Gabaculine does not inhibit cytokinin-stimulated biosynthesis of chlorophyll in *Pinus nigra* seedlings in the dark

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**Abstract**

Chlorophyll (Chl) accumulation was monitored during black pine (*Pinus nigra* L.) seed germination for 14 days in the light and in the dark in the presence of gabaculine (GAB) and cytokinin in order to elucidate the regulation of gymnosperm seedling greening in the dark, primarily at the level of aminolevulinic acid formation. In the light, GAB inhibited chlorophyll accumulation in a manner dependent on concentration and developmental stage, and in the dark it showed no effect. Cytokinin, 10⁻⁵ M benzyl adenine (BA) partly overcame GAB-induced inhibition in the light, mainly during earlier developmental stages. In the seedlings grown in the dark, an equal quantity of Chl accumulated in the presence of cytokinin with and without GAB and it was approximately 20–40% higher than in the control seedlings or in the seedlings grown only in the presence of GAB. 5-Amino-levulinic acid (ALA) synthesis was equal in the light and in the dark in seedlings of the same age and seedlings treated with GAB grown in the dark. In the light, GAB inhibited ALA synthetic activity. The results indicate that ALA synthesis is not a rate-limiting step within Chl biosynthesis in pine seedlings grown in the dark. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

**Keywords**: Aminolevulinic acid; Cytokinin; Cotyledons; Gymnosperms; Development

1. Introduction

Several species of pine have been studied with respect to the contributions of light-dependent and light-independent chlorophyll (Chl) biosyntheses to overall pigment accumulation under various conditions. The kinetics of chlorophyll accumulation in seedlings grown in the light and in the dark is much the same in *Pinus taeda* [1], *P. tunbergii* [2], *P. sylvestris* [3], *P. pinea* [4] and *P. nigra* [5,6]. Regulation of chlorophyll biosynthesis in gymnosperm seedlings is much less investigated than in angiosperms and photosynthetic bacteria. The most distinctive difference is the ability to synthesize chlorophyll in the dark, which is enabled by a light-independent protochlorophyll oxidoreductase (DPOR). As this is not the only difference, we wished to show by this work some further specificity of chlorophyll biosynthesis regulation, primarily at the level of 5-aminolevulinic acid (ALA) accumulation.

ALA formation is known to be a crucial regulatory step in chlorophyll biosynthesis in plants [7,8]. In plants and photosynthetic bacteria, ALA is formed via the C5 pathway that consists of three steps: synthesis of tRNA[^1]u, reduction of tRNA[^1]u to glutamate-1-semialdehyde (GSA) and subsequent transamination of GSA. The C5 pathway is the only pathway for ALA synthesis that has been unequivocally demonstrated in plants [9,10]. It is localized in the plastids. Phytochrome-regulated ALA synthesis is well documented at the initial stages of greening, while at the later stages, light is thought to exert its effect on ALA formation through a feedback inhibition by Pchlide [11]. Light apparently induces ALA formation in green algae by controlling the expression of the enzymes glutamate-1-semialdehyde aminotransferase

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(GSAT) and porphobilinogen deaminase (PBGS) [12–14] and in higher plants by coordinated transcriptional control of both glutamyl-tRNA reductase (GR) and GSAT [15]. Since no such increase in activity was found for other enzymes of the C5 pathway, light regulation of ALA synthesis must occur at the reductase step [9].

One of the most conspicuous effects of cytokinins on plant tissues is stimulation of chlorophyll synthesis during greening of seedlings or etiolated leaves [16]. The applied cytokinins shorten or eliminate the lag phases for Chl synthesis in angiosperms [17,18].

Gabaculine, 3-amino-2,3-dihydrobenzoic acid (GAB), a potent inhibitor of ALA synthesis at the aminotransferase-regulated site [19], has been used to study tetrapyrrole biosynthesis in plants [20–22]. Simultaneous influence of cytokinin and GAB on ALA synthesis has been investigated in angiosperms. In greening cucumber cotyledons, the accumulation of GSA in the presence of GAB was increased by BA to the same extent as the synthesis of ALA [23]. These results indicate that the site of action of BA should be located before the step catalyzed by GSA aminotransferase. The amount of total plastidic RNA was doubled in BA treated cotyledons. The results indicate that stimulation of the synthesis of ALA by BA is due to an increased level of tRNA Glu in plastids [24].

Therefore, chlorophyll biosynthesis in black pine cotyledons in the presence of GAB and BA was investigated in order to determine similarity or difference between the mechanisms by which cytokinin exerts an effect on chlorophyll biosynthesis in greening cotyledons in angiosperms and gymnosperms.

2. Material and methods

2.1. Plant material

Seeds of black pine were obtained from Tara mountains in the west part of Serbia. Pine seeds were imbibed in distilled water for 24 h in the dark and were germinated in Petri dishes (10 cm) on three layers of filter paper moistened with mineral nutrient solution [25] for control plants or in the presence of GAB or BA. Seedlings were grown in the dark or in white light (220 μmol cm−2 s−1, Sylvania cool White IF 72T12-CW-UHO, photoperiod 12/12) at 24 ± 1°C. All manipulations with dark-grown plants were carried out under a dim green safelight. The effectiveness of the safelight (a 25-W bulb covered with a Fotokemika TZ dark green filter) was indicated by its failure to convert Pchllide to Chl in young, dark grown wheat seedlings.

2.2. Treatments

In the first experiment, pine seedlings were germinated for 7 days in mineral nutrient solution in the dark and then incubated with different concentrations of GAB (50, 100 and 500 10−6 M) for 1, 3 and 7 days under the same light conditions as they had been kept before incubation. Chl content was measured immediately after incubation (8th, 9th and 14th day of germination).

In the second experiment the seeds were grown in the presence of: (A) nutrient solution-control plants; (B) nutrient solution + 10−5 M BA; (C) nutrient solution + 10−4 M GAB and (D) nutrient solution + 10−5 M BA + 10−4 M GAB for 5, 7, 9 and 14 days in the light or in the dark.

2.3. Chl determination

Chlorophyll was extracted in dimethylformamide and measured spectrophotometrically according to Moran [26]. Chl was determined in five replications, each consisting of 50 seedlings. Statistical evaluation of the results was performed by Student’s t-test.

2.4. Measurement of ALA synthesis

For the determination of ALA synthesis in the light-grown and dark-grown black pine seedlings, about 1 g of fresh weight of seedlings was incubated in 100 mM levulinic acid (LA) in 10 mM phosphate buffer (pH 7.2) to prevent ALA utilization. Seedlings were incubated for 3 h under the same conditions under which they had been grown. After the incubation, the seeds were dried with a paper towel, frozen and stored at −20°C. Weighed samples of frozen material were homogenized in 10% TCA. ALA was extracted according to Averina and Yaronskaya [27], and the concentration was determined spectrophotometrically after 15 min of heating at 100°C in the presence of ethyl acetoacetate with the addition of the same
quantity of Erlich reagent [28] into the cooled sample. The amount of porphobilinogen formed from ALA was calculated using the coefficient $7.45 \times 10^{-4}$ mol$^{-1}$ cm$^{-1}$ [29].

3. Results

During germination in the light and in the dark, GAB and BA at the applied concentrations did not influence the morphology and growth of the seedlings.

Incubation in the presence of GAB led to a significant inhibition of Chl accumulation in seedlings grown in the light in a manner dependent on concentration and incubation duration. After a 24-h incubation, no inhibition of Chl accumulation was detected at the applied GAB concentrations (below 500 μM), which may be due to a slow transport of the inhibitor into the tissue. Three days incubation with GAB significantly inhibited Chl accumulation at all the investigated concentrations. The highest inhibition of Chl accumulation (approximately 60%) was observed after 7 days in the presence of 500 μM GAB, when the chlorophyll content decreased to half of the initial value (Fig. 1).

When black pine seedlings develop in the dark, they accumulate a significantly lower quantity of chlorophyll compared to the seedlings grown exposed to the light (Fig. 2). The dynamics of chlorophyll accumulation is also different: under the light, during first 8 days, approximately 80% of chlorophyll found after 14 days of germination is accumulated, while in the dark, during the first 8 days only 40% of the quantity synthesized in the first 14 days is found. Another difference from the development under the light is the absence of GAB effect. Namely, chlorophyll content in seedlings incubated in the presence of GAB for 1, 3 and 7 days is equal to the controls at the same germination stage. The measured differences were not statistically significant (t-test).

In the second experiment, the influences of cytokinin and gabaculine on Chl accumulation in seedlings grown in the light and in the dark were monitored. Chlorophyll content was measured after 14 days of development. In the seedlings of *P. nigra* grown in the light, cytokinin ($10^{-5}$ M BA) slightly stimulated Chl accumulation during earlier developmental stages, while this effect became negligible after 14 days. GAB ($10^{-4}$ M) inhibited...
Chl accumulation significantly but not absolutely. Cytokinin partly overcame the effect of GAB, especially during the earlier developmental stages (Fig. 3).

During 14 days of development in the dark, cytokinin (10\(^{-5}\) M BA) stimulated Chl accumulation by 20–40%. The effect of GAB (10\(^{-4}\) M) was negligible (the measured differences were not significant (t-test)). The chlorophyll concentrations in the presence of cytokinin (BA) and in the presence of cytokinin + GAB were similar (Fig. 4).

ALA synthetic activity was monitored in the seedlings of *P. nigra* grown in the light and in the dark for 5, 7, 9 and 14 days. ALA accumulation was measured after 3 h of incubation in the presence of LA (competitive inhibitor of ALAD). ALA synthetic activity was the same in the light and in the dark in control plants (Fig. 5). In plants treated with 10\(^{-4}\) M GAB, ALA synthetic activity in seedlings grown in the dark equalled the control values. In the light, GAB inhibited ALA synthetic activity, but did not suppress it completely. Variations of ALA synthetic activity were not correlated with Chl accumulation.
4. Discussion

Chlorophyll biosynthesis in gymnosperm seedlings in the light and in the dark is enabled by POR and DPOR activities [29]. A plausible explanation seems to be that both light-dependent and light-independent Chl biosynthesis contribute significantly to total in vivo pigment accumulation, where DPOR activity dominates in young seedlings, but loses its importance with the progress of seedling development. This explanation is also supported by our results (Figs. 1 and 2) because the difference in quantity of chlorophyll accumulated in the light and in the dark grows during seedling development. Such a conclusion is in qualitative agreement with the Pchllide reducing activities measured in vivo by dark incubation or flash illumination of mountain pine cell extracts supplemented with [14C]Pchllide [30]. On the other hand, it is not certain that the positive effects of light on Chl accumulation in pine species are due solely to POR-mediated Pchllide photoreduction. Such an increase in the amount of Chl could conceivably result from positive light regulation of DPOR activity, or light-dependent differences in pigment accumulation not directly related to the overall capacity for Pchllide reduction [31].

ALA production and utilization limit PChl production. In order to detect possible specificity in ALA synthesis, black pine seedlings were grown in the presence of GAB (Fig. 1). An essential difference may be noticed between the GAB effect on Chl accumulation in the light and in the dark. While in the light GAB inhibits Chl accumulation in a manner dependent on concentration and incubation duration, it shows no effect in the dark. The absence of GAB inhibition of Chl biosynthesis in the dark may be interpreted in several ways. For instance, GAB transport may be light-dependent; thus, inhibitor may not penetrate into the tissue in the dark, which is not very likely. Alternatively, the inhibitor may have no target in the tissue, because the synthesis of ALA, as a key precursor of Chl, does not take place through the C5 pathway, but following some other pattern. The C4 pathway is the only pathway for ALA synthesis in mitochondria of fungi and animals. Both pathways have been reported to operate in Euglena gracilis [32]. It has been hypothesized that plants may contain the C4 pathway in the mitochondria besides the C5 pathway in plastids [9], but it has not been unequivocally established. Currently, all the experiments point to the C5 pathway as the only tetrapyrrole pathway in plants. It cannot be excluded at present, however, that there is a second pathway that becomes operative only when the C5 pathway of the plastids is blocked [9]. The third explanation could be that GAB reaches the target but the inhibition is not complete and the remaining GSAT activity is sufficient to produce ALA which is utilized for the production of a small quantity of chlorophyll. Or else, GAB affects ALA availability for Pchllide biosynthesis, as in wheat seedlings [33] and cucumber [34]. It is proposed that plants may contain two distinct pools of ALA with different metabolic destinations, different regulatory mechanisms and different degrees of susceptibility to GAB.

The variations of ALA synthetic activity during germination in the light and in the dark indicate both similarities and differences in comparison with angiosperms. ALA synthetic activity determined in the light (6 × 10⁻⁶ mol ALA/g FW) corresponds to the values found in angiosperm [27,35] and gymnosperm seedlings [34]. ALA synthetic activity is the same in the dark and in the light (Fig. 5). No correlation was found between ALA and Chl formation in the dark. This indicates that in dark-grown pine seedlings ALA formation is not a rate limiting step for Chl accumulation (ALA is accumulated in the dark but is not utilized for chlorophyll, but for synthesis of some other product, or is rapidly degraded) and that ALA production in gymnosperms is light-independent. In P. sylvestris, phytochrome regulates not only the elimination of the lag phase but also maintenance of a high Chl accumulation rate in P. sylvestris in continuous light. Since ALA accumulation responded in the same manner to the red light pretreatment, it is further concluded that ALA formation is the point where phytochrome regulates Chl biosynthesis in continuous light [36]. ALA synthetic activity was not inhibited by GAB in the dark, and the inhibition in the light was incomplete. This indicated a likely activity of the alternative pathway of ALA synthesis in the dark and a simultaneous activity of the C5 and the alternative pathways in the light.

Earlier investigations of cytokinin influence on Chl biosynthesis [37] had shown that exogenous cytokinin (BA) induced Chl synthesis in isolated embryos grown in the dark and stimulated it in those grown in the light and that the change of Chl accumulation rate coincided with the change
of content and activity of endogenous cytokinins in the embryo. Cytokinin slightly stimulated Chl accumulation in seedlings grown in the light and somewhat more intensively that in the dark (Fig. 4). In the light, cytokinin partly prevented the inhibition of Chl accumulation caused by GAB (Fig. 3), while in the dark an equal quantity of Chl was accumulated in the presence of GAB + BA and of BA alone (Fig. 4). This indicates that BA exerts an influence primarily on the alternative biosynthetic pathway which is GAB-insensitive, and also, that the regulation of greening is similar to that in Euglena, where the C4 biosynthetic pathway has been detected in mitochondria as a stage of heme biosynthesis, and the C5 pathway occurs in plastids as a stage of Chl production [38]. It is assumed that the activation of ALA synthesis by the C5 route in the light leads to suppression of ALA synthase activity (C4). GAB would block C5 ALA formation in the light and this may be a signal for the cells that more ALA formation is required via C4. Mitochondrial activity is important for the mobilization of nutritive compounds from the megagametophyte because in the dark there is no photosynthesis, and energy is needed for seedling growth. This is in accordance with an original theory [39] which states that greening of gymnosperm seedlings is limited by the available nutrients and also with our result showing that megagametophyte utilization slows down the chlorophyll biosynthesis rate and terminates it after 14 days when the resources become exhausted.

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References


