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Expression of genes for chloroplast proteins in pine yellow cotyledons grown at low temperature

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Introduction

Cotyledons of most gymnosperms synthesize chlorophylls (Chls) in complete darkness and differentiated stromal and granal lamellae are evident in their chloroplasts. The protein components of the two photosystems and the Cyt *b/f* complex are expressed and accumulate in the thylakoids of dark-grown cotyledons (Shinohara *et al*. 1992a) but the O_2-evolving enzyme remains latent (Inoue *et al*. 1976; Oku and Tomita 1976). The evolution of O_2 is activated by the brief illumination of dark-grown cotyledons or isolated thylakoid membranes (Inoue *et al*. 1976; Oku and Tomita 1976; Shinohara *et al*. 1992b). During photoactivation, 4 Mn atoms are integrated into the O_2-evolving apparatus, concomitantly with the integration of extrinsic proteins of 23 and 17 kDa, respectively (Shinohara *et al*. 1992b). Genes whose products are involved in photosynthesis are expressed in the dark-grown cotyledons of gymnosperms (Yamamoto *et al*. 1991; Mukai *et al*. 1991; Shinohara *et al*. 1992a; Kojima *et al*. 1994).

Various photosynthetic eukaryotes, such as gymnosperms, mosses and algae, as well as photosynthetic prokaryotes, synthesize an enzyme that reduces protochlorophyllide (Pchlide) independently on light, allowing these organisms to synthesize Chl in darkness (Fujita 1996). The synthesis of Chl in gymnosperms has been studied in cotyledons (Bogorad 1950; Sudyina 1963; Michel-Wolwertz 1977; Wolwertz and Brouers 1980) and in primary needles (Ou and Adamson 1995; Schoefs and Franck 1998). More recently, we have found that dark-grown pine cotyledons are yellow at low temperatures (Muramatsu *et al*. 2001). In the present study, we examined the expression of genes for chloroplast proteins and the accumulation of chloroplast proteins in the yellow cotyledons.

Materials and Methods

Cones of Japanese black pine (*Pinus thunbergii*) were dried and the seeds isolated from them were stored at 4°C in darkness until use (Shinohara et al. 1992b). The seeds were sown on 0.8% (w/v) agar and allowed to germinate at 8°C in continuous light from white fluorescent lamps (35 μmol m^{-2} s^{-1}) and in darkness for 4 months. After 4 months, the light- and dark-grown cotyledons were harvested. Some of the dark-grown seedlings were transferred to 25°C in darkness and incubated for one more week. Control dark-grown cotyledons were harvested

after seeds had been allowed to germinate at 25°C for 3 weeks in darkness, respectively. All manipulations of dark-grown cotyledons were performed, when possible, in complete darkness. However, when required, light was provided by a dim green safe light.

Total RNA was prepared from the cotyledons as described previously (Shinohara and Murakami 1996). For Northern blotting, 5 μg of total RNA were fractionated by formaldehyde gel electrophoresis. Probes for transcripts of *cab* and *rbc*S were 0.9-kb cDNAs derived from pine (Yamamoto *et al*. 1991). Probes for transcripts of *psb*A and *rbc*L were a 0.2-kb *Pst*I-*Pvu*II internal fragment of tobacco *psb*A (Sugiura 1992) and a 1.1-kb *Sma*I-*Hin*dIII fragment of pine *rbc*L (Mukai *et al*. 1991), respectively.

Crude protein extracts were prepared from the light- and dark-grown cotyledons as described previously (Yamamoto *et al*. 1991). Proteins composition was analyzed, on an equal fresh weight basis (2 mg), by SDS-PAGE on a 13% polyacrylamide slab gel that contained 0.1% (w/v) SDS. For immunoblotting analysis, the separated proteins were transferred electrophoretically to a nitrocellulose membrane (Shinohara *et al*. 1992b). The antisera raised against pine SSU, pine LHCPII, and tobacco RuBisCO were generous gifts from Dr. Naoki Yamamoto (Yamamoto *et al*. 1991).

Results and Discussion

Cotyledons of *P. thunbergii* synthesized Chl in complete darkness at 25°C under standard germination conditions, as reported previously (Shinohara *et al*. 1992a). By contrast, dark-grown cotyledons were yellow at low temperature (Muramatsu *et al*. 2001). The light-independent synthesis of Chl in dark-grown cotyledons was almost completely inhibited at 8°C and the Chl content of dark-grown cotyledons was less than one-twentieth of that of light-grown cotyledons. Thus, the light-independent synthesis of Chl in dark-grown cotyledons was sensitive to low temperature. The level of Chl that accumulated in darkness at 8°C increased upon transfer of seedlings from 8°C to 25°C and incubation for one week. In contrast, the level of Pchlide that accumulated in darkness at 8°C decreased by this treatment. These results suggest that the pathway for the light-independent synthesis of Chl is reversibly inactivated at low temperature, and that low temperature inhibits mainly the light-independent Pchlide reductase.

We examined whether or not transcripts of genes for photosynthetic proteins were detectable in yellow cotyledons grown at 8°C (Fig. 1). Transcripts of the *rbcL*, *rbcS*, *psbA*, and *cab* genes were found in the cotyledons at substantial levels. The levels of all four transcripts increased during the greening that occurred after transfer from 8°C to 25°C and incubation for one week. Thus, these four genes were expressed in the gymnosperm cotyledons, irrespective of light conditions and Chl content.

We also examined the presence of the LSU and SSU of RuBisCO and of the two apoproteins of LHCPII in dark-grown yellow cotyledons by immunoblotting with specific antibodies (Fig. 2). Both LSU and SSU were detectable in the extracts of yellow cotyledons, but the accumulation of the two apoproteins of LHCPII was very limited. The two apoproteins did accumulate during greening after cotyledons had been transferred to 25°C. This result suggests that the apoproteins might be unstable when there is a limited supply of Chl.

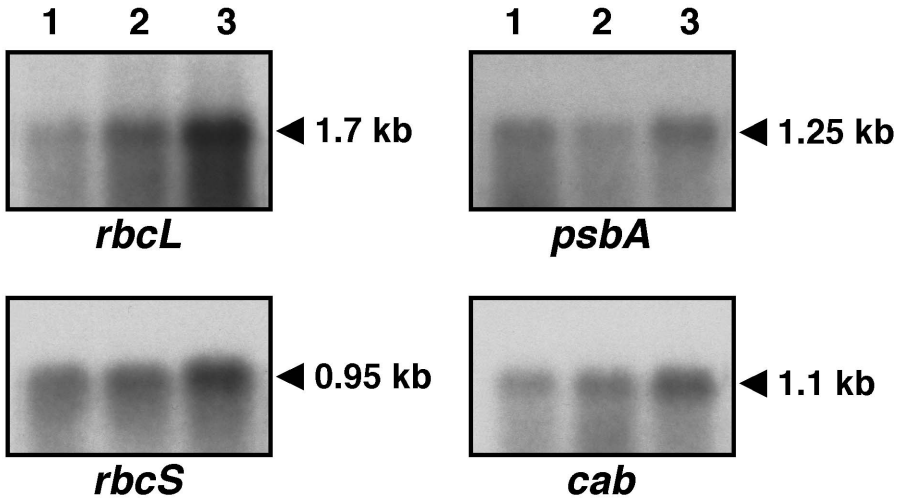

Fig. 1. Presence of transcripts of *rbcL*, *rbcS*, *psbA* and *cab* genes in dark-grown yellow cotyledons. Total RNA was prepared from dark-grown cotyledons cultured at 25°C for 3 weeks (lane 1), dark-grown yellow cotyledons cultured at 8°C for 4 months (lane 2) and dark-grown cotyledons cultured at 8°C for 4 months and then at 25°C for one week (lane 3).

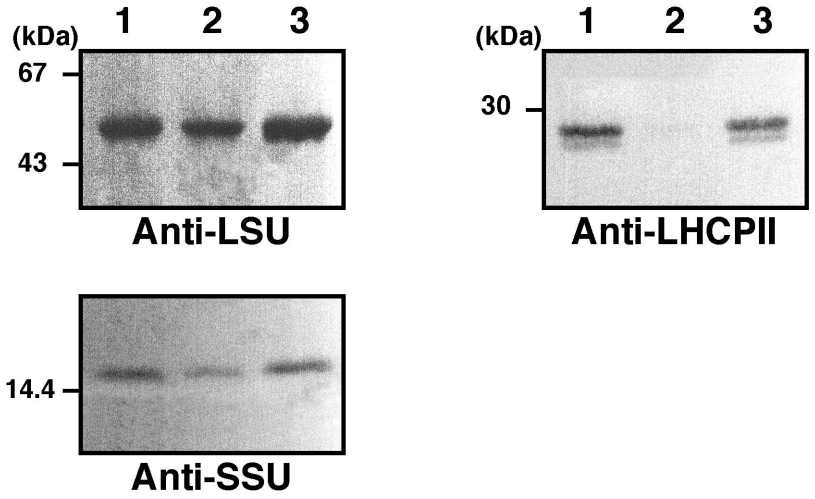

Fig. 2. Accumulation of the LSU and SSU of RuBisCO, and the apoproteins of LHCPII in dark-grown yellow cotyledons. Crude extracts of protein were prepared from dark-grown cotyledons cultured at 25°C for 3 weeks (lane 1), dark-grown yellow cotyledons cultured at 8°C for 4 months (lane 2) and dark-grown cotyledons cultured at 8°C for 4 months and then at 25°C for one week (lane 3).

Many gymnosperms have light-dependent and light-independent pathways for the synthesis of Chl in their chloroplasts. The light-independent synthesis of Chl in pine cotyledons is temperature-dependent and is markedly inhibited at 8°C (Muramatsu *et al*. 2001). Substantial levels of transcripts of the *cab*, *rbcS*, *rbcL* and *psbA* genes were found in these cotyledons (Fig. 1). Large amounts of the LSU and SSU of RuBisCO were also detected in the cotyledons, while only traces of the apoproteins of LHCPII were found (Fig. 2). The stable assembly of LHC, as well as that of the two photosystem complexes, requires the synthesis of both Chl and apoproteins. Many Chl-binding proteins are very unstable in the absence of Chl

(Mullet *et al*. 1990). Since relatively high levels of *cab* mRNA were found in the yellow cotyledons (Fig. 2), the imbalance between the supply of Chl and the synthesis of LHCPII apoproteins might have caused very limited accumulation of LHCPII apoproteins and smaller antenna size in the yellow cotyledons. Similar considerations must apply to the assembly of Chl-containing thylakoid components, the PSI and PSII complexes, in dark-grown yellow cotyledons, with resultant limited development of the thylakoid system. In conclusion, light and Chl appear not to be necessary for the expression of some members of *cab* and *rbcS* in the nuclear genome and the expression of *rbcL* and *psbA* in the chloroplast genome in pine cotyledons.

Acknowledgments

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