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Light-Independent Expression of *cab* and *rbcS* Genes in Dark-Grown Pine Seedlings[1]

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ABSTRACT

In angiosperms, light has been shown to induce the expression of *cab* and *rbcS* genes, which encode the apoprotein of light-harvesting chlorophyll *a/b* binding protein (LHCP) and the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), respectively. By contrast, chlorophylls are synthesized in the cotyledons of pine seedlings even if seeds are germinated in the dark. We have examined the expression of *cab* and *rbcS* genes in the cotyledons of pine (*Pinus thunbergii*) seedlings grown in darkness. The proteins of LHCP and the large subunit and the small subunit of Rubisco were detected in the cotyledons of dark-grown seedlings. The transcripts of *cab* and *rbcS* genes were present at substantial levels in dark-grown seedlings. However, the transcripts and the translated products of the genes were not found in the embryos of dry seeds. These results indicate that light is not required for the expression of *cab* and *rbcS* genes during the course of development of the cotyledons of pine seedlings. The processing of the precursor polypeptides of the mature proteins also appears to take place even in the dark.

Light is a modulator of complex developmental and regulatory mechanisms in higher plants. In the past decade, the regulation of gene expression by light has been studied extensively at the molecular level (23). Of the genes whose transcription is regulated by light, the *cab*[2] and *rbcS* genes are the ones whose regulation has been studied in greatest detail (23). These genes encode the LHCP and the SSU of Rubisco. Products of both genes are synthesized in the cytoplasm as precursor polypeptides of higher molecular masses than the mature functional peptides. Each precursor is transported into a chloroplast, processed to the size of the mature protein, and then assembled as part of the PSII complex or combined with LSU to form the holoenzyme of Rubisco.

Although light is generally required for the synthesis of Chl in angiosperms, coniferous plants can produce large amounts of Chl in cotyledons of dark-grown seedlings (15). This phenomenon suggests that the development of chloroplasts can proceed without illumination in conifers. The expression of *cab* and *rbcS* is positively regulated by a phytochrome-mediated response (5, 6, 11, 13, 20), but the fact that the cotyledons of dark-grown seedlings of coniferous plants contain chlorophylls suggests that some genes, such as *cab* and *rbcS*, are expressed even in darkness. In fact, Alosi *et al.* (1) recently showed the presence of LHCP and *cab* transcript in dark-grown Douglas-fir seedlings.

In the present study, we examined whether two genes, *cab* and *rbcS*, are expressed in the cotyledons of dark-grown pine seedlings. Our results indicate that, even in the dark, the levels of the transcripts increase and the translated products of each gene are accumulated in the course of germination.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Pine (*Pinus thunbergii*) seeds were germinated on moist vermiculite at 25°C in the dark and under white light. Seed embryos were isolated from 3-d-imbibed and 8-d-germinated seeds. The embryos in the former case did not develop, and they were yellow in color. They are denoted as imbibed embryos in the text. Protrusion of the root was observed approximately within 1 week of germination. The embryos of 8-d-imbibed seeds, denoted as germinated embryos, did not undergo extensive development and the hypocotyls were pale green. After 14 d, cotyledons were excised from dark- and light-grown seedlings. Embryos from dark-imbibed and dark-germinated seeds and cotyledons from dark-grown seedlings were collected under light from a dim green safelight (λ_{max}, 510 nm; 0.5 $\mu E \cdot m^{-2}\ s^{-1}$) after it had been passed through an interference filter. The plant materials were immediately frozen in liquid nitrogen and stored at −70°C prior to analysis. Seed embryos were also excised from nonimbibed, dry seeds and are denoted as dry embryos.

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[2] Abbreviations: *cab*, nuclear genes encoding LHCP; LHCP, apoprotein of light-harvesting Chl *a/b* binding protein; Rubisco, ribulose bisphosphate carboxylase/oxygenase; *RbcS*, nuclear genes encoding the small subunit of Rubisco; SSU, small subunit of Rubisco; LSU, large subunit of Rubisco; *rbcL*, a chloroplast gene encoding the LSU of Rubisco; SSPE, buffer containing 0.18 M NaCl, 0.01 M Na-phosphate (pH 6.8), and 0.001 M EDTA; SSC, buffer containing 0.15 M NaCl and 0.015 M Na-citrate (pH 7.0).

Light

White light was obtained from 20 fluorescent lamps (Toshiba FLR110H.W/A) and two kinds of metal halide lamp, 28 Yoko lamps (Toshiba DR-400) and 12 B.O.C. lamps (Mitsubishi MLRBOC400F-U), and its fluence was 580 $\mu E \cdot m^{-2} s^{-1}$. Red light ($\lambda_{max}$, 660 nm; 2.3 $\mu E \cdot m^{-2} s^{-1}$) and far-red light ($\lambda_{max}$, 730 nm; 2.3 $\mu E \cdot m^{-2} s^{-1}$) were obtained by use of interference filters (Vacuum Optics Corporation of Japan, W type).

Determination of Chl Content

Chl contents were determined by the methods of Ogawa and Shibata (14).

Antibodies

A thylakoid membrane fraction was prepared from fresh cotyledons of light-grown pine seedlings by the method of Delepelaire and Chua (4). The apoprotein of LHCP and the SSU of Rubisco were separated by electrophoresis on preparative denaturing polyacrylamide gels (8). Antisera were raised in rabbits by injection of each purified protein. Antiserum was also raised against the Rubisco holoenzyme isolated from tobacco leaves (3).

Electrophoresis and Immunological Detection of LHCP and Rubisco

Embryos and cotyledons were ground in an equal volume of buffer that contained 0.1 M Tris (pH 8.0) and 28 mM 2-mercaptoethanol. The crude extracts were fractionated by SDS-PAGE as described by Laemmli (8). After electrophoretic transfer of proteins, nitrocellulose filters were probed first with antisera and then with horseradish peroxidase-conjugated antibodies raised in goat against rabbit IgG (Bio-Rad).

Preparation of RNA

Total RNA was extracted from dry embryos, imbibed embryos, and cotyledons of seedlings with 0.1 M Tris buffer (pH 9.0) containing 1% SDS, 1% ascorbate, and 5% 2-mercaptoethanol and an equal volume of a mixture of phenol saturated with 0.1 M Tris (pH 9.0), chloroform, and isoamyl alcohol (25:24:1, v/v). The aqueous extract was loaded onto 10 mL of 5.7 M CsCl and centrifuged at 130,000*g* (in a Hitachi SRP 28 rotor) for 24 h. When RNA was prepared from small amounts of plant tissue, the aqueous extract was loaded on 0.5 mL of 5.7 M CsCl and centrifuged at 435,000*g* (in a Beckman TLA-100.2 rotor). From the precipitated total RNA, contaminating DNA, and low mol wt RNA were removed by precipitation with 2.0 M LiCl. Poly(A)$^+$ RNA was obtained by fractionation of total RNA on a column of oligo(dT) cellulose.

Translation *in Vitro* and Indirect Immunoprecipitation

Poly(A)$^+$ RNA was translated in a reticulocyte lysate, cell-free translation system in the presence of L-[^{35}S]Met (Amersham) at 30°C for 1.5 h. Indirect immunoprecipitation of the translation products with the antisera raised against LHCP and SSU was carried out by the method of Watanabe and Price (25).

Northern Blot Hybridization

Aliquots of poly(A)$^+$ (0.1 μg) or poly(A)$^-$ RNA (5 μg) were denatured in 33 mM Mops (pH 7.0), 8 mM sodium acetate, and 2 mM EDTA at 65°C for 15 min. The denatured RNAs were subjected to electrophoresis on 1% agarose gels that contained 0.6 M formaldehyde in 20 mM Mops (pH 7.0), 5 mM sodium acetate and 1 mM EDTA, and transferred to a Biodyne A nylon membrane (Pall Ultrafine Filtration Corporation). Prehybridization and hybridization were performed in the presence of 50% formamide, 5 × SSPE, 0.1% SDS, denatured salmon sperm DNA (1.0 mg/mL) and 5 × Denhardt's solution at 42°C. The cDNA inserts of pPDLHC2176 (26) and pPDSSU4 (27) were labeled with [α-^{32}P]dCTP (Amersham) using the Multiprime DNA labeling system (Amersham), and the cDNA probes were included in the hybridization mixture. The filters were washed with 0.1 × SSC and 0.1% SDS at 65°C.

RESULTS

Cotyledons of dark-grown pine seedlings contained approximately 10 mg of Chl *a* and 3 mg of Chl *b* per gram fresh weight. These levels were between one-third and one-half of those measured in the light-grown seedlings. To examine the presence of LHCP, crude extracts prepared from dry, imbibed, and germinated embryos and cotyledons were subjected to SDS-PAGE with subsequent Western blot hybridization. Substantial amounts of LHCP were detected as a doublet of 28 and 29 kD in the cotyledons of both light- and dark-grown seedlings, but LHCP was not detected in the dry embryos and imbibed embryos. The accumulation of LHCP was shown to occur at an earlier stage of seed germination, namely after 8 d, in the light (Fig. 1).

The presence of both LSU and SSU were also examined. When the nitrocellulose filter was reacted with antiserum against tobacco Rubisco after transfer of proteins in a preliminary experiment, the antiserum reacted only with the LSU of pine Rubisco, and not with pine SSU. This result may be due to the conservation of *rbcL* genes and the divergence of *rbcS* genes, *i.e.* a high degree of homology, 91%, was observed between the amino acid sequences deduced from the nucleotide sequences of pine (11a) and tobacco *rbcL* (19), whereas the amino acid sequence of mature SSU from pine (27) was not so similar (only 71% homology) to that of tobacco SSU (10). Therefore, the presence of SSU and LSU was confirmed by use of the antisera raised against tobacco Rubisco and pine SSU, respectively. Substantial amounts of both LSU and SSU were present in the cotyledons of dark-grown pine seedlings (Fig. 1). A small amount of SSU and LSU was detected in the germinated embryos. However, they were not detected in dry embryos. The results show that the accumulation of Rubisco occurred at an earlier stage of germination.

To determine at what level the expression of *cab* and *rbcS* genes is regulated in pine seedlings, the effect of white light on the level of the transcripts was examined. Poly(A)$^+$ RNAs were isolated from cotyledons of dark- and light-grown seed-

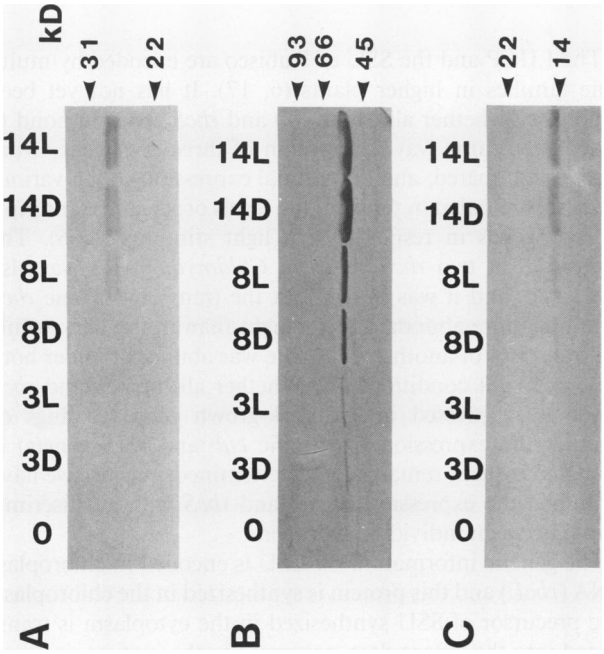

Figure 1. Accumulation of chloroplast proteins during the course of germination under dark conditions. Pine seeds were germinated for 3, 8, and 14 d in the dark (D) and in the light (L). Whole embryos were isolated from embryos of dry (0), 3-d-imbibed seeds (3D and 3L), and 8-d-germinated seeds (8D and 8L). Cotyledons were excised from 14-d-old dark-grown seedlings (14D) and from 14-d-old light-grown seedlings (14L). Crude extracts prepared from whole embryos and cotyledons were subjected to SDS-PAGE on an equal fresh-weight basis (1 mg). After electrophoretic transfer of proteins, nitrocellulose filters were reacted with antisera against pine LHCP (A), tobacco Rubisco (B), or the pine SSU (C). Antiserum against Rubisco from tobacco reacts only with pine LSU but not with pine SSU. Numbers to the right of lanes indicate molecular mass standards.

lings, translated in a reticulocyte cell-free system, and the precursor polypeptides of LHCP and SSU were immunoprecipitated from the cell-free translation mixtures (Fig. 2). Two precursors to LHCPs were observed as a doublet on SDS-PAGE and their molecular masses were estimated to be approximately 32 and 35 kD (Fig. 2). The molecular masses of the precursor to SSU was also estimated to be 26 kD. These values were also deduced to be 28.5 and 19.3 kD from the cDNA sequences that encode LHCP (26) and SSU (27) of pine. The molecular masses estimated on SDS-PAGE are larger than those deduced from the cDNA sequence. The reason for the discrepancy between the values is not known. However, the results demonstrate the presence of active transcripts of both *cab* and *rbcS* at a substantial level in the cotyledons of dark-grown seedlings.

We have cloned the cDNAs that encode LHCP and the SSU using poly(A)$^+$ RNA from dark-grown pine seedlings (26, 27). The isolation of the cDNA clones using mRNA from cotyledons of dark-grown seedlings itself demonstrates that the mRNAs for LHCP and SSU are present in the dark-grown pine seedlings. In the experiments whose results are shown in Figure 3, the two plasmids from the cDNA clones were used as hybridization probes to examine the accumulation of the respective transcripts in embryos and cotyledons during germination in the dark and light. Poly(A)$^+$ RNA was prepared from dry embryos, imbibed embryos, and cotyledons. After electrophoresis and transfer to a nylon membrane, RNA was hybridized to ^{32}P-labeled cDNA probes dissected from pPDLHC2176 (26) and from pPDSSU4 (27), which encode LHCP and SSU, respectively. The results shown in Figure 3 indicate that the transcripts from *cab* and *rbcS* genes were abundant in the cotyledons of dark-grown seedlings. No transcripts of the genes were detected in the poly(A)$^+$ (Fig. 3) and poly(A)$^-$ RNA (our unpublished data) from dry and imbibed embryos.

The transcription of *cab* (11, 13, 20) and *rbcS* (5, 6, 20) genes are regulated by phytochrome in angiosperms. To examine the involvement of phytochrome in the expression of the two genes of interest, in terms of levels of the respective transcripts, 14-d-old dark-grown seedlings were exposed to red light and far-red light. When different lengths of time, ranging one to 24 h, has elapsed after the treatment with red or far-red light, total RNA was prepared from the cotyledons and the relative level of transcripts of the genes were analyzed in a similar manner. We did not detect any distinct effect of red light or far-red light on the levels of the transcripts (our unpublished data).

DISCUSSION

The regulation of *cab* and *rbcS* genes by light has been studied most extensively at the molecular level in angiosperms (23), but little is known with respect to gymnosperms except for a recent report on dark-grown Douglas-fir plant (1). Positive regulation of the expression of *cab* and *rbcS* genes by light was not observed in pine seedlings, even though levels of the transcripts of these genes and their translated proteins

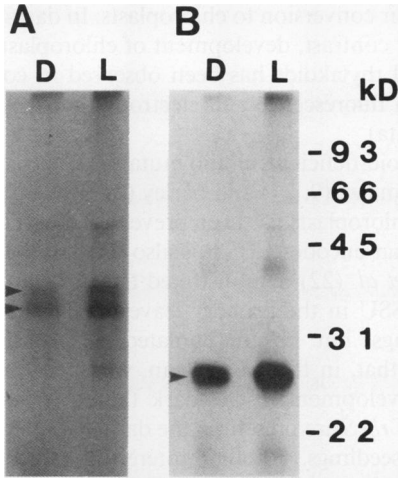

Figure 2. Fluorograms of immunoprecipitates of translation products showing the presence of mRNAs that encode LHCP and SSU. Pine seeds were germinated for 14 d in the dark (D) and in the light (L). Poly(A)$^+$ RNAs were prepared from the cotyledons of 14-d-old seedlings and translated at a concentration of 0.3 μg/μL in a reticulocyte lysate, cell-free system. Precursors to LHCP and SSU were identified among the products of translation by immunoprecipitation with antisera raised against LHCP (A) and the SSU (B). Immunoprecipitates were subjected to electrophoresis on SDS-PAGE. Numbers to the right of lanes indicate molecular mass standards.

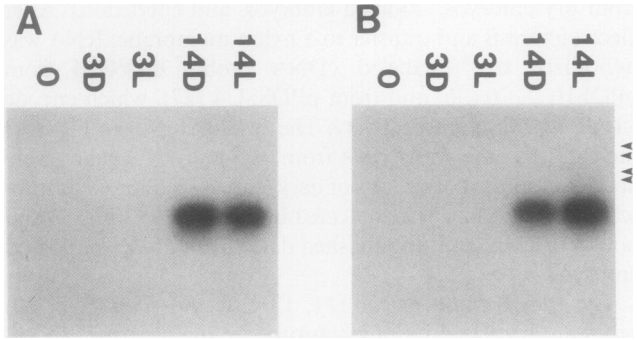

Figure 3. Accumulation of transcripts of *cab* and *rbcS* genes in the course of germination under dark conditions. Poly(A)$^+$ RNA was isolated from dry seeds embryos (0), 3-d-imbibed seeds kept in the dark (3D) or in the light (3L), and 14-d-old seedlings grown in the dark (14D) or in the light (14L). The samples of denatured RNA were subjected to electrophoresis, transferred to nylon membranes, and hybridized with the cDNAs that encode LHCP (A) and SSU (B). The mobilities of yeast 28S and 18S rRNA (3.4 and 1.8 kilobases), and those of *Escherichia coli* 23S and 16S rRNA (2.9 and 1.5 kilobases) are indicated by arrowheads.

are known to increase in response to light in many angiosperms. Expression of *cab* and *rbcS* genes in darkness was confirmed in pine seedlings by three experiments (Figs. 1, 2, and 3). It should be noted that light is not required for the expression of *cab* and *rbcS* genes. Expression of these genes may be controlled by factor(s) other than light signals.

The light-independent expression of *cab* and *rbcS* genes may be related to the relatively greater extent of development of chloroplasts in the dark-grown seedlings of pine than in those of angiosperms. In general, in etiolated plants, proplastids develop only to the etioplast stage and exposure to light results in their conversion to chloroplasts. In dark-grown pine seedlings, by contrast, development of chloroplasts that contain Chl and thylakoids has been observed in cotyledonous cells by both fluorescence and electron microscopy (our unpublished data).

In carotenoid-deficient, albino mutants or herbicide-treated seedlings of maize (9, 21) and barley (2), in which the development of chloroplasts has been prevented, the accumulation of mRNA that encodes LHCP is also prevented in the light. Thompson *et al.* (22) demonstrated the presence of mRNA specific for SSU in the primary leaves of dark-grown mung bean seedlings, but not in etiolated pea seedlings. They pointed out that, in the mung bean, which undergoes more extensive development in the dark than does the pea, the transcripts of *rbcS* are present in the dark at higher levels than in the pea seedlings. Another interesting observation was reported by Vernet *et al.* (24). White callus of *Nicotiana sylvestris* grown in the light did not show any Rubisco activity and contained only an extremely low level of the transcript of the *rbcS* gene (24). Similarly, in chloroplast-free parsley cells, cultured in the light, mRNA for the LHCP apoprotein was not detectable (12). These data suggest that the development of chloroplast is important or necessary for the expression of *cab* and *rbcS* genes. It is possible that the expression of *cab* and *rbcS* genes is responsible for the development of plastid.

The LHCP and the SSU of Rubisco are encoded by multigene families in higher plants (6, 17). It has not yet been established whether all of the *cab* and *rbcS* genes respond to light in the same way. Expression of three *rbcS* genes from pea was compared, and differential expression of the various genes was revealed in terms of the levels of specific transcripts of *rbcS* genes in response to a light stimulus (5, 6). The expression of two *rbcS* genes in *Chlamydomonas* was also compared, and it was shown that the transcript of one *rbcS* gene was more abundant in the light than in the dark, while the transcript of another *rbcS* gene was abundant under both dark and light conditions (7). Whether all the *cab* and *rbcS* genes are expressed in the dark-grown pine seedlings or whether the expression of specific *cab* and *rbcS* gene(s) is regulated by light remains to be determined, because we have examined the expression of *cab* and *rbcS* without discriminating between individual members.

The genetic information for LSU is encoded in chloroplast DNA (*rbcL*) and this protein is synthesized in the chloroplast. The precursor of SSU synthesized in the cytoplasm is transported into the chloroplast, processed to the mature size, and assembled with LSU to form the holoenzyme. However, in *Chlamydomonas*, when the pool of LSU is depleted, the imported SSU is selectively and rapidly degraded in the chloroplast (18). Expression of the chloroplast *rbcL* gene and the nuclear *rbcS* gene is probably coordinated, even in the dark.

With respect to the products of translation, LHCP and SSU were detected by immunoprecipitation as larger precursors with molecular masses of approximately 32 and 35 kD and 26 kD on SDS-PAGE, respectively (Fig. 2). By contrast, in the cotyledons of dark-grown seedlings, two LHCPs and SSU were detected as the mature proteins with molecular masses of 29 and 28 kD and 14 kD which were smaller than the precursor proteins (Fig. 1). This result suggests that, in dark-grown pine seedlings, the transport of precursors to chloroplasts and the processing to mature proteins also occurs so that the functional complexes can be formed. Oku *et al.* (15) reported that, in dark-grown pine (*P. silvestris*) seedlings, a functional PSI developed without any exposure of seedlings to light, while no PSII activity was detected. In dark-grown spruce seedlings, the PSII reaction center and the PSII-associated electron transfer system were assembled in the primary thylakoid membranes, but the oxygen-evolving system remained latent unless the seedlings were exposed to light (16). The mechanism of expression of *cab* and *rbcS* genes and the roles of LHCP and Rubisco in the chloroplast of dark-grown pine seedlings remain to be clarified.

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