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ORIGINAL ARTICLE

A novel insight into the regulation of light-independent chlorophyll biosynthesis in *Larix decidua* and *Picea abies* seedlings

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Abstract Light-independent chlorophyll (Chl) biosynthesis is a prerequisite for the assembly of photosynthetic pigment–protein complexes in the dark. Dark-grown *Larix decidua* Mill. seedlings synthesize Chl only in the early developmental stages and their Chl level rapidly declines during the subsequent development. Our analysis of the key regulatory steps in Chl biosynthesis revealed that etiolation of initially green dark-grown larch cotyledons is connected with decreasing content of glutamyl-tRNA reductase and reduced 5-aminolevulinic acid synthesizing capacity. The level of the Chl precursor protochlorophyllide also declined in the developing larch cotyledons. Although the genes *chlL*, *chlN* and *chlB* encoding subunits of the light-independent protochlorophyllide oxidoreductase were constitutively expressed in the larch seedlings, the accumulation of the ChlB subunit was developmentally regulated and ChlB content decreased in the fully developed cotyledons. The efficiency of *chlB* RNA-editing was also reduced in the mature dark-grown larch seedlings. In contrast to larch, dark-grown seedlings of *Picea abies* (L.) Karst. accumulate Chl throughout their whole development and show a different control of ChlB expression. Analysis of the plastid ultrastructure, photosynthetic proteins by Western blotting and photosynthetic parameters by gas exchange and Chl fluorescence measurements provide additional experimental proofs for differences between dark and light Chl biosynthesis in spruce and larch seedlings.

Keywords Chlorophyll biosynthesis · Light-independent protochlorophyllide oxidoreductase · Gymnosperms · Plastids · RNA-editing

Abbreviations

A	Net photosynthetic rate
ALA	5-Aminolevulinic acid
Chl	Chlorophyll
Chlide	Chlorophyllide
chlLNB	*chlL, chlN, chlB* genes
cpDNA	Plastid DNA
DPOR	Light-independent protochlorophyllide oxidoreductase
GluTR	Glutamyl-tRNA reductase
LHC2	Light-harvesting complexes associated with photosystem II
LPOR	Light-dependent NADPH-protochlorophyllide oxidoreductase
Pchlide	Protochlorophyllide
PLB	Prolamellar body
RD	Respiration rate
ΦPSII	Effective quantum yield of PSII

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Introduction

Light is the most important environmental modulator of plant morphogenesis including the control of chloroplast

biogenesis (Hudák et al. 2005; Jiao et al. 2007). Chl biosynthesis is strictly light-dependent in angiosperm plants at the photoreduction step of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) (Schoefs 1999). This reaction is catalyzed by the light-dependent NADPH:protochlorophyllide oxidoreductase (LPOR), which is encoded in the nuclear genome (Teakle and Griffiths 1993; Masuda and Takamiya 2004) and imported to the plastids as a cytosolic precursor (Reinbothe et al. 1995; Aronsson et al. 2003; Schemenewitz et al. 2007). In etiolated angiosperms Pchlide accumulates and at the same time the synthesis of Pchlide precursor 5-aminolevulinic acid (ALA) are repressed by an unknown feedback mechanism. It is assumed that the rate limiting ALA synthesis is transcriptionally and post-translationally regulated, and it is likely that this control takes place at the first committed enzymatic step in the tetrapyrrole biosynthesis, the reduction of glutamate by glutamyl-tRNA reductase (GluTR) (for review: Papenbrock and Grimm 2001; Tanaka and Tanaka 2006). It is likely that these control mechanisms function to coordinate Chl synthesis tightly with the expression and assembly of the pigment–proteins of the photosystems I and II (PSI and PSII), respectively. Moreover, this coordination involves complex anterograde and retrograde signaling between chloroplast and nucleus (Gálová et al. 2000; Beck 2005; Tanaka and Tanaka 2007).

Contrary to angiosperms, gymnosperm plants exhibit unique properties for a controlled assembly of Chl-binding proteins. Seedlings of many gymnosperm species are able to synthesize Chl and to develop etiochloroplasts in the dark (Walles and Hudák 1975; Fujita and Bauer 2003) using an additional Pchlide-reducing enzyme, the light-independent protochlorophyllide oxidoreductase (DPOR). DPOR consists of three subunits, which are encoded by the plastid genes *chlL*, *chlN* and *chlB,* respectively. Skinner and Timko (1999) confirmed a positive correlation between the level of *chlL*, *chlN* and *chlB* expression and the efficiency of Chl synthesis in dark-grown *Pinus taeda* seedlings. The most effective light-independent Chl biosynthesis in conifer seedlings occurs in their cotyledons. Generally, the light-independent Chl biosynthesis is also accompanied with the accumulation of proteins of the two photosystems as well as with the differentiation processes of the thylakoid membranes (Mariani et al. 1990; Yamamoto et al. 1991; Peer et al. 1996; Muramatsu et al. 2001; Hudák et al. 2005).

However, high diversity has been observed in the efficiency of Chl synthesis and in the biogenesis of the photosynthetic apparatus among dark-grown gymnosperm species. Among conifers *Larix decidua* seedlings show a low ability to synthesize Chl in the dark, while *P. abies* accumulates the highest amounts of Chl of all Pinaceae during skotomorphogenesis (Fujita and Bauer 2003).

Chl synthesis in the dark depends on tissue specificity and various environmental factors (Fujita and Bauer 2003; Kusumi et al. 2006). Karpinska et al. (1997) described species and tissue-specific differences in post-transcriptional modification of *chlB* transcripts in *P. abies*, *L. eurolepis* and *Pinus sylvestris*. RNA-editing is required for restoration of conserved amino acids in the central region of ChlB. These exchanges of nucleotides in codons by RNA-editing can be classified as nonsynonymous substitutions: polar hydrophilics are replaced by hydrophobic amino acid residues. Therefore, it was suggested that RNA-editing probably plays a role in the recovery of enzyme function (Karpinska et al. 1997; Tillich et al. 2006).

We aimed to unravel the regulatory mechanisms that limit the dark Chl synthesis in gymnosperms. We focused on the key regulatory steps in the Chl biosynthetic pathway: synthesis of ALA and reduction of Pchlide during the development of *L. decidua* and *P. abies* seedlings. Expression of *chlLNB* genes, *chlB* RNA-editing efficiency and accumulation of DPOR subunits were investigated in response to the developmental stages of seedlings growing in the dark and after illumination. This study shows that the presence of DPOR-ChlB subunit is posttranscriptionally and developmentally regulated in larch and spruce seedlings. We combined molecular and physiological approaches for improved characterization of species-specific variability in plastid biogenesis during skotomorphogenesis and initial photomorphogenesis of conifers. The acquired knowledge should be useful for recent and further attempts to manipulate the light-independent Chl biosynthesis in higher plants.

Materials and methods

Plant material and growth condition

Picea abies (L.) Karst. and *L. decidua* Mill. seeds obtained from Semenoles (Liptovský Hrádok, Slovakia) were soaked for 24 h and germinated in well-moistened vermiculite for 14 days in complete darkness at 25°C. For analysis of earlier developmental stages, 7-day-old seedlings were used. Subsequently, for light-treatment experiments, 14-day-old dark-grown seedlings were light exposed at 100 μmol photons m^{-2} s^{-1} PAR in a cultivation chamber at 25°C. All manipulations of dark-grown seedlings were performed under dim green light. The safety of the dim green light was checked in etiolated barley leaves and the light did not cause Pchlide phototransformation. In some experiments, the seedlings grown in light/dark regime (14 h light at 100 μmol photons m^{-2} s^{-1} per 10 h dark) were used.

Determination of ALA synthesizing capacity

ALA synthesizing capacity was determined according to Alawady and Grimm (2005). To inhibit ALA metabolism, 100 mg of cotyledon segments from dark-grown and illuminated seedlings were incubated with 40 mM levulinic acid in 20 mM phosphate buffer (pH 7.1) in light (100 μmol photons m^{-2} s^{-1} PAR) or in darkness for 4 h. After homogenizing the cotyledons in liquid nitrogen and centrifugation, the supernatant was boiled with ethylacetoacetate for 10 min and mixed with an equal volume of Ehrlich's reagent. ALA derivatives were spectrophotometrically quantified at λ 553 nm (Jenway 6400, London, UK) using an ALA calibration curve.

Pigment analysis

Samples (100 mg of cotyledons) were ground with mortar and pestle and extracted with 80% (v/v) chilled acetone and $MgCO_3$ to avoid acidification and phaeophytinization of pigment. After centrifugation the extracts were spectrophotometrically quantified using single beam spectrophotometer (Jenway 6400): Chl *a* at 663.2 nm, Chl *b* at 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 from 100 mg of hot steam treated (2 min at 70°C) cotyledons was extracted in 3 ml acetone:0.1 M NH_4OH (9:1, v/v). The extract was washed three times with 1 vol hexane. After this procedure esterified tetrapyrroles were removed by hexane. The amount of Pchlide was measured spectrofluorometrically (Hitachi, Tokyo, Japan) at λ_{ex} 438 nm and λ_{em} 633 nm (ex slit, 5 nm; em, 2.5 nm) in acetone phase and quantified using a Pchlide standard prepared from etiolated barley (*tigrina* mutant) 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 one-way analysis of variance was performed (ANOVA, Statgraphics). All measurements were carried out with 5 independent repetitions.

Isolation of proteins and Western-blot analysis

Extraction of total proteins from cotyledons, SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described in Kruse et al. (1995). Twenty microgram protein samples were electrophoresed in 12% (v/v) SDS-polyacrylamide gel followed by transfer to the Hybond-C membranes (Amersham, Freiburg, Germany). Antibodies against *Plectonema boryanum* ChlB and *Marchantia polymorpha* ChlL were provided by Y. Fujita, Nagoya, Japan, and *Pisum sativum* D1 protein by J. Komenda, Třeboň, Czech Republic. Antibodies against GluTR were raised in the laboratory of prof. B. Grimm. Antibodies against LHC2b were purchased from Agrisera (Vännäs, Sweden).

Isolation of nucleic acids and reverse-transcription PCR

Plastid DNA was isolated from *L. decidua* and *P. abies* cotyledons with a few modifications (Triboush et al. 1998). Total RNA was isolated from the dark-grown and illuminated *P. abies* and *L. decidua* cotyledons using a modified method of Wang et al. (2005). Cotyledons were homogenized in liquid nitrogen and resuspended in a 1 ml extraction buffer. Upon the addition of 3% (w/v) polyvinyl pyrrolidone to the extraction buffer, the samples were vigorously shaken for 15 min at room temperature before chloroform/isoamylalcohol was added for extraction. All RNA samples were treated with RQ1 RNase-free DNase I (Promega, Madison, WI, USA). Lack of contaminating DNA in the purified RNA samples was confirmed by PCR using the primers designed for RT-PCR. The first strand cDNA was synthesized from 2 μg of purified RNA using an ImProm-II™ Reverse Transcription System (Promega) and universal random primers. RT-PCR was performed using two-step PCR and gene-specific primers: for *chlL*: RTLf 5′-TGTATTAGGCGACGTGGTTTGT-3′ and RTLr 5′-CTGCAAATAATGCATCGAATCC-3′; for *chlN*: RTNf 5′-CCGGAATGGCTCATGCTAAC-3′ and RTNr 5′-TCTCGCATTGGCAAATCCA-3′; for *chlB*: RTBf 5′-CGTTTATTAAAAGATCTGGACATCAGA-3′ and RTBr 5′-GTAGATACATAAGGCATTCCAAATTC-3′; for 23S rRNA: 23SRTf 5′-GTTGCAACTTGCGAATGTGCAGA-3′ and 23SRTr 5′-GCTTAACATCCTCAGTGGCATTCG-3′. The template was denaturated at 94°C for 2 min, followed by 30 cycles at 94°C for 15 s, 60°C for 1 min. PCR products were examined on 2% (w/v) agarose gels and stained with ethidium bromide.

Sequencing

For amplification and sequencing of the *L. decidua chlL*, *chlN* and *chlB* gene homologs, primers were designed based on the available sequence data for *Pinus thunbergii* (NC_001631), *Pinus koraiensis* (NC_004677), *P. abies* (AJ001025) and *Marchantia polymorpha* (NC_001319). For amplification of *chlL* we used the following primers: LF1 5′-ATTGCCATCTGCCTAATGCC-3′, LF2 5′-GGC

TTAACCTGTCAATCCAC-3′, LR1 5′-CGGTACTTGG CATGAATTCT-3′; for amplification of *chlN*: LF3 5′-TG GTTTGAAATTCTGTCC-3′, NR2 5′-GGAGTACTGGTA ATAAACTCG-3′ and for *chlB*: BF4 5′-ATGAAATTAGC CCATTGGATG-3′, BR3 5′-GTCCCTTACAAAACGGG GGAT-3′. PCR fragments were subcloned into the pBluescript II KS + vector (Stratagene, La Jolla, CA, USA). Plasmid DNAs were purified by QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and sequenced with ABI 3100-Avant Genetic Analyser (Applied Biosystems, Foster City, CA, USA) using standard M13 sequencing primers. New sequences were submitted to the NCBI GenBank.

Restriction analysis of *chlB* editing sites

The editing of CGG to UGG in *chlB* transcripts of *L. decidua* and *P. abies* was examined by restriction digestion of 170 bp *chlB* cDNA amplicons (amplified by using the same primers and amplification program as for RT-PCR) by *Hin*dIII (Fermentas, St. Leon-Rot, Germany). An additional predicted site for C to U substitution in a CCA codon 19 bp upstream from the CGG codon was found by *Bam*HI (Fermentas) digestion of *L. decidua chlB* cDNA amplicons. As control PCR fragments derived from plastid DNA were also digested using the same primer combination. All cDNA amplicons were purified before digestion using QIAquick PCR purification Kit (Qiagen). After digestion the samples were examined on 8% (v/v) polyacrylamide gel and stained with ethidium bromide.

Gas exchange and chlorophyll fluorescence measurements

Gas exchange in cotyledons was measured using infrared gas analyser (CIRAS-2, PP-Systems, Hitchin, UK) in seedlings growing 14 days in the dark and after 6, 12, 24, 48 h of illumination (100 µmol m^{-2} s^{-1} PAR). Prior to the measurement, cotyledons from four to five seedlings were placed in the PLC6 automatic universal leaf cuvette, and after 10 min of stabilization, rate of photosynthesis was measured three-times independently at 500 µmol m^{-2} s^{-1} PAR, 360 vpm CO_2 and 25 ± 1°C. Subsequently, the rate of dark respiration (R_D) was measured after 10 min of stabilization in the dark. In-vivo Chl fluorescence was carried out with pulse amplitude modulated systems (FMS-2, Hansatech, Norfolk, UK). We used 7 and 14-day-old dark-grown seedlings, light/dark-grown seedlings and 14-day-old dark-grown seedlings after 1, 6, 12, 24, 48 h of illumination (100 µmol m^{-2} s^{-1} PAR). Maximal fluorescence in light adapted state (F'_m) was obtained after 10 min of actinic irradiance using a saturation flash of 800 ms duration at 8,000 µmol m^{-2} s^{-1} PAR. Steady state fluorescence in light adapted state (F_t) was measured at 100 µmol m^{-2} s^{-1} PAR actinic light. After 10 min of illumination, the Pchlide content decreased, so the contribution of Pchlide to F_t was zero. Effective quantum yield of PSII photochemistry (ΦPSII) was calculated as $(F'_m - F_t):F'_m$ (Maxwell and Johnson 2000).

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 ACM (Fluka, Seelze, Germany). Ultrathin sections were cut on ultramicrotome Reichert-Jung E (Vienna, Austria), stained with uranyl acetate and lead citrate and observed using an Jeol 2000 FX electron microscope (Tokyo, Japan).

Results

Synthesis of Chl, Chl precursors and protein analysis

Differences in pigment contents were examined in dark-grown and illuminated *P. abies* and *L. decidua* seedlings (Fig. 1). Dark-grown *P. abies* seedlings had a continuous synthesis of Chl during development. A small increase of the ALA synthesizing capacity and Chl content was observed in the 14-day-old seedlings compared to the 7-day-old seedlings (Table 1). In cotyledons of 7 and 14-day-old seedlings the levels of Pchlide showed similar values. Illumination of the dark-grown 14-day-old seedlings for 24 h stimulated the rate of ALA synthesis and the Chl content increased. After illumination of the seedlings the Chl *a*/*b* ratio decreased. No Pchlide was determined in cotyledons of illuminated seedlings (Table 1). Parallel to analysis of ALA synthesis we determined the light-independent presence of glutamyl-tRNA reductase (GluTR) in 7 and 14-day-old spruce seedlings. The GluTR content slowly increased after illumination of seedlings (Fig. 2). Consistent with the Chl content in dark-grown seedlings LHC2b and D1 proteins of PSII were present in the protein extract (Fig. 2).

In contrast, the plastid development differed in dark-grown *L. decidua* seedlings. They synthesized Chl only in their initial development in darkness and showed a decrease in Chl content in later stages (Fig. 1; Table 2). Both Chl *a* and Chl *b* decreased equally as indicated the similar Chl *a*/*b* ratio (Table 2). In etiolated cotyledons of the 14-day-old seedlings the level of Pchlide also declined in comparison with cotyledons of 7-day-old seedlings. A similar decline of ALA synthesis rate was observed

Fig. 1 Seedlings of *P. abies* and *L. decidua* at different developmental stages. **a** 7-day-old dark-grown *P. abies*. **b** 14-day-old dark-grown *P. abies*. **c** 14-day-old dark-grown *P. abies* after exposition to light for 24 h. **d** 7-day-old dark-grown *L. decidua*. **e** 14-day-old dark-grown *L. decidua*. **f** 14-day-old dark-grown *L. decidua* after exposition to light for 24 h. *Bar* 1 cm for each panel

(Table 2), which was confirmed by reduced GluTR content (Fig. 2). The amount of LHC2b and D1 proteins in 14-day-old dark-grown seedlings declined simultaneously with the Chl content. On the other hand, illumination of the etiolated seedlings led to increased Chl content and Chl *a*/*b* ratio, an elevated rate of ALA synthesis and increased contents of GluTR, LHC2b and D1 (Table 2; Fig. 2).

ChlB and ChlL levels in *P. abies* did not considerably change during the phase of seedling development in the dark and successive 24-h exposure to light (Fig. 2).

During *L. decidua* seedling development, the levels of the ChlB subunit of DPOR decreased. Maximal ChlB content was found in the 7-day-old dark-grown seedlings, while its content decreased in the 14-day-old dark-grown seedlings and upon subsequent 24 h illumination. ChlL

Table 1 ALA-synthesizing capacity, Pchlide and Chl content in *P. abies* seedlings

P. abies	7 days dark	14 days dark	14 days dark + 24 h illumination	14 days light/dark
ALA synthesizing capacity	13.7 ± 0.4a	17.4 ± 1.4b	25.9 ± 2.1c	22.4 ± 2.2c
Pchlide	15.8 ± 0.4a	15.6 ± 0.3a	ND	ND
Chl *a* + *b*	778.6 ± 54.6a	900.7 ± 43.5a	1313.8 ± 69.7b	2065.0 ± 87.0c
Chl *a*/*b*	3.98 ± 0.06a	3.95 ± 0.07a	2.20 ± 0.04b	2.15 ± 0.03b

P. abies: ALA synthesizing capacity (nmol ALA g^{-1} FW h^{-1}), Pchlide content (nmol Pchlide g^{-1} FW), Chl *a* + *b* content (nmol Chl g^{-1} FW) and Chl *a*/*b* ratio in cotyledons of 7-day-old and 14-day-old dark-grown seedlings, 14-day-old dark-grown seedlings after exposition to light for 24 h and for comparison 14-day-old light/dark-grown seedlings. *ND* not detectable. Values with different letters denote statistically significant differences at $P < 0.05$ (LSD-test), $n = 5$

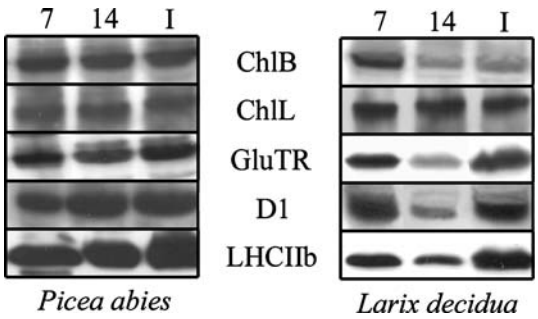

Fig. 2 Western-blot analysis of ChlB, ChlL, GluTR, D1 and LHC2b proteins in *P. abies* and *L. decidua* seedlings. *Lane 7*, 7-day-old dark-grown seedlings; *lane 14*, 14 day old-dark-grown seedlings; *lane I*, 14-day-old dark-grown seedlings after exposition to light for 24 h

subunit was present in all investigated developmental stages of the seedlings (Fig. 2).

The primary structure of the *chlLNB* genes in *L. decidua*

Using primers to amplify *L. decidua* DPOR encoding sequences we sequenced 876 bp of the entire *chlL* coding sequence, and partial sequences of the *chlN* and *chlB* genes (1,398 bp and 1,446 bp fragments, respectively). The indicated sequences were submitted to the NCBI GeneBank under accession numbers AY603408, AY587603 and AY560614 for *chlL*, *chlN* and *chlB*, respectively. The nucleotide sequence of *L. decidua chlB* and the deduced amino acid sequence of the ChlB polypeptide suggest the presence of RNA-editing sites essential for maintaining conserved amino acids leucine and tryptophane at positions 210 and 217 of ChlB polypeptide, respectively (Table 3). The sequence of *L. decidua* ChlB was compared with the homologous sequences of *L. kaempferi* and the hybrid of both species *L. eurolepis* as well as with other species of gymnosperms and lower plants (Table 3). In contrast to both parental species (*L. decidua* and *L. kaempferi*), the hybrid *L. eurolepis* does not need RNA-editing for restoration of conserved leucine at position 210 of the ChlB sequence. It was striking that the conserved leucine of the hybrid *L. eurolepis* ChlB is correctly encoded in cpDNA, while both parental species contain a codon for the amino acid proline at the corresponding position of *chlB* gene (Table 3).

Expression of *chlL*, *chlN* and *chlB* genes

Referring to the different pigment contents during the development of *P. abies* and *L. decidua* seedlings, it is suggested that the changes in Chl contents during the studied developmental phases correlate with changes in the expression of important genes involved in the Chl synthesis. Transcript levels of *chlL*, *chlN* and *chlB* genes were analyzed in both species. However, semiquantitative RT-PCR did not

Table 2 ALA-synthesizing capacity, Pchlide and Chl content in *L. decidua* seedlings

L. decidua	7 days dark	14 days dark	14 days dark + 24 h illumination	14 days light/dark
ALA synthesizing capacity	15.8 ± 0.3a	7.7 ± 0.9b	92.7 ± 4.6c	66.0 ± 2.5d
Pchlide	13.5 ± 0.6a	7.8 ± 0.4b	ND	ND
Chl *a* + *b*	303 ± 8.2a	60.7 ± 3.4b	406.4 ± 21.9c	1530 ± 28.0d
Chl *a*/*b*	2.25 ± 0.05a	2.20 ± 0.07a	2.41 ± 0.03b	2.46 ± 0.06b

L. decidua: ALA synthesizing capacity (nmol ALA g^{-1} FW h^{-1}), Pchlide content (nmol Pchlide g^{-1} FW), Chl *a* + *b* content (nmol Chl g^{-1} FW) and Chl *a*/*b* ratio in cotyledons of 7-day-old and 14-day-old dark-grown seedlings, 14-day-old dark-grown seedlings after exposition to light for 24 h and for comparison 14-day-old light/dark-grown seedlings. *ND* not detectable. Values with different letters denote statistically significant differences at $P < 0.05$ (LSD-test), $n = 5$

Table 3 Comparison of deduced amino acid sequences of a central region of ChlB polypeptides

Species	Deduced amino acid sequences
	210 **217**
Larix decidua	EDPKNLPKARFN CCA CGG
Larix kaempferi	EDPKNLPKARFN CCA CGG
Larix eurolepis	EDLKNLPKARFN CTA CGG
Picea abies	EDSKNLPKARFN
Pinus thunbergii	EDPKNLPKARFN
Abies alba	EDLKNLPKAWFN
Taxus baccata	EDLKNLPKAWFN
Taxodium distichum	EDLKNLPKAWFN
Thuja occidentalis	EDLQNLPKAWFN
Cupressus sempervirens	EDLQNLPKAWFN
Juniperus chinensis	EDLKNLPKAWFN
Sequoiadendron giganteum	EDLKSLPKAWFN
Sequoia sempervirens	EDLQNLPKAWFN
Cycas revoluta	KDLINLPRAWFN
Ginkgo biloba	EDLKNLPKAWLN
Marchantia polymorpha	ENLHELPKAWFN
Physcomitrella patens	ENLHELPKAWFN

Positions of conserved amino acids (*bolded* and *underlined*) are signed by position numbers in *Larix* species. Appertaining cpDNA triplet sequences (with underlined cytidine to be edited at RNA level) are under the corresponding amino acids. In the case of *L. decidua*, *L. kaempferi*, *P. abies* and *P. thunbergii,* RNA-editing is required for restoration of codons for conserved leucine (L) and tryptophane (W). In the case of hybrid *L. eurolepis*, the triplet sequence coding for conserved L is already present in the DNA. All sequences were obtained from GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/). The GenBank accession numbers for particular sequences: *Larix decidua* (AY560614), *Larix kaempferi* (AB232500), *Larix eurolepis* (X98679), *Picea abies* (X98685), *Pinus thunbergii* (NC_001631), *Abies alba* (X98570), *Taxus baccata* (X98571), *Taxodium distichum* (AB232474), *Thuja occidentalis* (AB232496), *Cupressus sempervirens* (AB232482), *Juniperus chinensis* (AB232486), *Sequoiadendron giganteum* (AB232466), *Cycas revoluta* (X98572), *Ginkgo biloba* (U01531), *Marchantia polymorpha* (NC_001319), *Physcomitrella patens* (NC_005087)

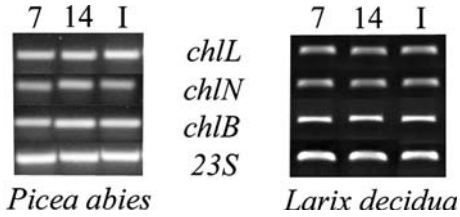

Fig. 3 RT-PCR analysis of *chlL*, *chlN* and *chlB* gene expression in cotyledons of *P. abies* and *L. decidua*. *Lane 7*, 7-day-old dark-grown seedlings; *lane 14*, 14-day-old dark-grown seedlings; *lane I*, 14-day-old dark-grown seedlings after exposition of the seedlings to light for 24 h

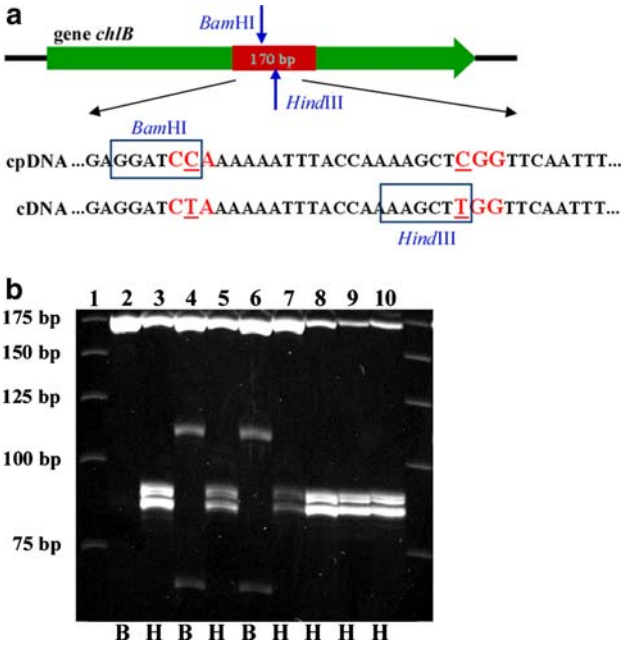

Fig. 4 Restriction analysis of *chlB* RNA-editing in *L. decidua* and *P. abies* seedlings. **a** Schematic representation of central region of *L. decidua chlB* gene with cpDNA sequence (*Bam*HI-recognition site is present) and corresponding cDNA sequence after efficient RNA-editing of underlined cytidines (*Bam*HI-recognition site disappear and *Hin*dIII-recognition site arise). **b** Electrophoretic separation of *Bam*HI and *Hin*dIII-digested 170 bp amplicons of cpDNA and cDNA (represented by *red panel* in **a**. *Lane 1*, DNA ladder; *lanes 2–3*, 7-day-old dark-grown *L. decidua* seedlings; *lanes 4–5*, 14-day-old dark-grown *L. decidua* seedlings; *lanes 6–7*, 14-day-old dark-grown *L. decidua* seedlings after exposition to light for 24 h; *lane 8*, 7-day-old dark-grown *P. abies* seedlings; *lane 9*, 14-day-old dark-grown *P. abies* seedlings; *lane 10*, 14-day-old dark-grown *P. abies* seedlings after exposition to light for 24 h. B, *Bam*HI (do not cleaves "edited" cDNA amplicons, cleaves 170 bp "unedited" cDNA amplicons to 60 and 110 bp fragments); H, *Hin*dIII (cleaves the 170 bp "edited" cDNA amplicons to 80 and 90 bp fragments, do not cleaves "unedited" cDNA amplicons)

confirm any significant differences in the expression of all three genes in the 7 and 14-day-old dark-grown seedlings (Fig. 3), although pronounced limitations in Chl and Pchlide contents and the decreased rate of ALA synthesis were observed during the development of dark-grown *L. decidua* seedlings (Table 2). Similarly, illumination of the 14-day-old etiolated seedlings for 24 h had no substantial effect on the level of *chlLNB* gene expression.

Editing of *chlB* transcripts

Restriction analysis of *chlB* amplicons was performed to reveal the efficiency of RNA-editing in *chlB* transcripts of *L. decidua* cotyledons. A 170 bp fragment of *chlB* cDNA was amplified, which contained the two potential C-U editing sites (Fig. 4a). Correct substitution of cytidine for uridine in the second position of CCA codon resulted in a disappearance of the recognition site for *Bam*HI in cDNA sequence. The amplicons representing the edited transcript sequences in the CCA codon could not be digested with *Bam*HI. Conversely, correctly performed C–U substitution

in the CGG codon (19 bp downstream from the CCA codon) gave rise to a new recognition site in the cDNA sequence for *Hin*dIII and the edited transcripts were represented by *Hin*dIII-cleavable cDNA fragments (Fig. 4b). For the *P. abies chlB* transcripts only editing of the CGG codon was revealed by this restriction digestion with *Hin*dIII because there is no *Bam*HI restriction site in the upstream-located edited codon. Although a pronounced change in the amount of the *chlB* transcript was not observed, we found an apparent decrease in the editing efficiency of the CGG codon in the *chlB* transcripts depending on the developmental stage of the larch seedlings. The highest efficiency of *chlB* editing was found in the 7-day-old dark-grown seedlings. In cotyledons of etiolated 14-day-old seedlings the portion of edited transcripts decreased and remained low even after 24 h of illumination (Fig. 4b). The CCA codon in larch *chlB* transcripts was edited efficiently in all three developmental stages examined, with only minimal decrease in 14-day-old dark-grown seedlings and after exposition of seedlings to light for 24 h.

Restriction analysis of RNA-editing efficiency in CGG codon of *P. abies chlB* transcripts indicated high effective C to U substitution both during skotomorphogenesis and after exposition of seedlings to light for 24 h (Fig. 4b).

Gas exchange and chlorophyll fluorescence measurements

Picea abies had a low net CO_2 uptake early after the transition from the dark to the light, but the net photosynthetic rate (A) was close to zero (Fig. 5a). This is consistent with the higher values of ΦPSII in spruce (Fig. 5b). Efficient quantum yield represents the proportion of absorbed energy used in photochemistry of PS II, and it may thus reflect the rate of photosynthesis (Maxwell and Johnson 2000). On the contrary, etiolated dark-grown *L. decidua* seedlings initially released CO_2 after being transferred from dark to light conditions (Fig. 5a).

Picea abies had similar ΦPSII in the 7 and 14-day-old seedlings (0.43 ± 0.01, 0.44 ± 0.01; *t* test, $P > 0.05$). On the other hand, 7-day-old dark-grown larch seedlings exhibited much higher ΦPSII than the 14-day-old seedlings (0.40 ± 0.01, 0.15 ± 0.05; *t* test, $P < 0.01$). After transferring the seedlings from the dark to the light, photosynthetic parameters substantially increased, especially in larch (Fig. 5a, b). After 12 h of illumination, the A-values of the two species were similar (3.9–4.6 nmol CO_2 g^{-1} DW s^{-1}); but upon extended illumination, the A-value was much higher for larch (104 nmol CO_2 g^{-1} DW s^{-1}) than for spruce (37 nmol CO_2 g^{-1} DW s^{-1}). Noteworthily, a higher

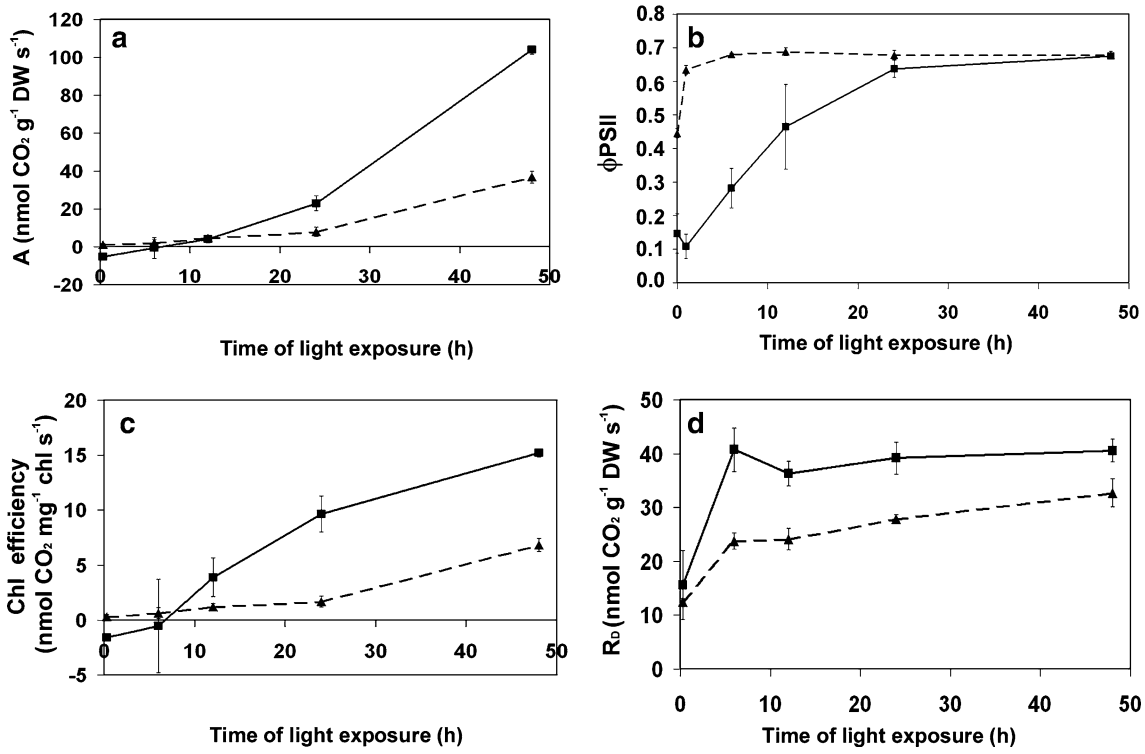

Fig. 5 Chlorophyll fluorescence and gas exchange measurements in *P. abies* and *L. decidua* seedlings. Seedlings were grown 14 days in the dark (0 h) and than were exposed to light. **a** Net photosynthetic rate. **b** Effective quantum yield of PSII. **c** Chlorophyll efficiency. **d** Respiration rate. *P. abies* (*full triangle/broken lines*), *L. decidua* (*full square/solid lines*)

Chl efficiency was determined in larch despite its much lower Chl content (Fig. 5c). The PSII quantum efficiency in spruce achieved within 6 h of illumination was comparable with that of seedlings growing in the light/dark regime (0.67 ± 0.01, 0.67 ± 0.02; t test, $P > 0.05$). An extended time of illumination (48 h) was necessary for similar balancing of PSII quantum efficiency in etiolated larch seedlings (0.68 ± 0.01, 0.69 ± 0.01; t test, $P > 0.05$). The rate of respiration also increased after illumination in both species, although a higher respiration rate was observed in larch (Fig. 5d).

Plastid ultrastructure

Similar ultrastructural features of plastids were observed in cotyledons of 7-day-old and 14-day-old dark-grown *P. abies* seedlings. These plastids contained prominent starch grains, which filled the stroma with their considerable large volume making the plastid shape irregular. Numerous thylakoid membranes including grana stacks composed of two to ten membranes were the most remarkable structural feature in these plastids (Fig. 6a, b). Thylakoid membranes were frequent and evenly spread through the plastidic stroma. Spruce plastids also contained prolamellar bodies (PLBs) and were typical etiochloroplasts (Fig. 6a, b).

The ultrastructure of *L. decidua* plastids from dark-grown seedlings changed depending on the length of cultivation. The green cotyledons of the 7-day-old seedlings possessed plastids with a typical thylakoid structure containing grana membrane stacks composed of two to six lamellae (Fig. 6c). In plastid stroma there were starch grains of variable size and a prominent PLBs.

The 14-day-old dark-grown seedlings were pale yellow (Fig. 1e). The membrane system of plastids from these cotyledons was poorly developed (Fig. 6d). The grana stacks were minute and consisted of only two or three thylakoid membranes. PLBs are typical for their narrow spacing tubules (Fig. 6d). Stroma frequently contained starch grains. Plastids from *L. decidua* seedlings represented typical etiochloroplasts in the 7-day-old seedlings but resembled rather etioplasts in 14-day-old seedlings (Fig. 6c, d).

Discussion

It is assumed that in phototrophic organisms different control mechanisms contribute to light-independent Chl biosynthesis including expression and activity of DPOR (Fujita and Bauer 2003; Yamazaki et al. 2006). In our experiments, we used *L. decidua* and *P. abies* seedlings, which both clearly differ in their ability to synthesize Chl in the dark, and focused on regulatory mechanisms in this metabolic pathway. Drazic and Bogdanovic (2000) reported on similar ALA synthesizing activities in both light- and dark-grown *Pinus nigra* seedlings with gradual increase of ALA levels during seedling development. This previous finding resembles more our recent data obtained with spruce seedlings. It seems that in some conifer species light does not always significantly induce GluTR accumulation and ALA synthesis rate. The equal cellular abundance of

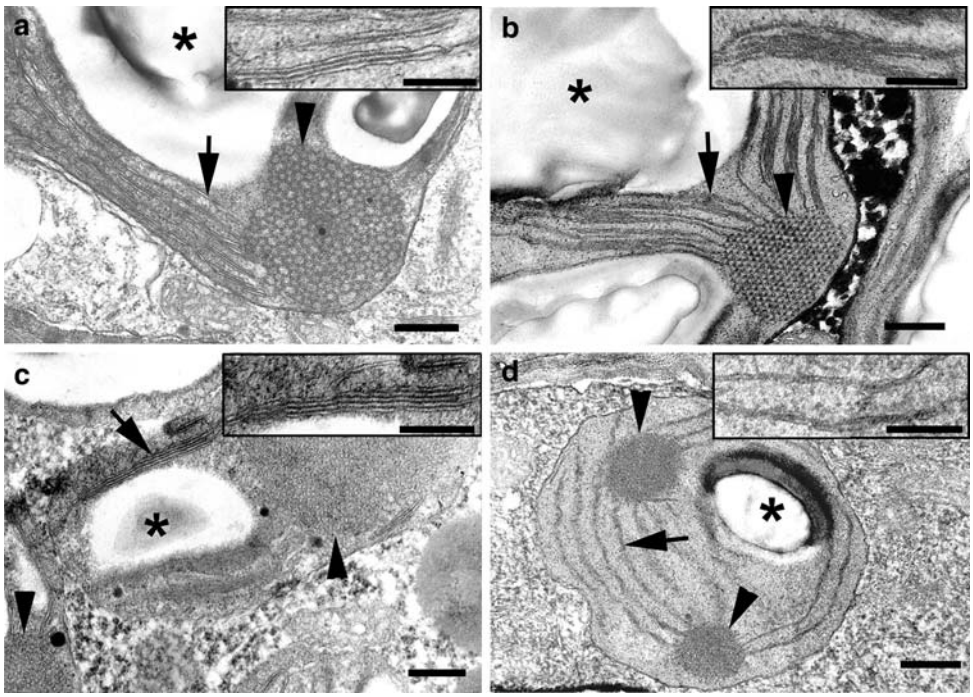

Fig. 6 Plastid ultrastructure in dark-grown *P. abies* and *L. decidua* seedlings. **a** 7-day-old *P. abies*. **b** 14-day-old *P. abies*. **c** 7-day-old *L. decidua*. **d** 14-day-old *L. decidua*. *Arrow heads* indicate prolamellar bodies, *arrows* indicate thylakoid membranes, *stars* indicate starch grains. The inserted panels show thylakoid membranes of corresponding plastids in detail. *Bar* 400 nm

GluTR was also described in both dark and light-grown *Chlamydomonas reinhardtii* (Nogaj et al. 2005), although posttranslational modifications of GluTR cannot be excluded. These observations are in contrast with the light induced expression of the GluTR-encoding *HEMA* gene and ALA synthesis in the photosynthetic tissue of angiosperms (Ilag et al. 1994; Kruse et al. 1997).

We have proved that GluTR accumulation and ALA synthesis in larch seedlings depend on their developmental stage and are markedly enhanced by illumination after transition of etiolated seedlings from the dark to the light (Fig. 2; Table 2). In early developmental stages of dark-grown larch seedlings a high light-independent accumulation of GluTR correlates with elevated ALA synthesis and Chl content. DPOR subunits are present for the catalytic reduction of the accumulating Pchlide, enabling to complete Chl synthesis for stabilizing the LHC2b and D1 proteins in developing thylakoid membranes. The well-developed plastid thylakoid membranes concord with the higher ΦPSII in this stage (Fig. 5b). However, in the larch seedlings the Chl level declines with prolonged skotomorphogenesis. This physiological switch is assumed to be accompanied by decrease of GluTR followed by reduced ALA synthesizing capacity, Pchlide and Chl content and as a consequence decreased ΦPSII. So, in this respect, mature larch seedlings resemble angiosperm plants in their behavior.

Another important regulatory step in the light-independent Chl biosynthetic pathway is reduction of Pchlide catalyzed by the DPOR complex. It has been suggested that low Chl synthesis of dark-grown larch species is a consequence of inappropriate processing of the *chlLN* polycistronic transcripts or synthesis of inactive ChlB polypeptide due to inefficient *chlB* RNA-editing (Karpinska et al. 1997; Armstrong 1998). The explanations imply that enough Pchlide is available, but DPOR is not sufficiently active. We showed that transcript levels of the *chlL*, *chlN* and *chlB* genes are similar in both 7 and 14-day-old dark-grown larch seedlings and remain constant after illumination of etiolated seedlings (Fig. 3). Constitutive expression of genes encoding DPOR has been reported in several other conifer species (Armstrong 1998; Skinner and Timko 1999; Kusumi et al. 2006).

Surprisingly, the main differences in the control of the *chlLNB* genes between young green and pale yellow mature larch cotyledons were the changes in efficiency of *chlB* RNA-editing and abundancy of the ChlB subunit during development in the dark (Figs. 2, 4b). While the possible role of *chlB* RNA-editing for developmentally regulated ChlB stability remain a speculation for now, there are certain experimental indications for energy state- and redox potential-dependent modifications in the synthesis and the assembly of the DPOR subunits. As the stoichiometric composition of the DPOR complex in plants still remains unclear (Fujita and Bauer 2003), it requires further studies to assign a precise contribution of ChlB to active DPOR in plants.

In *Chlamydomonas reinhardtii* the translation of ChlL is negatively photoregulated and this suppression can be overcome by DCMU-inhibition of photosynthetic electron flow (Cahoon and Timko 2000). Decreased ChlB accumulation has been reported in photoautotrophically grown *Chlorella protothecoides* in comparison with heterotrophically and mixotrophically grown cells (Shi and Shi 2006). It seems that differential expression of the DPOR subunits represent an important species-specific regulatory mechanism, which controls DPOR function in response to developmental and environmental stimuli.

Correlations between Chl content in dark-grown conifer seedlings and plastid ultrastructure were previously reported (Mariani et al. 1990; Hudák et al. 2005). In developing thylakoids Chl is necessary for stabilization of the LHC2 and other protein components of the two photosystems (Eichacker et al. 1990; Kim et al. 1994). Our electron microscopic analysis of the plastid ultrastructure in cotyledons of the 7-day-old dark-grown larch seedlings confirmed a presence of grana in comparison with 14-day-old seedlings, in which the thylakoid membranes are poorly developed (Fig. 6c, d). Also the higher accumulation of LHC2b and D1 as well as higher ΦPSII in 7-day-old seedlings is related to the formation of a complex thylakoid membrane structure. Prominent PLBs in plastids of both larch and spruce dark-grown seedlings represent potential reservoirs of Pchlide. However, it has been shown previously that even after a rapid decrease of Pchlide in illuminated seedlings, some of the plastids may still retain small PLBs several days after transferring them to light. This phenomenon was described both in conifers and angiosperm plants (Walles and Hudák 1975; Solymosi et al. 2007).

Two distinct forms of Pchlide have been identified in plastids of dark-grown tissues both in angiosperm and gymnosperm plants—photoactive and non-photoactive Pchlide (reviewed in Schoefs 1999). Interestingly, the non-photoactive Pchlide is preferentially located in prothylakoids and photoactive Pchlide in PLBs (Selstam et al. 1987; Böddi et al. 1989). The photoactive Pchlide could form complexes with NADPH and LPOR proteins and is fastly phototransformed in response to the illumination. Then the non-photoactive Pchlide can be progressively used to reload LPOR (Schoefs 2001).

The situation is more complicated in etiochloroplasts of conifer seedlings where two Pchlide-binding enzymes are present, LPOR and DPOR, respectively. Unfortunately, it is not known whether these two enzymes compete for Pchlide pools or Pchlide distribution is precisely regulated between LPOR and DPOR. Moreover, in contrast to LPOR, there

are no data for precise localization of the DPOR complex inside the plastids. As we showed the Pchlide and Chl content is significantly lower in 14-day-old dark-grown larch seedlings compared to 7-day-old seedlings. At the same time, the activity of DPOR is probably reduced, as the content of ChlB subunits decreased. Then, it is expected that enzymatic reduction of the remaining Pchlide will be processed predominantly by LPOR upon illumination. Exposure to light after the widely expanded dark period can cause light stress to the larch seedlings. Prolonged acclimation could be required to recover for photoperiodic growth. Similar situation was thoroughly investigated in etiolated *Ginkgo biloba* stems, which are not able to synthesized Chl in darkness (Skribanek et al. 2008).

Increased Chl content after illumination of dark-grown larch and spruce seedlings reflects higher A and ΦPSII (Fig. 5a, b). Shinohara et al. (1992a) founded that all electron carriers of the photosynthetic apparatus in *P. thunbergii* cotyledons are present in the dark, but in low abundance. The reaction center of PSII in dark-grown seedlings is functionally active, but evolving O_2 remains latent and Mn integration into the O_2 evolving enzymes is strictly light-dependent (Shinohara et al. 1992a, b). This explains the fact that despite the high Chl content in dark-grown spruce cotyledons, the A was close to zero after immediate illumination. Although light did not dramatically stimulate ALA synthesis and GluTR accumulation in spruce seedlings, it is obvious from Fig. 5 that A, ΦPSII and Chl efficiency gradually increased after extended light exposure. Synthesis of different components of the photosynthetic apparatus was accompanied with more intensive metabolism, which requires higher R_D (Fig. 5d).

In conclusion, although the transcriptional control of the genes for DPOR leads to constant transcript levels, the amount of ChlB subunit clearly decreased in etiolated larch cotyledons. Our results indicate that efficient *chlB* RNA-editing is attenuated in 14-day-old larch seedlings in comparison with green cotyledons of 7-day-old dark-grown seedlings (Fig. 4). After illumination of etiolated larch seedlings photosynthesis, ALA accumulation and Chl biosynthesis are highly activated. These dramatic changes were not observed in developing spruce seedlings, which accumulate Chl at least 14 days in the dark. The etiolation of initially green larch cotyledons is not only the result of impaired Pchlide reduction, but also the consequence of a complex developmentally regulated decrease of metabolic flow into the Chl biosynthetic pathway.

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