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Light-independent accumulation of essential chlorophyll biosynthesis- and photosynthesis-related proteins in *Pinus mugo* and *Pinus sylvestris* seedlings

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Abstract

Dark-grown seedlings of *Pinus mugo* Turra and *Pinus sylvestris* L. accumulate chlorophyll (Chl) and its precursor protochlorophyllide (Pchlide). Pchlide reduction is a key regulatory step in Chl biosynthesis. In the dark, Pchlide is reduced by light-independent Pchlide oxidoreductase (DPOR) encoded by three plastid genes *chlL*, *chlN*, and *chlB* (*chlLNB*). To investigate the differences in *chlLNB* gene expressions, we compared the dark-grown and 24-h illuminated seedlings of *P. mugo* and *P. sylvestris*. Expression of these genes was found constitutive in all analyzed samples. We report light-independent accumulation of important proteins involved in Chl biosynthesis (glutamyl-tRNA reductase) and photosystem formation (D1 and LHCI). Chl and Pchlide content and plastid ultrastructure studies were also performed.

Additional key words: chlorophyll biosynthesis; conifer seedlings; plastid gene expression; plastid ultrastructure; protochlorophyllide reduction.

Introduction

Seedlings of many gymnosperms are able to synthesize Chl as well as to form etiochloroplasts with developed thylakoid membrane system in the dark (Hudák *et al.* 2005). Chlorophylls are the major group of photosynthetic pigments associated with thylakoid membranes and required for light absorbtion during photosynthesis (Buchanan *et al.* 2000). In oxygenic phototrophs, Chl is synthesized from glutamate *via* a complex pathway that consists of at least fifteen enzymatic steps (Fujita 1996). Glutamyl-tRNA reductase (GluTR) is involved in the synthesis of 5-aminolevulinic acid (ALA), the first comitted precursor of tetrapyrrole biosynthesis (Tanaka and Tanaka 2007). The Chl-specific biosynthetic pathway, known as the Mg-branch, consists of six enzymatic reactions, with the reduction of Pchlide to chlorophyllide (Chlide) being the key regulatory step (Armstrong 1998). Two genetically and biochemically distinct strategies to reduce Pchlide have arisen during evolution. The first of them relies on light-dependent Pchlide oxidoreductase (LPOR), a nuclear-encoded and light-activated enzyme. Reduction of Pchlide in the dark is catalysed by light-independent Pchlide oxidoreductase (DPOR), the enzyme complex encoded by three plastid genes *chlL*, *chlN*, and *chlB* (*chlLNB*) (Armstrong 1998, Fujita and Bauer 2003). Integration of the newly synthesized Chl into developing thylakoids is tightly coupled with the biogenesis of the photosynthetic apparatus and the development of chloroplasts (Mariani *et al.* 1990, Muramatsu *et al.* 2001, Hudák *et al.* 2005). In contrast to angiosperms, expression of *Lhcb* and *psbA* genes as well as accumulation of LHC and D1 proteins is thought to be light-independent in gymnosperms. On the other hand, illumination of dark-grown gymnosperm seedlings can induce the accumulation of *Lhcb* and *psbA* mRNAs and corresponding proteins (Yamamoto *et al.* 1991, Shinohara *et al.* 1992, Peer *et al.* 1996).

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Abbreviations: ALA – 5-aminolevulinic acid; Chl – chlorophyll; Chlide – chlorophyllide; *chlLNB* – *chlL*, *chlN*, and *chlB* genes; cpDNA – plastid DNA; DPOR – light-independent protochlorophyllide oxidoreductase; GluTR – glutamyl-tRNA reductase; LHC – light-harvesting complex; LPOR – light-dependent protochlorophyllide oxidoreductase; Pchlide - protochlorophyllide.
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In the present study, we focused on the light-independent Chl biosynthesis in *P. mugo* and *P. sylvestris* seedlings. We studied expression of genes encoding DPOR subunits, alongside with Western blot analysis of ChlL, ChlN, ChlB, GluTR, D1, and LHCI proteins. Pigment quantification and plastid ultrastructure studies were also performed.

Materials and methods

Plant material: Seeds of *P. mugo* Turra and *P. sylvestris* L. obtained from Semenoles (Liptovský Hrádok, Slovakia) were soaked for 24 h in distilled water. Seedlings were grown in perlite in a phytochamber at 23°C under complete darkness. For analysis of early developmental stages, cotyledons of 7-day-old (*P. sylvestris*) and 10-day-old (*P. mugo*) seedlings were used. Cotyledon samples of mature dark-grown seedlings were collected after 12 days (*P. sylvestris*) and 16 days (*P. mugo*) of cultivation in the dark (Fig. 1). Mature seedlings of *P. sylvestris* and *P. mugo* were exposed to light at 100 µmol(photon) m^{-2} s^{-1} PAR for 24 h and cotyledons were collected. Manipulations with dark-grown seedlings were performed under dim-green light.

RNA isolation, reverse transcription and RT-PCR: Total RNA was isolated using *Spectrum™ Plant Total RNA Kit* (*Sigma-Aldrich*, St. Louis, MO, USA). RNA samples were treated with RNase-free DNAse I (*Fermentas*, St. Leon-Rot, Germany) for 1 h at 37°C. The absence of DNA in the RNA preparations was confirmed by PCR with primers specific to the *rrn23* gene, coding for plastid ribosomal 23S RNA (23SRTf 5′-AATGAGCCGGCGACTTATAGG-3′, 23SRTr 5′-GGG TCCATAAGCAGTGACAATTG-3′). The first strand of cDNA was synthesized from 1.5 µg of the purified RNA using *ImProm-II™ Reverse Transcription System* (*Promega*, Madison, WI, USA) and universal random primers. Subsequent RT-PCR was performed using specific primers for: *chlL*: LRTf 5′-TGTATTAGGCGA CGTGGTTTGT-3′ and LRTr 5′-CTGCAAATAATGCA TCGAATCC-3′; for *chlN*: NRTf 5′-CCGGAATGGCT CATGCTAAC-3′ and NRTr 5′-TC TCGCATTGGCA AATCCA-3′; for *chlB*: BRTf 5′-CG TTTATTAAAA GATCTGGACATCAGA-3′ and BRTr 5′- GTAGATA CATAAGGCATTCCAAATTC-3′. PCR were performed as follows: 2 min at 94°C and 30 cycles of 15 s at 94°C and 1 min at 60°C. Amplification products were separated on 2% agarose gels and stained with ethidium bromide. PCR amplifications were performed with DreamTaq™ DNA polymerase (*Fermentas*, St. Leon-Rot, Germany).

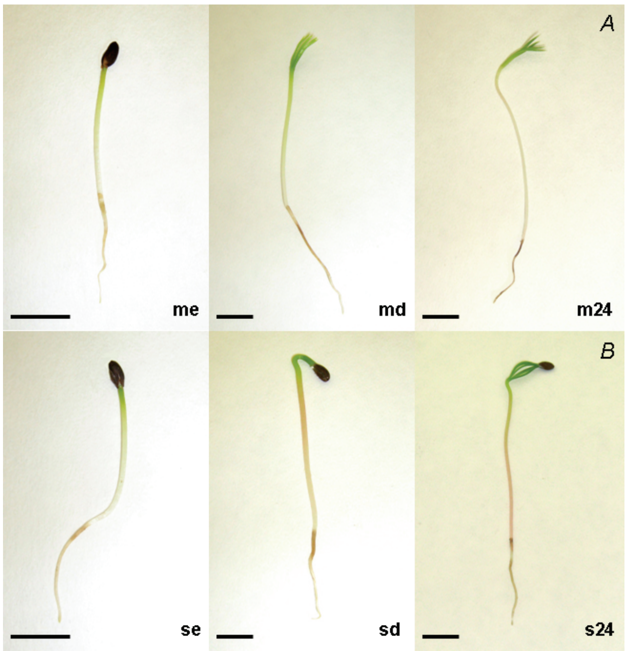

Fig. 1. Seedlings of *P. mugo* (*A*): 10-day-old dark-grown seedlings (me), 16-day-old dark-grown seedlings (md) and 17-day-old 24-h illuminated seedlings (m24) are shown. Seedlings of *P. sylvestris* (*B*): 7-day-old dark-grown seedlings (se), 12-day-old dark-grown seedlings (sd) and 13-day-old 24-h illuminated seedlings (s24) are shown. Bars represent 1 cm.

DNA isolation and sequencing: Plastid DNA was isolated according to Triboush *et al*. (1998). Central part of the *chlB* gene (170 bp) was amplified using *Pfu* DNA polymerase (*Fermentas*, St. Leon-Rot, Germany) with the same primers and amplification program as for RT-PCR. The PCR product was purified using *Wizard SV Gel* and *PCR Clean-Up System* (*Promega*, Madison, WI, USA) and directly sequenced for both strands on an *ABI 3100-Avant Genetic Analyser* (*Applied Biosystems*, Foster City, CA, USA).

Protein isolation and Western blot analysis: Cotyledons (100 mg) were ground under liquid nitrogen and suspended in protein extraction buffer (28 mM dithiothreitol, 5% SDS, 175 mM sucrose, 28 mM Na_2CO_3, 10 mM EDTA). After 30 min incubation at 70°C and 15 min centrifugation (12,100 × *g*), supernatant was used for determination of protein concentration using *Bichinonic Acid Kit for Protein Determination* (*Sigma-Aldrich*, St. Louis, MO, USA). Protein samples (25 µg) were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane using *Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell* (*Bio-Rad*, Hercules, CA, USA). For protein immunodetection, specific primary antibodies were used. Antibodies against ChlL, ChlN, and ChlB were provided by Y. Fujita, Nagoya University, Nagoya, Japan and the antibody against GluTR was provided by B. Grimm, Humboldt

University, Berlin, Germany. Antibodies against D1 and LHCI were purchased from *Agrisera*, Vännäs, Sweden. Secondary antibody Goat Anti-Rabbit IgG (H+L)-HRP Conjugate (*Bio-Rad*, Hercules, CA, USA) was used. Signal was revealed using chemiluminiscent kit *Immobilon Western* (*Millipore*, Bedford, MA, USA).

Pigment analysis: Chl was extracted with 80% chilled acetone, spectrophotometrically (*Jenway 6400*, London, UK) quantified: Chl *a* at 663.2 nm, Chl *b* at 646.8 nm and calculated according to Lichtenthaler (1987). Pchlide from 100 mg of cotyledons was extracted in 3 ml acetone: 0.1 M NH_4OH (9:1, v/v). The extract was washed three times with equal volume of hexane. After this procedure esterified tetrapyrroles were removed by hexane. The amount of Pchlide was measured spectrofluorometrically (*RF-5301 PC, Shimadzu*, Kyoto, Japan) at λ_{ex} 438 nm and λ_{em} 633 nm (slit widths: ex. 10 nm, em. 3 nm) in acetone phase and quantified using a Pchlide standard. Pchlide standard was prepared from etiolated barley fed with 10 mM aminolevulinic acid according to Koski and Smith (1948) and spectrophotometrically quantified at 623 nm using molar extinction coefficient in diethyl ether $\varepsilon = 3.56\ 10^4\ M^{-1}\ cm^{-1}$ (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 constructed. To evaluate the significant differences, *t*-test was performed (*Microsoft Office Excell 2003*).

Electron microscopy: Cotyledon samples were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and postfixed in 2% osmium tetroxide in the same buffer (Karnovsky 1965). Fixed specimens were dehydrated in acetone series and embedded in *DURCUPAN ACM* (*Fluka*, St. Louis, MO, USA). Ultrathin sections were cut on ultramicrotome *Reichert-Jung Ultracut E*, stained with uranyl acetate and lead citrate and observed with *JEOL 2000 FX* electron microscope (*Jeol Ltd*., Tokyo, Japan).

Results

Expression of *chlL*, *chlN*, and *chlB* genes: Using RT-PCR, we have studied the expression of *chlLNB* genes in dark-grown and 24-h illuminated seedlings of *P. mugo* and *P. sylvestris*. Expression of *chlLNB* genes was constitutive in all analyzed seedlings. Constitutively expressed plastid gene *rrn23,* encoding the 23S rRNA, was used as a corresponding gene (Fig. 2).

DNA sequence analysis: After sequencing of the cpDNA fragment containing the central part of the *chlB* gene of *P. mugo,* 102 bp long sequence was obtained and submitted to GenBank database (http://www.ncbi.nlm.nih.gov/, accession number GQ283002). We have identified two codons (CCG coding for proline and CGG coding for arginine) that probably undergo RNA-editing in the posttranscriptional processing of *chlB* primary transcripts. RNA-editing modifies codons for proline and arginine to codons for leucine and tryptophan, respectively. We compared deduced amino-acid sequences corresponding to central part of ChlB polypeptide. *P. mugo* and other gymnosperm species *P. sylvestris, Pinus thunbergii*, *Pinus koraiensis*, *Pinus strobus* and *Pinus longaeva* encode proline and arginine in their cpDNA. On the contrary, *Thuja occidentalis*, *Ginkgo biloba*, *Cupressus sempervirens* and *Sequoiadendron giganteum* encode amino acids leucine and tryptophan directly in their cpDNA (Table 1).

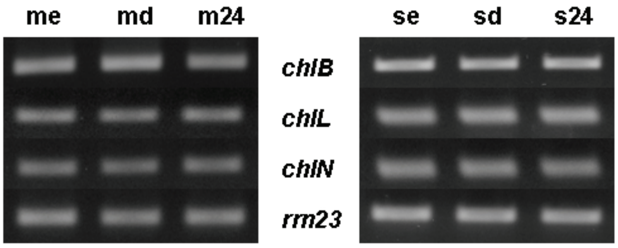

Fig. 2. Expression of *chlL*, *chlN*, and *chlB* genes. Constitutively expressed *rrn23* gene, coding for ribosomal RNA 23S, was used as a corresponding gene. 10-day-old dark-grown seedlings *P. mugo* (me), 16-day-old dark-grown *P. mugo* seedlings (md), 17-day-old 24-h illuminated *P. mugo* seedlings (m24), 7-day-old dark-grown *P. sylvestris* seedlings (se), 12-day-old dark-grown *P. sylvestris* seedlings (sd), 13-day-old 24-h illuminated *P. sylvestris* seedlings (s24).

Western blot analysis: The levels of ChlB protein were highest in the early developmental stages and mature dark-grown seedlings of *P. mugo* and slightly decreased after 24-h illumination. *P. sylvestris* seedlings accumulated ChlB at the highest rate in early developmental stages. We have observed highest ChlL accumulation in early developmental stages of both species. We did not determine dramatic changes in the accumulation of ChlN protein during development of the seedlings in the dark and after their illumination. Dark-grown and illuminated seedlings of *P. mugo* and *P. sylvestris* accumulated similar amounts of D1 protein. Accumulation of the LHCI complex polypeptides increased during the development in the dark. However, illumination of seedlings induced LHCI complex protein accumulation in both examined conifers. We report light-independent accumulation of the GluTR enzyme in the early developmental stages of *P. mugo* and *P. sylvestris* seedlings in the dark. The level of GluTR increased after 24-h illumination of the seedlings in comparison to the dark-grown mature seedlings (Fig. 3).

Pigment analysis: Dark-grown and 24-h illuminated seedlings of *P. mugo* and *P. sylvestris* are able to accumulate Chl (Table 2). Early developmental stages and mature dark-grown seedlings of both conifers have shown no statistical difference in the Chl (*a*+*b*) content. Statistically significant increase in the Chl (*a*+*b*) content was observed after 24-h illumination of the seedlings (Fig. 4). Pchlide content was similar in early developmental stages and mature dark-grown in *P. mugo* and *P. sylvestris* seedlings. Pchlide content significantly declined after illumination of the seedlings in both studied species (Fig. 5).

Plastid ultrastructure: Plastids of dark-grown *P. mugo* and *P. sylvestris* seedlings are developed as typical etiochloroplast. The prominent structure of these plastids are prolamellar bodies with narrow spacing of the tubules. From prolamellar bodies thylakoids radiate. Numerous membranes are present in plastid stroma, where the grana made from 2–5 thylakoids are present. Plastids of both conifer species contain large starch grains that often make plastid shape irregular (Fig. 6.).

Discussion

For more than a century, it has been known that gymnosperms have the ability to synthesize Chl during the early development in the dark (Fujita and Bauer 2003). Dark-grown conifer seedlings are able to form etiochloroplasts with developed membrane system (Hudák *et al.* 2005) and to accumulate proteins and assimilation pigments needed for the assembly of photosynthetic apparatus (Demko *et al.* 2009). In this study, we have shown that light-independent accumulation of GluTR protein and DPOR subunits in *P. mugo* and *P. sylvestris* led to the accumulation of Chl during the development in the dark. Both conifer species differentiate etiochloroplasts with thylakoid membranes and contain significant levels of proteins essential for photosystem formation (D1 and LHCI).

Glutamyl-tRNA reductase plays a crucial role in the regulation of ALA formation. In contrast to angiosperms, expression of *Hema* genes and accumulation of corresponding GluTR protein is thought to be light-independent in gymnosperms and lower plants (Kruse *et al.* 1997, Drazic and Mihailovic 1998, Nogaj *et al.* 2005, Vasileuskaya *et al.* 2005, Demko *et al.* 2009). We have observed the highest level of GluTR protein in the early developmental stages of dark-grown *P. mugo* and *P. sylvestris* seedlings. Increase in the GluTR content was apparent after 24-h illumination. Our results indicate that light is not primarily essential for GluTR accumulation. On the other hand, illumination positively regulated GluTR levels in the conifer seedlings.

Reduction of the D-ring of Pchlide, catalysed by DPOR complex in the dark, is an important regulatory step in Chl biosynthesis (Armstrong 1998). Constitutive expression of plastid genes *chlL*, *chlN*, and *chlB*, encoding the DPOR subunits, has been reported in various organisms, irrespective of the developmental stage or environmental factors. Shi and Shi (2006) observed that *chlLNB* genes are expressed in photoautotrophically, mixotrophically and heterotrophically grown *Chlorella protothecoides* cells in dark- and light-grown cultures. Conifer species *Pinus taeda*, *P. strobus*, *T. occidentalis, Chamaecyparis obtusa*, *Picea abies* and *Larix decidua* express *chlLNB* genes constitutively when grown in the dark (Spano *et al.* 1992, Skinner and Timko 1999, Kusumi *et al.* 2006, Demko *et al.* 2009). Previously published data support our results of constitutive expression of *chlLNB* genes in dark-grown and 24-h illuminated pine seedlings.

Sequencing of the central part of the *chlB* gene in *P. mugo* revealed presence of two codons, CCG and CGG, that are posttranscriptionally modified by RNA-

Table 1. Deduced amino-acid sequence of the central part of the ChlB polypeptide. *P. mugo* and *P. sylvestris*, similarly as some other conifer species, encode proline and arginine in their cpDNA. Codons for these amino acids are posttranscriptionally edited and afterwards encode leucine and tryptophan, respectively. Many gymnosperms encode conserved amino acids leucine and tryptophan in their cpDNA. Accesion numbers to GenBank NCBI Database (http://www.ncbi.nlm.nih.gov/): *P. mugo* GQ283002, *P. sylvestris* X98682, *Pinus thunbergii* FJ899562, *Pinus koraiensis* A4228468, *Pinus strobus* U02533, *Pinus contorta* EU998740, *Pinus nelsonii* EU998746, *Pinus longaeva* EU998744, *Pinus monophylla* EU998745, *Picea mariana* L25773, *Cryptomeria japonica* AP009377, *Larix decidua* A4560614, *Thuja occidentalis* AB232496, *Gingko biloba* U01531, *Cupressus sempervirens* AB232482, *Sequoiadendron giganteum* AB232466, *Chamaecyparis lawsoniana* AB232480.

Species	Amino-acid sequence
Pinus mugo	ED**P**KNLPKA**R**FN
Pinus sylvestris	ED**P**KNLPKA**R**FN
Pinus thunbergii	ED**P**KNLPKA**R**FN
Pinus koraiensis	ED**P**KNLPKA**R**FN
Pinus strobus	ED**P**KNLPKA**R**FN
Pinus contorta	ED**P**KNLPKA**R**FN
Pinus nelsonii	ED**P**KNLPKA**R**FN
Pinus longaeva	ED**P**KNLPKA**R**FN
Pinus monophylla	ED**P**KNLPKA**R**FN
Picea mariana	ED**S**KNLPKA**R**FN
Cryptomeria japonica	EE**L**QNLPKA**L**FN
Larix decidua	ED**P**KNLPKA**R**FN
Larix kaempferi	ED**P**KNLPKA**R**FN
Thuja occidentalis	ED**L**QNLPKA**W**FN
Ginkgo biloba	ED**L**KNLPKA**W**LN
Cupressus sempervirens	ED**L**QNLPKA**W**FN
Sequoiadendron giganteum	ED**L**KSLPKA**W**FN
Chamaecyparis lawsoniana	ED**L**QNLPKA**W**FN

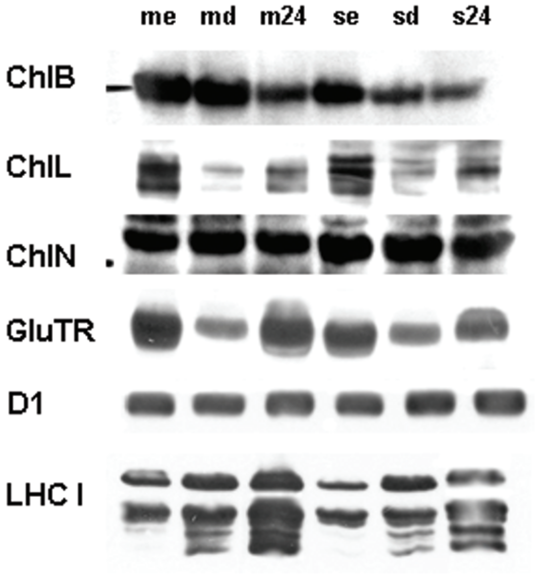

Fig. 3. Western blot of the DPOR subunits, GluTR, D1 and LHCI complex proteins in *P. mugo* and *P. sylvestris* cotyledon samples. 10-day-old dark-grown seedlings *P. mugo* (me), 16-day-old dark-grown *P. mugo* seedlings (md), 17-day-old 24-h illuminated *P. mugo* seedlings (m24), 7-day-old dark-grown *P. sylvestris* seedlings (se), 12-day-old dark-grown *P. sylvestris* seedlings (sd), 13-day-old 24-h illuminated *P. sylvestris* seedlings (s24).

editing. RNA-editing (deamination of cytidines to uridines) modifies the nucleotide sequence at the mRNA level, thus leading to a change in amino acid sequence (Bock 2000). Our preliminary analyses of *chlB* RNA-editing indicate that *P. mugo* and *P. sylvestris* seedlings partialy process these codons (data not shown). Both *P. mugo* and *P. sylvestris* encode proline and arginine at the 210 and 217 amino acid positions, respectively. In the first case, CCG codon for proline is changed to CUG for leucine. In the latter codon, CGG is modified to UGG, resulting in arginine to tryptophan codon substitution. Karpinska *et al.* (1997) reported editing of *chlB* transcripts in *P. sylvestris* and *Picea abies* in both codons, while only in the second codon in *Larix eurolepis*. Many conifers encode leucine and tryptophan primarily in their plastid DNA. These conserved amino acids might play an essential role in proper ChlB protein function (Karpinska *et al.* 1997).

Table 2. Contents of Chl and Pchlide in *P. mugo* and *P. sylvestris* seedlings. Means ± standard errors of three experiments. 10-day-old dark-grown seedlings *P. mugo* (me), 16-day-old dark-grown *P. mugo* seedlings (md), 17-day-old 24 h illuminated *P. mugo* seedlings (m24), 7-day-old dark-grown *P. sylvestris* seedlings (se), 12-day-old dark-grown *P. sylvestris* seedlings (sd), 13-day-old 24-h illuminated *P. sylvestris* seedlings (s24).

	me	md	m24	se	sd	s24
Pchlide [nmol g^{-1}(FM)]	3.8±0.3	4.6±0.2	0.7±0.1	5.3±0.8	4.4±0.7	0.9±0.2
Chl *a*+*b* [nmol g^{-1}(FM)]	340±18	325±9	400±15	355±18	352±13	410±8

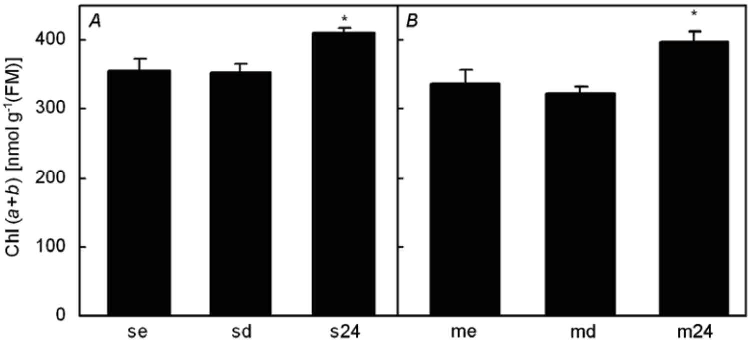

Fig. 4. Chl ($a + b$) content [nmol g^{-1}(FM)] in *P. mugo* (A) and *P. sylvestris* (B) seedlings. Means ± standard errors of three experiments. *Star* denotes statistically significant difference at $P < 0.05$ (*t*-test). 10-day-old dark-grown seedlings *P. mugo* (me), 16-day-old dark-grown *P. mugo* seedlings (md), 17-day-old 24-h illuminated *P. mugo* seedlings (m24), 7-day-old dark-grown *P. sylvestris* seedlings (se), 12-day-old dark-grown *P. sylvestris* seedlings (sd), 13-day-old 24-h illuminated *P. sylvestris* seedlings (s24). FM – fresh mass.

Western blot analysis has shown minor differences in ChlB accumulation in analyzed seedlings. The level of ChlB was highest in early stages and mature seedlings grown in the dark and decreased after 24-h illumination. The ChlL subunit abundance decreased rapidly during the development in the darkness, while the level of ChlN did not seem to change significantly in all cotyledon samples. Similar declines in ChlL and ChlB were observed in *C. protothecoides* cultures after transition from darkness to light (Shi and Shi 2006). Since the expression of *chlLNB* genes was constitutive in our experiments, the changes in protein accumulation could have been caused by posttranscriptional or posttranslational processes. The presence of DPOR subunits after illumination may indicate that DPOR takes part in Pchlide reduction also in the first hours of deetiolation.

Proteins of the light-harvesting antennae and photosystem reaction centers are essential for the assembly of the photosystem apparatus. Accumulation of these proteins is light-dependent in angiosperms but in gymnosperms does not require light (Canovas *et al.*

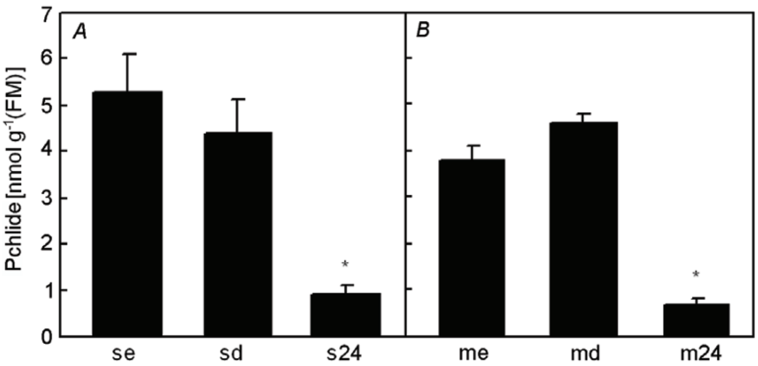

Fig. 5. Pchlide content [nmol g^{-1} FM] in *P. mugo* (A) and *P. sylvestris* (B) seedlings. Means ± standard errors of three experiments. *Star* denotes statistically significant difference at $P < 0.05$ (*t*-test). 10-day-old dark-grown seedlings *P. mugo* (me), 16-day-old dark-grown *P. mugo* seedlings (md), 17-day-old 24-h illuminated *P. mugo* seedlings (m24), 7-day-old dark-grown *P. sylvestris* seedlings (se), 12-day-old dark-grown *P. sylvestris* seedlings (sd), 13-day-old 24-h illuminated *P. sylvestris* seedlings (s24). FM – fresh mass.

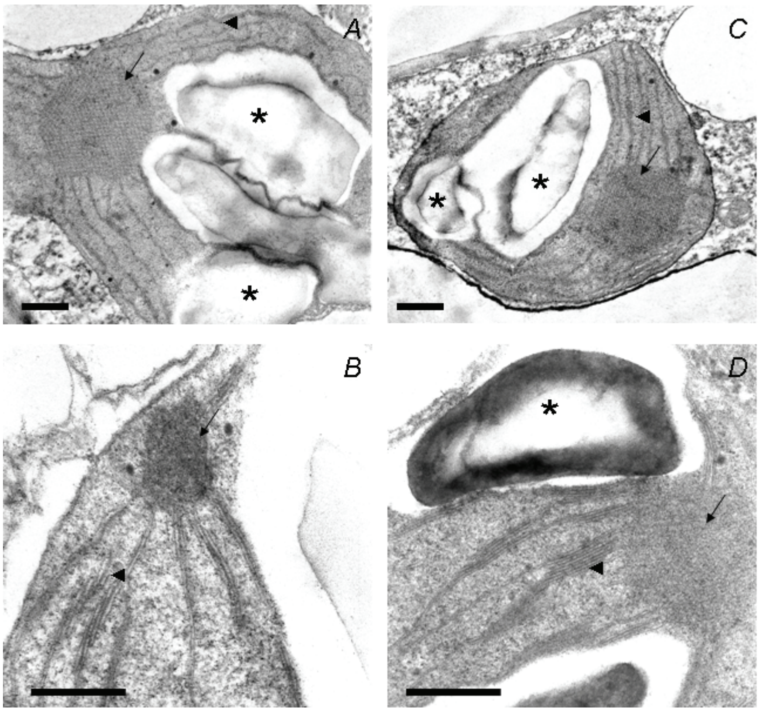

Fig. 6. Plastid ultrastructure in 16-day-old dark-grown *P. mugo* (*A*, *B*) and 12-day-old dark-grown *P. sylvestris* (*C*, *D*) seedlings. *Stars* indicate starch grains, *arrows* indicate prolamellar bodies and *arrow heads* indicate thylakoid membranes. Bars represent 500 nm.

1993, Peer *et al.* 1996). Yamamoto *et al.* (1991) have observed light-independent D1 and LHCBIIb protein accumulation in dark-grown *P. thunbergii* seedlings. *Lhcb* and *psbA* (coding for D1 protein) gene expression and LHCB protein accumulation was proven in *Pseudotsuga menziesii* dark-grown seedlings (Alosi *et al.* 1990). The accumulation of D1 protein was similar in all analyzed *P. mugo* and *P. sylvestris* cotyledons. Accumulation of LHCI complex proteins rose during the development in the dark. We observed the highest LHCI abundance after 24-h of illumination. Our data suggest that accumulation of proteins needed for the formation of photosystems is light-independent in *P. mugo* and *P. sylvestris*.

Chlorophylls are a crucial part of photosystems. Dark-grown *P. mugo* and *P. sylvestris* seedlings accumulated reasonable amounts of Chl *a* and *b* and their precursor Pchlide. Pchlide content declined dramatically after seedling illumination, which was probably caused by fast photoreduction catalysed by LPOR. The reduction of Pchlide resulted in statistically significant increase of the Chl content.

To summarize, *P. mugo* and *P. sylvestris* seedlings, grown under continuous darkness, synthesize Pchlide and Chl. They contain enzymes involved in early and final steps of Chl biosynthesis (GluTR and DPOR, respectively) and accumulate proteins necessary for the photosystem formation (D1 and LHCI). *P. mugo* and *P. sylvestris* dark-grown mature seedlings differentiate thylakoid membrane system, which is essential for the formation of photosynthetic apparatus. Further studies may reveal the complex and complicated regulatory mechanisms that control light-independent Chl biosynthesis in gymnosperms.

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