BRIEF COMMUNICATION

Feeding with aminolevulinic acid increased chlorophyll content in Norway spruce (*Picea abies*) in the dark

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Abstract

In contrast to angiosperms, which accumulate protochlorophyllide after application of aminolevulinic acid in the dark, feeding with aminolevulinic acid (0.01–20 mM) *via* the roots in the 18-d-old seedlings of Norway spruce (*Picea abies*) stimulated not only protochlorophyllide but also chlorophyll accumulation.

Aminolevulinic acid (ALA) is a general precursor for tetrapyrrole pigments of both the chlorophyll (Chl) and heme pathways. Treatment of etiolated angiosperm leaves with exogenous ALA gives rise to accumulation of protochlorophyllide (Pchlide). It was concluded that all enzymes necessary for Pchlide biosynthesis were already active and present in nonlimiting amounts in etiolated plant tissue and that only the amount and activity of enzymes involved in ALA synthesis limited the synthesis rate (Granick 1959, Papenbrock and Grimm 2001). Pchlide accumulates in the dark because the angiosperms reduced Pchlide to chlorophyllide (Chlide) by photoenzyme, light-dependent protochlorophyllide oxidoreductase (LPOR). This reaction represents a key regulatory step in the strictly light-dependent biogenesis of angiosperm chloroplasts. In contrast to the dependence on light for the Chl biosynthesis of angiosperms, some photosynthetic organisms such as anoxygenic photosynthetic bacteria, cyanobacteria, algae, mosses, ferns ,and gymnosperms are capable of synthesizing Chl and bacteriochlorophylls in the dark by light-independent Pchlide oxidoreductase (DPOR) (for review *see* Armstrong 1998).

Whereas positive effect of ALA feeding on Chl accumulation in the light is well known in angiosperms (Al-Thabet 2006, Awad 2008, Memon *et al*. 2009), the effect of exogenously added ALA on Chl accumulation in the dark-grown seedlings of gymnosperms is ambiguous. Some results indicate that ALA synthesis is not a rate-limiting step in Chl biosynthesis in the dark-grown pines (Fujita and Bauer 2003). We decided to feed with ALA the Norway spruce [*Picea abies* (L.) Karst.] - species with the highest ability of Pinaceae to synthesize Chl in the dark. We tried to find out the answer to the following question: Is the limiting step in Chl biosynthesis in the dark the rate of ALA synthesis or the reduction of Pchlide to Chlide by DPOR?

Seeds of *Picea abies* (Zakamenné, Slovakia) were first imbibed 36 hours in water and then germinated and cultivated on cotton moistened with 20 mM phosphate buffer with pH 6.8 (control plants) or with different concentration of ALA (0.01, 0.1, 1, 10, 20 mM ALA) in 20 mM phosphate buffer in the dark or in the light (100 μmol m^{-2} s^{-1} photosynthetic active radiation). Every fourth day 50 ml of solution was applied to the seedlings (total 200 ml of solution). All manipulations in the dark were performed under a dim green safety light. The safety of the light was indicated by no Pchlide reduction in young dark-grown seedlings of barley. Chls from 18-d-old conifer cotyledons were extracted with chilled 80% acetone (v/v). Small amount of sand and $MgCO_3$ was added to avoid acidification and phaeophytinization of

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Abbreviations: ALA – aminolevulinic acid; Chl – chlorophyll; Chlide – chlorophyllide; DPOR – light-independent protochlorophyllide oxidoreductase; FM – fresh mass; LHC – light-harvesting complex; LPOR – light-dependent protochlorophyllide oxidoreductase; Pchlide – protochlorophyllide; PS – photosystem.
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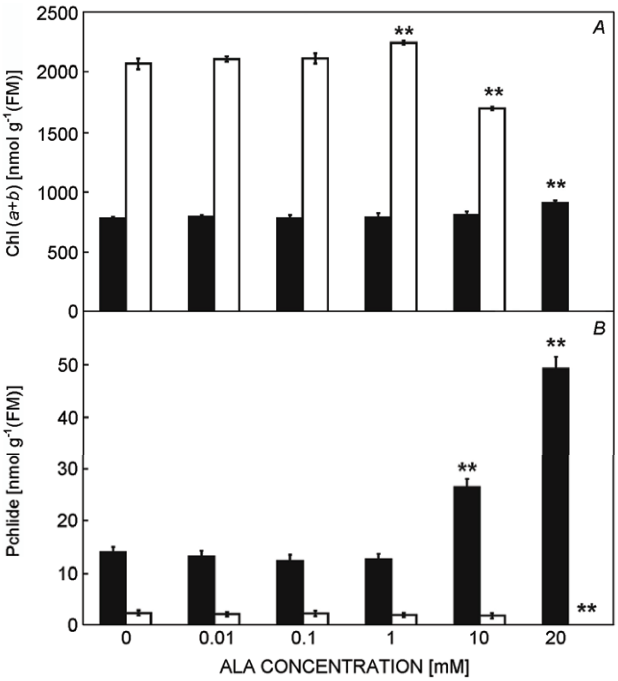

Fig. 1. Amounts of chlorophyll (Chl) (*a*+*b*) in nmol g^{-1}(FM) (*A*) and protochlorophyllide in nmol g^{-1}(FM) (*B*) in the light (*white bars*) and in the dark (*black bars*) at different concentration of ALA in cotyledons from 18-d-old seedlings of *Picea abies*. Means ± SD, n = 5, The asterisks above bars indicate significance of difference from control plants at $P<0.05$ (*) and $P<0.01$ (**). FM – fresh mass.

pigments. After centrifugation the Chl (*a*+*b*) content was determined spectrophotometrically (*Jenway 6400*, London, UK) and calculated according to Lichtenthaler (1987). Prior to the extraction of Pchlide, cotyledons were heated in hot steam for 2 min to inactivate enzymes of Chl biosynthesis. Than the pigments were extracted with 100% acetone: 0.1 N NH_4OH (9:1 v/v). The amounts of Pchlide in hexane-washed acetone extracts were measured spectrofluorometrically (*RF-5301 PC, Shimadzu*, Kyoto, Japan) at λ_{ex} 438 nm/ λ_{em} 632 nm and quantified using standard according to Koski and Smith (1948). The presence of protoporphyrine IX was detected in the same extracts at λ_{ex} 402 nm/ λ_{em} 595 nm. Fluorescence emission spectra at low temperature (77 K) were measured with the Spex fluorolog spectrofluorometer *(Horiba, Jobin Yvon Inc*., Edison, NJ, USA). The needles were immersed in liquid nitrogen in an optical Dewar flask when measured at 77 K. The raw data were corrected on the PMT photocathode sensitivity. Statistical differences between control and ALA-treated seedlings were performed by Student *t*-test, n = 5.

Basically, the control dark-grown *P. abies* seedlings synthesized a lower amount of Chl and accumulated higher amount of Pchlide than the light-grown ones (Fig. 1*A,B*). We also detected the characteristic fluorescence emission of pigment-protein complexes belonging to PSI (735 nm) and PSII (686, 697 nm) in dark-grown *P. abies* (Fig. 2*A*). This confirmed assembly of both photosystems in the absence of light, although the ratio between PSII and PSI fluorescence emission bands is lower in light-grown plants (Fig. 2*B*), indicating that the amount of PSI must be substantially reduced in the dark. Reduced PSI relative to PSII was confirmed in dark-grown *P. palustris* seedlings by second dimension analysis of thylakoid complexes by SDS-PAGE (Canovas *et al.* 1993). Hovewer we can not exclude the contribution of higher reabsorption of the short-wavelength peak inside the leaf tissue by Chl in light-grown seedlings. The emission bands observed at 632 and 658 nm in dark-grown seedlings correspond to the free Pchlide and Pchlide bound in prolamelar bodies, respectively (Fig. 2*A*).

In the dark, higher Chl content was found only after application of 20 mM ALA (Fig. 1*A*). The seedlings accumulated also significantly higher protoporphyrine IX (Fig. 2*A*, *inset*) and Pchlide at 10 and 20 mM ALA (Fig. 1*B*). Both forms of Pchlide, phototransformable (F632 nm) and non-phototransformable (F658 nm) accumulated after ALA-feeding (Fig. 2*A*). This indicates that surplus Pchlide molecules caused by ALA-feeding appear to be free and also bound in LPOR phototrans-

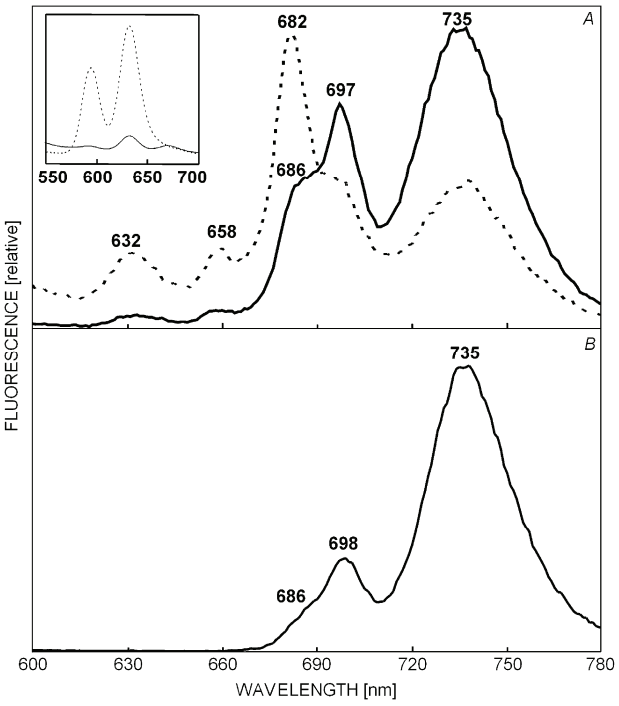

Fig. 2. The 77-K fluorescence emission spectra of 18-d-old dark-grown cotyledons of *Picea abies* without ALA (*solid line*) and at 20 mM ALA (*dashed line*) (*A*) and control light-grown seedlings (*B*). Excitation wavelength (λ_{ex}) = 440 nm. Emission spectra are normalized at their maxima. Inset shows room-temperature emission spectra of hexane-washed acetone extract of dark-grown cotyledons of *Picea abies* without ALA (*solid line*) and at 20 mM ALA (*dashed line*). Excitation wavelength = 402 nm. Emission at 595 nm corresponds to ProtoIX.

formable complex. The surplus Chl increased 77-K fluorescence at 682 nm (Fig. 2*A*), indicating rather increase amount of LHCII protein complex and decrease of efficiency of the energy migration from LHCII to the reaction center or increased concentration of free Chl(ide) molecules. In angiosperms, Chl synthesis and transcription of the genes encoding light-harvesting Chl-binding protein (*Lhcb*) is induced by light (reviewed by Tobin and Silverthorne 1985). In gymnosperms, the transcription of the Chl-binding protein is light-independent, even in species with restricted ability to synthesize Chl in darkness (Mukai *et al*. 1992). Because it is known that sufficient amounts of total and translatable mRNA for LHCII and of LHCII apoproteins themselves are present in dark-grown pine cotyledons, the assembly of LHCII is limited by the supply of Chl in the dark (Yamamoto *et al.* 1991, Mukai *et al.* 1992, Shinohara *et al.* 1992, Muramatsu *et al.* 2001). Therefore surplus Chls after ALA feeding are probably bound to LHCII proteins and retain them in chloroplast. This is consistent with lower Chl *a*/*b* ratio in seedlings growing at 20 mM ALA in comparison to control (3.97 *vs*. 3.72), because Chl *b* is bound in LHCII and not in the reaction center and core antennas of PSII. However we can not exclude also the contribution of Chl(ide) molecules to fluorescence at 682 nm.

In the light, Chl content increased at 1 mM ALA (Fig. 1*A*). We detected also low concentration of Pchlide, but its content was reduced in comparison to dark-grown plants (Fig. 1*B*). Positive effect of low concentration of ALA on growth and Chl content in the light in angiosperms has been well documented by several authors (Al-Thabed 2006, Awad 2008, Memon *et al*. 2009). However, higher concentration of ALA (10–20 mM ALA) had negative effect on growth and Chl accumulation and at 20 mM the seedlings were not able to survive (Fig. 1*A*). Accumulation of free Chl precursors in the light leads to photooxidative damage (Papenbrock *et al*. 2001).

It was found that ALA feeding in the dark in *Pinus jeffreyi* resulted in accumulation of Pchlide, mainly its non-phototransformable form (Michel-Wolwertz and Brouers 1974). Despite higher Pchlide, lower Chl content after 10 mM ALA feeding in the dark-grown *Pinus nigra* was found (Dražič and Mihailovič 1998). These observations indicated that the DPOR is able to cope only with the natively supplied Pchlide and that ALA synthesis is not a rate limiting step in Chl synthesis in the dark. Moreover, Fujita and Bauer (2003) suggested that after ALA feeding large pool of Pchlide bound to LPOR suppressed the DPOR synthesis in *Pinus jeffreyi.* The Norway spruce (*P. abies*) has the highest ability of Pinaceae to synthesize Chl in the dark (Fujita and Bauer 2003) and Chl synthesis was clearly stimulated after ALA feeding in our experiment, although the seedlings did not reach Chl concentration determined in light-grown seedlings. Thus, ALA synthesis is partially the rate-limiting step in Chl synthesis in the dark, hovewer the reduction of Pchlide to Chlide is also ineffective and limiting step, as indicates higher Pchlide/Chl ratio after ALA feeding in the dark.

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