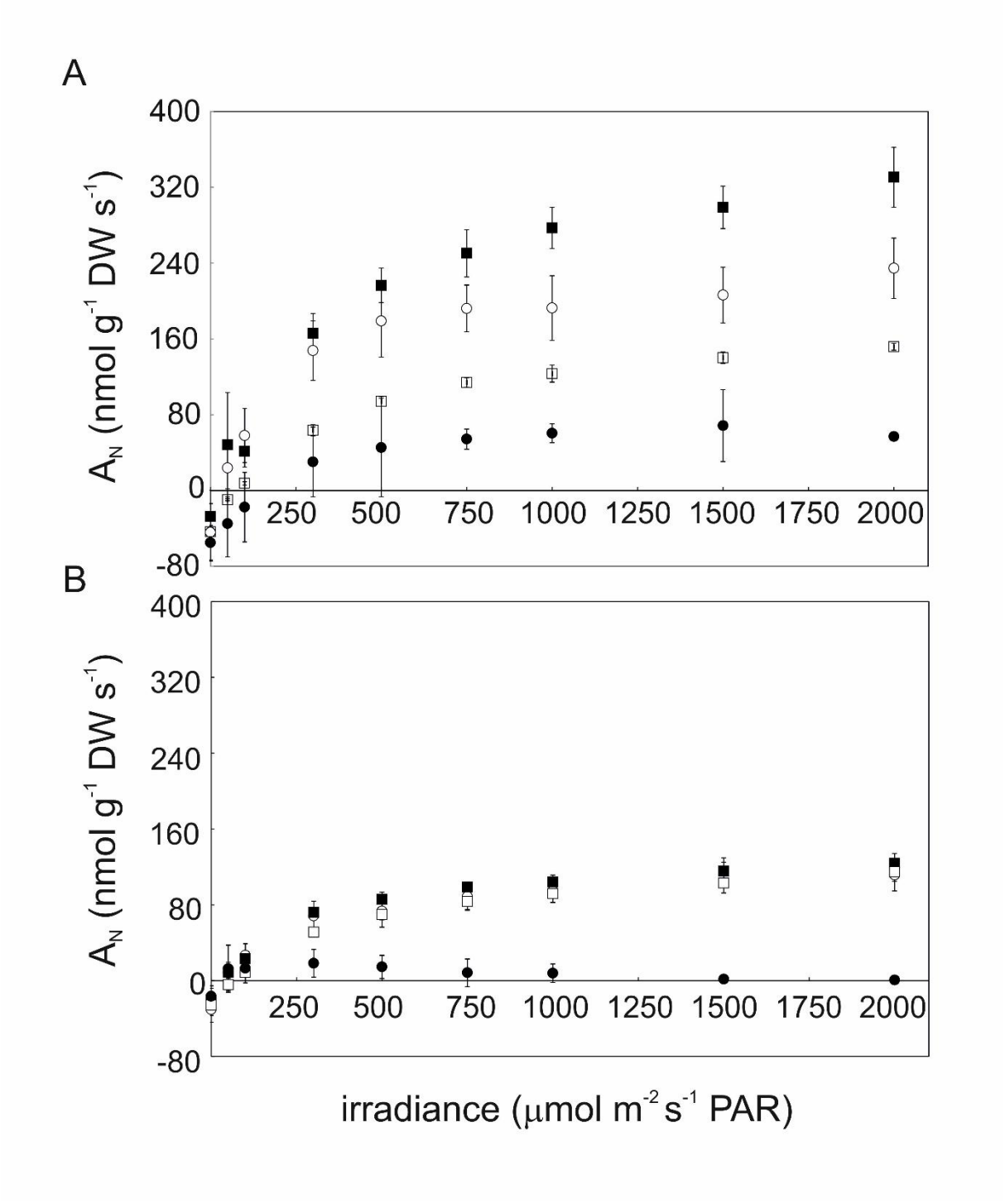
A
B
400
320
240
160
80
0
-80
250
500
750
1000
1250
1500
1750
2000
A_N (nmol g^{-1} DW s^{-1})
irradiance (μmol m^{-2} s^{-1} PAR)

Fig. 7

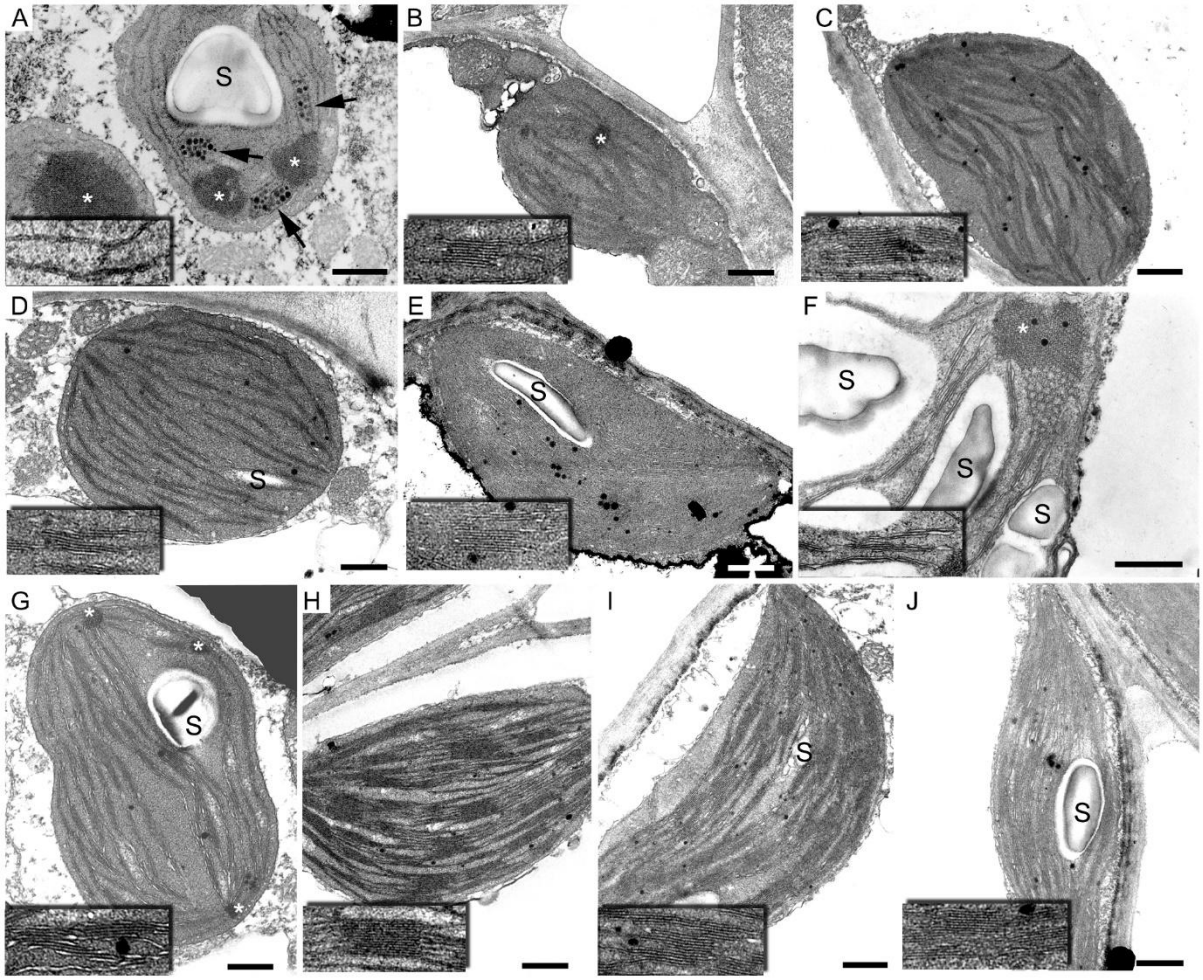

Fig. 8

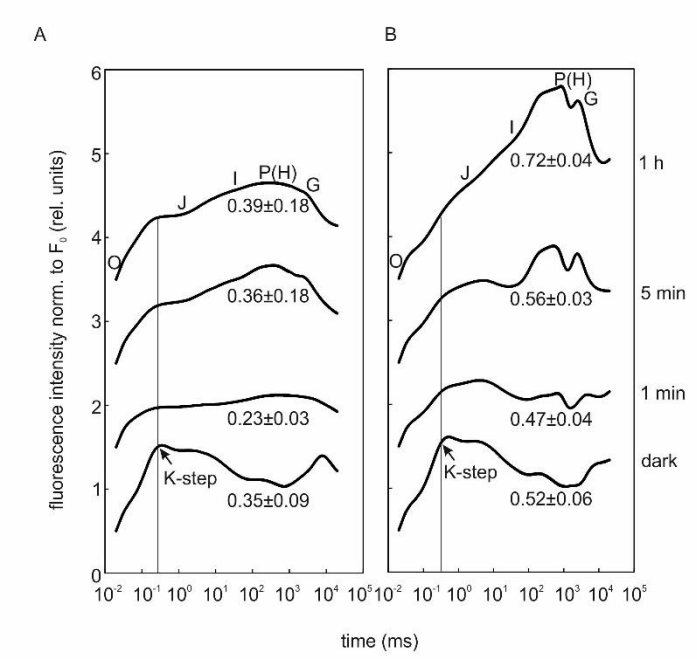
A
B
fluorescence intensity norm. to F0 (rel. units)
time (ms)
O
J
I
P(H)
G
K-step
0.39±0.18
0.36±0.18
0.23±0.03
0.35±0.09
0.72±0.04
0.56±0.03
0.47±0.04
0.52±0.06
1 h
5 min
1 min
dark

Fig. S1

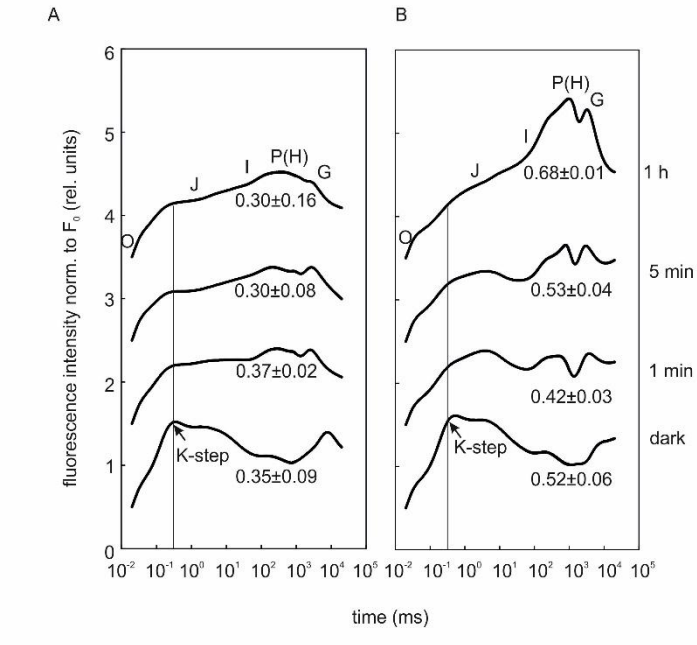
A
B
fluorescence intensity norm. to F0 (rel. units)
time (ms)
O
J
I
P(H)
G
K-step
0.30±0.16
0.30±0.08
0.37±0.02
0.35±0.09
0.68±0.01
0.53±0.04
0.42±0.03
0.52±0.06
1 h
5 min
1 min
dark

Fig. S2

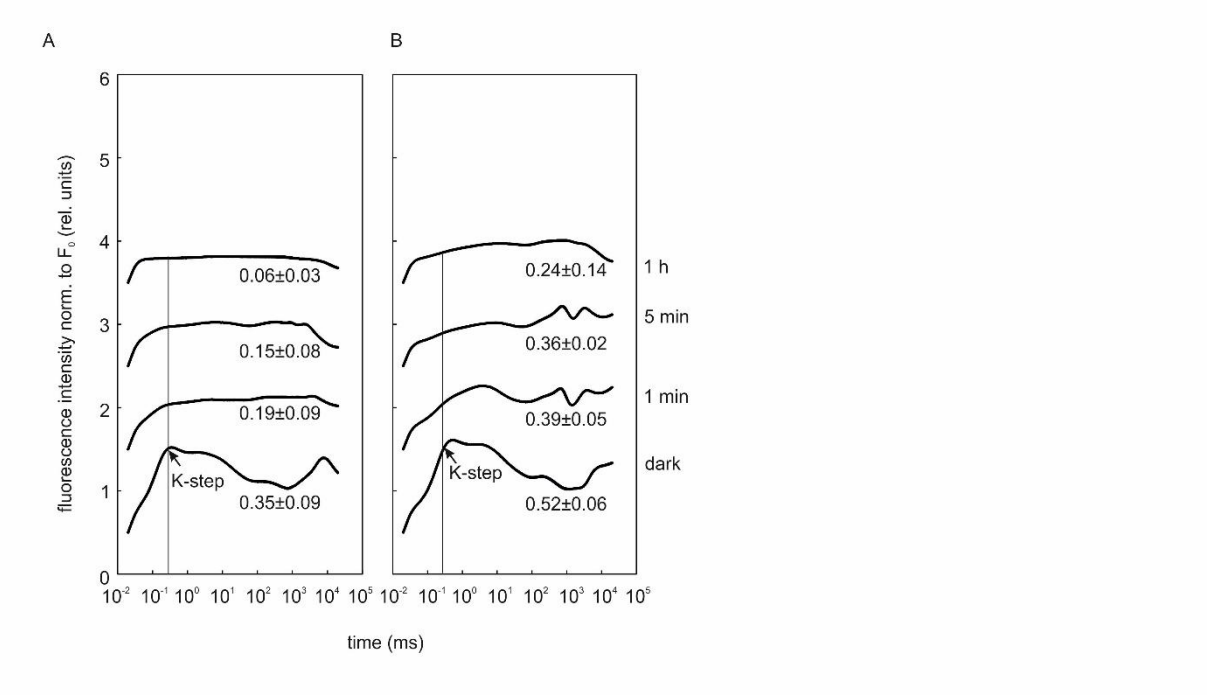
A
B
fluorescence intensity norm. to F0 (rel. units)
time (ms)
0.06±0.03
0.15±0.08
0.19±0.09
K-step
0.35±0.09
0.24±0.14
1 h
5 min
0.36±0.02
1 min
0.39±0.05
dark
K-step
0.52±0.06

Fig. S3