Plant Physiol. (1992) 98, 39–43
0032-0889/92/98/0039/05/$01.00/0

Received for publication June 5, 1991
Accepted August 5, 1991

Biochemical Characteristics of Thylakoid Membranes in Chloroplasts of Dark-Grown Pine Cotyledons[1]

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ABSTRACT

Japanese black pine (*Pinus thunbergii*) cotyledons were found to synthesize chlorophylls in complete darkness during germination, although the synthesis was not as great as that in the light. The compositions of thylakoid components in plastids of cotyledons grown in the dark and light were compared using sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of polypeptides and spectroscopic determination of membrane redox components. All thylakoid membrane proteins found in preparations from light-grown cotyledons were also present in preparations from dark-grown cotyledons. However, levels of photosystem I, photosystem II, cytochrome b_6/f, and light-harvesting chlorophyll-protein complexes in dark-grown cotyledons were only one-fourth of those in light-grown cotyledons, on a fresh weight basis. These results suggest that the low abundance of thylakoid components in dark-grown cotyledons is associated with the limited supply of chlorophyll needed to assemble the two photosystem complexes and the light-harvesting chlorophyll-protein complex.

Cotyledons of most gymnosperms, especially conifers, can synthesize Chl in complete darkness (4). Chloroplasts developed in the dark contain characteristic paracrystalline prolamellar bodies, stromal, and granal lamellae (15). The reaction center of PSII in spruce chloroplasts developed in the dark is functionally active, but the O_2-evolving enzyme remains latent until cotyledons are exposed to light (13, 22). Because PSI in dark-grown spruce seedlings is active (22), activation of the O_2-evolving enzyme in PSII seems to be the only light-dependent process in the development of the photosynthetic system in coniferous plants. However, details of thylakoid assembly in dark-grown seedlings of coniferous plants have not been documented. As a first approach to understanding the effect of light on the development of the photosynthetic apparatus in coniferous plants, we compared the composition of electron transport components in chloroplasts of *Pinus thunbergii* cotyledons grown in darkness and in the light. Reported here are the results indicating that both photosystems and Cyt b_6/f complex are formed in a functional state in dark-grown pine cotyledons.

[1] This work was supported by a grant from Science and Technology Agency of Japan to Forestry and Forest Products Research Institute and Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

MATERIALS AND METHODS

Plant Material

Japanese black pine (*Pinus thunbergii*) seeds (20) were sown on moist vermiculite, germinated, and grown in an artificial light room (Phytotron) maintained at 25°C and 70% RH with a 14-h photoperiod of about 370 $\mu E \cdot m^{-2}\ s^{-1}$ (light-grown seedlings) or without illumination (dark-grown seedlings). Illumination from metal halide lamps consisted of 42 bulbs of Toshiba Yoko lamps (D400) and 12 bulbs of Mitsubishi BOC lamps (MLRBOC400F). Additional illumination was from 20 tubes of Toshiba fluorescent lamps (FLR110H). Cotyledons were harvested on the 18th day after germination and stored at −80°C until use. All manipulations of dark-grown seedlings were performed in the dark or under a dim green light.

Preparation of Thylakoid Membranes

Frozen cotyledons (10 g fresh weight) were powdered with a mortar and pestle in liquid nitrogen. Thylakoid membranes were prepared from powdered cotyledons by homogenizing with a Polytron in 50 mL of 50 mM Hepes-KOH (pH 7.6), 10 mM EDTA, and 10% (w/v) PEG-4000, and filtering through two layers of Miracloth. Debris was resuspended in the same buffer, homogenized, and filtered. Combined filtrates were centrifuged at 20,000*g* for 60 min, and precipitated membrane fractions were suspended in 5 mL of 50 mM Hepes-KOH (pH 7.6) and 10 mM EDTA. Suspensions were layered over a three-step sucrose gradient (8 mL of 2 M sucrose, 15 mL of 1.3 M sucrose, and 8 mL of 0.4 M sucrose) containing 50 mM Hepes-KOH (pH 7.6) and 10 mM EDTA, and centrifuged at 80,000*g* for 60 min. Thylakoid membrane fractions were collected from the interface between 1.3 M and 2 M sucrose. They were diluted about six times with distilled water and centrifuged at 20,000*g* for 60 min. Pellets were stored at −80°C until use. All treatments were carried out at 4°C under a dim green light.

Electrophoresis

For electrophoretic analysis of thylakoid membrane polypeptides, stored membranes were suspended in 10 mM Na-

Table I. *Size and Chl Content of Light- and Dark-Grown Pine Cotyledons*

Over a hundred cotyledons were used in determining the length and average fresh weight of a cotyledon. Values of Chl content are means ± SE of three measurements.

Cotyledon	Length	Fresh Weight[a]	Chl Content			Chl *a*/Chl *b* Ratio
			Chl *a*	Chl *b*	Total	
	cm	*mg*	*mg/g fresh wt*			
Light-grown	3.46 ± 0.59	6.91	1.00 ± 0.03	0.35 ± 0.01	1.35 ± 0.04	2.89 ± 0.03
Dark-grown	2.34 ± 0.40	3.67	0.33 ± 0.00	0.10 ± 0.01	0.43 ± 0.01	3.25 ± 0.13

[a] Average fresh weight of a cotyledon.

PPi (pH 7.3) and washed three times with the same medium. Washed membranes were solubilized in the SDS sample buffer (17) at room temperature and subjected to SDS-PAGE on a slab gel of 13%, or 12 to 18% linear gradients of acrylamide containing 0.1% (w/v) SDS, using the discontinuous buffer system of Laemmli (17). Gels were stained with Coomassie brilliant blue R-250.

Peptide Sequencing

NH_2-terminal amino acid sequences were determined as described previously (16). After electrophoresis, peptides in the gel were blotted onto a polyvinylidine difluoride membrane (Immobilon, Millipore). The transferred peptides were stained with 0.1% (w/v) Amido black 10B (Bio-Rad) in 50% (v/v) methanol and 10% (v/v) acetic acid for 1 min and destained with distilled water. The stained bands were cut out, treated with 0.6 N HCl for 24 h at 25°C to release blockage at the NH_2-terminus, and subjected to protein sequencing (model 477A, Applied Biosystems).

Determination of the Abundance of Thylakoid Components

The abundance of P700,[2] Cyt b_{559}, Cyt *f*, and Cyt b_6 in thylakoid membranes was determined spectrophotometrically using a Hitachi 557 spectrophotometer as described previously (6). After suspending in 50 mM Tricine-NaOH (pH 7.5), the membranes were treated with 10 mM ferricyanide for 10 min at 4°C and used for determination of each component.

The abundance of P700 was determined by the absorption difference at 700 nm between ascorbate-reduced and ferricyanide-oxidized samples in a two-wavelength mode (reference wavelength, 730 nm). For determination of Cyts, the difference spectra (540–580 nm) between hydroquinone-reduced and ferricyanide-oxidized (Cyt *f*), ascorbate-reduced and hydroquinone-reduced (Cyt b_{559}), and dithionite-reduced and ascorbate-reduced (Cyt b_6) samples were measured sequentially. Abundance was calculated using the absorption difference coefficients of Hiyama and Ke (12) for P700, Böhme *et al.* (5) for Cyt *f*, Garewal and Wasserman (8) for Cyt b_{559}, and Stuart and Wasserman (24) for Cyt b_6.

The amount of Chl and the Chl *a*/*b* ratio were determined spectrophotometrically with acetone extracts (80%) according to Arnon (3). Total protein and Fe-S center (Center X, B, and A in PSI [4Fe-4S] and Rieske Fe-S center in Cyt b_6/f complex [2Fe-2S]) were determined by the method of Lowry *et al.* (18) and by the method of Golbeck and San Pietro (10), respectively.

[2] Abbreviations: P700, reaction center in photosystem I; LHC, light-harvesting Chl-protein complex.

RESULTS

Accumulation of Chl in Dark-Grown Pine Cotyledons

Table I shows the sizes of cotyledons of light- and dark-grown seedlings on the 18th day after germination and the abundance of Chl in each cotyledon. Cotyledons synthesized Chl in complete darkness under ordinary conditions for germination. The total amount of Chl in a dark-grown cotyledon was 0.17 of that in a light-grown one. The ratio of Chl *a* to Chl *b* found in dark-grown cotyledons was always higher than that in light-grown cotyledons, although values were somewhat variable (data not shown). These results suggest that although Chl can be synthesized in the dark, synthesis is limited. The higher Chl *a*/*b* ratio in dark-grown cotyledons suggests that the assembly of LHC is more limited than that of the two photosystem complexes.

Composition of Thylakoid Proteins in Dark-Grown Pine Cotyledons

The protein composition of thylakoid preparations from light- and dark-grown cotyledons was analyzed by SDS-PAGE (Fig. 1). The protein amounts in membranes prepared from light- and dark-grown cotyledons were 10.7 and 19.0 mg/mg Chl, respectively. However, the protein per fresh weight in dark-grown cotyledons was 0.57 of that in light-grown cotyledons. All thylakoid membrane proteins found in light-grown cotyledons, except for the 14.5 kD protein (band I), could also be detected in the preparations from dark-grown cotyledons. The 37, 36, and 22 kD proteins (bands B, C, and E) were found only in the thylakoids of dark-grown cotyledons. The proteins (indicated by black dots) found in O_2-evolving PSII membranes isolated from light-grown cotyledons (K. Shinohara, T. Ono, Y. Inoue, in preparation), such as the two apoproteins of core antenna pigment proteins, the D1 and D2 reaction center proteins, the two LHC II apoproteins, and the three extrinsic proteins (so-called 33, 24, and 18 kD proteins), were detected in both types of thylakoids. A similar observation was reported by Jansson *et al.* (14) using an immunoblotting technique with antibodies against different

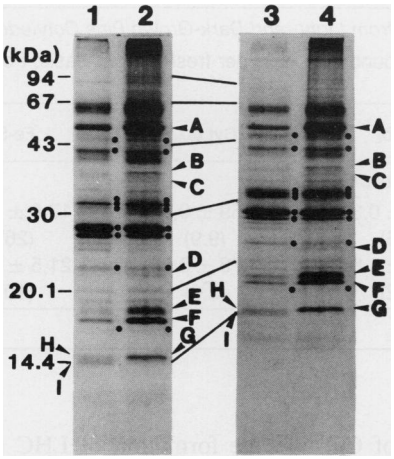

Figure 1. Comparison of thylakoid membrane proteins in light- and dark-grown pine cotyledons. Solubilized thylakoid membranes (2.5 μg Chl) were subjected to SDS-PAGE on 13% (lanes 1 and 2) and 12 to 18% gradients (lanes 3 and 4) of polyacrylamide. Lanes 1 and 3, thylakoid membranes of light-grown cotyledons; lanes 2 and 4, thylakoid membranes of dark-grown cotyledons. Bands A to I were subjected to protein sequencing. Each band with a black dot was found in the preparation of PSII membranes. For details, see the text.

PSII polypeptides. These results indicate that most proteins of the PSII complex are synthesized in dark-grown cotyledons.

We determined the NH_2-terminal amino acid sequences of the major pine thylakoid proteins to help identify the proteins accumulated in the dark (54, 37, 36, 22, 18.5, 17.5, and 15 kD proteins; bands A, B, C, D, E, F, and G) and in the light (15 and 14.5 kD; bands H and I). Although the number of known NH_2-terminal amino acid sequences of pine chloroplast proteins is limited, we succeeded in identifying four polypeptides (A, G, H, and I bands) by comparison with protein sequences in the National Biomedical Research Foundation Protein Sequence Data Bank. The NH_2-terminal sequence (Ala-Gly-Val-?-Asp-Tyr-Arg-Leu-Thr-Tyr-Tyr) of the major protein in band A is the same as that of the NH_2-terminus deduced from the pine *rbcL* on the chloroplast genome (20). The NH_2-terminal sequences (Met-Gln-Val-Trp-Pro-Pro-Phe-Gly-Asn-Pro-Lys) of bands G, H, and I are identical to the putative NH_2-terminus of the mature Rubisco small subunit deduced from the pine cDNA nucleotide sequence (30). These results indicate there are at least two types of Rubisco small subunit in pine cotyledons, which have different electrophoretic mobilities, and that the synthesis of one of them (band I) is light-dependent. This feature may be explained by developmental and light-dependent expression of some members of the *rbcS* gene family (25).

Abundance of Electron Transport Components in Thylakoid Membranes of Dark-Grown Pine Cotyledons

We spectrophotometrically measured the amounts of P700 and Cyts in pine thylakoid membranes. Figure 2 shows typical oxidation-reduction difference spectra of P700, Cyt *f*, Cyt b_{559}, and Cyt b_6 in the thylakoids of light-grown cotyledons. Except for Cyt b_6, each spectrum appears to exhibit the pattern of a single component. There is no difference in the spectral patterns found in the light- and dark-grown thylakoid preparations. The high redox potential form of Cyt b_{559} was lost in the frozen preparations.

Table II summarizes the contents of P700, Cyts, and Fe-S centers per Chl in thylakoid membranes prepared from light- and dark-grown cotyledons. The thylakoid components, P700, Cyt *f*, and Cyt b_{559}, were present in the same proportion in the two different thylakoid preparations. However, the levels of P700, Cyt *f*, and Cyt b_{559} in dark-grown cotyledons were about one-fourth of those in light-grown cotyledons on a per fresh weight basis, and about one-seventh, on a per cotyledon basis. These features indicate that development of the thylakoid system in dark-grown cotyledons is limited. The abundance of Rubisco in the membrane preparations from dark-grown cotyledons is considerably higher (bands A and G in Fig. 1). If the occurrence of Rubisco in our preparations is not an experimental artifact, this suggests that in dark-grown tissues a higher proportion of Rubisco is membrane-bound than is found in light-grown tissues, or that the synthesis of stroma enzymes in dark-grown cotyledons is not affected by the limited development of thylakoids. It has been

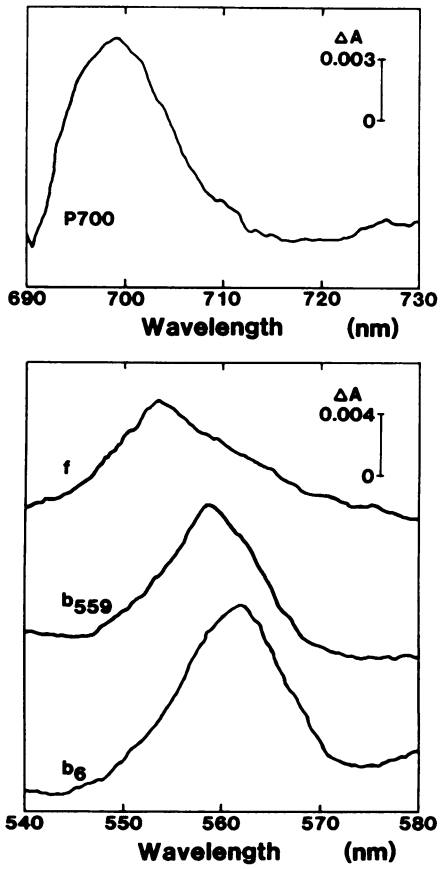

Figure 2. Typical oxidation-reduction difference spectra of P700, Cyt *f*, Cyt b_{559}, and Cyt b_6. The thylakoid membranes (equivalent to 100 nmol Chl/mL) prepared from light-grown pine cotyledons were used. For details, see "Materials and Methods."

Table II. *Abundance of P700, Cyts and Fe-S Centers in Thylakoid Membranes Prepared from Light- and Dark-Grown Pine Cotyledons*

Values are means ± SE of three replicates. Figures in parentheses indicate average abundance on a per fresh weight basis. For details, see the text.

Source of Thylakoid Membranes	P700	Cyt *f*	Cyt b_6	Cyt b_{559}	Fe-S
			mmol/mol Chl		
Light-grown cotyledons	1.93 ± 0.04	2.07 ± 0.31	6.36 ± 0.38	6.59 ± 0.53	17.0 ± 1.34
	(2.9)[a]	(3.1)	(9.5)	(9.9)	(26)
Dark-grown cotyledons	1.69 ± 0.09	1.76 ± 0.06	10.82 ± 1.84	4.73 ± 0.54	21.5 ± 1.84
	(0.81)	(0.84)	(5.2)	(2.3)	(10)

[a] (pmol/g fresh weight).

reported that the amounts of the large and the small subunits of Rubisco are the same in crude extracts prepared from light- and dark-grown cotyledons (31).

The level of Cyt b_6 in dark-grown cotyledons was not as low as those of P700, Cyt *f*, and Cyt b_{559}. This suggests that Cyt b_6 in pine cotyledons is synthesized independently of these other components in the early stages of chloroplast development. The presence of relatively large amounts of Cyt *f*, Cyt b_6, and Rieske Fe-S center proteins in etiolated barley seedlings has been reported (27). It is possible that the Cyt b_6 levels in our case were overestimated due to contamination with mitochondrial Cyt *b*. The contamination may be more significant in preparations from dark-grown cotyledons because of a limited development of the thylakoid system. The amount of Fe-S centers in light-grown cotyledons was 0.63 of the value expected from the amounts of P700 and Cyt *f* present, whereas the amount of Fe-S centers found in dark-grown cotyledons was 0.91. Errors involved in the experimental determination probably underestimate the actual amounts. Despite such errors, these results indicate that Fe-S centers are also formed independent of illumination.

DISCUSSION

The present study shows that all the electron transport components of the photosynthetic apparatus in *P. thunbergii* cotyledons are present in the dark (Fig. 1 and Table II). These results are consistent with previous findings for spruce seedlings by Inoue *et al.* (13) and Oku *et al.* (22), who found that the reaction centers of PSI and PSII are functionally active, although the O_2-evolving system remains latent.

The presence, but relatively low abundance, of all thylakoid components so far tested in dark-grown cotyledons (Table II) indicates that the development of thylakoid system is arrested in the dark. The Chl *a*/*b* ratio in dark-grown cotyledons is always higher (Table I), indicating that the total size of LHC is smaller than those of the two photosystem complexes in the cotyledons. The stable assembly of LHC as well as the two photosystem complexes requires the formation of both Chl and apoproteins. Because it is known that sufficient amounts of total and translatable mRNA for LHC II and of LHC II apoproteins themselves are present in dark-grown pine cotyledons (31), the supply of Chl for the assembly of LHC must be limited in the dark, and the imbalance between the supply of Chl and the formation of LHC apoproteins causes a smaller antenna size in dark-grown cotyledons. Similar phenomena have been reported for conditional Chl *b*-deficient angiosperm mutants (1, 9), for bean seedlings grown under limited light (29), and for cells of *Chlorella* growing under high light intensity (7). Similar limitation must occur in the assembly of Chl-containing thylakoid components, PSI and PSII complexes, and LHC in dark-grown pine cotyledons, resulting in limited development of the thylakoid system.

The pattern within the thylakoid system in dark-grown pine cotyledons is similar to that found in the greening of bean seedlings under limited light (29), except for the loss of O_2 evolution. We have recently succeeded in preparing PSII membranes, which can evolve O_2, from light-grown pine cotyledons, and have examined the difference in protein composition and Mn and Ca abundance between PSII membranes obtained from the dark-grown cotyledons before and after photoactivation of O_2-evolving system (K. Shinohara, T. Ono, Y. Inoue, in preparation). The PSII membranes obtained before photoactivation have no activity for O_2-evolution and contain no Mn atoms in the O_2-evolving enzyme. We have also found that Mn integration into the O_2-evolving enzyme is strictly light-dependent, possibly due to the energy requirement of the process.

It has been recently reported that the transcripts of *cab, rbcS, rbcL,* and *psbA* and the mature proteins of the first three are abundant in total RNA fraction and in crude extracts prepared from dark-grown seedlings of *P. thunbergii* (20, 31). A similar phenomenon is found in dark-grown Douglas-fir seedlings (2). The expression of these nuclear and chloroplast genes is highly tissue-specific but not strongly regulated by light (2, 20, 31). In addition, the present study suggests that the expression of *psaA, psaB, psaC, psbB, psbC, psbD, psbE, psbF, petA,* and *petB* in the chloroplast genome (23), and the genes for Rieske Fe-S protein and the three extrinsic proteins of PSII (11, 19) in the nuclear genome, occur in dark-grown pine cotyledons. Even in angiosperms, the expression of photosynthesis-related genes in the nuclear and the chloroplast genomes has been reported for etiolated seedlings, although generally detected in low amounts (21, 25, 26, 28). The expression of nuclear and chloroplast genes in dark-grown seedlings of gymnosperms and angiosperms may be related to the developmental stage of seedlings and plastids (21), unless the expression is strongly regulated by light.

ACKNOWLEDGMENTS

We are grateful to Prof. H. Sakurai and Mr. T. Fujii of Waseda University for their technical advice on the determination of Fe-S centers, and to Prof. T. Oku of Kyushu University for stimulating discussions. We also wish to thank to Dr. Paul Fons of Tsukuba University for his language reviewing.

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