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Initial Characterization of a Pea Mutant with Light-Independent Photomorphogenesis

Shannon Frances,[a] Michael J. White,[b] Michael D. Edgerton,[c] Alan M. Jones,[c] Robert C. Elliott,[b,1] and William F. Thompson[a,2]

[a] Department of Genetics, North Carolina State University, Raleigh, North Carolina 27695
[b] Department of Botany, North Carolina State University, Raleigh, North Carolina 27695
[c] Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27514

We have identified a mutant of pea cultivar Alaska that has many of the characteristics normally associated with light-grown seedlings even when grown in complete darkness. We have designated this mutant *lip*1, for *l*ight *i*ndependent *p*hotomorphogenesis. Etiolated wild-type pea seedlings are white to slightly yellow in color and have a distinct morphology characterized by elongated epicotyls and buds containing unexpanded leaves with small, undifferentiated cells. In contrast, mutant seedlings grown under the same conditions are yellow in color and have short epicotyls and expanded leaves showing clear cellular differentiation. Transmission electron microscopy revealed partially developed, agranal plastids in the dark-grown mutant, unlike wild-type seedlings that contain etioplasts with prolamellar bodies. The mutant also exhibits a much shorter lag period for chlorophyll accumulation when etiolated seedlings are transferred from darkness to white light. The dark-grown mutant has 10-fold less spectrally detectable phytochrome, which can be attributed to a 10-fold reduction in the level of the PHYA polypeptide. *Cab*, *Fed*1, and *Rbc*S transcripts are present in dark-grown mutant seedlings at levels comparable to those produced in light-grown material. The levels of these transcripts show a normal decrease when green plants grown for 15 days in a light/dark cycle are transferred to continuous darkness. However, transcript levels remain high during dark treatment of seedlings grown for 9 days in continuous light, indicating that the dark adaptation response in this mutant is developmentally plastic. The *lip*1 mutant has several features in common with the deetiolated Arabidopsis mutants *det*1, *det*2, and *cop*1. However, there are also several important differences, including varying effects on phytochrome levels, organ-specific gene expression, plastid development, and response to dark adaptation.

INTRODUCTION

In seedlings of many plant species, the first exposure to light initiates a dramatic switch from skotomorphogenesis to photomorphogenesis (Mohr and Shropshire, 1983). These two profoundly different developmental programs affect a wide variety of molecular, biochemical, and morphological processes. The control mechanisms involved in such a dramatic transition are of fundamental importance in plant development. These mechanisms are also likely to be complicated with regard to the types of photoreceptors involved as well as interactions between photoreceptors. Several aspects of the switching process, such as photoreceptor function and mechanisms of gene regulation, are being investigated in many laboratories (for a review, see Thompson and White, 1991). However, there is as yet no regulatory pathway for which all the components are known, and it remains unclear how many separate pathways are involved in the overall developmental response.

Various photomorphogenetic mutants have been useful in investigating these pathways (Kendrick and Kronenberg, 1986). Among the best characterized are photoreceptor mutants. For example, the *aurea* mutation of tomato (Sharrock et al., 1988), which results in a light-grown phenotype of yellow-green leaves and elongated hypocotyls, causes a deficiency of type I phytochrome. Complementary to *aurea* are *lh* of cucumber (López-Juez et al., 1992) and *hy*3 of Arabidopsis (Somers et al., 1991), which are long hypocotyl mutants deficient in functional type II phytochrome. *hy*1, *hy*2, and *hy*6 are chromophore mutants (Chory et al., 1989b; Parks et al., 1989; Parks and Quail, 1991), which presumably reduce the levels of all types of active phytochrome (Chory et al., 1989a; Parks and Quail, 1991). In Arabidopsis *blu* mutants, blue light fails to inhibit hypocotyl elongation, whereas there is a normal response to excitation of the phytochrome system (Liscum and Hangarter, 1991). Khurana and Poff (1981) described an Arabidopsis mutant (strain JK218) that has no phototropic response to blue

[1] Current address: John Innes Institute, Colney Lane, Norwich NR4 7UH, England.
[2] To whom correspondence should be addressed.

light and may represent a lesion in the blue light receptor. These data, together with data from crosses between *blu* and *hy* mutants, provide unequivocal evidence for distinct red and blue light signal transduction pathways. (For reviews emphasizing photoreceptor mutants, see Tomizawa et al., 1990; Kendrick and Nagatani, 1991; Okada and Shimura, 1992.)

Other photomorphogenetic mutants appear to affect signal transduction events subsequent to photoperception. For example, the high pigment mutant of tomato, *hp*, exhibits responses that reflect an increased sensitivity to phytochrome rather than a mutation in phytochrome itself (Peters et al., 1989). The long internode pea mutant *lv* has a phenotype similar to that of the phytochrome-deficient long hypocotyl mutants but has normal levels of both type I and type II phytochrome, suggesting that it is also a signal transduction mutant (Nagatani et al., 1990).

An additional set of regulatory loci have recently been defined by a series of Arabidopsis mutants producing a "deetiolated" phenotype (Chory et al., 1989b, 1991; Chory and Peto, 1990; Chory, 1991; Deng et al., 1991; Deng and Quail, 1992). These mutants develop many of the characteristics normally associated exclusively with light-grown plants, even when germinated and grown in complete darkness. Thus, it is possible to bypass the normal requirement for light induction by blocking the action of one or more repressors of photomorphogenesis (Chory, 1991).

We report here the discovery and initial characterization of a deetiolated mutant of pea that we have called *lip*1 because it exhibits light independent photomorphogenesis. Mutant seedlings show decreased epicotyl elongation and express several otherwise light-dependent traits in dark-grown seedlings. This mutant thus provides an opportunity to study a genetically defined control point for photomorphogenetic development in a large-seeded plant species amenable to biochemical analysis.

RESULTS

Isolation of a Spontaneous Deetiolated Mutant

Pea seedling development differs in some respects from that of other dicots used in photomorphogenetic studies (Sutcliffe and Pate, 1977). The cotyledons, which comprise most of the volume of the seed, remain in the substrate, and it is the epicotyl rather than the hypocotyl that elongates upon germination. The apical bud, or plumule, contains approximately six leaf primordia in the seed (Sutcliffe and Pate, 1977). During seedling growth in darkness, these leaves remain unexpanded while the first few internodes of the epicotyl elongate rapidly (Sutcliffe and Pate, 1977). Light stimulates leaf expansion, visible initially as an increase in bud size, as well as a sharp decrease in the rate of epicotyl elongation.

The *lip*1 mutant was identified in a population of nonmutagenized, dark-grown seedlings of pea cultivar Alaska by its short epicotyl and enlarged, yellow apical bud, as shown in Figure 1. Segregation studies indicate *lip*1 is a recessive mutation at a single locus. For example, 81 F_2 progeny from six independent crosses between *lip*1 and wild-type Alaska showed a segregation ratio of 2.9:1 wild-type to mutant.

Although light-grown *lip*1 and wild-type plants are nearly indistinguishable at the seedling stage, differences become apparent in mature plants, as shown in Figure 2. When wild-type and *lip*1 plants were grown together under several different conditions (artificial or natural light/dark cycles, with or without a night interrupt, 18°C/14°C or 22°C/18°C), mature *lip*1 plants were ~30% as tall as the wild type, had up to 20% fewer nodes, and were darker green. Under our normal conditions for growth and seed production (see Methods), wild-type and *lip*1 plants flower and senesce at approximately the same time, but *lip*1 plants produce only 30% as much seed.

Greening Is Potentiated in Mutant Seedlings

Exposure to light is normally required for significant accumulation of chlorophylls, carotenoids, and xanthophylls. Dark-grown *lip*1 seedlings accumulate pigments that give them a distinctive yellow color (Figure 1), but do not become green. The lack of chlorophyll accumulation presumably reflects the well-known light requirement for conversion of protochlorophyllide to chlorophyllide (Forreiter et al., 1991). However, chlorophyll accumulation occurs rapidly in response to illumination with white light, as shown in Figure 3. The initial rate of accumulation is greater in *lip*1 seedlings, which lack the lag

Figure 1. Morphology of Dark-Grown Pea Seedlings.

The gross morphology of dark-grown wild-type pea seedlings is shown at right and of *lip*1 pea seedlings is shown at left. Seedlings were grown in continuous darkness for 6 days.

Figure 2. Adult Wild-Type and *lip*1 Plants.

Wild-type (right) and *lip*1 (left) plants were grown for 2 months in a controlled light/dark cycle (9-hr light at 22°C/15-hr dark at 18°C). Similar results were obtained under other growing conditions, as described in the text.

phase seen for chlorophyll accumulation in wild-type seedlings. We presume chlorophyll accumulation is potentiated in dark-grown seedlings by a prior accumulation of essential precursors, mRNAs, and/or enzymatic machinery. This potentiation is correlated with the more extensive morphological development of the plastids in mutant seedlings and accumulation of mRNAs for plastid proteins.

Dark-Grown *lip*1 Seedlings Have Anatomical Features Resembling Light-Grown Plants

Figures 4A and 4B show that leaves of both *lip*1 and wild-type seedlings grown in the light contain normal complements of differentiated tissues. Furthermore, light-grown *lip*1 and wild-type mesophyll cells are indistinguishable at the subcellular levels. A comparison of fully developed chloroplasts is shown in Figures 5A and 5B.

The unexpanded leaves of etiolated wild-type seedlings exhibit less cellular organization. These leaves contain smaller, more closely packed cells that are not as clearly differentiated into distinct types (Figure 4C) and that contain typical etioplasts, many of which contain prolamellar bodies (Figure 5C). In contrast, Figure 4D shows that leaves of dark-grown *lip*1 seedlings are similar to those of light-grown seedlings with respect to both cell size and tissue differentiation. In addition, mesophyll cells of dark-grown *lip*1 leaves contain plastids that are approximately as large as the chloroplasts in the light-grown material and have unstacked membranes resembling thylakoids (Figure 5D). These plastids appear to represent a developmental stage intermediate between chloroplasts and etioplasts.

The mutant can be grown in the dark for an extended period of time. One-month-old dark-grown *lip*1 seedlings have two to three nodes, each with completely expanded leaves (data not shown). Seedlings grown in this manner do not require the addition of nutrients other than water. Presumably, nutrients are supplied to the seedling from the seed, which in normal pea provides nutrients to the first 15 nodes of the plant (Sutcliffe and Pate, 1977).

Gene Expression in Etiolated Seedlings

We have examined transcripts encoded by four different light-regulated genes. Figure 6 shows results from experiments in which 6-day-old dark-grown seedlings were transferred to continuous white light and the accumulation of each mRNA was

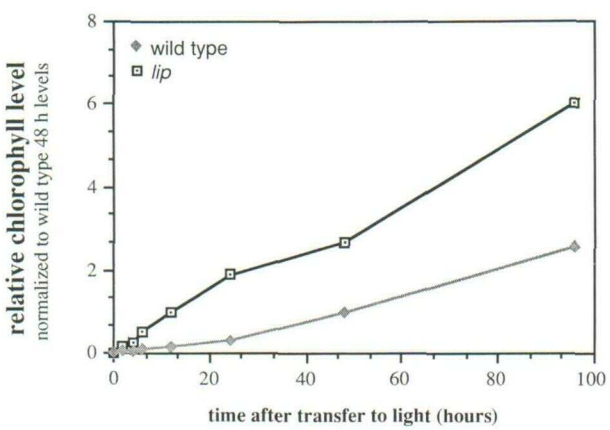

Figure 3. Time Course of Chlorophyll Accumulation in Wild-Type and *lip*1 Seedlings after Transfer to Light.

Wild-type (◇) and *lip*1 (⊡) seedlings were grown in complete darkness for 6 days, then transferred to continuous light. Buds were harvested into dimethylformamide at the indicated times after transfer. Total chlorophyll content was calculated as described in Methods.

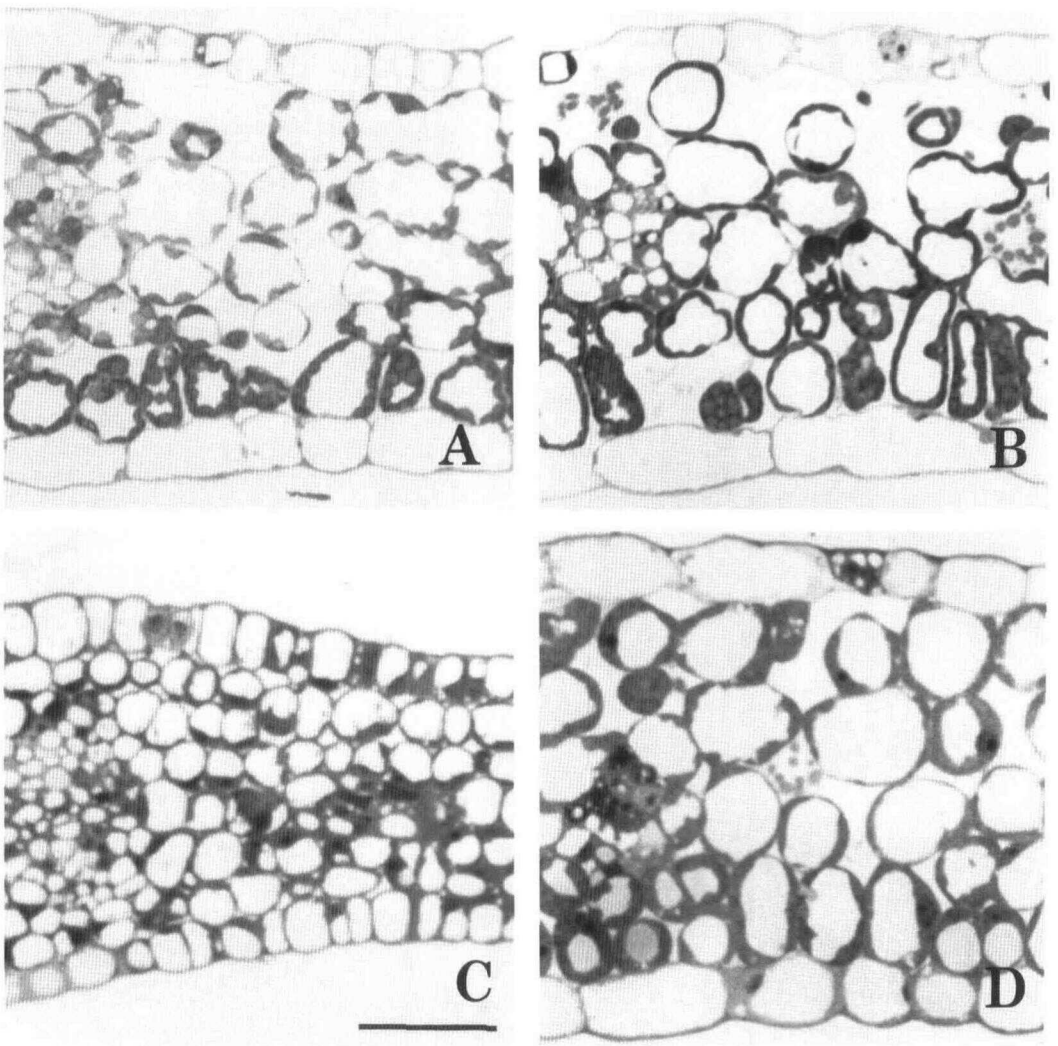

Figure 4. *Light Micrographs of Leaf Cross-Sections from 6-Day-Old Light- and Dark-Grown Seedlings.*

(A) Light-grown wild type.
(B) Light-grown *lip*1.
(C) Dark-grown wild type.
(D) Dark-grown *lip*1.
Bar = 100 μm.

measured at the indicated time points. In wild-type seedlings, transcripts from the chlorophyll *a/b* binding *Cab* gene family, the ribulose bisphosphate carboxylase small subunit *Rbc*S gene family (Wehmeyer et al., 1990), and the ferredoxin gene *Fed*1 (Elliott et al., 1989) were initially present at low levels and increased in response to illumination. Transcripts of *Blec*1, which encodes a putative pea bud lectin (Dobres and Thompson, 1988), decreased with prolonged light exposure following an initial transient increase. In *lip*1 seedlings, however, the levels of *Cab*, *RbcS*, and *Fed*1 transcripts were already elevated prior to illumination. Although these levels increased somewhat with prolonged illumination, the effect was much less dramatic than in wild-type seedlings. The *Blec*1 transcript levels in dark-grown *lip*1 seedlings were reduced as compared to wild-type and remained low throughout the experiment.

To determine whether the *lip*1 mutation alters the organ specificity of gene expression, we compared transcript levels for buds, stems, and roots of light- and dark-grown seedlings. As shown in Figure 7, the absolute levels of expression of *Cab*, *Fed*1, and *Rbc*S were somewhat higher in mutant seedlings. However, the relative abundance of these mRNAs in different organs was unaffected.

Gene Expression in Green Leaves

When light-grown plants are transferred into the dark for a prolonged period, many light-regulated transcripts decline in abundance but return to high levels when the plants are reilluminated. We refer to this response as "dark adaptation" to distinguish it from the initial response of an etiolated seedling to light. The Arabidopsis deetiolated mutants described so far exhibit a spectrum of altered dark adaptation responses. Green seedlings of deetiolated *det*1 show normal dark adaptation

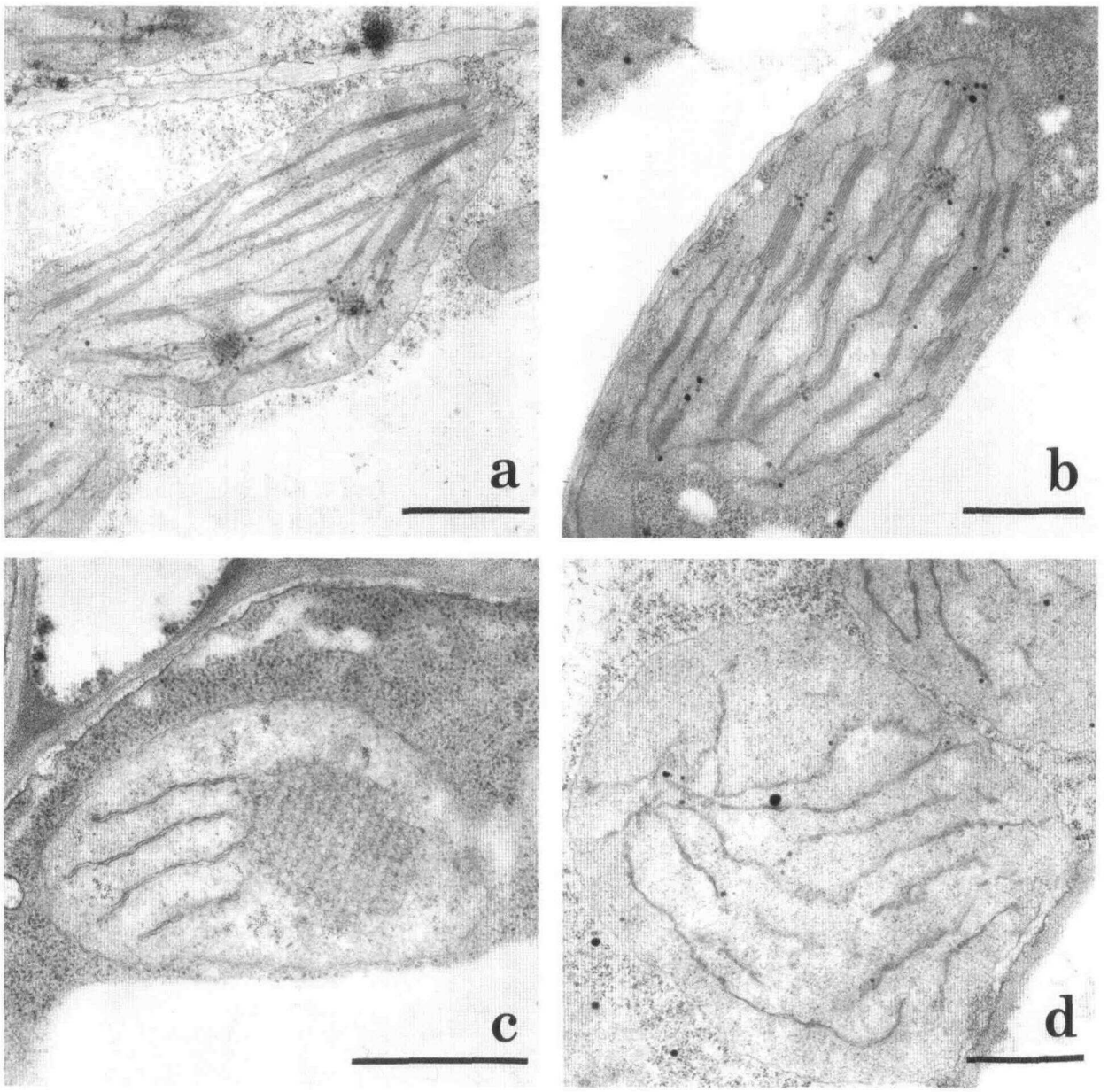

Figure 5. Electron Micrographs of Representative Plastids from 6-Day-Old Light- and Dark-Grown Seedlings.

(A) Light-grown wild type.
(B) Light-grown *lip*1.
(C) Dark-grown wild type.
(D) Dark-grown *lip*1.
Bars = 1 μm.

(Chory et al., 1989b), while dark-treated constitutive photomorphogenic *cop*1 seedlings continue to produce high levels of *Rbc*S and other normally light-regulated mRNAs (Deng et al., 1991). The *det*2 mutant has an intermediate response, in which transcript levels are only partially reduced in the dark (Chory et al., 1991).

In *lip*1 plants, the dark adaptation response varies with the conditions used to grow the seedlings. Figure 8 illustrates the responses obtained with *Fed*1 transcripts; similar data were obtained for *Cab* and *Rbc*S transcripts (data not shown). Dark adaptation occurred normally in *lip*1 plants grown for 15 days in a light/dark cycle before being transferred to darkness, with transcript levels decreasing in the dark and increasing again upon reillumination. However, if seedlings were grown for 9 days in continuous light prior to the beginning of the experiment, transcript levels remained high in the mutant even though they decreased in the wild-type controls.

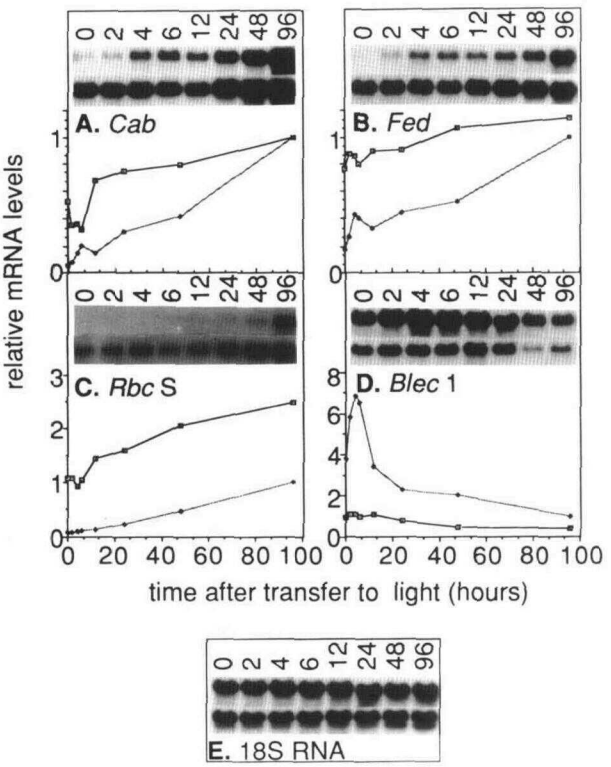

Figure 6. RNA Gel Blot Analysis of Transcripts in Wild-Type and *lip*1 Seedlings after Transfer to Light.

Wild-type (◇) and *lip*1 (⊡) seedlings were grown for 6 days in complete darkness, then transferred to continuous light. Buds were harvested at the indicated times after transfer. Total RNA was analyzed as described in Methods. A representative autoradiogram is shown for each probe (top row of bands in **[A]** to **[E]** represent wild type; bottom row of bands, *lip*1); the graphs present data averaged from three independent experiments. Hybridization signals were quantitated with the AMBIS Radioanalytic Imaging System and normalized to the value for wild-type seedlings at 96 hr.

(A) *Cab*, chlorophyll *a/b* binding protein, light-harvesting complex II.
(B) *Fed*1, ferredoxin I.
(C) *Rbc*S, Rubisco small subunit.
(D) *Blec*1, pea bud lectin.
(E) 18S rRNA. (The lower band results from cross-hybridization with chloroplast rRNA.)

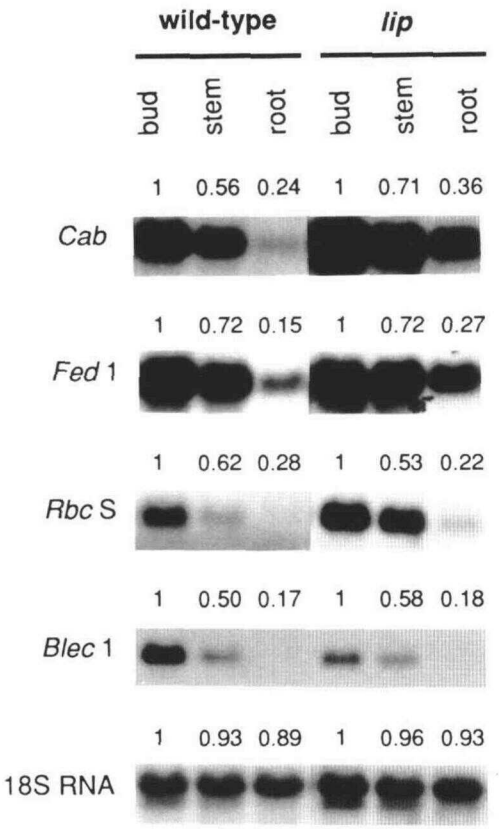

Figure 7. Organ-Specific Expression of *Cab*, *Fed*1, and *Rbc*S in Wild-Type and *lip*1.

Seedlings were grown in continuous light for 6 days. Total RNA from buds, stems, and roots was isolated and analyzed as described in Methods. A representative autoradiogram is shown for each probe, while the numbers above each lane present data averaged from two independent experiments. Hybridization signals were quantitated with the AMBIS Radioanalytic Imaging System and normalized to the value for buds. Abbreviations are as given for Figure 6.

Phytochrome Abundance Is Reduced in Mutant Seedlings

Phytochrome levels are high in etiolated pea and low in light-grown pea (Otto et al., 1984). The level of phytochrome in dark-grown mutant seedlings was examined by both spectral and immunoassay techniques and results are shown in Figure 9. In vivo difference spectra showed that epicotyls of etiolated *lip*1 seedlings have 10-fold less photoreversible

phytochrome than wild-type seedlings (Figure 9A). Immunoblots of protein extracted from the same material and probed with two different antibodies to phytochrome showed that the decrease in spectral activity can be attributed to a corresponding decrease in the amount of phytochrome apoprotein (Figure 9B). In replicate experiments, *lip*1 phytochrome levels were 11 and 14% of the wild-type level. Our estimates were calculated from the slopes of the lines relating signal on the immunoblot to the amount of sample loaded (Figure 9C), thus avoiding the problems associated with nonlinear responses in protein quantitation.

Light and Hormone Treatments

Many features of the mutant phenotype including the reduced phytochrome level could be explained if significant amounts of Pfr were present in young *lip*1 seedlings. Persistence of Pfr in the seed, for example, might produce responses similar to those normally produced by exposure to red light (R) early in germination. To address this possibility, mutant and wild-type seedlings were given R and far-red light (FR) treatments as described in Methods. R treatments stimulated photomorphogenesis and potentiated chlorophyll accumulation in wild-type seedlings. These effects were reversible by FR in the wild-type, but similar FR irradiation did not repress the constitutive photomorphogenesis of *lip*1 seedlings (data not shown). Therefore, we believe it is unlikely that the *lip*1 phenotype results solely

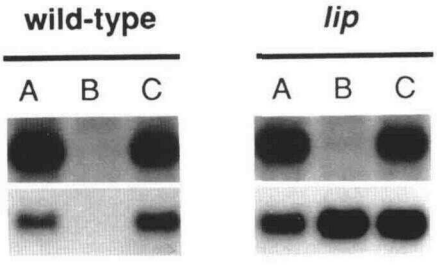

Figure 8. Changes in *Fed*1 mRNA Abundance after Transfer of Light-Grown Plants into Darkness.

Prior to the start of the experiment, plants were grown in either continuous light for 9 days (bottom panels) or in a controlled light/dark cycle (9-hr light at 22°C/15-hr dark at 18°C) for 15 days (top panels). Leaves from one set of seedlings were harvested into liquid nitrogen, and the remaining seedlings were transferred into complete darkness for 2 days (from continuous light) or 3 days (from cycle). As before, tissue was harvested from some seedlings and the remainder were returned to the light for 24 hr. Each harvest consisted of the two developmentally youngest fully expanded leaves. All plant material was harvested at the same time of day to avoid differences in transcript levels due to circadian rhythms. Total RNA was prepared and analyzed as described in Methods. Lanes A, light-grown seedlings; lanes B, light-grown seedlings transferred to darkness for an extended period; lanes C, light-grown seedlings transferred to darkness for an extended period and returned to the light for 24 hr.

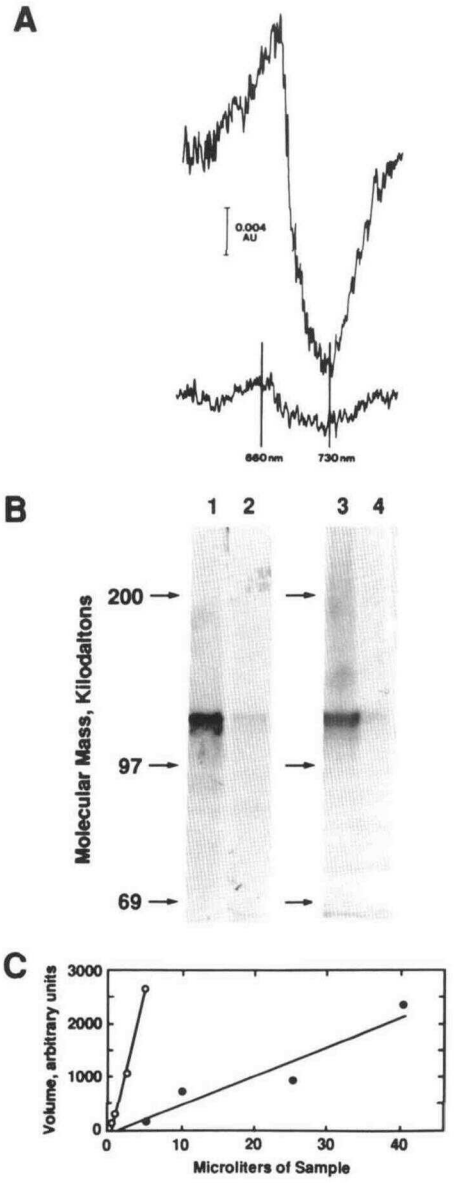

Figure 9. Etiolated *lip*1 Seedlings Containing Reduced Levels of Phytochrome.

(A) In vivo difference spectra for wild-type (top) and *lip*1 (bottom) seedlings.

(B) Immunoblot analysis. Wild-type (lanes 1 and 3) and *lip*1 (lanes 2 and 4) protein extracts were fractionated by SDS-PAGE and probed with anti-etiolated oat phytochrome antiserum 4032 (lanes 1 and 2) or anti-phytochrome monoclonal antibody Pea25 (lanes 3 and 4).

(C) Densitometric quantitation of Pea25 immunoblot in **(B)**. Phytochrome levels in *lip*1 (closed circles) seedlings were found to be 11 to 14% of wild-type (open circles) levels.

from the presence of Pfr in embryos or seedlings. The fact that *lip*1 also affects the morphology of mature, light-grown plants (Figure 2) is consistent with this interpretation.

Gibberellins and cytokinins are known to affect internode elongation, leaf development, and pigment biosynthesis (Salisbury and Ross, 1992). In particular, it is known that application of cytokinin to dark-grown Arabidopsis seedlings will cause them to deetiolate (Chory, 1991, 1992). Therefore, we treated wild-type seedlings with various concentrations of cytokinin and gibberellin. These treatments failed to produce morphological responses similar to the *lip*1 phenotype, although at high concentrations both hormones caused strikingly abnormal growth (data not shown).

DISCUSSION

Pea mutants offer several advantages for studies of light signal transduction. Pea is a large-seeded plant that grows vigorously for extended periods in the dark, produces significant quantities of dark-grown tissue, and has a particularly dramatic developmental response to illumination. Pea genetics are well established (Mendel, 1866; Weeden and Wolko, 1990), pea transformation has recently been reported (Puonti-Kaerlas et al., 1990; Nauerby et al., 1991), and biochemical analysis is relatively unhindered by hydrolytic enzymes and reactive compounds such as phenolics. These features of the pea system make it especially useful for biochemical studies of signal transduction, and mutants such as *lip*1 will eventually provide points of contact between such studies and the extensive genetic analysis being carried out in Arabidopsis.

The *lip*1 phenotype, including the reduction in type I phytochrome, resembles that which would result from constitutive phytochrome action. Formally, it remains possible that the phytochrome gene(s) may be affected, or that factors affecting phytochrome synthesis or photoconversion may be altered. However, the inability of FR irradiation to affect the phenotype of *lip*1 seedlings as well as the existence of a clear mutant phenotype in mature light-grown plants both argue against explanations that assume persistence of Pfr from developing embryos. We know of no precedent for the synthesis of phytochrome as Pfr in the dark, and most mutations leading to such a result should be dominant rather than recessive. Thus, we prefer the hypothesis that the *LIP*1 gene product affects an early step in phytochrome signal transduction. In this respect, *lip*1 seems similar to Arabidopsis deetiolated mutants for which the lesions have been shown to be epistatic to mutations affecting *PHYA*, *PHYB*, and phytochrome chromophore biosynthesis (Chory, 1992; Deng and Quail, 1992).

Table 1 compares the phenotypic characteristics of the *lip*1 mutant described here and three previously characterized deetiolated mutants of Arabidopsis. Dark-grown seedlings of all four mutants exhibit a variety of characteristics normally associated with light-grown plants, such as leaf development, reduced stem elongation, and expression of normally light-induced genes, whereas mature light-grown plants show similar differences from the wild type in height, color, and fertility. However, there are also specific and striking differences among the mutants. For example, the roots of light-grown *det*1 and *cop*1 (but not *det*2) seedlings contain developed chloroplasts (Chory and Peto, 1990; Deng et al., 1991), demonstrating a reduction in the normal organ-specific repression of plastid development. The *det*2 mutant shows less derepression of photosynthetic genes in dark-grown seedlings, and light-grown seedlings do not develop chloroplasts in their roots. In contrast, *lip*1 seedlings show complete derepression of photosynthetic genes in leaf cells of dark-grown seedlings, but the roots of light-grown seedlings do not develop chloroplasts or accumulate chlorophyll. In addition, the relative abundance of photosynthesis-related transcripts in roots was not dramatically affected by the *lip*1 mutation. Thus, *lip*1 is unique among these four mutants in its ability to completely derepress photosynthetic genes without affecting organ specificity.

Dark adaptation responses also differ among the four mutants. Wild-type plants reduce the abundance of mRNAs encoding photosynthetic proteins when they are transferred from light to darkness, and light-grown *det*1 seedlings respond similarly (Chory et al., 1989b). However, *cop*1 seedlings lack this response, maintaining high levels of mRNA in darkness (Deng et al., 1991), and in *det*2 seedlings there is only a partial decrease in mRNA levels (Chory et al., 1991). The effect of different growth regimes on these responses has not been explored in previous investigations, but our data show that the response of *lip*1 seedlings is quite plastic. This mutant shows no dark adaptation response when seedlings are grown for 9 days under constant light prior to the experiment. However, a normal response is observed in 15-day-old *lip*1 seedlings grown in a light/dark cycle. Because the dark-adaptation experiments with the Arabidopsis mutants were all done with young seedlings grown in continuous light, it is not possible to determine if a similar developmental plasticity might also characterize their responses.

Segregation studies show that *lip*1 is a recessive mutation at a single locus. In this respect, it is identical to the *det* and *cop* mutations (Chory et al., 1989b; Deng et al., 1991). To account for the recessive nature of these mutations, it is attractive to suppose that the wild-type gene products act to suppress developmental pathways associated with photomorphogenesis (Chory et al., 1989b; Chory, 1991). According to this hypothesis, light would stimulate photomorphogenesis by interfering with the synthesis or activity of these gene products, and photomorphogenesis would become constitutive when mutations block repressor production or reduce repressor activity.

In Arabidopsis, the *DET*1 and *COP*1 gene products are required not only to prevent photomorphogenetic development in the dark but also to repress plastid development in nonphotosynthetic tissues of light-grown seedlings. This conclusion derives from the fact that both *det*1 and *cop*1 mutants show ectopic photosynthetic gene expression and chloroplast development in roots (Chory et al., 1989b; Deng and Quail, 1992).

Table 1. Comparison of Deetiolated Mutants

	*lip*1	*cop*1[a]	*det*1[b,c]	*det*2[c,d]
Phenotypic Characteristics				
In the dark				
Leaf/cotyledon development	Yes	Yes	Yes	Partial
Chloroplast development	Partial	Partial	Partial	None
Epicotyl/hypocotyl	Short	Short	Short	Short
Type I phytochrome levels	Reduced	Unknown	Normal	Normal
In the light				
Height	Short	Short	Short	Short
Color	Dark green	Normal	Pale	Dark green
Flowering time	Normal	Unknown	Normal	Late
Fertility	Reduced	Reduced	Reduced	Male sterile
Senescence	Normal	Normal	Normal	Late
Green roots	No	Yes	Yes	No
Light-Specific Gene Expression				
mRNA levels in dark-grown seedings				
*Rbc*S	High	High	High	High
Cab	High	High	High	High
*Fed*1	High	High	Unknown	Unknown
*Phy*A	Unknown	Low	Unknown	Unknown
Dark adaptation response				
Continuous light to continuous dark	Absent	Absent	Normal	Reduced
Light/dark cycle to continuous dark	Normal	Unknown	Unknown	Unknown

[a] Deng et al. (1991), Deng and Quail (1992), and X.-W. Deng, personal communication.
[b] Chory et al. (1989b) and Chory and Peto (1990).
[c] J. Chory, personal communication.
[d] Chory et al. (1991).

In this context, it is noteworthy that the *lip*1 mutation does not cause extensive ectopic gene expression or plastid development in the roots of pea seedlings. Thus, if we assume *LIP*1 to be equivalent to *DET*1 or *COP*1, we must also assume that pea seedlings have an additional system for repressing plastid development in root cells. For the present, it is simpler to assume that *LIP*1 controls a regulatory step different from those controlled by either *DET*1 or *COP*1.

There are striking similarities between the *lip*1 phenotype and the photomorphogenesis-like effects that Kloppstech and his collaborators have reported in dark-grown pea seedlings subjected to periodic temperature shifts or cyclic heat shock treatments (Kloppstech et al., 1991; Otto et al., 1992). Like mutant seedlings, heat-shocked seedlings have shortened internodes, expanded leaves, and partially developed plastids when compared to controls grown in the dark without heat treatments. Heat-shocked seedlings also express several normally light-regulated genes in the dark and accumulate chlorophyll immediately upon transfer to light. They also accumulate an elevated level of the yellow xanthophyll pigment lutein, which may also be responsible for the striking yellow color of dark-grown *lip*1 seedlings.

Kloppstech et al. (1991) and Otto et al. (1992) have suggested that heat shock may substitute for a light signal that normally induces or synchronizes circadian rhythmicity, proposing that synchronization per se can bring about photomorphogenesis. Our results with *lip*1 do not speak to this question directly, but they do show that a very similar or identical developmental process can be induced in the absence of either light or temperature signals. Thus, the fact that heat shock can induce photomorphogenesis may simply indicate that a crucial negative regulator of photomorphogenesis is sensitive to thermal denaturation in vivo. *LIP*1 itself would be a good candidate for such a regulator.

METHODS

Plant Material, Growth Conditions, and Genetic Methods

The *lip*1 mutant is a spontaneous mutation of *Pisum sativum* cv Alaska isolated from a population of seed purchased from W. Atlee Burpee Company (Warminster, PA). The original mutant individual was identified in a group of dark-grown seedlings by virtue of its expanded leaves and short epicotyl. This individual was grown to maturity in a greenhouse and allowed to self-fertilize to produce seeds for subsequent experiments.

For most experiments, seedlings were grown in either continuous light or continuous darkness at 22°C. Wild-type and *lip*1 seedlings were grown in adjacent containers, exposed to the same environment. Prior

to planting, seeds were surface sterilized in 10% Clorox (0.6% sodium hypochlorite) for 10 min, rinsed three times in distilled water, and imbibed under the same light conditions in which they were to be grown. Seedlings were grown in plastic trays containing three layers Kimpak (Kimberley Clark, Roswell, GA). To prevent fungal infection, the Kimpak was wetted with a suspension of Benomyl (methyl 1-butylcarbamoyl-2 benzimidazole-carbamate from Charles H. Lilly Co., Portland, OR) at 1 g/L. No effects on growth or development were observed at this concentration of fungicide. Trays were placed in a covered plastic box in a completely dark growth room, or were loosely wrapped in clear plastic wrap and placed in a Conviron growth chamber (model No. CMP 3023) ~135 cm from a bank of four 40-W cool-white fluorescent lights supplemented with two 100-W incandescent lights. The resulting light intensity was ~60 μmol m^{-2} sec^{-1}. Dark-grown tissue was harvested under complete darkness or under a dim, green safe light.

Plants for seed or for use in genetic crosses were grown in a temperature-controlled greenhouse on a 22°C day/18°C night cycle. Natural lighting was supplemented with a 3-hr night interruption with low intensity incandescent lamps from 11 PM to 2 AM to give the photomorphogenetic equivalent of a long day. Seeds were surface sterilized as above, sown in a standard perlite/gravel mixture, and watered once with the Benomyl suspension and subsequently with half-strength Hoagland's solution.

Plants were grown in a light/dark cycle for dark adaptation experiments in the Southeastern Plant Environment Laboratory in an A-type growth chamber (8 × 12 × 7 ft, as described by Downs and Thomas, 1991) under a mixture of fluorescent and incandescent lamps at an intensity of 700 μmol m^{-2} sec^{-1} (400 to 800 nm). Temperature and day length were controlled on a cycle of 9-hr light at 22°C and 15-hr dark at 18°C. Seeds were surface sterilized and grown in a standard perlite/gravel mixture as described above.

Pigment Analysis

Seedlings were grown in darkness for 6 days and then transferred into continuous light. At various time points after transfer, buds were harvested into dimethylformamide and extracted in the dark at 4°C for 2 days. Total chlorophyll content was calculated from the absorbance at 664.5 nm, using an absorption coefficient of 83.39 g^{-1} L (Moran and Porath, 1980).

Microscopy

Outer leaves from the apical buds of 6-day-old seedlings grown in continuous light or darkness were removed and fixed in 2% glutaraldehyde, 0.1 M sodium phosphate buffer, pH 7.0, on ice for 1 hr and then post-fixed with 2% osmium tetroxide in 2% glutaraldehyde, 0.1 M sodium phosphate buffer, pH 7.0, for 1.5 hr at 4°C. After dehydration in an acetone series, the tissue was embedded in Spurr's resin (Hirsch et al., 1983). Light micrographs were taken of 1-μm sections stained with toluidine blue. Electron micrographs of thin sections stained with uranyl acetate and lead citrate were taken with a JEOL 100S transmission electron microscope.

RNA Gel Blot Analysis

Total RNA was isolated and glyoxylated as previously described (Elliott et al., 1989). Five micrograms of glyoxylated RNA per lane was fractionated on 1.25% agarose gels, transferred to a Tropilon (Tropix, Bedford, MA) membrane by capillary blotting with 25 mM Na_2HPO_4 buffer, and cross-linked to the filter with 120 μJ of UV light (254 nm) in a Stratagene Stratalinker. RNA probes were prepared with RNA polymerases T3 or T7 (Promega). Probes prepared in this manner were *Fed*1 (an AluI-AvaII fragment of *Fed*1 [Elliott et al., 1989] inserted into pBSM13−, Stratagene) and *Cab* (a PstI fragment of pAB96 [Coruzzi et al., 1983] inserted into the same vector). DNA probes for *Rbc*S were prepared from a PstI fragment of pSS15 (Broglie et al., 1981) with the Amersham MultiPrime kit.

When RNA probes were used, filters were prehybridized and hybridized at 55°C in a buffer containing 50% formamide, 5 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate), 50 mM Tris, 5 mM EDTA, 0.1% sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, and 40 μg/mL calf liver RNA. When DNA probes were used, the same protocol was followed except that the hybridization solution contained 30% formamide and the temperature for hybridization was 42°C. After ≥12 hr of hybridization, filters were washed twice at room temperature in 2 × SSC, 0.1% SDS and then twice at 55°C in 1 × SSC, 0.1% SDS.

Blots were reprobed with an oligonucleotide complementary to the 18S ribosomal RNA (Gallo-Meagher et al., 1992) to control for variations in loading and transfer efficiency. Hybridization signals were quantitated with an AMBIS Radioanalytic Imaging System. Autoradiographic images were then obtained by exposing the filters at −80°C to Kodak X-OMAT AR film with Kodak Lanex intensifying screens.

Phytochrome Measurements

Wild-type and *lip*1 seedlings were grown in complete darkness to a height of ~5 cm (4 days for wild-type, 7 days for *lip*1 seedlings). In vivo difference spectra were recorded by packing 14 epicotyls (without plumules) into a 2 × 10 mm quartz cuvette. Phytochrome was first converted to Pfr by a 5-min irradiation with red light (R) (Corion S10-660 filter, λ_{max} 658 nm, half-band width = 10 nm), and absorption spectra were recorded on a Shimadzu UV3000 spectrophotometer. Phytochrome was then converted to Pr by a 5-min irradiation with FR (Corion S10-750 filter, λ_{max} 748 nm, half-band width = 34 nm) and difference spectra were recorded. Measurements were repeated several times on each sample.

For immunoblot analysis, etiolated epicotyls were frozen in liquid nitrogen and lyophilized. The frozen material was then extracted by grinding in hot SDS sample buffer (2% SDS, 125 mM Tris, 750 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 10% glycerol, pH 6.8). DNA content was determined by a Hoechst dye-binding assay (Cesarone et al., 1979) using a Perkin-Elmer LS-5B luminescence spectrophotometer, and protein content was determined by an Amido Schwartz dye-binding assay (Schaffner and Weissmann, 1973). DNA and protein yields per gram fresh weight or per epicotyl were similar for wild-type and mutant peas. Aliquots adjusted to equal DNA content were separated by 6% SDS-PAGE and transferred to nitrocellulose filters. The filters were probed with either a polyclonal antibody to etiolated oat phytochrome (antiserum No. 4032) or with the anti-phytochrome monoclonal antibody Pea25 (Cordonnier et al., 1986). The signals on the immunoblots were quantified using a Molecular Dynamics (Sunnyvale, CA) image analyzer. The signals reported are the volumes (signal area and intensity). Every experiment was performed at least twice.

Red and Red-Far-Red-Light Treatments of Embryos and Seedlings

For irradiation of seedlings, seeds were sterilized, imbibed, and sown on Kimpak as described above. Seeds were germinated in complete darkness for 4 days. Seedlings were given 20 min R, 20 min far-red light (FR) followed by 20 min R, or 20 min FR once a day for 5 days (the R and FR sources used are described in Gallo-Meagher et al., 1992). They were then examined for various morphological characteristics.

Hormone Treatments

For cytokinin treatment, seeds were imbibed and grown on Kimpak as described above but in the presence of various concentrations (0 to 300 μM) of benzyladenine (6-benzyl aminopurine, Sigma). Benzyladenine was prepared as a 2.2 mM stock solution in 1.0 M HCl and diluted into water for use. The final benzyladenine solution was adjusted to pH 7.0. Gibberellin (GA_3, Sigma) was prepared as a 50 mM stock solution in ethanol and diluted into water containing 0.1% Tween 20. Solutions containing up to 500 μM GA_3 were applied to dark-grown seedlings by spraying to runoff. Control seedlings were sprayed with a similar water/ethanol/Tween solution.

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