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Chlorophyll biosynthesis and chloroplast development in etiolated seedlings of *Ginkgo biloba* L.

A. PAVLOVIČ[*,+], Ľ. SLOVÁKOVÁ[*], V. DEMKO[*], M. DURCHAN[**], K. MIKULOVÁ[*], and J. HUDÁK[*]

Department of Plant Physiology, Faculty of Natural Sciences, Comenius University in Bratislava, Mlynská dolina B2, SK-842 15, Bratislava, Slovak Republic[*]
Biology Centre, Academy of Sciences, Branišovská 31, 37005, České Budějovice, Czech Republic[**]

Abstract

Ginkgo biloba L. is a large tree native in China with evolutionary affinities to the conifers and cycads. However unlike conifers, the gymnosperm *G. biloba* is not able to synthesize chlorophyll (Chl) in the dark, in spite of the presence of genes encoding subunits of light-independent protochlorophyllide oxidoreductase (DPOR) in the plastid genome. The principal aims of the present study were to investigate the presence of DPOR protein subunits (ChlL, ChlN, ChlB) as well as the key regulatory step in Chl formation: aminolevulinic acid (ALA) synthesis and abundance of the key regulatory enzyme in its synthesis: glutamyl-tRNA reductase (GluTR). In addition, functional stage of photosynthetic apparatus and assembly of pigment-protein complexes were investigated. Dark-grown, illuminated and circadian-grown *G. biloba* seedlings were used in our experiments. Our results clearly showed that no protein subunits of DPOR were detected irrespective of light conditions, what is consistent with the absence of Chl and Chl-binding proteins (D1, LHCI, LHCIIb) in the dark. This correlates with low ALA-synthesizing capacity and low amount of GluTR. The concentration of protochlorophyllide (Pchlide) in the dark is low and non-photoactive form (Pchlide_{633}) was predominant. Plastids were developed as typical etioplasts with prollamelar body and few prothylakoid membranes. Continual illumination (24 h) only slightly stimulated ALA and Chl synthesis, although Pchlide content was reduced. Prollamelar bodies disappeared, but no grana were formed, what was consistent with the absence of D1, LHCI, LHCIIb proteins. Light-induced development of photosynthetic apparatus is extremely slow, as indicated by Chl fluorescence and gas exchange measurements. Even after 72 h of continuous illumination, the values of maximum (F_v/F_m) and effective quantum yield (Φ_{PSII}) and rate of net photosynthesis (P_N) did not reach the values comparable with circadian-grown plants.

Additional key words: aminolevulinic acid; chlorophyll; *Ginkgo biloba*; gymnosperms; light-independent chlorophyll biosynthesis; protochlorophyllide; protochlorophyllide oxidoreductase.

Introduction

Chlorophylls are the most abundant tetrapyrrole molecules that are essential for photosynthesis. 5-aminolevulinic acid (ALA) is the first committed precursor in the tetrapyrrole biosynthetic pathway. Plants generate ALA from glutamate (C_5 - pathway) in a three-step process (Beale 1999). On the other hand, the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) is the last step of the biosynthetic pathway leading to the formation of the chlorins. This reaction can be catalysed by two different Pchlide reductases: light-dependent protochlorophyllide oxidoreductase (LPOR) and light-independent protochlorophyllide oxidoreductase (DPOR) (Schoefs and Franck 2003). Whereas LPOR is a single peptide enzyme encoded by nuclear genome, three plastid

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[+] Corresponding author; phone:+421 2 60296646, e-mail: pavlovic@fns.uniba.sk

Abbreviations: ALA – aminolevulinic acid; Chl – chlorophyll; Chl (*a*+*b*) – chlorophyll (*a*+*b*); Chlide – chlorophyllide; DPOR – light-independent protochlorophyllide oxidoreductase; F_m – maximal fluorescence in the dark; F'_m – maximal fluorescence in the light adapted state; F_v – variable fluorescence; F_0 – minimal fluorescence; GluTR – glutamyl-tRNA reductase; LHC – light harvesting complex; LPOR – light-dependent protochlorophyllide oxidoreductase; PAR – photosynthetic active radiation; Pchlide – protochlorophyllide; PLB – prolamellar body; P_N – rate of net photosynthesis; PS – photosystem; Φ_{PSII} – effective quantum yield of PSII.

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genes (*chlL*, *chlB*, *chlN*) encode structural subunits of DPOR. The subunits of DPOR exhibit sequence similarity to the nitrogenase subunits NifH, NifD and NifK, suggesting that the mechanism of reducing D-ring of Pchlide may be similar to the reduction of dinitrogen by nitrogenase (Nomata *et al.* 2005). Whereas anoxygenic photosynthetic bacteria use DPOR and angiosperms use LPOR as their only Pchlide reductases, most oxygenic phototrophs employ both DPOR and LPOR. To this group of plants belong gymnosperms, ferns, mosses and cyanobacteria. The noticeable exception, the gymnosperm *G. biloba*, is dependent on light for Chl synthesis and chloroplast development (Armstrong 1998). Dark-grown seedlings of *G. biloba* show complete etiolation, including no or little Chl accumulation (Chinn and Silverthorne 1993, Skribanek *et al.* 2008) and plastids develop as typical etioplasts (Mariani and Rascio 1982). Despite these facts, *Ginkgo* retains intact *chlB* and *chlL* genes in the plastid genome (Suzuki and Bauer 1992, Burke *et al.* 1993, Richard *et al.* 1994, Boivin *et al.* 1996, Fujita and Bauer 2003, Kusumi *et al.* 2006). Also other gymnosperm species *Welwitschia mirabilis* does not synthesize Chl in the dark. However in this case *chlL* gene has been lost (Burke *et al.* 1993). The reduced ability to synthesize Chl in the dark was documented also in conifers *Larix decidua* and *Thuja occidentalis*, despite the expression of the genes for DPOR subunits (Mariani *et al.* 1990, Kusumi *et al.* 2006, Demko *et al.* 2009). Since free excited Chl molecules may react with oxygen and form reactive oxygen species, the ability to synthesize Chl in the dark is synchronized with expression of apoproteins of light-harvesting Chl *a*/*b* binding proteins (Yamamoto *et al.* 1991, Mukai *et al.* 1992, Muramatsu *et al.* 2001, Demko *et al.* 2009). In the case of *G. biloba*, its *Lhcb* mRNA is expressed in a highly light-dependent manner (Chinn and Silverthorne 1993). In this respect, the genus *Ginkgo* is of great evolutionary interest, because the ability of Chl biosynthesis in the dark was lost, although the genes encoding the subunits of DPOR are still present in the plastid genome.

The aim of the present study was to investigate Chl biosynthesis in the key regulatory steps: ALA formation in the dark-grown, 24-hours illuminated and in the circadian-grown *G. biloba* seedlings. Although the genes encoding subunits of DPOR (*chlL* and *chlB*) were found in *G. biloba*, interesting question arises, whether the protein subunits are expressed. The slow greening process in relation to assembly of photosynthetic apparatus in the light was monitored using *in vivo* and low temperature (77 K) Chl fluorescence, gas exchange measurements, Western-blot analysis of crucial PSII and PSI proteins, and electron microscopy.

Materials and methods

Plant material and culture condition: The seeds of *G. biloba* L. were collected in the forest park in Bratislava. The seeds were imbibed in water for 24 h and germinated in perlit for 2–3 months. Seedlings were grown in the dark and in the light under circadian illumination 12 h dark/12 h light (100 μmol m^{-2} s^{-1} PAR). For greening experiments, dark-grown seedlings were exposed to continuous illumination of the same intensity as mentioned above. All manipulations in the dark were performed under a dim green safelight. All samples were taken 2 cm from shoot apex. Etiolated angiosperm *Hordeum vulgare* and dark-grown cotyledons from gymnosperm *Picea abies* were used as negative and positive controls, respectively, in Western-blot analysis.

Pigment extraction and quantification: Chlorophylls were extracted with mortar and pestle in 80% chilled acetone plus $MgCO_3$ and purified sea sand. After centrifugation at 4°C the resultant solution of Chl *a* and *b* was determined spectrophotometrically at 663 nm (Chl *a*) and 646 nm (Chl *b*) (*Jenway 6400, Krackeler Scientific*, London, UK). The concentration was calculated according to Lichtenthaler (1987).

Pchlide was extracted with 3 ml of acetone: 0.1 M NH_4OH (9:1, v/v) using mortar and pestle. The extracts were washed out three times with equal volume of hexane. Pchlide remained in the hexane-extracted acetone fraction and was determined by spectrofluorimeter *Perkin Elmer LS45* (Norwalk, CT, USA). Slit widths for both monochromators were set at 10 nm. Fluorescence was excited at 438 nm and emission at 633 nm corresponds to Pchlide. Pchlide standard was prepared from barley growing for a week in darkness on 10 mM ALA (*Fluka*, Steinheim, Germany). Cuttings were fixed by water vapour (steam) and homogenated in acetone: 0.1N NH_4OH (9:1, v/v). Carotenoids were washed out three times by adding 1 vol of 100 % hexane. To increase the stability of Pchlide, it was transferred to diethyl ether. Pchlide concentrations in extracts were determined photometrically at wavelength 623 nm using the molar extinction coefficient in diethyl ether $\varepsilon = 3.56\ 10^4\ M^{-1}\ cm^{-1}$ (Koski and Smith 1948).

Measurement of ALA-synthesizing capacity: 100 mg of fresh mass (FM) of *G. biloba* epicotyls were incubated in 40 mM levulinic acid in 20 mM phosphate buffer (pH = 7.1) to prevent ALA depletion. Seedlings were incubated for 4 h under the same conditions under which they had been grown. After incubation the samples were quickly dried and finally frozen in liquid nitrogen. The plant material was ground in liquid nitrogen to fine powder and resuspended in 20 mM phosphate buffer (pH = 7.1). After centrifugation, 100 μl ethylacetate was added to the 400 μl of supernatant. The samples were boiled at 100°C for 10 min, subsequently cooled for 5 min, mixed with 500 μl of modified Ehrlich reagent and absorption was measured at 525 and 553 nm (*Jenway 6400, Krackeler Scientific*, London, UK). The calibration curve for ALA (*Fluka*, Steinheim, Germany) was measured and ALA-synthesizing capacity was calculated as nmol(ALA) g^{-1}(FM) h^{-1} (Alawady and Grimm 2005).

Isolation of proteins and Western-blot analysis: Extraction of total proteins from plant samples, SDS-polyacrylamide gel electrophoresis and immunobloting were performed as described in Kruse *et al.* (1995). 20 μg of protein samples were electrophoresed in 12% (v/v) SDS-polyacrylamide gel followed by transfer to the *Hybond-C* membranes (*Amersham*, Freiburg, Germany). Antibodies against ChlB, ChlN (both from *Plectonema boryanum*) and ChlL (from *Marchantia polymorpha*) were provided by Y. Fujita. Antibodies against GluTR and LHCIIb were raised in the laboratory of B. Grimm. Antibodies against D1 and LHCI proteins were purchased from *Agrisera* (Vännäs, Sweden). As secondary antibody the Goat Anti-Rabbit IgG (H+L)-HRP conjugate (*BioRad*, Hercules, CA, USA) was used. Blots were visualized using *Immobilon Western* chemiluminiscent kit (*Millipore*, Billerica, MA, USA) and medical X-ray film (*Foma biochemia*, Hradec Králové, Czech Republic).

Gas exchange and Chl fluorescence measurements: Photosynthetic rate (P_N) was measured using infrared gas analyser *CIRAS-2* (*PP-Systems*, Hitchin, UK) after 1, 6, 12, 24, 48, and 72 h since transferring dark-grown seedlings to light to monitor the greening process. Simultaneously, the Chl fluorescence was monitored by *Fluorcam FC1000-LC* (*Photon Systems Instruments*, Brno, Czech Republic) attached to *CIRAS-2*. One cm of shoot apex (30 min adapted in the dark) was enclosed in the universal automatic *PLC6* leaf cuvette (*PP-Systems*, Hitchin, UK). After 10 min of stabilisation in the dark, minimal fluorescence (F_0) was recorded. Then maximal fluorescence (F_m) was measured using saturation pulse (5 000 μmol m^{-2} s^{-1} PAR, 800 ms duration) and maximal quantum yield of PSII (F_v/F_m) was calculated as $(F_m - F_0)/F_m$. Than actinic light was switched on for 15 min (100 μmol m^{-2} s^{-1} PAR) and 10 saturation pulses were triggered to measure maximal Chl fluorescence in the light adapted state (F'_m). The effective quantum yield (Φ_{PSII}) was calculated as $(F'_m - F_t)/F'_m$ according to Maxwell and Johnson (2000). Simultaneously at the end of the light period (15 min) steady state P_N was recorded at 360 ppm CO_2, leaf temperature 23 ± 1°C and relative air humidity 65 – 70%.

Low temperature fluorescence: Fluorescence emission spectra of epicotyls were measured at low temperature (77 K) with the *Spex* fluorolog spectrofluorometer (*Horiba, Jobin Yvon Inc*., Edison, NJ, USA) using the spectral bandwidth of emission and excitation monochromator of 2 nm. The epicotyls were immersed in liquid nitrogen in an optical Dewar flask when measured at 77 K. The raw data were corrected on the PMT photocathode sensitivity. The excitation wavelength was 440 nm. The effect of fluorescence reabsorption is very weak due to low pigment concentration.

Electron microscopy: Samples from cotyledons were fixed in a mixture of 5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde, postfixed in 2% (v/v) osmium tetroxide at room temperature (Karnovsky 1965). Fixed specimens were dehydrated in acetone series and embedded in *Durcupan* (*Fluka*, Neu Ulm, Germany). Ultrathin sections were cut with *Ultracut E* ultramicrotome (*Reichert-Jung*, Vienna, Austria), stained with uranyl acetate and lead citrate and examined in an electron microscope *Jeol 2000 FX* (Tokyo, Japan).

Statistical analysis: All of the measurements were performed four times, and the means and calculated standard deviations (SD) are reported.

Results

Synthesis of Chl, Chl precursors and protein analysis: Dark-grown seedlings of *G. biloba* did not contain Chl. After 24 h of irradiance, Chl was detected but at very low concentration. Seedlings contained low concentration of Pchlide, which was not detected after 24 h of illumination due to action of LPOR (Table 1). 77-K fluorescence emission spectra showed that Pchlide accumulated in the dark mainly in nonphotoactive form ($Pchlide_{633}$). Weak fluorescence at 656 nm corresponds to photoactive $Pchlide_{656}$ bound in ternary complex with LPOR and $NADPH^+$. No peaks of Chl fluorescence behind 656 nm were detected in the dark indicating that PSI and PSII were not assembled. After 24 h of illumination neither $Pchlide_{633}$ nor $Pchlide_{656}$ were detected. Instead of

Table 1. ALA-synthesizing capacity, Pchlide and Chl (*a*+*b*) concentration in dark-grown seedlings after 24 h of illumination and circadian-grown (12 h light/12 h dark) *Ginkgo biloba* seedlings, n.d. – not detected, mean ± SD, *n* = 4.

	dark-grown	24 h illumination	light/dark cycle
ALA syntesizing capacity [nmol g^{-1}(FM) h^{-1}]	2.8 ± 0.3	4.1 ± 0.6	34.3 ± 1.2
Pchlide [nmol g^{-1}(FM)]	1.4 ± 0.2	n.d	0.3 ± 0.1
Chl *a* + *b* [nmol g^{-1}(FM)]	n.d.	23.1± 2.5	852.0 ±181.7

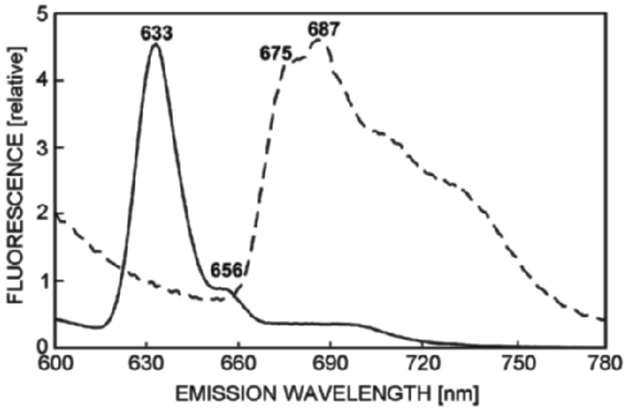

Fig. 1. The 77 K fluorescence emission spectra of dark-grown (*solid line*) and 24-h-illuminated epicotyls of *G. biloba* (*dashed line*). Excitation wavelength = 440 nm. Emission spectra are normalized at their maxima.

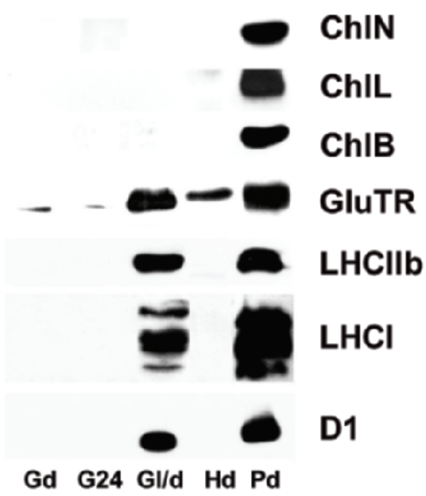

Fig. 2. Protein gel blot analysis of ChlN, ChlL, ChlB, GluTR, LHCIIb, LHCI and D1 proteins in *G. biloba* epicotyls. 20 µg protein samples were electrophoresed and blotted, Gd – dark-grown *G. biloba*, G24 – 24-h-illuminated *G. biloba*, Gl/d – circadian (light/dark cycle) grown *G. biloba*, Hd – dark-grown *Hordeum vulgare* (negative control), Pd – dark-grown *Picea abies* (positive control).

Pchlide, new emission peaks at 687 and 675 nm appeared, indicating the presence of PSII and free Chl and chlorophyllide [Chl(ide)], respectively. The assembly of PSI was delayed as indicates low fluorescence at 735 nm (Fig. 1). This is consistent with nondetectable LHCI proteins in dark-grown and in 24-h illuminated seedlings. Also D1 and LHCIIb proteins from PSII were not detected (Fig. 2). ALA-synthesizing capacity and GluTR content in dark-grown seedlings were very low in comparison to the circadian-grown seedlings. Even after 24 h of illumination, ALA-synthesizing capacity increased only slightly (Table 1). In circadian-grown seedlings, ALA-synthesizing capacity and GluTR content is much higher (Table 1, Fig. 2). The protein subunits of DPOR were not detected in dark-grown, 24-h illuminated and in circadian-grown *G. biloba* epicotyls (Fig. 2).

Gas exchange and *in vivo* Chl fluorescence: F_v/F_m, which is proportional to the quantum yield of O_2 evolution and Φ_{PSII}, which represents the portion of energy absorbed by PSII used in photochemistry, were low within the first hours of illumination, but gradually increased during the illumination to the subsequent lag phase between 48–72 h. Even after 72 hours of continuous illumination, the values of F_v/F_m and Φ_{PSII} did not reach the values of circadian-grown plants (Fig. 3*A,B*). Rate of net photosynthesis (P_N) during the greening

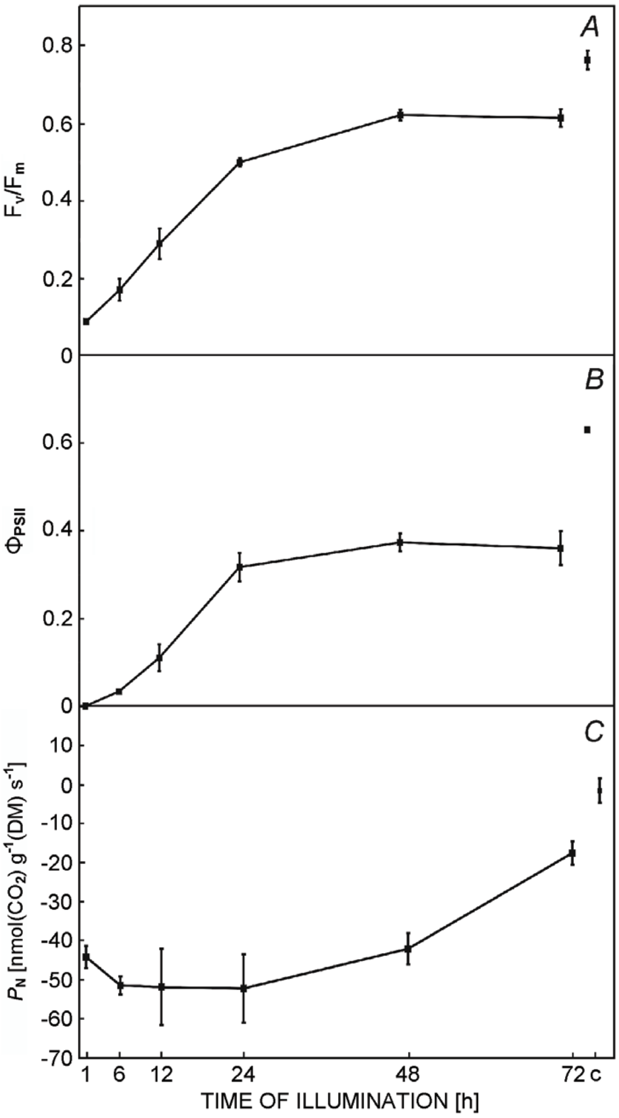

Fig. 3. Greening process in *G. biloba* epicotyls after 1–72 h of illumination. Maximum (*A*) and effective quantum yield of PSII (*B*) and rate of net photosynthesis (*C*), c – circadian (light/dark cycle) grown seedlings. The measurements were performed at 100 µmol m^{-2} PAR, means ± SD, *n* = 4.

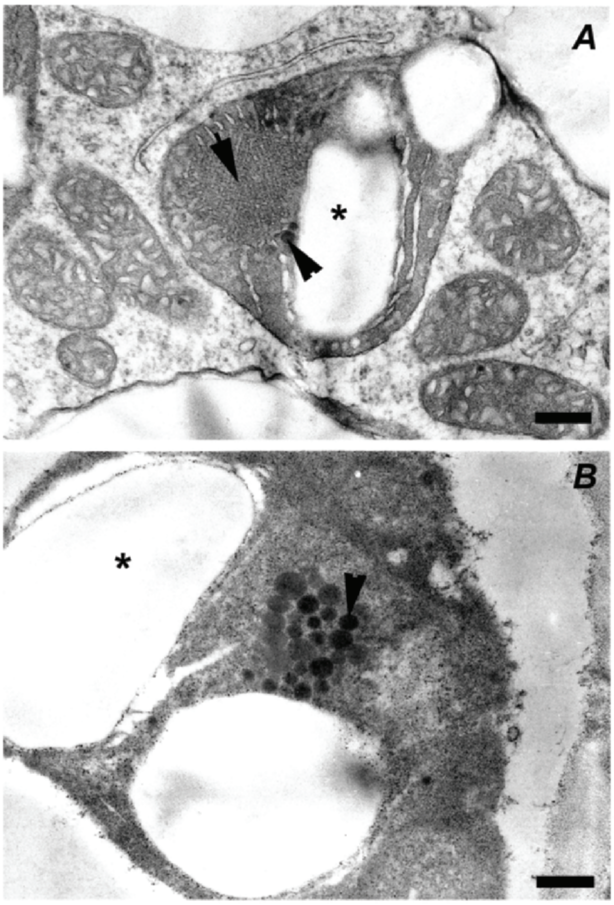

Fig. 4. Plastids from dark-grown (*A*) and 24-h-illuminated epicotyls of *G. biloba* (*B*). Note prollamelar body (*arrow*), plastoglobuli (*arrowhead*) and starch grains (*asterisk*). No grana are formed neither in darkness nor after 24 h of illumination. Bar = 200 nm.

process was negative due to the intensive respiration (Fig. 3*C*). The low net CO_2 uptake (P_{N} different from rate of respiration) was recorded after 24 h of illumination (data not shown). It looks surprisingly that P_{N} of light-grown plants was close to zero, but P_{N} was measured on shoot apex and not on the leaves to avoid comparison of different organs (dark-grown *G. biloba* has not differentiated leaves).

Electron microscopy: Dark-grown epicotyls of *G. biloba* have differentiated plastids as typical etioplasts with prollamelar body (PLB) and few prothylakoid membranes. Large starch grains and plastoglobuli were also present. After 24 h of illumination, PLB disappeared and the plastids contained only large starch grains and plastoglobuli. No grana were observed after 24 h of illumination.

Discussion

In spite of the presence of intact *chlB* and *chlL* genes in the plastid genome of *G. biloba* (Suzuki and Bauer 1992, Burke *et al.* 1993, Richard *et al.* 1994, Boivin *et al.* 1996, Fujita and Bauer 2003, Kusumi *et al.* 2006), the protein subunits of DPOR were not found irrespective of light condition (Fig. 2). This is consistent with the results that seedlings of *G. biloba* germinated and grown in darkness showed complete etiolation with no Chl (*a*+*b*) (Table 1). Chinn and Silverthorne (1993) and Skribanek *et al.* (2008) detected also no or little Chl (*a*+*b*) in some individuals in dark-grown *Ginkgo*. Skribanek *et al.* (2008) supposed that little Chl in some individuals came from the embryo *via* cell division. Durchan *et al.* (1992) and Böddi *et al.* (1999) also found traces of Chl in etiolated angiosperms, which appear to be deposited into embryos during its formation rather than synthesized during dark period. We agree with this interpretation because of the absence of DPOR protein subunits in *G. biloba* epicotyls (Fig. 2). It is unknown why *G. biloba* does not accumulate Chl in the dark. Fujita and Bauer (2003) predicted several possibilities. According to them the genes for DPOR have become pseudogenes (no information is available for *G. biloba chlN* genes and *chlL* genes was detected only by Southern blot analysis) or have some mutations causing catalytic activity of DPOR to be decreased or that the *chlL*, *chlN*, and *chlB* genes are highly downregulated in the dark. We can exclude the last two possibilities as we did not find any protein subunit of DPOR neither in the light nor in the dark. We supposed that the genes encoding DPOR subunits are not expressed and if so, post-transcription or post-translation processing is failed. It was proposed that editing of *chlB* transcripts in conifers might play an important role in determining the content of Chl in the dark (Fujita and Bauer 2003). It is known that RNA-editing in *chlB* transcripts occurs in dark-grown seedlings of *Picea abies* and *Larix decidua*. Karpinska *et al.* (1997) suggest that conservation of the correct amino acids, either by encoding them in DNA or by subsequent RNA-editing is important for the function of ChlB protein. Two editing sites in *chlB* transcripts were found in *P. abies*, but in the case of *G. biloba* correct aminoacids are encoded in plastid DNA (Richard *et al.* 1994, Karpinska *et al.* 1997). Thus it can be excluded that insufficient RNA-editing in *G. biloba* may be the reason for inability to synthesize Chl in the dark. *L. decidua* and *T. occidentalis,* which are also etiolated in the dark but contain low concentration of Chl, transcribed all three subunits of

DPOR and in the case of *L. decidua* two protein subunits were detected in the dark (Kusumi *et al.* 2006, Demko *et al.* 2009). It appears that species of *Thuja* have lost DPOR enzyme activity because of nonsynonymous mutation of some of the *chl* genes (Kusumi *et al.* 2006). Because Chl is essential for stabilisation of the proteins for photosystems in thylakoid membranes, it is not surprising that D1, LHCIIb and LHCI proteins were not detected by Western-blot analysis in dark-grown and illuminated seedlings (Fig. 2), although the *Lhcb* mRNA was detected in the dark with 10-fold lower expression in comparison to light-grown seedlings (Chinn and Silverthorne 1993).

The Pchlide concentration is very low in comparison to angiosperms as well as to other gymnosperms (Table 1). Selstam *et al.* (1987) found that Pchlide was present in dark-grown seedlings of Scots pine and Jeffrey pine to the same extent as in dark-grown wheat [10–15.8 nmol g^{-1}(FM)]. In our previous study we determined the similar amount of Pchlide as Selstam *et al.* (1987) in *L. decidua* and *P. abies* (Demko *et al.* 2009). In the case of *Ginkgo*, the concentration of Pchlide is very low, as was also found by Mariani and Rascio (1982) and Skribanek *et al.* (2008). ALA-synthesizing capacity is poor in the dark. In gymnosperm conifers with ability to synthesize Chl in the dark, the rate of ALA synthesis is much more higher and in *P. abies* and *Pinus nigra* is almost comparable with light-grown plants and stimulation of ALA synthesis by light is negligible (Drazic and Bogdanovic 2000, Demko *et al.* 2009). In angiosperms, the regulation of ALA synthesis is under the control of phytochrome (Huang *et al.* 1989). The enzyme involved in ALA synthesis that is most under the control of light is GluTR (Kruse *et al.* 1997, Goslings *et al.* 2004). After exposure to the light, angiosperm *Cucumis sativus* cotyledons rapidly increased ALA synthesis and Chl increased linearly at the rate 23 μg(Chl) g^{-1}(FM) h^{-1} (Huang and Castelfranco 1989). The greening process in etiolated *Helianthus annuus* cotyledons is also fast and is completed within 12 h of illumination (Lebkuecher *et al.* 1999). The rapid stimulation of ALA and Chl synthesis, as well as GluTR abundance after illumination of dark-grown seedlings was also documented recently in gymnosperm *L. decidua* cotyledons with decreased ability to synthesize Chl in the dark (Demko *et al.* 2009). In the case of *G. biloba* epicotyls, the low cellular abundance of GluTR and ALA synthesis after illumination showed its weak response to light. Thus, the greening process of *Ginkgo* is much slower than that of angiosperms and larch seedlings as it was also concluded by Chinn and Silverthorne (1993). Skribanek *et al.* (2008) supposed that greening process in angiosperms is different in the leaves and the stems. This is probably because of the difference in the ratio of the various Pchlide forms, *i. e.* the photoactive, long-wavelength Pchlide forms bound with LPOR in PLB are dominating in the leaves. Therefore, the greening of the leaves is faster than that of the stems, in which nonphotoactive forms are predominant. Another evidence for slow greening process is documented by measuring the functional stage of photosynthetic apparatus using gas exchange and *in vivo* Chl fluorescence. *G. biloba* needs more than 72 h to achieve the values of F_v/F_m, Φ_{PSII}, and P_N comparable with seedlings growing under light/dark regime (Fig. 3*A,B,C*). Also the transition of etioplasts to chloroplasts is extremely slow, as was also documented in this (Fig. 4) and previous study (Skribanek *et al.* 2008). For comparison, the Chl fluorescence parameters of dark-grown *Helianthus annuus* cotyledons followed by exposure to 12 h of 100 μmol m^{-2} s^{-1} PAR did not differ significantly from cotyledons of light-grown seedlings (Lebkuecher *et al.* 1999). Norway spruce (*P. abies*) with the highest capacity for Chl synthesis in the dark within Pinaceae showed even shorter period of illumination (1–6 h) to achieve the Chl fluorescence values comparable with circadian-grown seedlings (Mariani *et al.* 1990, Demko *et al.* 2009). This is in accordance with fast chloro-etioplast to chloroplast transition after illumination in this species. In contrast to *G. biloba*, the dark-grown *P. abies* seedlings after 24 h of illumination have completely formed grana without prollamelar bodies (Walles and Hudák 1975).

In conclusion, in spite of the presence of the genes *chlL* and *chlB* in the plastid genome, the protein subunits of DPOR were not detected in *G. biloba*. This is in accordance with the incapability of Chl synthesis and assembling of PSI and PSII in the dark. As a result of impaired Pchlide reduction in the dark, ALA-synthesizing capacity and GluTR content are rapidly down-regulated. The slow greening process of *G. biloba* had been attributed to high ratio of $Pchlide_{633}/Pchlide_{655}$, but it may also be due to weak response of GluTR and ALA synthesis to light. Further analyses are required to resolve the etiolation phenomenon in this ancient tree.

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