The Plant Cell, Vol. 11, 901–910, May 1999, www.plantcell.org

Plastid Translation Is Required for the Expression of Nuclear Photosynthesis Genes in the Dark and in Roots of the Pea *lip1* Mutant

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The expression of nuclear photosynthesis genes in pea seedlings requires both light and a postulated signal produced by developing plastids. The requirement for the plastid signal for the accumulation of transcripts of *Lhcb1*, *RbcS*, *PetE*, and *AtpC* genes was investigated in the pea mutant *lip1*, which shows light-independent photomorphogenic development. Lincomycin and erythromycin, inhibitors of plastid translation, decreased the accumulation of transcripts of nuclear photosynthesis genes in shoots of light-grown wild-type and *lip1* seedlings, indicating that the plastid signal is required in the *lip1* mutant. Treatment with lincomycin or erythromycin also reduced the accumulation of transcripts in shoots of dark-grown *lip1* seedlings, indicating that light is not an obligate requirement for the synthesis or activity of the plastid signal. Lincomycin had a similar effect on the accumulation of *Lhcb1* transcripts in dark-grown *cop1-4* seedlings of Arabidopsis. Accumulation of transcripts of nuclear photosynthesis genes was also observed in roots of light-grown *lip1* seedlings, and this accumulation, which was associated with the development of chloroplasts, was again dependent on plastid translation. The plastid signal therefore regulates the expression of nuclear photosynthesis genes in the dark and in roots of the *lip1* mutant.

INTRODUCTION

During early development, higher plants can follow one of two developmental patterns (McNellis and Deng, 1995). In continuous darkness, seedlings follow skotomorphogenic (etiolated) development: etiolated seedlings have long hypocotyls, apical hooks, small unopened cotyledons that contain etioplasts, and low levels of expression of nuclear photosynthesis genes. If seedlings are grown in the light, they follow photomorphogenic (deetiolated) development. In contrast to etiolated seedlings, light-grown seedlings have short hypocotyls, no apical hooks, open and expanded cotyledons containing photosynthetically competent chloroplasts, and high levels of expression of nuclear photosynthesis genes (Staub and Deng, 1996).

This increased expression of nuclear photosynthesis genes also requires the presence of intact chloroplasts. It has been proposed that a plastid-derived signal is required for the expression of nuclear photosynthesis genes (reviewed in Oelmüller, 1989; Taylor, 1989; Susek and Chory, 1992). Treatment of mustard seedlings with norflurazon, a noncompetitive inhibitor of phytoene desaturase (Young, 1991), resulted in photooxidation of chloroplasts without the disruption of cytosolic ribosomes (Frosch et al., 1979; Reiss et al., 1983). Norflurazon-treated mustard seedlings showed a decrease in transcripts of *Lhcb* (for light-harvesting chlorophyll *a*/*b* binding proteins) and *RbcS* (for small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) genes (Oelmüller et al., 1986). Similar effects on *Lhcb* and *RbcS* transcripts were observed if chloramphenicol (an inhibitor of plastid translation) was applied to mustard seedlings during early development (Oelmüller et al., 1986). Treatment of barley seedlings with tagetitoxin, an inhibitor of chloroplast transcription, also reduced the levels of *Lhcb* and *RbcS* transcripts (Rapp and Mullet, 1991), suggesting a role for plastid RNA in signaling.

Because the plastid signal is required continuously (Oelmüller et al., 1986; Burgess and Taylor, 1988) and inhibitors of plastid translation, such as chloramphenicol (Oelmüller et al., 1986), lincomycin (Gray et al., 1995), and erythromycin (Gray et al., 1995), have no effect on nuclear photosynthesis gene expression if applied later than 48 to 72 hr after germination, the plastid signal cannot be a direct product of plastid gene expression. However, because such inhibitors decrease the expression of nuclear photosynthesis

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genes if applied within 48 to 72 hr of germination, the synthesis of the plastid signal must involve a product of early plastid gene expression (Gray et al., 1995).

Light and the development of plastids are both crucial factors in the expression of nuclear photosynthesis genes (Taylor, 1989; Thompson and White, 1991; Argüello-Astorga and Herrera-Estrella, 1998; Leon et al., 1998). Analysis of light-regulated promoters has identified many *cis*-acting light-responsive elements, although no universal element has been found in light-regulated promoters (Terzaghi and Cashmore, 1995; Argüello-Astorga and Herrera-Estrella, 1998). Analysis of promoters of nuclear photosynthesis genes has shown that elements required for light regulation cannot be separated from those required for the response to the developmental state of plastids (Bolle et al., 1994; Kusnetsov et al., 1996; Puente et al., 1996). It has been proposed that the light and plastid signal regulatory pathways merge before the regulation of gene expression, and these pathways may act through a common *trans*-acting factor (Kusnetsov et al., 1996; López-Juez et al., 1996; Puente et al., 1996).

This study was designed to investigate in greater detail the proposed link between light and the plastid signal. In particular, we decided to determine whether the plastid signal regulates photosynthesis gene expression in the dark. However, under normal skotomorphogenic development, nuclear photosynthesis genes are expressed only at low levels. Therefore, we have used the pea *lip1* mutant, which shows light-independent photomorphogenesis (Frances et al., 1992). This mutant has many of the characteristics associated with photomorphogenic mutants previously identified in other species, including Arabidopsis (Wei and Deng, 1996). Dark-grown *lip1* peas have short stems, expanded leaves containing partially developed plastids, and an abundance of nuclear photosynthesis gene transcripts (Frances et al., 1992). The recessive *lip1* mutation, which maps to a single locus, also produces pleiotropic effects throughout plant development. These include dwarfism, an inability of green plants to respond to darkness, and a change in the gibberellin GA_{20}-to-GA_{19} ratio when compared with wild-type plants (Frances et al., 1992; Sponsel et al., 1996; Frances and Thompson, 1997).

To investigate the relationship between light and production of the plastid signal, we examined the effect of lincomycin and erythromycin on wild-type and *lip1* seedlings. The inhibition of plastid translation decreased the transcripts of several nuclear photosynthesis genes in shoots of wild-type and *lip1* light-grown seedlings. Treatment with lincomycin or erythromycin also reduced the accumulation of transcripts in shoots of dark-grown *lip1* seedlings and in roots of light-grown *lip1* seedlings. Lincomycin treatment also reduced the accumulation of *Lhcb1* transcripts in dark-grown seedlings of the Arabidopsis photomorphogenic mutant *cop1-4.* This clearly demonstrates that the plastid-derived signal is synthesized and is able to regulate nuclear gene expression in the absence of light in both pea and Arabidopsis.

RESULTS

Inhibition of Plastid Translation Does Not Affect the Photomorphogenic Development of Wild-Type or *lip1* Seedlings

Preliminary experiments were performed to examine the effect of the inhibition of plastid translation on pea plant development and morphology. Wild-type and *lip1* seedlings were grown on either water or 0.5 mM lincomycin for 5 days in darkness followed by growth for either 2 days in the light or 2 days in the dark. Representative seedlings for each treatment are shown in Figure 1. Wild-type seedlings grown on water showed photomorphogenic development in the light; the seedlings had short stems and an expanded green shoot apex. Wild-type seedlings grown in continuous darkness on water showed skotomorphogenic development; the seedlings had long stems and a closed, unexpanded, yellow apical hook. Both light- and dark-grown *lip1* seedlings germinated on water showed photomorphogenic development, as has been previously reported (Frances et al., 1992). Dark-grown *lip1* seedlings did not accumulate chlorophyll, due to the essential light requirement for the conversion of pro-

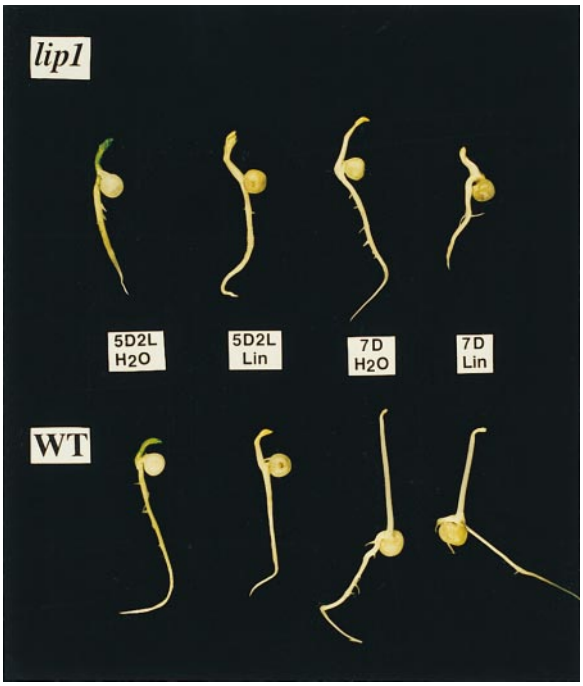

Figure 1. Phenotype of Light- and Dark-Grown Wild-Type and *lip1* Seedlings Germinated on Water or Lincomycin.

Peas were germinated on water or 0.5 mM lincomycin (Lin) and grown for either 5 days in the dark followed by 2 days in the light (5D2L) or for 7 days in continuous darkness (7D). WT, wild-type.

tochlorophyllide to chlorophyllide by protochlorophyllide reductase (Forreiter et al., 1991). Treatment with lincomycin (or 0.5 mM erythromycin; data not shown) caused complete chlorosis of light-grown wild-type and *lip1* seedlings but had no significant effect on the morphology of either light- or dark-grown wild-type or *lip1* seedlings. This suggests that any changes in gene expression are likely to be due to the inhibition of plastid translation rather than to changes in seedling development.

Plastid Translation Is Required for the Expression of Nuclear Photosynthesis Genes in Shoots of Light-Grown Wild-Type and *lip1* Seedlings

To investigate the effect of plastid translation on the accumulation of transcripts of photosynthesis genes, we treated wild-type and *lip1* seedlings with either water or 0.5 mM lincomycin. Lincomycin has been shown previously to inhibit chloroplast protein synthesis (Ellis and Hartley, 1971) but has little effect on mitochondrial protein synthesis in vitro (Pope, 1976). Seedlings were grown for 5 days in darkness followed by 2 days in light, and total RNA was extracted from shoot tissue. Figure 2 shows the results of an RNA gel blot analysis using probes for the nuclear photosynthesis genes *Lhcb1*, *RbcS*, *PetE* (plastocyanin), and *AtpC* (γ subunit of chloroplast ATP synthase). Hybridization to an rRNA probe was used as a loading control for quantification (Figure 3).

Increased amounts of photosynthesis gene transcripts were observed in the shoots of light-grown *lip1* seedlings grown on water compared with the shoots of wild-type seedlings. Transcripts were 1.6- to 10.6-fold higher in water-grown *lip1* shoots than in the shoots of wild-type seedlings (Figure 3). Treatment with lincomycin consistently reduced the accumulation of transcripts of all photosynthesis genes examined in both light-grown wild-type and *lip1* shoots but had no effect on the mRNA for polyubiquitin or high-mobility-group protein HMG-I/Y in four replicate treatments (data not shown). Essentially identical results to those presented in Figures 2 and 3 were obtained in all repeated experiments. The decrease in transcript abundance due to lincomycin treatment varied among the genes examined and between wild-type and *lip1* seedlings. In light-grown lincomycin-treated wild-type seedlings, transcripts decreased to 24 to 55% of the amounts in untreated seedlings (Figure 3). In light-grown *lip1* seedlings, the decreases were slightly smaller (to 40 to 65% of the amounts in control seedlings).

Treatment with 0.5 mM erythromycin produced effects similar to those of lincomycin on *Lhcb1* mRNA abundance in shoots of light-grown wild-type and *lip1* seedlings (Figure 4). Treatment with erythromycin inhibits plastid protein synthesis but has no effect on mitochondrial translation (Tassi et al., 1983). Inhibition of plastid translation results in decreased accumulation of transcripts of several photosynthesis genes in shoots of light-grown wild-type and *lip1* seedlings. This indicates that pea has a requirement for plastid protein synthesis for the expression of nuclear photosynthesis genes similar to that found in mustard and tobacco (Oelmüller et al., 1986; Gray et al., 1995).

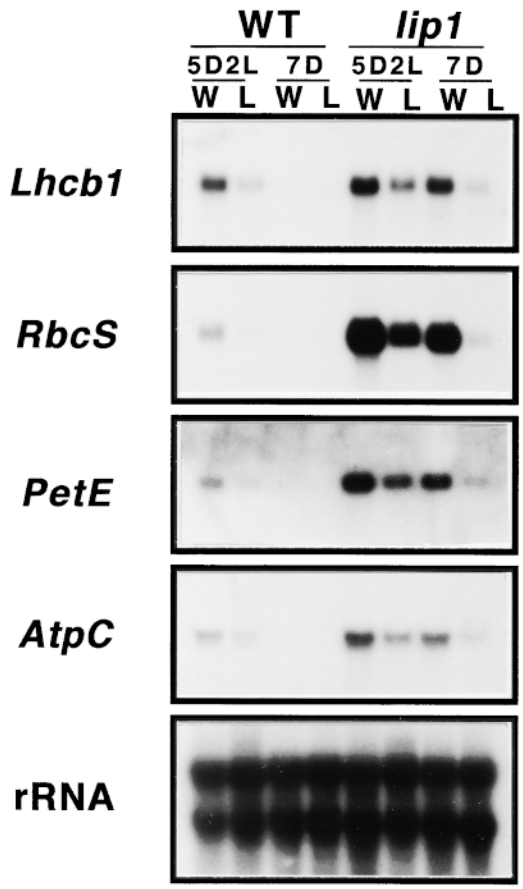

Figure 2. Effect of Lincomycin on the Accumulation of Transcripts of Nuclear Photosynthesis Genes in Wild-Type and *lip1* Shoots.

Wild-type Alaska (WT) and *lip1* seeds were germinated on either water (W) or 0.5 mM lincomycin (L) and grown for 5 days in the dark followed by 2 days in the light (5D2L) or in continuous darkness for 7 days (7D). Shoot tissue was excised from the seedlings, and total RNA was extracted and subjected to RNA gel blot analysis using ^{32}P-labeled probes from the nuclear photosynthesis genes *Lhcb1*, *RbcS*, *PetE*, and *AtpC*. Hybridization with a probe for rRNA was used as a loading control.

Plastid Translation Is Required for the Accumulation of Transcripts of Photosynthesis Genes in Shoots of Dark-Grown *lip1* Seedlings

To investigate whether plastid translation is required for the accumulation of transcripts of photosynthesis genes in the

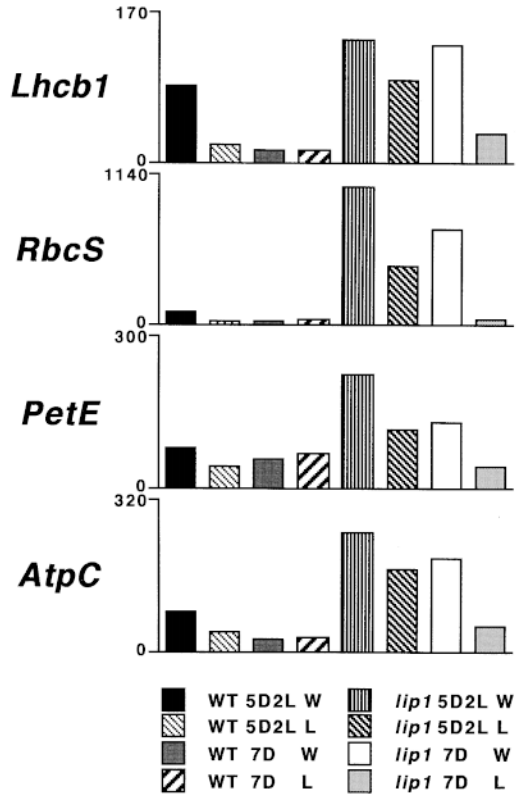

Figure 3. Quantification of the Effect of Lincomycin on Transcript Accumulation in Wild-Type and *lip1* Shoots.

Hybridization signals from the autoradiographs shown in Figure 2 were quantified using a laser scanning densitometer and normalized to rRNA to account for any small differences in the loading of total RNA. For each probe, the amounts of transcripts are expressed relative to wild-type plants grown on water for 5 days in the dark and 2 days in the light (5D2L), which was given an arbitrary value of 100. Abbreviations are as given in the legend to Figure 2.

dark, we germinated both wild-type and *lip1* seedlings on water or 0.5 mM lincomycin and grew them for 7 days in continuous darkness. Shoot tissue was excised, and total RNA was extracted and subjected to RNA gel blot analysis. The results obtained are shown in Figures 2 and 3.

Only very small amounts of transcripts of photosynthesis genes were detected in shoots of dark-grown wild-type seedlings grown on either water or lincomycin. However, much greater amounts of all photosynthesis gene transcripts examined were detected in shoots of dark-grown *lip1* seedlings germinated on water. Transcripts were 2.3- to 47.6-fold higher in *lip1* seedlings compared with wild-type seedlings. In all cases, the amounts of transcripts of photosynthesis genes in shoots of dark-grown *lip1* seedlings were reduced by treatment with lincomycin. The water/lincomycin ratios for transcripts in shoots of dark-grown *lip1* seedlings were 4.0 for *Lhcb1*, 3.1 for *PetE*, 3.8 for *AtpC*, and 17.0 for *RbcS.* Essentially identical results were obtained in four replicate experiments. A similar decrease in *Lhcb1* mRNA was observed in shoots of dark-grown *lip1* seedlings after treatment with 0.5 mM erythromycin (Figure 4). This clearly demonstrates that plastid translation is required for the expression of nuclear photosynthesis genes in the dark in shoots of *lip1* seedlings.

Plastid Translation Is Required for the Accumulation of Transcripts of Photosynthesis Genes in Roots of *lip1* Seedlings

Because the organ specificity of photosynthesis gene expression has been shown to be altered in some of the photomorphogenic mutants previously identified in Arabidopsis (Chory and Peto, 1990; Deng et al., 1991), the accumulation of transcripts of photosynthesis genes in the roots of wild-type and *lip1* seedlings was investigated. Both wild-type and *lip1* seedlings were grown on water or 0.5 mM lincomycin, and total RNA was extracted from root tissue and subjected to RNA gel blot analysis with probes for the nuclear photosynthesis genes *Lhcb1, RbcS, PetE*, and *AtpC.* The hybridization signal of rRNA was used as a loading control. The results obtained are shown in Figures 5 and 6.

Transcripts of nuclear photosynthesis genes accumulated to a much greater extent in roots of light-grown *lip1* seedlings compared with the roots of light-grown wild-type seedlings. Transcripts were 1.2- to 9.3-fold higher in *lip1*

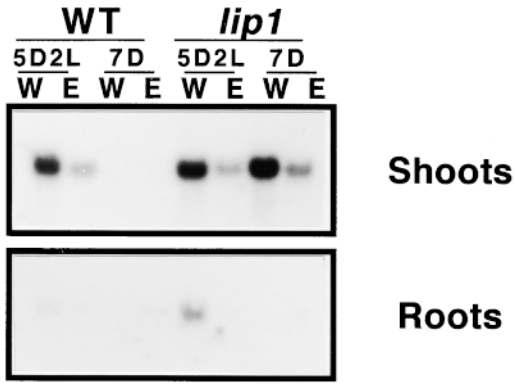

Figure 4. Effect of Erythromycin on the Accumulation of *Lhcb1* Transcripts in the Shoots and Roots of Wild-Type and *lip1* Seedlings.

Wild-type Alaska (WT) and *lip1* seedlings were grown on either water (W) or 0.5 mM erythromycin (E) for 5 days in the dark followed by 2 days in the light (5D2L) or in continuous darkness for 7 days (7D). Shoot and root tissues were excised from the seedlings, and total RNA was extracted and subjected to RNA gel blot analysis using a ^{32}P-labeled probe for pea *Lhcb1.*

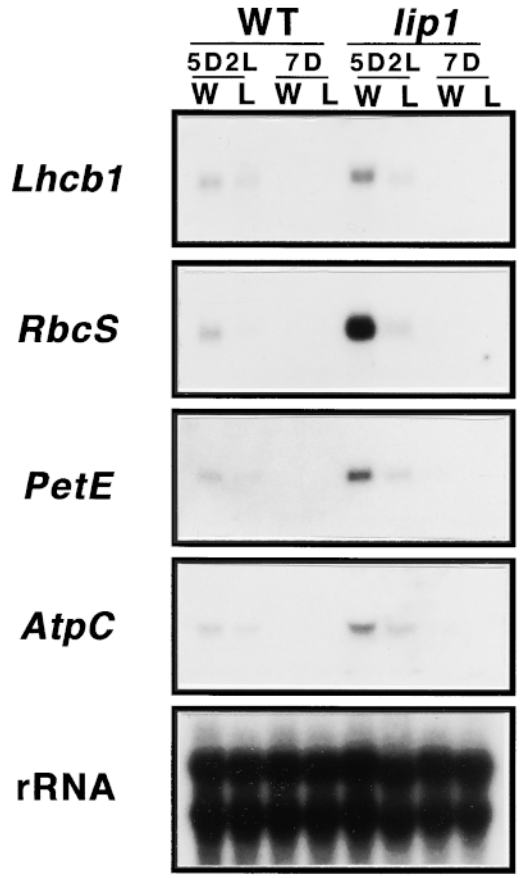

Figure 5. Effect of Lincomycin on the Accumulation of Transcripts of Nuclear Photosynthesis Genes in the Roots of Wild-Type and *lip1* Seedlings.

Wild-type Alaska (WT) and *lip1* seedlings were grown on either water (W) or 0.5 mM lincomycin (L) for 5 days in the dark followed by 2 days in the light (5D2L) or in continuous darkness for 7 days (7D). Root tissue was excised from the seedlings, and total RNA was extracted and subjected to RNA gel blot analysis using ^{32}P-labeled probes for *Lhcb1*, *RbcS*, *PetE*, and *AtpC*. Hybridization with a probe for rRNA was used as a loading control.

seedlings than in wild-type seedlings (Figure 6). This increase in transcript abundance in *lip1* roots was associated with the development of chloroplasts, which showed both a stacked thylakoid membrane system and the accumulation of starch granules (Figure 7A). Although the plastids in the roots of light-grown wild-type seedlings contained internal membranes, these were unstacked, and starch granules were not observed (cf. Figures 7A and 7E).

In the roots of dark-grown wild-type or *lip1* seedlings grown on water or lincomycin, transcripts were detectable only after extended exposure of autoradiographs. Plastids in roots of dark-grown wild-type and *lip1* seedlings contained little internal membrane structure and were smaller than the plastids observed in roots of light-grown *lip1* seedlings (Figures 7C, 7D, 7G, and 7H). The accumulation of transcripts of photosynthesis genes in roots of light-grown *lip1* seedlings was reduced after lincomycin or erythromycin treatment (Figures 4 and 5). This decrease in mRNA abundance was associated with the loss of a stacked thylakoid membrane system and of internal starch grains (Figures 7B and 7F). The water/lincomycin ratios for transcripts in roots of light-grown *lip1* seedlings were 4.4 for *Lhcb1*, 5.5 for *PetE*, 2.4 for *AtpC*, and 12.8 for *RbcS*, similar to the effect on transcripts in shoots of dark-grown seedlings (Figure 3). Essentially identical results were obtained in three repeated experiments. This clearly demonstrates that plastid translation is required for the expression of nuclear photosynthesis genes in the roots of light-grown *lip1* seedlings.

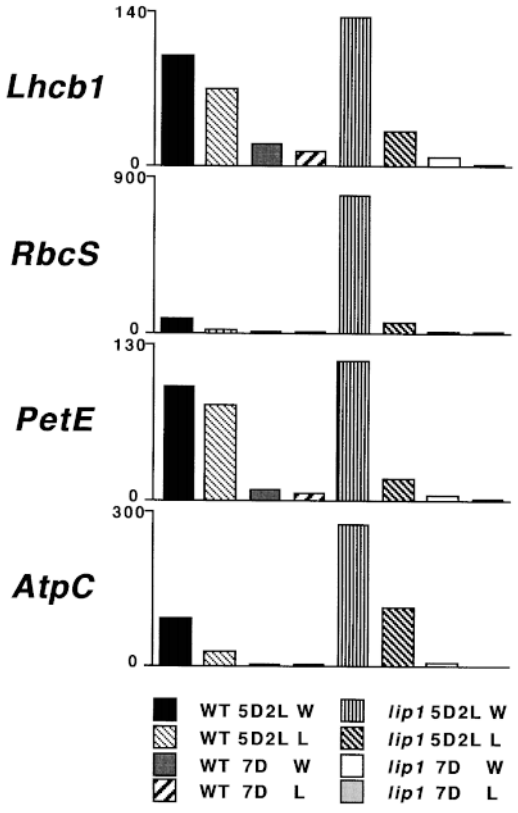

Figure 6. Quantification of the Effect of Lincomycin on Transcript Accumulation in Wild-Type and *lip1* Roots.

Hybridization signals from the autoradiographs shown in Figure 5 were quantified using a laser scanning densitometer and normalized to rRNA to account for any small differences in the loading of total RNA. For each probe, the amounts of transcripts are expressed relative to wild-type plants grown on water for 5 days in the dark followed by 2 days in the light (5D2L), which was given an arbitrary value of 100. Other abbreviations are as given in the legend to Figure 5.

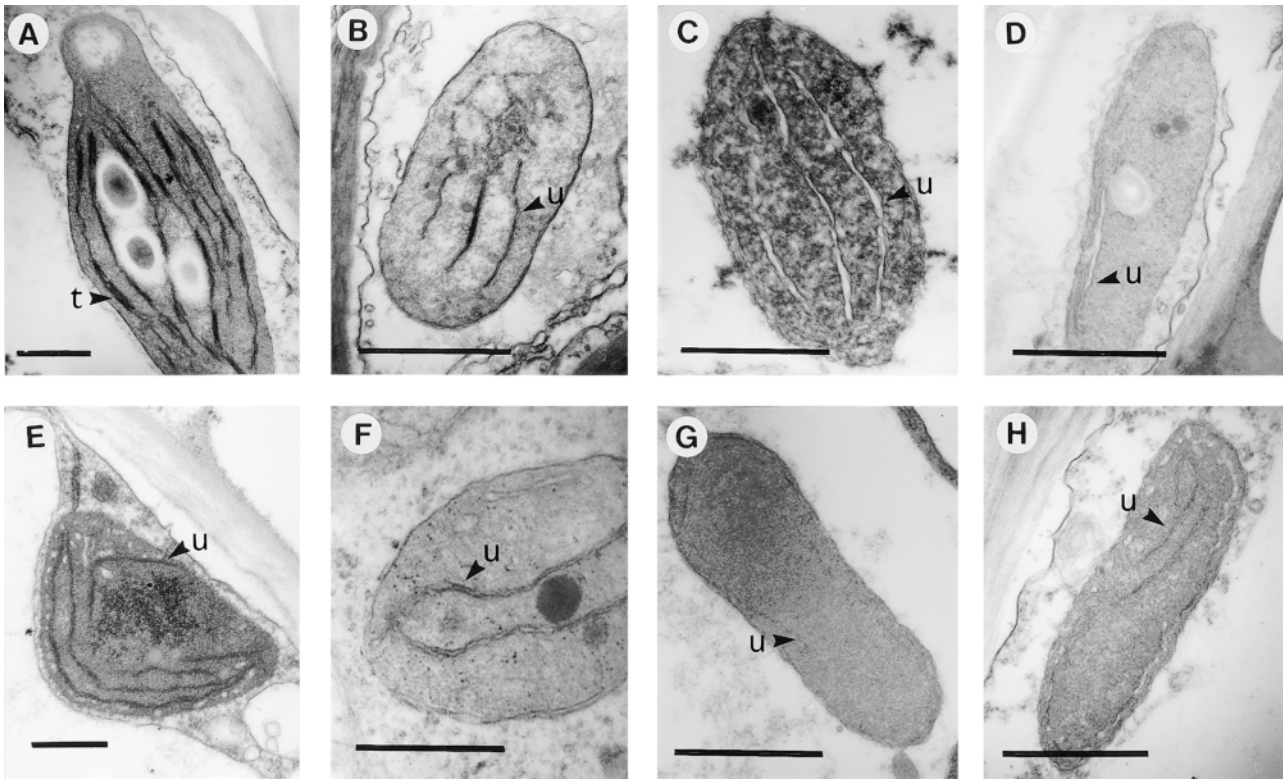

Figure 7. Transmission Electron Microscopy of Plastids from Wild-Type and *lip1* Roots.

Wild-type Alaska and *lip1* seedlings were grown on water or 0.5 mM lincomycin as given below. Thin sections (40 to 50 nm) of root tissue were fixed in glutaraldehyde and hydrogen peroxide, embedded in Araldite, and stained with uranyl acetate before being viewed at 80 kV in a Philips C100 transmission electron microscope. t, stacked thylakoids; u, unstacked membranes. Bars = 500 nm.
(A) and **(B)** Plastids from *lip1* seedlings grown on water **(A)** or lincomycin **(B)** for 5 days in the dark and 2 days in the light.
(C) and **(D)** Plastids from *lip1* seedlings grown on water **(C)** or lincomycin **(D)** for 7 days in continuous darkness.
(E) and **(F)** Plastids from wild-type seedlings grown on water **(E)** or lincomycin **(F)** for 5 days in the dark and 2 days in the light.
(G) and **(H)** Plastids from wild-type seedlings grown on water **(G)** or lincomycin **(H)** for 7 days in continuous darkness.

Inhibition of Plastid Translation Reduces the Accumulation of *Lhcb1* mRNA in the Arabidopsis *cop1-4* Photomorphogenic Mutant

To determine whether a requirement for plastid translation could be demonstrated in another photomorphogenic mutant, we investigated the effect of lincomycin on *Lhcb1* mRNA accumulation in the Arabidopsis *cop1-4* mutant (Deng and Quail, 1992). Approximately 200 seeds of *cop1-4* and wild-type Arabidopsis (Wassilewskija) were sown on either water or 0.5 mM lincomycin, vernalized at 4°C overnight, and allowed to germinate in the light for 24 hr before being grown for 7 days in the light or in darkness. Total RNA was extracted from the seedlings, and RNA gel blot analysis was performed using a probe from the Arabidopsis *Lhcb1*2* gene. The hybridization signal of rRNA was used as a loading control. The results obtained are shown in Figure 8.

Growth of wild-type and *cop1-4* seedlings on 0.5 mM lincomycin resulted in complete chlorosis of light-grown seedlings, as has been shown previously with wild-type and *lip1* peas, but there was no apparent effect on the morphology of either light- or dark-grown seedlings (data not shown). Treatment with lincomycin decreased the accumulation of *Lhcb1* mRNA in both light-grown wild-type and *cop1-4* seedlings (Figure 8). *Lhcb1* mRNA decreased to $<$10% of the amount found in untreated light-grown wild-type and *cop1-4* seedlings, and it was detected only after extended exposure of autoradiographs. In dark-grown wild-type seedlings, *Lhcb1* mRNA was barely detectable. In contrast, *Lhcb1* mRNA accumulated in dark-grown *cop1-4* seedlings, although only to $\sim$5% of the amount observed in light-grown *cop1-4* seedlings germinated on water. Lincomycin treatment of dark-grown *cop1-4* seedlings markedly decreased the accumulation of *Lhcb1* mRNA, resulting in a water/lincomycin ratio of 3.7 (when corrected for rRNA loading), similar to that for dark-grown *lip1* seedlings. This in-

dicates that plastid translation also is required for the accumulation of *Lhcb1* mRNA in the Arabidopsis *cop1-4* photomorphogenic mutant.

DISCUSSION

The pea photomorphogenic mutant *lip1* aberrantly expresses nuclear photosynthesis genes in the shoots of dark-grown seedlings and in the roots of light-grown seedlings. Treatment of *lip1* seedlings with inhibitors of plastid translation reduced the accumulation of transcripts of all nuclear photosynthesis genes examined in the light and the dark. Plastid translation is required during early development for the production and/or activity of the plastid signal (Oelmüller et al., 1986; Gray et al., 1995), which indicates that the plastid signal regulates the expression of nuclear photosynthesis genes under all conditions examined, in the light and the dark, and in roots.

Previous studies have demonstrated that the plastid signal is required continuously for the expression of nuclear photosynthesis genes (Oelmüller et al., 1986; Burgess and Taylor, 1988). Coupled with the observation that inhibition of plastid translation only during the first 48 to 72 hr after germination affects nuclear photosynthesis gene expression (Oelmüller et al., 1986; Gray et al., 1995), this suggests that the plastid signal is not a direct product of plastid translation (Oelmüller et al., 1986; Gray et al., 1995). However, the requirement for plastid translation during early development indicates that the production and/or activity of the plastid signal requires plastid gene expression (Gray et al., 1995). Indeed, it has been suggested previously that the signal synthetase may be a stable chloroplast-encoded protein (Susek and Chory, 1992), although this remains to be proven.

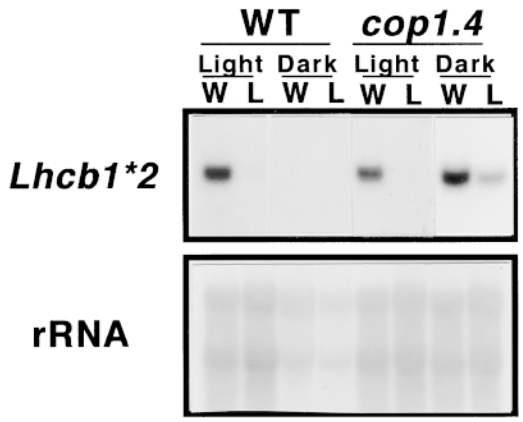

Figure 8. Effect of Lincomycin on *Lhcb1* mRNA Accumulation in the Arabidopsis Photomorphogenic Mutant *cop1-4*.

Wild-type Arabidopsis (WT) and *cop1-4* seedlings were grown on either water (W) or 0.5 mM lincomycin (L) for 7 days in the light or for 7 days in continuous darkness. Total RNA was extracted from the seedlings and subjected to RNA gel blot analysis using a ^{32}P-labeled probe from the Arabidopsis *Lhcb1*2* gene. Hybridization signals from RNA extracted from dark-grown seedlings are the result of a fivefold increase in exposure time relative to light-grown samples. Hybridization with a probe for rRNA was used as a loading control.

Because inhibition of plastid translation in shoots of light-grown wild-type and *lip1* seedlings is capable of decreasing the accumulation of transcripts of several nuclear photosynthesis genes, the plastid signal must be required in both wild-type and *lip1* shoots. It is interesting that lincomycin is not as effective in *lip1* compared with the wild type for inhibition of transcript accumulation from some of the photosynthesis genes examined. Similar partial uncoupling of nuclear photosynthesis gene expression from chloroplast control has been reported in the Arabidopsis photomorphogenic mutants *deetiolated2* (*det2*) and *cop4* (Chory et al., 1991; Hou et al., 1993). Because there is still a requirement for plastid translation in *lip1*, it seems unlikely that *Lip1* is directly involved in the synthesis of the plastid signal, although *Lip1* still may have some role in the regulation of nuclear photosynthesis genes by the plastid signal.

The observation that the accumulation of transcripts of nuclear photosynthesis genes in shoots of dark-grown *lip1* seedlings also requires plastid translation and, by inference, activity of the plastid signal, demonstrates that light is not an obligate requirement for production or activity of the plastid signal. The shoots of dark-grown *lip1* seedlings contain plastids in a state intermediate between wild-type etioplasts and chloroplasts (Frances et al., 1992). These plastids lack prolamellar bodies and contain an unstacked membrane system that has some resemblance to thylakoid membranes (Frances et al., 1992). Because these plastids are still capable of producing the plastid signal, its synthesis therefore does not require the presence of photosynthetically active chloroplasts, a stacked thylakoid membrane system, or the synthesis of chlorophyll. Previous work has shown that the first 2 days after germination are critical for production of the plastid signal in tobacco seedlings (Gray et al., 1995). Inhibition of plastid translation only during this 2-day period in dark-grown seedlings prevents the expression of nuclear photosynthesis genes when the seedlings subsequently are transferred to light (Gray et al., 1995).

The observation that light is not an obligate requirement for production of the plastid signal in *lip1* suggests that the plastid signal may be produced by developing plastids in the dark in wild-type plants rather than at the dark–light transition, as has been previously suggested (Mochizuki et al., 1996). The recent observation that inhibition of plastid protein synthesis with streptomycin prevents expression of *RbcS* and *POR* (protochlorophyllide reductase) in dark-grown rice seedlings (Yoshida et al., 1998) supports this view. It has been proposed that light and the plastid signal may act through a common *trans*-acting factor that binds to the promoter regions of nuclear photosynthesis genes (Bolle et al., 1994; Kusnetsov et al., 1996; López-Juez et al., 1996;

Puente et al., 1996). Although the experiments presented here have not addressed this hypothesis directly, the separation of the effects of the plastid signal from that of light in the *lip1* mutant indicates that the signaling pathways are, at least during some stages of signal transduction, distinct from one another.

The observation that transcripts of nuclear photosynthesis genes did not accumulate in roots of dark-grown *lip1* seedlings indicates that the *lip1* mutation does not affect the organ specificity of nuclear photosynthesis gene expression. It is therefore likely that the mechanism of organ-specific expression of nuclear photosynthesis genes is distinct from those involved in light and plastid signal regulation. This is supported by the observation that the tissue-specific regulation of the pea plastocyanin gene (*PetE*) takes place at the transcriptional level, whereas light-regulated expression appears to involve a post-transcriptional mechanism (Pwee and Gray, 1993; Helliwell et al., 1997). The increased accumulation of transcripts of photosynthesis genes in roots of light-grown seedlings, however, suggests that *lip1* may affect the development of plastids in roots. Indeed, this increase in transcript abundance in roots of light-grown *lip1* seedlings was associated with the development of chloroplasts containing a stacked thylakoid membrane system. The plastids in the roots of light-grown wild-type seedlings also showed partial development of a thylakoid membrane system, although the membranes were not stacked; this may represent an earlier stage of chloroplast development. Therefore, *lip1* appears to affect the development of plastids in light-grown roots. However, because plastids in roots of dark-grown *lip1* seedlings are indistinguishable from those found in wild-type seedlings, some degree of organ specificity in the control of plastid development must remain in *lip1* seedlings.

The observation that the accumulation of *Lhcb1* mRNA in dark-grown Arabidopsis *cop1-4* seedlings was also reduced by inhibition of plastid translation suggests that the plastid signal may be acting downstream of COP1 in the light-regulatory signal transduction pathway. Because treatment with inhibitors of plastid translation had no observable effect upon the morphological development of pea or Arabidopsis seedlings, it is unlikely that the plastid signal acts directly on COP1 function. One possible explanation is that the plastid signal acts to modulate the activity of a CIP (for COP1-interacting protein) either directly, through protein–protein interactions, or by binding to the promoter regions of target genes and preventing the CIP from activating transcription.

Dark-grown *lip1* and *cop1* seedlings provide useful experimental systems in which to investigate production of the plastid signal in the absence of light. Many of the previous studies investigating the plastid signal used the herbicide norflurazon, an inhibitor of carotenoid biosynthesis (Young, 1991), to examine the effects of the plastid signal on gene expression (e.g., Oelmüller et al., 1986; Bolle et al., 1994; Kusnetsov et al., 1996; Puente et al., 1996). After growth in light, the absence of carotenoids causes photooxidation of chloroplasts and downregulation of nuclear photosynthesis genes (Mayfield and Taylor, 1984; Oelmüller et al., 1986). However, by using norflurazon, one cannot address the effects of, or the requirement for, the plastid signal during growth in darkness, because the destruction of chloroplasts (and presumably the plastid factor) will take place only upon transfer to light. The treatment of dark-grown *lip1* and *cop1* seedlings with inhibitors of plastid translation, such as lincomycin or erythromycin, allows the effects of the plastid signal in the dark to be investigated. The identification and cloning of *Lip1* may provide further insight into how the light and plastid signal transduction pathways interact during development.

METHODS

Plant Material and Growth Conditions

Seeds of wild-type pea plants (*Pisum sativum* cv Alaska) and the photomorphogenic mutant *lip1* were obtained from the John Innes germ-plasm collection (John Innes Centre, Norwich, UK) and from W.F. Thompson (North Carolina State University, Raleigh, NC). Seeds of Arabidopsis *cop1-4* were obtained from X.-W. Deng (Yale University, New Haven, CT). Seeds from these stocks were grown to maturity in a greenhouse and allowed to self-fertilize to produce seed for subsequent experiments.

Before germination, pea seeds were surface sterilized for 2 min with 70% (v/v) ethanol followed by a 10-min treatment in 10% (v/v) sodium hypochlorite solution. The seeds were washed several times in sterile distilled water before being soaked for 4 hr at room temperature in sterile distilled water, 0.5 mM lincomycin (Melford Laboratories Ltd., Ipswich, UK), or 0.5 mM erythromycin (Melford Laboratories Ltd.). After soaking, the seeds were sown onto Whatman 3MM chromatography paper in sterile magenta vessels containing 10 mL of water, lincomycin, or erythromycin solutions, as described above. The vessels were wrapped in two layers of aluminium foil, and the seeds were allowed to imbibe overnight at 4°C before being placed in a constant temperature growth room at 22°C for 5 days. After 5 days, the vessels were either unwrapped and placed for 2 days in photosynthetically active irradiance of 100 μmol m^{-2} sec^{-1} (5 days of dark and 2 days of light) or left in the dark for another 2 days (7 days of dark).

For experiments with Arabidopsis, seeds were surface sterilized as described above before being sown onto half-strength Murashige and Skoog medium (Sigma) containing 1% (w/v) agar and either water or 0.5 mM lincomycin in 9-cm Petri dishes. Seeds were vernalized overnight at 4°C and allowed to germinate in the light for 24 hr before being grown for an additional 7 days in photosynthetically active irradiance of 100 μmol m^{-2} sec^{-1} or left in continuous darkness for 7 days.

Total RNA Extraction and Gel Blot Analysis

Total RNA was extracted separately from shoot and root tissues from pea seedlings or from whole Arabidopsis seedlings by using Tripure isolation reagent (Boehringer Mannheim) according to the manufacturer's protocol. Approximately 10 μg of total RNA was separated by electrophoresis on a 1.2% agarose gel and blotted to GeneScreen Plus

membrane (New England Nuclear Research Products, Boston, MA), as previously described (Helliwell et al., 1997). Radiolabeled probes were produced from cDNAs encoding pea RbcS (Anderson and Smith, 1986), pea Lhcb1 (Cline et al., 1989), pea PetE (Last and Gray, 1989), pea AtpC (Napier et al., 1992), pea polyubiquitin (Watts and Moore, 1992), and pea HMG-I/Y (Webster et al., 1997), and from an Arabidopsis genomic DNA fragment containing *Lhcb1*2* (Leutwiler et al., 1986), by using random hexanucleotide primers and α-^{32}P-dATP (Feinberg and Vogelstein, 1983). Hybridization with each of these ^{32}P-labeled probes was followed by probe removal and rehybridization, as described by Helliwell et al. (1997). As a control for loading and transfer efficiency, the membranes were incubated with a ^{32}P-labeled probe for the pea rRNA gene cluster (Jorgensen et al., 1987).

Autoradiographic images were obtained by exposing the membranes to X-ograph Blue X-ray autoradiography film (X-ograph Ltd., Malmesbury, UK). Several exposures for different lengths of time were made for each membrane. Measurements of the hybridization signals were made using a laser scanning densitometer (model 300s; Molecular Dynamics, Sunnyvale, CA) with Imagequant software (Molecular Dynamics). The values obtained were normalized to the intensity of the 25S and 18S rRNA bands. Quantitative analysis was performed only on those autoradiographs within the linear response range.

Transmission Electron Microscopy

Root tissue from wild-type and *lip1* seedlings was fixed in glutaraldehyde and hydrogen peroxide, as described by Peracchia and Mittler (1972). Thick tissue sections were washed in Pipes buffer, treated with 2% osmium ferricyanide and 2 mM calcium chloride, and bulk stained with uranyl acetate before being embedded in araldite, as described by Navaratnam et al. (1998). Thin sections (40 to 50 nm) were cut using a diamond knife on a microtome (Ultracut E; Reichert, Vienna, Austria) and viewed at 80 kV in a transmission electron microscope (model CM100; Philips, Eindhoven, The Netherlands).

ACKNOWLEDGMENTS

We thank Bill Thompson and those at the John Innes pea germplasm center for their gifts of wild-type Alaska and *lip1* peas, David Lonsdale and Tony Moore for the gift of plasmids, and Xing-Wang Deng for the Arabidopsis *cop1-4* seed. We also thank Jeremy Skepper and Janet Powell at the Cambridge Multi-Imaging Centre for the preparation of tissue and assistance with transmission electron microscopy, and Martyn Seekings for his invaluable assistance with the growth of the peas used for these experiments. J.A.S. was supported by a Biotechnology and Biological Sciences Research Council research studentship.

Received August 19, 1998; accepted February 18, 1999.

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