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BRIEF COMMUNICATION

Influence of irradiance on chlorophyll synthesis in *Picea abies* calli cultures

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Abstract

Dark-grown seedlings of *Picea abies* (L) Karst. are able to accumulate the highest amounts of chlorophyll (Chl) and its precursor protochlorophyllide (Pchlide) in all *Pinaceae*, but calli derived from 14-d-old green cotyledons of *P. abies* are completely white during the cultivation in the dark. Pchlide reduction is catalysed in the dark by light-independent protochlorophyllide oxidoreductase (DPOR). This enzyme complex consists of three protein subunits ChlL, ChlN and ChlB, encoded by three plastid genes *chlL*, *chlN* and *chlB*. Using semiquantitative RT-PCR, we observed very low expression of *chlLNB* genes in dark-grown calli. It seems, that *chlLNB* expression and thus Chl accumulation could be modulated by light in *P. abies* calli cultures. This hypothesis is supported by the fact, that we observed low contents of glutamyl-tRNA reductase and Flu-like protein, which probably affected Chl biosynthetic pathway at the step of 5-aminolevulinic acid formation. ChlB subunit was not detected in dark-grown *P. abies* calli cultures. Our results indicated limited ability to synthesize Chl in callus during cultivation in the dark.

Additional key words: chlorophyllide, light-independent protochlorophyllide oxidoreductase, Norway spruce, plastid gene expression, protochlorophyllide reduction.

Plants depend on light signals to modulate many aspects of their development and to optimize their photosynthetic activity (Hudák *et al.* 2005). Chloroplast differentiation is connected with biosynthesis of chlorophylls (Chl; Tanaka and Tanaka 2007). In the first phase of tetrapyrrole biosynthesis, glutamate is reduced by glutamyl-tRNA reductase (GluTR) and converted to 5-aminolevulinic acid (ALA). In oxygenic phototrophs, the *trans*-reduction of protochlorophyllide (Pchlide) and its conversion to chlorophyllide (Chlide) is a key regulatory step of Chl biosynthesis (Fujita 1996). Two distinct ways of Chl synthesis have been described, depending on whether the enzymatic reduction of Pchlide to Chlide requires light as an essential factor (Armstrong 1998). In angiosperms the reduction of Pchlide is catalyzed by nuclear encoded, light-dependent NADPH:protochlorophyllide oxidoreductase (LPOR) (Schoefs 1999). However, in some plant species the development of functional chloroplasts and Chl synthesis are not exclusively dependent on light. For example, gymnosperms possess a light-independent protochlorophyllide oxidoreductase (DPOR) that reduces Pchlide to Chlide irrespective of the presence of light. DPOR provides these organisms with the ability to synthesize Chl in the dark (Suzuki and Bauer 1992, Fujita 1996, Armstrong 1998, Fujita and Bauer 2003). This enzymatic complex consists of three protein subunits ChlL, ChlB and ChlN, encoded by three plastid genes *chlL*, *chlB* and *chlN*. Various conifers species display differences in their ability of chlorophyll accumulation. *Picea abies* seedlings accumulate the highest amounts of Chl of all *Pinaceae* during skotomorphogenesis (Fujita and Bauer 2003). At the other extreme, dark-grown cotyledons of *Larix decidua* produce very small amounts of Chl and hence green poorly. Vigorous light-independent Chl biosynthesis is usually said to be restricted to the cotyledons of gymnosperms (Von Wettstein *et al.* 1995). Activity of DPOR and Chl synthesis in the dark depends on the stage of ontogenesis,

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Abbreviations: ALA - 5-aminolevulinic acid; Chl - chlorophyll; Chlide - chlorophyllide; *chlLNB* - *chlL*, *chlN*, *chlB* genes; DPOR - light-independent NADPH-protochlorophyllide oxidoreductase; FLP - Flu-like protein; GluTR - glutamyl-tRNA reductase; NAA - 1-naphthaleneacetic acid; LPOR - light-dependent NADPH-protochlorophyllide oxidoreductase; Pchlide - protochlorophyllide.

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tissue specificity and various environmental factors (Fujita and Bauer 2003, Kusumi *et al.* 2006). It is mostly limited to young seedlings (Mariani *et al.* 1990).

The aim of this study was to determine, whether the genes encoding DPOR are expressed in the *P. abies* calli cultures, and establish what is the influence of light on their expression. We focused on GluTR and FLP protein accumulation, which are important in the early steps of Chl biosynthesis.

Picea abies (L.) Karst. seeds were soaked for 24 h and germinated in well-moistened *Perlite* in the dark at 23 ± 2 °C. Callus cultures were derived from cotyledons of 14-d-old seedlings. Cotyledones were sterilized with 2 % NaOCl for 10 min and washed several times with sterile distilled water. The explants were then transfered on Murashige and Skoog (1962; MS) medium containing naphthaleneacetic acid (NAA; 2 mg dm^{-3}), kinetin (1 mg dm^{-3}) and sucrose (20 g dm^{-3}). The medium was solidified with agar (7 g dm^{-3}). Calli were grown in cultivation chamber at 23 ± 2 °C, either with 16-h photoperiod (irradiance of 40 μmol m^{-2} s^{-1}) or in the dark.

Samples (over 200 mg of callus or cotyledones) were ground with mortar and pestle and extracted with chilled acetone (80 %) and $MgCO_3$. After centrifugation the extracts were then quantified spectrophotometrically (spectrophotometer *Jenway 6400*, London, UK; Chl *a* at 663.2 nm, Chl *b* at 646.8 nm) and calculated according to Lichtenthaler (1987).

After extraction of total proteins from dark and light grown callus, SDS-polyacrylamide gel electrophoresis and immunobloting were performed as described by Kruse *et al.* (1995). Protein content was determined using the bichinonic acid kit for protein determination (*Sigma-Aldrich*, St. Louis, MO, USA). Twenty-five microgram protein samples (25 μg) were electrophoresed in a 12 % SDS-polyacrylamide gel. The proteins were then transferred to nitrocellulose membrane (pores 0.45 μm) using *Trans Blot SD* cell (*Bio-Rad*, Hercules, USA). Specific primary antibodies against ChlB, ChlL and ChlN proteins were provided by Y. Fujita, Nagoya, Japan, against Flu-like protein by J.-D. Rochaix, Geneva, Switzerland and against GluTR by B. Grimm, Berlin, Germany. Signal was revealed using chemiluminiscent kit *Immobilon*™ *Western Chemiluminiscent HRP* substrate (*Millipore*, Bedford, MA, USA).

Plastid DNA was isolated from *P. abies* callus according to Triboush *et al.* 1998. Total RNA was isolated using *Spectrum*™ plant total RNA kit (*Sigma-Aldrich*). All RNA samples were treated with RNase-free Dnase I (*Fermentas*, Burlington, Canada). First strand cDNA was synthesized from 2.2 μg purified RNA using *ImProm-II*™ reverse transcription system (*Fermentas*) and random primers. RT-PCR was performed using gene specific primers: for *chlB*: BRTf 5′-CGTTTATTAAAA GATCTGGACATCAGA-3′ and BRTr 5′-GTAGAT ACATAAGGCATTCCAAATTCC-3′, for *chlN*: NRTf 5′-CCGGAATGGCTCATGCTA AC-3′ and NRTr 5′-TCTCGCATTGGCAAATCC A-3′, for *chlL*: LRTf 5′-TGTATTAGGCGACGTGGTTTG T-3′ and LRTr 5′-CTGCAAATAATGCATCGAATCC-3′, for *rrn23*: 23SRTf 5′-AATGAGCCGGCGACTTATAGG-3′ and 23SRTr 5′-GGGTCCATAAGCAGTGACAATTG-3′ (PCR program: denaturation at 94 °C for 2 min, followed by 30 cycles: 15 s at 94 °C, 1 min. at 60 °C). PCR products were examined on 2 % agarose gel with ethidium bromide.

After the cotyledons had been placed onto MS medium, callus appeared on cutting surfaces. The first symptoms of callus proliferation were observed approximately 21 d after primary explant incubation. After 4 weeks, callus appeared on the whole surface of the cotyledons. When the explants were grown under irradiance 40 μmol m^{-2} s^{-1}, compact green-pigmented callus was formed. Calli growing in the dark were friable without green pigmentation.

Pigment analysis showed differences between light- and dark-grown calli cultures, and also between calli and intact 14-d-old cotyledones. The highest content of Chl was observed in cotyledons isolated from light-grown seedlings [12.47 mg g^{-1}(d.m.)]. Dark-grown seedlings contained approximately a quarter of Chl amount of light-grown seedlings [2.91 mg g^{-1}(d.m.)]. Trace amounts of Chl were spectrophotometrically quantified in dark-grown calli [0.29 mg g^{-1}(d.m.)]. On the contrary, light-grown calli synthesized greater amounts of Chl [1.25 mg g^{-1}(d.m.)].

Semiquantitative RT-PCR confirmed the expression of *chlL*, *chlN* and *chlB* genes in light- and dark-grown calli of *P. abies*. The expression of all three genes was very low in dark-grown calli compared with light-grown calli. The *rrn23*, encoding plastid 23S rRNA, was used as a corresponding gene (Fig. 1).

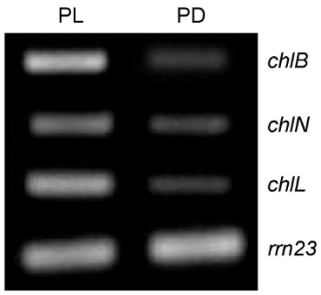

Fig. 1. RT-PCR analysis of *chlB*, *chlN*, *chlL* gene expression in calli cultures of *Picea abies* light-grown green callus (PL) and dark-grown white callus (PD). The *rrn*23 gene encoding plastid 23S rRNA was used as corresponding control gene.

Using Western blot analysis we have confirmed the presence of all three protein subunits of the DPOR enzyme (ChlL, ChlN, ChlB) in *P. abies* green calli growing under the low irradiance. In the dark-grown white calli, the contents of ChlN and ChlL subunits were low and ChlB subunit was not detected. The GluTR and FLP proteins were identified in the green light-grown callus, but their abundance declined when the calli growed in the dark (Fig. 2).

During evolution, phototrophs had to evolve highly efficient strategies to control tetrapyrrole biosynthesis and to prevent overaccumulation of free Chl intermediates, that are potentially extremely destructive. Chl biosynthesis is regulated at the step of ALA synthesis. This regulation has been attributed to feedback control of GluTR (Meskauskiene *et al.* 2001). Flu protein is thought to bind to GluTR and repress its activity to prevent overproduction of Pchlide. Flu-like protein (FLP) is structurally similar to Flu and probably plays a similar regulatory role in tetrapyrrole biosynthetic pathway (Falciatore *et al.* 2005). In dark-grown calli, we observed lower level of GluTR and FLP accumulation. In green light-grown calli, GluTR and FLP levels were higher compared to white calli cultures. Demko *et al.* (2009) described presence of GluTR in 7- and 14-d-old *P. abies* dark-grown seedlings. They observed increase of the Chl content, ALA synthesis and GluTR content after 24 h of irradiance of 14-d-old seedlings. Similar cellular abundance of GluTR was described in both dark and light-grown *Chlamydomonas reinhardtii* cultures (Nogaj *et al.* 2005). 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).

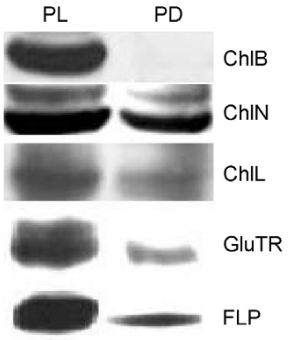

Fig. 2. Western-blot analyses of ChlB, ChlN, ChlL, GluTR and FLP proteins in *Picea abies* calli cultures. PL - light-grown green callus, PD - dark-grown white callus.

Enzymatic reduction of Pchlide to Chlide, catalyzed by DPOR enzyme in the dark, represents another regulatory step in the Chl biosynthesis (Fujita 1996). Constitutive expression of plastid genes *chlL*, *chlN* and *chlB* has been reported in photoautotrophically, mixotrophically and heterotrophically cultivated *Chlorella protothecoides* cells and in various conifer species grown in the dark (Spano *et al.* 1992, Skinner and Timko 1999, Kusumi *et al.* 2006, Shi and Shi 2006, Demko *et al.* 2009). In contrast to previous studies, we observed very low expression of *chlLNB* genes in dark-grown calli suggesting that *chlLNB* expression can be modulated by light in *P. abies* calli.

Western blot analysis confirmed the presence of all three protein subunits of the DPOR enzyme in *P. abies* green calli growing in the low irradiance. Similarly, Shi and Shi (2006) observed accumulation of DPOR subunits in light-grown *Chlorella protothecoides* cells. Their findings indicate that DPOR, which was thought to work only in darkness, may partially account for the Pchlide reduction in the light. Under low irradiace the *chlL*-disrupted mutant (DPOR-less), and a *por*-disrupted mutant of *Leptolyngbia boryana* (LPOR-less) both grew photoautotrophically in the same way as the wild type cells. This indicates that both LPOR and DPOR are themselves sufficient for Chl biosynthesis and cell growth under low irradiance (10 - 25 μmol m^{-2} s^{-1}; Fujita *et al.* 1998). In dark-grown spruce calli, low contents of ChlL and ChlN proteins were observed and the ChlB subunit was not detected. It is possible, that insufficiency of DPOR enzyme may repress Chl biosynthesis in dark-grown *P. abies* calli.

Karpinska *et al.* (1997) described differences in posttranscriptional modification of *chlB* transcripts in *P. abies*, *Pinus sylvestris* and *Larix eurolepis*. They demonstrated the presence of two C to U editing sites in the central region of *chlB* transcript in *P. sylvestris* and *P. abies* and one editing site in *L. eurolepis*. The presence of two editing sites was also detected in *L. decidua* (Demko *et al.* 2009). Our preliminary results indicate, that these previously described codons of *chlB* transcripts can be edited also in *P. abies* calli cells (data not shown). RNA-editing leads to restitution of conserved amino acids (leucine and tryptophan) and it has been suggested, that editing of *chlB* transcripts may be necessary for proper DPOR function.

It is generally suggested, that plastids of dark-grown calli cultures do not contain developed thylakoid system, which is a prerequisite for the assembly of photosynthetic apparatus components (Salajová *et al.* 1998). Chloroplasts of *Stevia rebaudiana* plants grown in *in vitro* conditions had poorly developed membrane system. Calli cultures of *S. rebaudiana* growing in light contained proplastids of almost round shape and their thylakoid system was represented by short thylakoids. In dark-grown calli only proplastids practically lacking the membrane system were observed (Ladygin *et al.* 2006) Low GluTR level probably limited Chl biosynthetic pathway at the step of ALA formation. Lower abundance of ChlL, ChlN proteins and the absence of ChlB subunit in the dark-grown calli may be the limiting factors during the Pchlide reduction catalyzed by DPOR. These ultrastructural and biochemical characteristics and the fact, that cells of dark-grown calli are completely white, indicate limited ability to synthesize Chl. This hypothesis is supported by the fact, that we quantified only trace amounts of Chl in dark-grown calli cultures.

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