Journal of Plant Physiology 167 (2010) 693–700

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Gabaculine alters plastid development and differentially affects abundance of plastid-encoded DPOR and nuclear-encoded GluTR and FLU-like proteins in spruce cotyledons

Viktor Demko*, Andrej Pavlovič, Ján Hudák

Department of Plant Physiology, Faculty of Natural Sciences, Comenius University, Mlynská dolina, 84215 Bratislava, Slovakia

ARTICLE INFO

Article history:
Received 4 June 2009
Received in revised form
9 December 2009
Accepted 13 December 2009

Keywords:
Chlorophyll biosynthesis
Chloroplast development
Picea abies
Protochlorophyllide oxidoreductase

ABSTRACT

Synthesis of 5-aminolevulinic acid (ALA) represents a rate limiting step in the tetrapyrrole biosynthetic pathway, and is regulated by metabolic feedback control of glutamyl-tRNA reductase (GluTR) activity. The FLU protein has been attributed to this regulation. Later in the biosynthetic pathway, reduction of protochlorophyllide (Pchlide), catalyzed by protochlorophyllide oxidoreductase (POR), ensures another important regulatory step in the chlorophyll biosynthesis. In the present work, we investigated the expression and cellular abundance of nuclear-encoded and plastid-encoded proteins involved in ALA synthesis and Pchlide reduction in Norway spruce (*Picea abies* L. Karst.) as a representative of plant species with high ability to synthesize chlorophyll in the dark. Using dark-grown, light/dark-grown and gabaculine-treated seedlings, we demonstrated that gabaculine-impaired etiochloroplast and chloroplast development has no negative effect on GluTR accumulation in the cotyledons. However, in contrast to control plants, the relative amount of GluTR was similar both in the dark-grown and light/dark-grown gabaculine-treated seedlings. We identified a partial sequence of the *FLU*-like gene in Norway spruce, and using antibodies against the FLU-like protein (FLP), we showed that FLP accumulated mostly in the dark-grown control seedlings and gabaculine-treated seedlings. In contrast to nuclear-encoded GluTR and FLP, accumulation of plastid-encoded light-independent POR (DPOR) was sensitive to gabaculine treatment. The levels of DPOR subunits were substantially lower in the light/dark-grown control seedlings and gabaculine-treated seedlings, although the corresponding genes *chlL*, *chlN* and *chlB* were expressed. Since we analyzed the samples with different plastid types, plastid ultrastructure and physiological parameters like Pchlide and chlorophyll contents, *in vivo* chlorophyll fluorescence and photosynthetic efficiency of the seedlings were characterized. Apart from etiochloroplast-specific accumulation of the DPOR subunits, we described, in some detail, additional specific features of chlorophyll biosynthesis in the spruce seedlings that differ from those known in angiosperms.

Introduction

Plastids represent highly heterogenic plant cell organelles capable of forming different types in response to developmental and environmental stimuli (Lopez-Juez and Pyke, 2005). Precise regulation of chlorophyll (Chl) biosynthesis is required for proper chloroplast development. The key regulatory steps in the Chl biosynthetic pathway are formation of 5-aminolevulinic acid (ALA) and reduction of protochlorophyllide (Pchlide). It is assumed that the rate of ALA synthesis from glutamate is controlled through the regulation of glutamyl-tRNA reductase (GluTR, EC: 1.2.1.70) activity at the beginning of the tetrapyrrole biosynthetic pathway (Papenbrock and Grimm, 2001; Tanaka and Tanaka, 2007). Particularly in etiolated plants, a negative feedback regulation of ALA synthesis is necessary to prevent overaccumulation of the later Chl precursor Pchlide, which would otherwise cause photodamage after illumination of the seedlings. The FLU protein has been identified in *Arabidopsis thaliana* as a negative regulator of tetrapyrrole biosynthesis involved in the feedback inhibition of ALA synthesis through interaction with GluTR in photosynthetically active tissues (Meskauskiene and Apel, 2002; Goslings et al., 2004). Falciatore et al. (2005) identified another regulator of tetrapyrrole biosynthesis in *Chlamydomonas reinhardtii*, the FLU-like protein (FLP). Two *FLP* transcripts were

Abbreviations: ALA, 5-aminolevulinic acid; A_N, net photosynthetic rate; Chlide, chlorophyllide; DPOR, light-independent protochlorophyllide oxidoreductase; FLP, FLU-like protein; F_v/F_m, maximum quantum yield of PSII; GluTR, glutamyl-tRNA reductase; LPOR, light-dependent NADPH:Pchlide oxidoreductase; Pchlide, protochlorophyllide; R_D, respiration rate; ΦPSII, effective quantum yield of PSII

* Corresponding author: Tel.: +421 2 60296646; fax:+ 421 2 65429064.
E-mail address: demko@fns.uniba.sk (V. Demko).

0176-1617/$ - see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2009.12.008

generated from the *FLP* pre-mRNA by alternative splicing, and the product of longer transcript interacted strongly with GluTR *in vitro*. Moreover, the FLPs partially complemented the *flu* mutation in *Arabidopsis* (Falciatore et al., 2005).

The reduction of Pchlide to chlorophyllide (Chlide) is a next crucial enzymatic step in the Chl biosynthetic pathway. In angiosperms, Pchlide reduction is catalyzed by the light-dependent NADPH:Pchlide oxidoreductase (POR, EC: 1.3.1.33), a nuclear-encoded and plastid-localized enzyme (Teakle and Griffiths, 1993; Schoefs, 1999). Three isoforms of LPOR with different expression patterns, PORA, PORB and PORC have been identified in *Arabidopsis* (Armstrong et al., 1995; Oosawa et al., 2000; Su et al., 2001).

In contrast to angiosperms, many anoxygenic bacteria, cyanobacteria, algae, nonvascular plants, ferns and gymnosperms possess an additional Pchlide-reducing enzyme, the light-independent Pchlide oxidoreductase (DPOR), which allows them to synthesize Chl in the darkness. DPOR is encoded by the three plastid genes *chlL*, *chlN* and *chlB* (Armstrong, 1998; Fujita and Bauer, 2003). The level of DPOR-encoding gene expression positively correlates with the ability to synthesize Chl in different tissues of dark-grown *Pinus taeda* seedlings (Skinner and Timko, 1999). Karpinska et al. (1997) reported species and tissue-specific *chlB* RNA-editing in *Picea abies*, *Larix eurolepis* and *Pinus sylvestris* that indicated posttranscriptional control of DPOR expression in some conifers. It is assumed that DPOR-mediated Chlide synthesis is ultimately needed for differentiation of the thylakoid membrane system inside the etiochloroplasts of conifer seedlings (Hudák et al., 2005; Demko et al., 2009).

Recent work addressing tetrapyrrole biosynthesis and plastid-to-nucleus signaling has been devoted to physiological and molecular mechanisms that control organelle biogenesis in response to metabolic demands of a whole plant (Gálová et al., 2000; Bräutigam et al., 2007; Tanaka and Tanaka, 2007; Moulin et al., 2008). In plants, the neurotoxin gabaculine inhibits tetrapyrrole biosynthesis by blocking the glutamate-1-semialdehyde aminotransferase (GSA-AT, EC: 5.4.3.8) activity (Hill et al., 1985; Grimm et al., 1991). The inhibitory effect of gabaculine has been used in several investigations to study compensatory mechanisms operating in the tetrapyrrole biosynthetic pathway (May et al., 1987; Masuda et al., 2002).

To investigate characteristic features of tetrapyrrole biosynthesis in Norway spruce, as a representative of plant species with high ability to synthesize chlorophyll in a light-independent manner, we searched for the genes encoding GluTR and FLU-like proteins (FLP) in the spruce seedlings and analyzed their expression in response to different experimental growth conditions. Using dark-grown, light/dark-grown and gabaculine-treated spruce seedlings, we were able to analyze correlations between different plastid developmental state and abundance of the nuclear-encoded GluTR and FLP proteins as well as plastid-encoded DPOR subunits ChlL, ChlN and ChlB.

Materials and methods

Plant material and growth conditions

Picea abies (L.) Karst. seeds (Semenoles, Liptovský Hrádok, Slovakia) were soaked for 24 h in distilled water and germinated on well-moistened filter paper in darkness or under standardized light/dark conditions (12 h light at 100 μmol m^{-2} s^{-1} PAR/12 h dark) at 24 °C. Control seedlings and gabaculine (3-amino-2,3-dihydrobenzoic acid)-treated seedlings were grown for 14 d in the dark or under the light/dark conditions on filter paper moistened with water or 300 μM gabaculine (Sigma, USA) solution, respectively. All manipulations of dark-grown seedlings were performed under dim green light. All samples were harvested at the same time in the morning (within the 3rd h of the light phase for light/dark-grown seedlings).

Pigment analysis

Samples (100 mg of cotyledons) were ground with a mortar and pestle and extracted with 80% (v/v) chilled acetone and $MgCO_3$ to avoid acidification and phaeophytinisation of pigments. After centrifugation, the extracts were spectrophotometrically quantified using a single beam spectrophotometer (Jenway 6400, UK): 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). To separate Pchlide from the esterified tetrapyrroles the extract was washed three times with an equal volume of hexane. The amount of Pchlide was measured spectrofluorometrically (Perkin-Elmer, LS 45, USA) at λ_{ex} 438 nm and λ_{em} 633 nm in the hexane-washed acetone phase and quantified using a Pchlide standard. The Pchlide standard was prepared from etiolated barley *tigrina* mutant according to Koski and Smith (1948) and spectrophotometrically quantified at 623 nm using the molar extinction coefficient in diethyl ether $\varepsilon = 3.56 \times 10^4$ M^{-1} cm^{-1} (Dawson et al., 1986). One-way analysis of variance (ANOVA, Statgraphics) was performed to evaluate the significant differences.

Gas exchange and Chl fluorescence measurements

Rates of photosynthesis (A_N), respiration (R_D) and Chl fluorescence were measured simultaneously with the infrared gas analyzer CIRAS-2 (PP-Systems, UK) and fluorcam FC 1000-LC (Photon Systems Instruments, Czech Republic) attached to the infrared gas analyzer. Prior to the measurements, cotyledons from three seedlings were enclosed in the PLC6 automatic universal leaf cuvette (PP-Systems, UK). After 15 min in the dark, minimal fluorescence (F_0) was recorded. Then, maximal fluorescence (F_m) was measured using a saturation pulse (4000 μmol m^{-2} s^{-1} PAR, 800 ms duration) and maximal quantum yield of PSII (F_v/F_m) was calculated as $F_m–F_0/F_m$. An induction curve of 15 min duration was then obtained by switching on the actinic light of 100 μmol m^{-2} s^{-1} PAR. Maximal fluorescence in the light-adapted state (F'_m) was obtained after 15 min of actinic irradiance using a saturation flash of 800 ms duration at 4000 μmol m^{-2} s^{-1} PAR. Simultaneously, steady state A_N was recorded at a CO_2 concentration of 360 μmol mol^{-1}, 100 μmol m^{-2} s^{-1} PAR and leaf temperature 23 ± 1 °C. Effective quantum yield of PSII photochemistry (ΦPSII) was calculated as $(F'_m-F_t)/F'_m$ (Maxwell and Johnson, 2000). One-way analysis of variance (ANOVA, Statgraphics) was performed to evaluate the significant differences.

Protein gel blot analysis

Extraction of total proteins from cotyledons, SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described by Kruse et al. (1995). Protein concentration was determined spectrophotometrically (Biophotometer plus, Eppendorf, Germany) using the Bicinchoninic Acid Kit for Protein Determination (Sigma, Germany). 20 μg protein samples were electrophoresed in a 12% (v/v) SDS-polyacrylamide gel followed by transfer to Hybond-C membranes (Amersham, Germany) using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, USA). Antibodies against *Plectonema boryanum* ChlL, ChlN and *Marchantia polymorpha* ChlB were provided by Y. Fujita, Nagoya, Japan. Antibodies against *Chlamydomonas reinhardtii* FLU-like protein were provided by J.D Rochaix, Geneva, Switzerland and against *Arabidopsis thaliana* GluTR by B. Grimm, Berlin, Germany. Antibodies against *Arabidopsis thaliana* LHCI proteins were purchased from Agrisera, Sweden. An IgG Fab-specific-peroxidase (Sigma, Germany) was used as a secondary antibody. Immobilon™ Western Chemiluminiscent HRP substrate