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Different Roles for Phytochrome in Etiolated and Green Plants Deduced from Characterization of *Arabidopsis thaliana* Mutants

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We have isolated a new complementation group of *Arabidopsis thaliana* long hypocotyl mutant (*hy*6) and have characterized a variety of light-regulated phenomena in *hy*6 and other previously isolated *A. thaliana hy* mutants. Among six complementation groups that define the *HY* phenotype in *A. thaliana*, three (*hy*1, *hy*2, and *hy*6) had significantly lowered levels of photoreversibly detectable phytochrome, although near wild-type levels of the phytochrome apoprotein were present in all three mutants. When photoregulation of chlorophyll *a*/*b* binding protein (*cab*) gene expression was examined, results obtained depended dramatically on the light regime employed. Using the red/far-red photoreversibility assay on etiolated plants, the accumulation of *cab* mRNAs was considerably less in the phytochrome-deficient mutants than in wild-type *A. thaliana* seedlings. When grown in high-fluence rate white light, however, the mutants accumulated wild-type levels of *cab* mRNAs and other mRNAs thought to be regulated by phytochrome. An examination of the light-grown phenotypes of the phytochrome-deficient mutants, using biochemical, molecular, and morphological techniques, revealed that the mutants displayed incomplete chloroplast and leaf development under conditions where wild-type chloroplasts developed normally. Thus, although phytochrome may play a role in gene expression in etiolated plants, a primary role for phytochrome in green plants is likely to be in modulating the amount of chloroplast development, rather than triggering the initiation of events (e.g., gene expression) associated with chloroplast development.

INTRODUCTION

Alteration of the growth and form of plants by light is one of the most dramatic ways in which the environment affects plants. Plants have several regulatory photoreceptors, the best studied of which is phytochrome. This soluble chromoprotein exists as a dimer of two polypeptides of approximately 120 kD, each with a covalently attached linear tetrapyrrole chromophore (Smith, 1983; Lagarias, 1985). The pigment-protein can undergo a reversible photo-induced conversion between two spectrally distinct forms, a red-absorbing form (Pr) and a far-red-absorbing form (Pfr) (Lagarias, 1985; Colbert, 1988). The mode of action of phytochrome is best characterized for dark-grown seedlings that are irradiated with low fluences of red light, which converts Pr to Pfr. Under these conditions, Pfr is involved in a variety of inductive responses, including the transcriptional regulation of nuclear genes for several chloroplast-destined proteins, such as the chlorophyll *a*/*b* binding proteins (Silverthorne and Tobin, 1987). This induction can be reversed by a subsequent phototransformation of Pfr back to Pr, triggered by a brief exposure to far-red light. Although it has been clearly shown that phytochrome plays a pivotal role in light perception, the primary mode of action of Pfr in light-regulated gene expression has not been determined. Also, because phytochrome-mediated light effects on gene expression have been studied primarily in etiolated seedlings, it is not known to what extent the results are applicable to the regulation of phytochrome-responsive genes in green plants grown under physiological conditions, i.e., light-dark diurnal cycles.

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In addition to the inductive effects on gene expression seen when etiolated seedlings are given pulses of red light, short red light pretreatments can decrease the lag time for chlorophyll formation when etiolated plants are transferred to light as well as induce the synthesis of certain chloroplastic enzymes (Kirk and Tilney-Basset, 1978; Mullet, 1988). These effects are reversed by a pulse of far-red light. Thus, it has been widely believed that Pfr is involved in the primary events associated with chloroplast biogenesis. However, Akoyunoglou (1980) showed that, although phytochrome is involved in greening when etioplasts differentiate to chloroplasts, phytochrome does not appear to be involved in the proplastid to chloroplast transition more typical of plants grown under physiologically relevant light conditions. Therefore, a role for phytochrome in chloroplast development during growth in bright light has not been directly demonstrated.

We are interested in the signal transduction pathways that lead to chloroplast biogenesis, a complex and highly regulated process that involves the coordinated regulation of many nuclear and chloroplast encoded genes. Physiological data have indicated that phytochrome plays a fundamental role in the regulation of gene expression for chloroplast-destined proteins when etiolated seedlings are given a pulse of red light (Silverthorne and Tobin, 1987). However, these light-limiting conditions do not allow chloroplast development. Mutants would allow a direct examination of the role that phytochrome plays in chloroplast biogenesis in vivo under conditions where wild-type chloroplasts develop normally.

Long hypocotyl (*hy*) mutants of *Arabidopsis thaliana* were the first recognized phytochrome photoreceptor mutants of higher plants (Koornneef, Rolff, and Spruit, 1980). These mutants were isolated on the basis of their increased hypocotyl growth relative to wild-type when grown in high photon fluence rate white light. Of the five complementation groups of *hy* mutants that were originally described, two, *hy*1 and *hy*2, had low levels of photoreversibly detectable phytochrome in etiolated seedlings (Koornneef, Rolff, and Spruit, 1980); however, very recently, it was shown that *hy*1 and *hy*2 contained the phytochrome apoprotein (Parks et al., 1989). With the exception of one additional study on the photocontrol of seed germination in the *hy* mutants (Cone, Jaspers, and Kendrick, 1985), no further analyses of the *hy* mutants have been performed. Long hypocotyl mutants of tomato and cucumber have also been described (Burdick, 1958; Koornneef et al., 1985; Parks et al., 1987; Adamse et al., 1988).

In this paper, we describe the isolation of 40 new long hypocotyl mutants of *A. thaliana* and the identification of one additional complementation group (*hy*6). We show that *hy*6, in addition to *hy*1 and *hy*2, has reduced or negligible levels of photoreversible phytochrome, even though the apoprotein is present in all three mutants. Expression of light-regulated genes is defective in *hy*1, *hy*2, and *hy*6 mutants when plants are etiolated and subjected to a pulse of red light. Because a role for phytochrome in gene expression in light-grown plants has been inferred from studies with etiolated seedlings but has not been directly demonstrated, we took advantage of the phytochrome-defective mutants to analyze the effect of phytochrome on gene expression in bright white light-grown plants. In contrast to the results seen with etiolated plants, several light-regulated genes are expressed normally when the *hy*1, *hy*2, and *hy*6 mutant plants are grown in bright light, even though these mutants display incomplete chloroplast and leaf development when grown in light. These studies suggest that the role played by phytochrome under physiologically relevant conditions may be quite different from the role deduced from treating etiolated plants with an inductive red light pulse.

RESULTS

*hy*6 Defines a New Complementation Group of Long Hypocotyl Mutant

We isolated 40 new long hypocotyl (*hy*) mutants of *A. thaliana* from a population of ethyl methanesulfonate (EMS)-mutagenized M2 seeds. The long hypocotyl variants occurred at a frequency of approximately 1 in 1000 in a batch of EMS-mutagenized M2 seeds in which alcohol dehydrogenase null mutants (a single genetic locus) occurred at 1 in 2500. The frequency of appearance of long hypocotyl mutants is consistent with the fact that a small number of distinct genetic loci can give rise to this phenotype. We initially concentrated our efforts on 10 long hypocotyl variants that looked paler than wild-type. Each of the 10 new *hy* mutants chosen for further analysis was individually crossed to each of the five *hy* complementation groups previously isolated (see Methods for details). Among the 10 mutants, four were alleles of *hy*1, four were alleles of *hy*2, one was an allele of *hy*3, and one defined a new complementation group, which we called *hy*6. We have examined an additional 10 mutants for allelism to *hy*6, but still have identified only one allele of the *hy*6 complementation group.

Like *hy*1 and *hy*2, the *hy*6 mutant exhibits many pleiotropic effects as adult plants (Figure 1; Koornneef, Rolff, and Spruit, 1980). It is pale in color, and has increased apical dominance and reduced leaf size, i.e., like *hy*1 and *hy*2, *hy*6 has an etiolated appearance in the light. However, *hy*6 plants have the most severe phenotype of the previously described *hy* mutants, including the most elongated hypocotyls of all the *hy* mutants, regardless of the light conditions used to grow the plants (Figure 1A). *hy*3 is distinguished by its somewhat paler color than wild-type, and by leaves that are expanded to a larger size than wild-

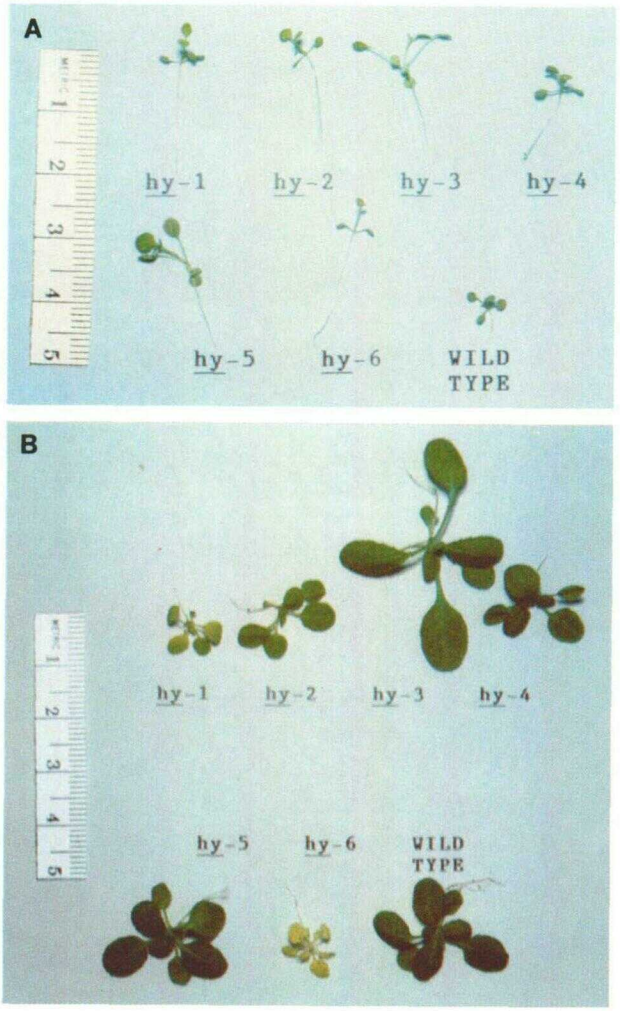

Figure 1. Phenotypes of the Six Long Hypocotyl Mutant Classes of *A. thaliana*.

(A) The long hypocotyl phenotype. The seedlings are shown 10 days after planting. The larger sizes of the *hy*3 and *hy*5 seedlings reflect faster early seedling growth as measured by fresh weight analysis (data not shown).

(B) Pleitrophy of mutations in adult plants. The plants are 3 weeks old.

Mutant and wild-type seeds were germinated at the same time and grown together under identical light (300 $\mu E/m^2/sec$) and humidity conditions.

type. *hy*4 and *hy*5, in contrast, look like wild-type plants as adults in that their leaves are fully expanded and green.

The *hy*6 mutant was characterized further by crossing the *hy*6 mutant line to wild-type and scoring each of the various phenotypes associated with the *hy*6 mutant (see Figure 1) in the F1 and F2 generations (Table 1). The heterozygous F1 plants did not have long hypocotyls, and segregation of the long hypocotyl phenotype in the F2 generation fit the 3:1 ratio expected for a single recessive mutation. In all the F2 plants examined, the multiple phenotypes seen in the adult plants co-segregated with the long hypocotyl phenotype.

Phytochrome Analysis in the *hy* Mutants

Koornneef, Rolff, and Spruit (1980) showed by dual wavelength spectrophotometric assay that *hy*1 and *hy*2 contained little to no photoreversible phytochrome, whereas *hy*3, *hy*4, and *hy*5 contained wild-type levels. We confirmed these observations using etiolated plants grown in total darkness for 5 days (Table 2), with the exception that we detected about 25% of the wild-type phytochrome level in the *hy*2 To76 allele previously analyzed. Because the photoreversibility levels we obtained were eightfold greater than those observed by Koornneef and co-workers, and because the values that they reported were close to the lower limit of detection of their spectrophotometer, the apparent discrepancy most likely reflects the increased resolution of our measurements. We found that the new mutant type, *hy*6, contained little to no photoreversible phytochrome, similar to *hy*1.

In contrast to the spectrophotometric assays, immunoblot analysis using an antiserum against pea phytochrome (Pea-25) (Cordonnier, Greppin, and Pratt, 1986) carried out on the same plants indicated that all of the mutants, including those with little to no detectable photoreversible phytochrome, contained approximately equivalent amounts of phytochrome protein with a monomer size of approximately 120 kD (Figure 2A). The following observations make it likely that Pea-25 was immunostaining *A. thaliana* phytochrome: (1) Pea-25 has previously been shown to be highly specific for phytochrome (Cordonnier, Greppin, and Pratt, 1986). (2) Pea-25 recognizes a highly conserved epitope found on phytochrome from every plant

Table 1. Complementation of *hy* Mutants by *hy*6

Cross	No. Short Hypocotyl Plants	No. Long Hypocotyl Plants
*hy*1/*hy*1 × *hy*6/*hy*6	34	0
*hy*2/*hy*2 × *hy*6/*hy*6	46	0
*hy*3/*hy*3 × *hy*6/*hy*6	52	0
*hy*4/*hy*4 × *hy*6/*hy*6	47	0
*hy*5/*hy*5 × *hy*6/*hy*6	56	0
HY/*HY* × *hy*6/*hy*6	60	0
HY/*hy*6 × *HY*/*hy*6 (F2)	178	64 ($\chi^2 = 0.27$)

χ^2 calculated for an expected segregation of three short hypocotyls to one long hypocotyl. $P > 0.05$.

Table 2. Photoreversible Phytochrome Content of *hy* Mutants

Line	Phytochrome	Content[a]
Wild-type	8.6	7.8
*hy*1	0.5	0.1
*hy*2	2.3	2.2[b]
*hy*3	7.1	7.4
*hy*4	7.4	6.4
*hy*5	8.6	7.1
*hy*6	0.5	0.2

[a] Phytochrome photoreversibility was measured at 664 nm and 728 nm for tissue samples of 325 mg, fresh weight, packed gently into a vertical light-path cuvette with a cross-sectional area of 0.33 cm^2. Entries are $\Delta\Delta A$ values multiplied by 1000. Each mutant, as well as wild-type, was independently assayed twice. Both measurements are presented here.

[b] This sample was 120 mg, fresh weight, measured in a cuvette of 0.1 cm^2 cross-sectional area.

tested, whether grown in the dark or in the light (Cordonnier, Greppin, and Pratt, 1986). (3) The *A. thaliana* polypeptide detected by Pea-25 is the correct size to be phytochrome. (4) If the 120-kD polypeptide undergoes partial degradation in vivo, a product about 4 kD smaller than the full-size polypeptide appears (Figure 2B), which is what happens with phytochrome from other species (Vierstra et al., 1984). (5) As reported below, the amount of polypeptide detected by Pea-25 is dramatically reduced when *A. thaliana* is grown in the light, as is expected if it is phytochrome. A more quantitative measurement of the phytochrome protein levels in *hy*1, *hy*2, and *hy*6 using a dilution analysis indicated that *hy*1 and *hy*6 contained somewhat less phytochrome protein than did wild-type (Figure 2B). This latter immunoblot, which was prepared with the same tissue used for spectrophotometric assay (Table 2), was repeated three times with the same results as those shown in Figure 2B. The appearance of a 116-kD putative degradation product and additional faint bands at lower molecular masses presumably arose from proteolysis that occurred during the extended handling of the tissue prior to its being frozen in the lyophilization chamber.

In addition to the studies carried out on etiolated plants, immunoblot analyses were also performed on SDS extracts of lyophilized, light-grown *A. thaliana* seedlings, as well as with seedlings that had been grown in the light for 3 weeks and then transferred to darkness for 4 days. The *A. thaliana* phytochrome apoprotein was not detected in any of these samples (data not shown). The failure to detect phytochrome was not the consequence of a technical difficulty with the immunoblot protocol since 10 ng of phytochrome from etiolated oat shoots, when mixed with any one of these samples, could be detected readily (data not shown). Because phytochrome could barely be detected in samples from etiolated seedlings after an eight-fold dilution (Figure 2B), it appeared that the phytochrome content of the light-grown seedlings was no more than about 10% that of etiolated seedlings.

Biochemical Characterization of the Phenotypes of the Phytochrome-Defective Mutants

We characterized the phytochrome-defective mutants *hy*1, *hy*2, and *hy*6 with respect to known light-regulated traits to assess directly the role that phytochrome plays in etiolated and green plants; however, the emphasis here is

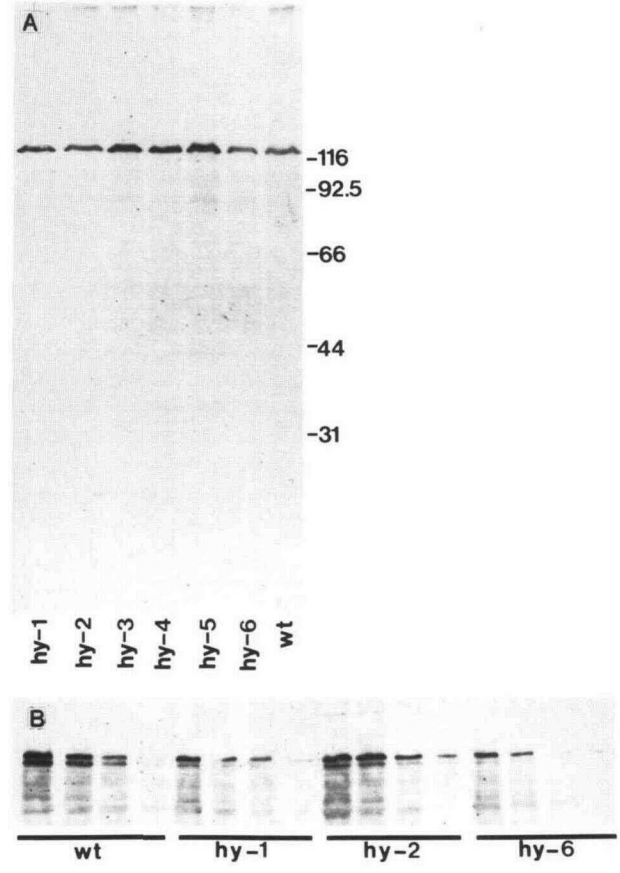

Figure 2. Immunoblot Analyses of Phytochrome from Wild-Type and Mutant *A. thaliana*.

(A) Ten-microliter aliquots of each of the six long hypocotyl mutants (*hy*1 through *hy*6) and of the Landsberg erecta wild-type. Positions and sizes (in kilodaltons) of molecular mass markers are as indicated.

(B) Each of three mutants (*hy*1, *hy*2, *hy*6) and wild-type were analyzed in a dilution series using SDS sample buffer extracts of the same tissue used for photoreversibility assays (Table 2). For each strain, sample loads from left to right were 10 μL, 5 μL, 2.5 μL, and 1.25 μL.

placed on green plants because the role of phytochrome in plants grown under high light fluence rates is not understood.

Analysis of Pigment Content in the Phytochrome-Deficient Mutants

We examined the specific chlorophyll content of the mutants under different light fluences, using either 6-hr, 12-hr, or 18-hr periods of light in a 24-hr day (Table 3). In each phytochrome-defective mutant (*hy*1, *hy*2, and *hy*6), the chlorophyll content per milligram of protein was lower than in wild-type; this value was up to 5 × lower for the *hy*6 mutant. Furthermore, the chlorophyll *a*/*b* ratios in these mutants were high, a possible indication that these mutants were deficient in light-harvesting complex. [Chlorophyll *b* is found exclusively bound to the light-harvesting chlorophyll *a*/*b* proteins (Kaplan and Arntzen, 1982).] *hy*6 is not simply a chlorophyll *b* mutant since a previously described chlorophyll *b* mutant of *A. thaliana* did not have a long hypocotyl phenotype (Hirono and Redei, 1963), and complementation analysis with the *ch*-1 mutant of *A. thaliana* indicated that *hy*6 and *ch*1 were non-allelic (data not shown).

Because the accumulation of anthocyanin pigments is also regulated by phytochrome and blue light (Mancinelli and Rabino, 1978; Beggs et al., 1987), we looked at the levels of anthocyanins produced in each of the phytochrome-deficient mutants. Anthocyanins accumulated at wild-type levels in the three *hy* mutants when the plants were grown for 3 weeks in white light (data not shown). This result implies that anthocyanin accumulation in mature *A. thaliana* plants requires very little or no phytochrome activity or that other photoreceptors can compensate for lack of photoreversible phytochrome.

Table 3. Chlorophyll Content and Chlorophyll *a*/Chlorophyll *b* Ratio of *hy* Mutants

Line and Photoperiod	Chlorophyll Content	Chlorophyll *a*/ Chlorophyll *b* Ratio
	mg chlorophyll/mg protein	mol/mol
Wild-type		
6 hr	0.55	2.3
12 hr	0.50	2.8
18 hr	0.22	2.9
*hy*1		
6 hr	0.13	3.0
12 hr	0.10	4.9
18 hr	0.18	3.8
*hy*2		
6 hr	0.37	2.3
12 hr	0.36	3.3
18 hr	0.17	3.4
*hy*6		
6 hr	0.12	3.1
12 hr	0.08	9.0
18 hr	0.08	6.1

Measurements were made on plant tissue grown for 3 weeks in white light.

Analysis of Chloroplasts and Cell Size in the Phytochrome-Deficient Mutants

To define further the phenotypes of the phytochrome-defective mutants, we carried out a comparative morphometrical and stereological analysis by light and electron microscopy of chloroplasts in mesophyll leaf cells of the three phytochrome-deficient mutants and wild-type. Care was taken to allow leaves to expand fully prior to taking samples for cell size estimations. We harvested plants when buds had formed in the rosette, but prior to bolting. To quantitate the number of chloroplasts per cell, the epidermal layer was peeled from the underside of several leaves, mesophyll protoplasts were isolated, and the number of chloroplasts per mesophyll cell was quantitated for 50 to 100 cells per mutant (Figure 3A). *hy*1, *hy*2, and *hy*6 had significantly fewer chloroplasts per cell compared with wild-type. In turn, the reduced number of chloroplasts per cell appeared to be correlated with a reduction in cell size in the small leaves of these plants (Figure 3B).

Because chlorophyll is associated with pigment-protein complexes in the thylakoid membranes of chloroplasts, we estimated the volume density of granal (stacked) thylakoids in the chloroplasts of each of the phytochrome-defective mutants (Figure 4). In *hy*1, *hy*2, and *hy*6, the percent of the total chloroplast volume that was occupied by grana was significantly lower than for wild-type chloroplasts (Table 4). In addition, there was a correlation with the amount of photoreversibly detectable phytochrome, the total specific chlorophyll content, and the volume of stacked thylakoid membranes, i.e., *hy*6 had undetectable phytochrome levels, the lowest chlorophyll content, the lowest volume density of grana per chloroplast, and the fewest number of chloroplasts per cell. Taken together, these results imply that the leaves and chloroplasts of the phytochrome-defective mutants do not develop to wild-type levels.

Expression of Known Light-Regulated Nuclear and Chloroplast Genes at High Photon Fluence Rates in *hy*1, *hy*2, and *hy*6

Because changes in gene expression underlie the complex developmental phenomenon of greening (Silverthorne and Tobin, 1987), and because *hy*1, *hy*2, and *hy*6 are defective

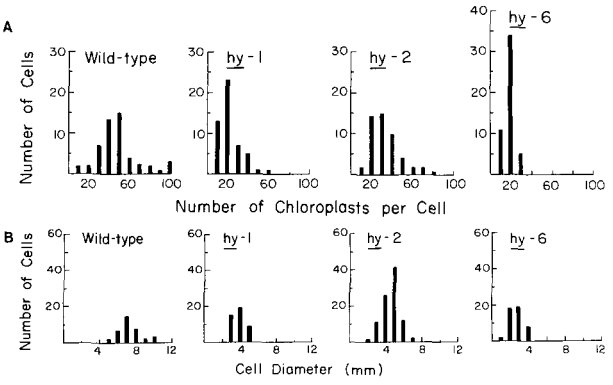

Figure 3. Characterization of Mesophyll Cells in the *hy* Mutants.

(A) Effect of the different *hy* mutations on the distribution of the number of chloroplasts in 50 to 100 leaf cells of each genotype. The mean number of chloroplasts per mesophyll cell was: wild-type, 53 ± 11; *hy*1, 26 ± 6; *hy*2, 37 ± 5; and *hy*6, 12 ± 4.
(B) Effect of the different *hy* mutations on average mesophyll cell size.

in photoreversible phytochrome, we reasoned that they may exhibit aberrant expression of genes known to be light-regulated. We looked at the expression of known light-regulated nuclear and chloroplast genes in plants that had been grown in an 18-hr day/6-hr night cycle to assess the role that phytochrome plays in plant development under physiologically relevant light conditions (Methods). Total RNA was prepared and RNA gel blot analysis performed (Figure 5, Table 5). The data represent a ratio of the light-regulated gene to the rRNA gene which is then normalized to the wild-type ratio. For every gene tested in each of the phytochrome-defective mutants, the accumulation of mRNA was within a factor of 2 of wild-type (Table 5), implying, at the very least, that very few molecules of photoreversible phytochrome are necessary for expression of these genes in bright white light.

Accumulation of Photosynthetic Complexes in the Phytochrome-Deficient Mutant Backgrounds

The phytochrome-deficient *hy* mutants were defective at chloroplast development, yet had wild-type expression of genes for chloroplast-destined and chloroplast-encoded proteins. Therefore, we examined the levels of accumulation of several photosynthetic proteins using antibodies specific for purified proteins or complexes. The same plants that were used for the RNA gel blot experiments were used for these studies. A representative protein gel blot for light-harvesting chlorophyll *a*/*b* proteins (LHCPII) (which are coded for by the *cab* genes) and the ribulose-bisphosphate carboxylase/oxygenase (Rubisco) (coded for by *rbc*S and *rbc*L) is shown in Figure 6. The data are summarized in Table 6. In contrast to the results obtained with mRNA in the previous section, some structural protein components associated with both photosystem I and photosystem II did not accumulate to wild-type levels in the *hy*1 and *hy*6 mutants. This was most pronounced for *hy*6, in which the level of LHCPII accumulated to less than 10% of wild-type protein levels. [In contrast, the *cab* mRNA was induced twofold over the levels of wild-type *cab* mRNA (Table 5).] Moreover, we could not detect any immunoreactive LHCPI polypeptides in the *hy*6 mutant (Table 6). In contrast to the LHCPs, the Rubisco subunits were accumulated at wild-type levels. The accumulation of all of the photosynthetic complexes in the *hy*2 mutant, by contrast, was similar to wild-type, implying that 25% of phytochrome activity is all that is necessary for accumulation of LHCPs.

Phytochrome Regulation of *Arabidopsis cab* mRNA Levels in Etiolated *hy*1, *hy*2, and *hy*6 Mutants

The results presented above clearly show that the phytochrome detectable in wild-type etiolated *Arabidopsis* seedlings is either not required or required only in very small levels to induce expression of photoregulated genes in high fluence rate white light. We also performed a more typical phytochrome assay of red light induction/far-red light reversal of gene expression using the phytochrome-deficient mutants. *cab* gene expression is under phytochrome control in etiolated wild-type *A. thaliana* seedlings (Karlin-Neumann, Sun, and Tobin, 1988). Those results are confirmed here (Figure 7), and analyses extended to the phytochrome-deficient *hy*1, *hy*2, and *hy*6 mutants. Two major conclusions can be drawn from the data in Figure 7A. First, *cab* gene induction in the *hy*6 mutant by a pulse of red light is about 20% of the levels in wild-type *A. thaliana* treated simultaneously. A pulse of far-red light reverses the inductive effect, indicating that the small red light response seen may be due to phytochrome action. A far-red light pulse given alone gives similar levels as those shown here for the red/far-red reversal experiments, indicating a slight induction by the far-red source alone (data not shown). Second, the *hy*2 allele (To76) that we have used here contains 25% of wild-type photoreversible phytochrome levels and shows complete induction of the *cab* mRNAs by a pulse of red light. In Figure 7B, a similar experiment with *hy*1 is shown that indicates that *hy*1 contained about 40% of wild-type *cab* mRNA levels. A mutant allele of *hy*1 (*hy*1-d412), previously isolated by Koornneef, Rolff, and Spruit (1980) and shown to contain approximately 25% of wild-type phytochrome activity, had similar levels of *cab* mRNA as wild-type after an inductive red light pulse (data not shown). As with the *hy*2 (To76) allele, these results imply that only 25% of wild-type pho-

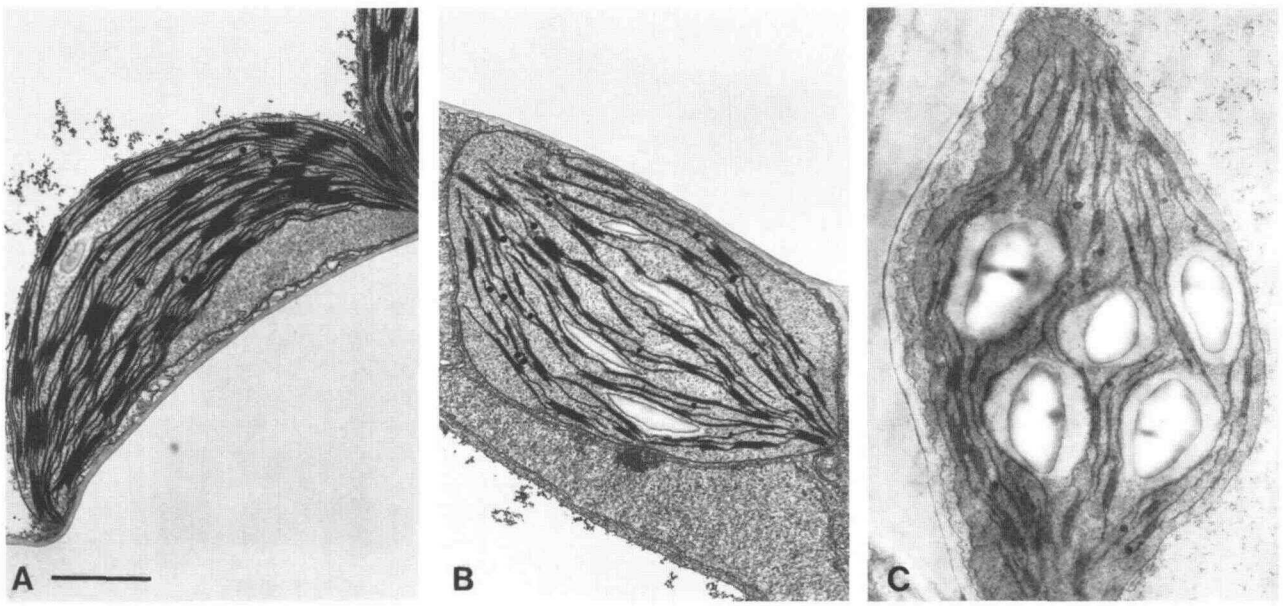

Figure 4. Illustrations of Chloroplasts.

(A) Representative electron micrograph of wild-type chloroplast.
(B) Representative electron micrograph of *hy*1 chloroplast.
(C) Representative electron micrograph of *hy*6 chloroplast.
The bar corresponds to 1 μm.

toreversible phytochrome levels are needed for wild-type levels of gene expression in etiolated *A. thaliana*.

DISCUSSION

Three Different *hy* Loci Are Deficient in Photoreversible Phytochrome Activity but Contain Phytochrome Apoprotein

Analysis of 20 new, long hypocotyl alleles has allowed us to identify a new *hy* complementation group in *A. thaliana*, bringing the total to six different *hy* loci. Alleles from three of these six groups, *hy*1, *hy*2, and *hy*6, are deficient in photoreversible phytochrome (Table 2); however, in each case, the defect is not associated with a significant decrease in the levels of phytochrome apoprotein itself (Figure 2). Similar results for *hy*1 and *hy*2 were also recently reported by Parks et al. (1989). Previous work with inhibitors of chromophore biosynthesis have indicated that phytochrome apoprotein can accumulate in the absence of normal levels of chromophore (e.g., Jones et al., 1986). *hy*1, *hy*2, and *hy*6 are, therefore, unlike the tomato mutant, *aurea*, where it was recently shown that a loss of phytochrome activity was accompanied by a decrease in the level of phytochrome apoprotein (Parks et al., 1987). Possible explanations for the defects in these *Arabidopsis*

Table 4. Volume Densities of Grana, Chloroplasts, and Cells in Leaves

Line	Chloroplast/Cell	Chloroplast/Leaf	Cell/Leaf	Grana/Chloroplast	Grana/Leaf
Wild-type	0.114 ± 0.043	0.083 ± 0.033	0.724 ± 0.061	0.300 ± 0.05	0.025 ± 0.005
*hy*1	0.199 ± 0.020[a]	0.135 ± 0.021[a]	0.677 ± 0.045	0.185 ± 0.02[a]	0.025 ± 0.003
*hy*2	0.175 ± 0.051[a]	0.123 ± 0.033[a]	0.708 ± 0.028	0.224 ± 0.03[a]	0.027 ± 0.004
*hy*6	0.206 ± 0.043[a]	0.131 ± 0.021[a]	0.637 ± 0.043[a]	0.175 ± 0.02[a]	0.022 ± 0.003

Values are v/v, mean ± SD.
[a] Denotes a significant difference from the wild-type, $P < 0.05$.

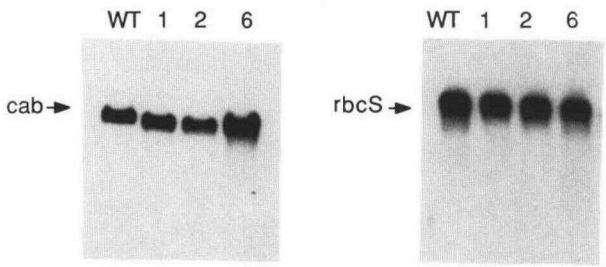

Figure 5. Autoradiograph of RNA Blot Probed with Radioactively Labeled *cab* or *rbc*S DNA.

Mutant and wild-type plants were grown side-by-side in high fluence rate white light for 3 weeks on an 18-hr day/6-hr night cycle and harvested at mid-day. Two micrograms of total RNA was loaded per lane. The numbers 1, 2, and 6 refer to the mutants *hy*1, *hy*2, and *hy*6.

mutants are that they have aberrant or absent chromophore. Specifically, the lesions in *hy*1, *hy*2, and *hy*6 may be related to chromophore biosynthesis, chromophore attachment to the apoprotein, or defective chromophore that is unable to undergo photoconversion with reasonable quantum yield even though it is attached to the apoprotein. We have explored the possibility that the mutants are chromophore auxotrophs by trying to rescue the mutant phenotypes with chromophore intermediates; however, we did not see reversion of the long hypocotyl phenotype. Experiments are in progress to ascertain whether there is chromophore attached to the apoprotein in these mutants. At present, we have not identified the specific lesion in any of these mutants; however, for the reasons outlined below, we believe the mutations are directly related to phytochrome itself and are not the indirect consequence of another biochemical defect.

It could be argued that the decreased levels of photoreversible phytochrome in these mutants are a consequence of a defective chloroplast system, rather than the other way around. This interpretation is consistent with the results of Atkinson, Bradbeer, and Frankland (1980), who found that chloroplast-defective white barley mutants had only 40% of the phytochrome activity of wild-type. Three points can be made in favor of our argument that the defect is in phytochrome rather than a nonspecific chloroplast effect. First, in the case of the barley mutants, a very severe chlorophyll deficiency was correlated with a moderate loss of phytochrome activity, whereas the opposite is the case for the *A. thaliana hy*1, *hy*2, and *hy*6 mutants. Second, *cab* mRNA did not accumulate to wild-type levels in the *hy*1 and *hy*6 mutants when etiolated seedlings were subjected to an inductive pulse of red light, conditions where there is not sufficient light for chloroplast development (Slovin and Tobin, 1982). Phytochrome is known to regulate expression of *cab* genes under these conditions in *Arabidopsis* (Karlin-Neumann, Sun, and Tobin, 1988; and our results), and in many other plants (Silverthorne and Tobin, 1987). *cab* mRNA accumulation, therefore, is a sensitive and reliable assay for phytochrome function in the absence of leaf and chloroplast development. Third, there is a correlation of "leakiness" of the observed phenotypes with the amount of photoreversible phytochrome. For instance, the *hy*2 (To76) allele analyzed here had 25% of the levels of wild-type photoreversible phytochrome, showed normal *cab* mRNA accumulation, and exhibited the least severe symptoms. When we examined a different *hy*2 allele, *hy*2-2, which was isolated during the course of this work, it was paler and showed reduced levels of *cab* mRNA compared with either wild-type or the To76 allele after a pulse of red light (J. Chory, unpublished data). Similar results were obtained with two different *hy*1 alleles, one of which was previously shown to contain 25% of wild-type photoreversible levels (Koornneef, Rolff, and Spruit, 1980), while the other had no spectroscopically detectable phytochrome (Table 2). Based on these three arguments, we are proceeding with the working hypothesis that the primary lesion in these mutants is related to phytochrome itself.

Phytochrome and Gene Expression

The *hy*1 and *hy*6 mutant alleles examined here are defective in the phytochrome regulation of *cab* genes when etiolated plants were subjected to inductive pulses of red light; however, these mutants contained wild-type levels of *cab* and other light-regulated mRNAs ("photogenes") when seeds were germinated and seedlings developed in high fluence rate white light. Based on previous results with etiolated plants, it is widely believed that a phytochrome signal transduction pathway is the primary regulator of photogene expression in plants. Our results with the phytochrome-deficient *hy* mutants are in agreement with this belief when etiolated plants are given inductive pulses of red light. Plants that are grown in high light, however, initiate leaf and chloroplast development and are

Table 5. Expression of Light-Regulated Genes in the *hy* Mutant Backgrounds

	Ratio of Expression of Photogene/rDNA Gene/Wild-Type					
Line	*cab*	*chs*	*rbc*S	*psa*A-B	*psb*A	*rbc*L
Wild-type	1.0	1.0	1.0	1.0	1.0	1.0
*hy*1	0.8	1.0	1.0	0.7	1.1	0.9
*hy*2	1.1	1.1	1.0	0.8	1.2	1.0
*hy*6	1.8	0.9	1.0	0.8	1.2	2.1

Signal was quantitated as described in Methods.

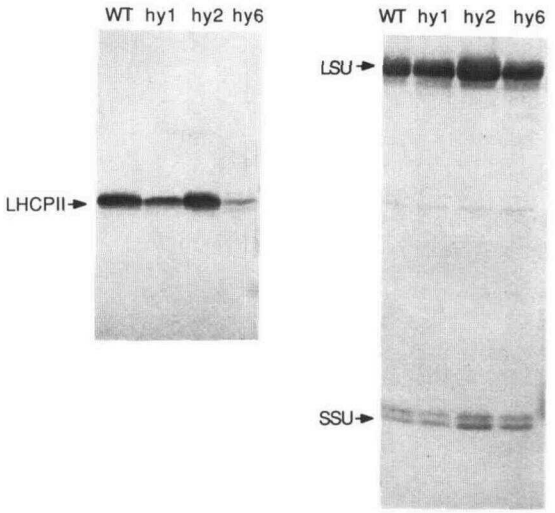

Figure 6. Immunoblot Analyses of the Levels of LHCPII or Rubisco Proteins Accumulated in the Three Mutants and Wild-Type.

LSU and SSU refer to the large and small subunits of the Rubisco, respectively.

developmentally very different from etiolated plants. Under these conditions, we deduced, using the *hy*1, *hy*2, and *hy*6 mutants, that phytochrome does not exert an effect on photogene expression.

cab mRNA levels are strongly reduced in the *hy*1 and *hy*6 alleles that we analyzed if etiolated seedlings are used as the source of material; however, about 20% to 40% of the wild-type mRNA levels are accumulated in response to a pulse of red light. Since we have not yet shown that these alleles are null, we do not know whether the residual amount of *cab* mRNA accumulated is due to a reduced number of Pfr molecules in the mutants or to the action of a different transduction pathway. Recent results with the *aurea* mutant of tomato showed similar dramatic effects on *cab* gene expression (Sharrock et al., 1988). It should be noted that the *cab* probe used in these studies recognizes all three *cab* genes of *A. thaliana*, and it may be that the expression of one of these genes is severely affected in the *hy* mutants, whereas the others are not. The recent observation that mRNA from one member of the family is accumulated at sixfold higher levels after a brief pulse of red light, whereas mRNAs from either or both of the other two *cab* genes are accumulated at higher levels during later stages of plastid development (Karlin-Neumann, Sun, and Tobin, 1988) is in agreement with this hypothesis. Use of gene-specific probes might aid in the dissection of the phytochrome-specific response in the *hy* mutants.

Since *hy*1, *hy*2, and *hy*6 contain approximately wild-type levels of light-regulated mRNAs (Table 5), it is evident that little if any of the photoreversible phytochrome detected in wild-type plants is needed for their transcription in *Arabidopsis* grown under normal daylight conditions. At the very least, far fewer molecules of phytochrome are required for wild-type gene expression levels in green plants than in etiolated plants. Given the recent observation that there are at least two kinds of phytochrome, one of which predominates in etiolated tissue and the other in light-grown tissue (Shimazaki and Pratt, 1985; Tokuhisa, Daniel, and Quail, 1985), it is attractive to speculate that these mutants are deficient in only etiolated-plant phytochrome. This suggests that green-plant phytochrome may be sufficient to exert photocontrol on accumulation of these light-regulated mRNAs in high fluence rate white light. However, if the defects were in chromophore synthesis, as argued above, one might expect all pools of phytochrome to be depleted, in which case another signal transduction pathway may bypass a phytochrome-mediated pathway.

Phytochrome and Chloroplast Development

Although photogenes were expressed at wild-type levels in the phytochrome-deficient mutants when grown in bright white light, the mutants clearly displayed a variety of aberrant properties associated with phytochrome or light-regulated phenomena. These abnormal phenotypes included reduced specific chlorophyll content (Table 3), higher chlorophyll *a*/*b* ratios (Table 3), smaller leaves as a result of less cell expansion (Figure 1 and Figure 3B), fewer chloroplasts per mesophyll cell (Figure 3A), and reduced grana volume density (Table 4) when compared with wild-type. LHCPI and LHCPII also did not accumulate to levels comparable with wild-type (Table 6).

The development of a proplastid to a fully photosynthetically competent chloroplast (greening) is a stepwise process that has been monitored both ultrastructurally and biochemically in several different plant species (Kirk and

Table 6. Accumulation of Photosynthetic Complexes in *hy* Mutant Backgrounds

	Normalized Ratio of Protein Accumulation			
Line	LHCP[a]	PSI/19KD	OEC	Rubisco
Wild-type	1.0	1.0	1.0	1.0
*hy*1	0.6	0.6	0.9	0.9
*hy*2	0.9	0.9	0.9	1.0
*hy*6	0.1	ND	0.5	1.0

Ratios were quantitated as described in Methods.

[a] Abbreviations: LHCP, light-harvesting protein complex associated with photosystem II; OEC, oxygen-evolving complex associated with photosystem II; PSI, 19-kD protein associated with light-harvesting complex I; Rubisco, large and small subunits of the ribulosebisphosphate carboxylase.

ND, not detected.

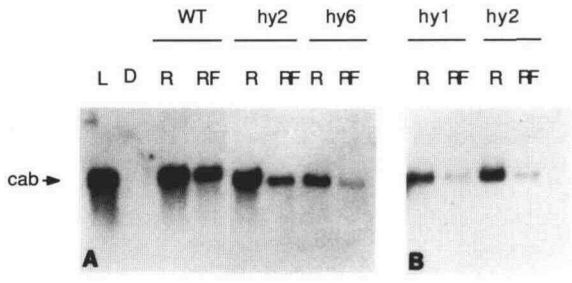

Figure 7. Phytochrome Regulation of *cab* mRNA Levels in Etiolated Plants.

(A) L (1 μg of RNA) refers to RNA prepared from wild-type *A. thaliana* grown for 3 weeks in high fluence rate white light. For all the other lanes, the seedlings were grown for 5 days in complete darkness (D) and irradiated for 10 min with red light (R) or red light followed by a 10-min pulse of far-red light (RF). The RNA load per lane in these samples was 9 μg.
(B) A separate experiment showing *hy*1 and *hy*2 grown and irradiated as in **(A)**. RNA load per lane was 5 μg. Similar results were obtained in two additional experiments.

Tilney-Basset, 1978; Mullet, 1988). After a few hours of light, the following phenotypes, indicative of early stages of chloroplast development, have been observed: (1) smaller and fewer leaf mesophyll cells than in older leaves; (2) fewer and smaller plastids than in older cells; (3) accumulation of chlorophyll *a*, but not much chlorophyll *b*, resulting in high chlorophyll *a*/*b* ratios; (4) formation of some granal stacks in chloroplasts, but not nearly so many or as stacked as in older chloroplasts; (5) high levels of accumulation of both nuclear and chloroplast mRNAs for components of the photosynthetic apparatus; and (6) little accumulation of LHCPs due to limiting amounts of a stromal factor that is necessary for stable insertion of LHCPs into membranes (Chitnis et al., 1986). These are the exact phenotypes that we observed in the phytochrome-deficient *hy* mutants under conditions where wild-type chloroplasts and leaves developed normally.

Based on the phenotypes of the *hy*1, *hy*2, and *hy*6 mutants and these former studies of chloroplast development, we propose that chloroplasts in the phytochrome-deficient mutants do not develop completely from proplastids in white light. The fact that leaf cells are not fully expanded in these mutants is also consistent with this hypothesis since leaf and chloroplast development are probably linked in dicots (Dale, 1988). Thus, phytochrome-deficient mutants never complete the developmental program associated with growth in high light fluence rates in wild-type plants. It has been hypothesized that phytochrome is the "trigger" for chloroplast development (e.g., Kirk and Tilney-Basset, 1978); however, based on the molecular phenotypes of these phytochrome-deficient mutants, we now believe that the primary role for phytochrome in greening *A. thaliana* plants is in modulating the amount of chloroplast development rather than triggering events associated with the initiation of chloroplast development.

METHODS

Plant Material, Growth Conditions, and Genetic Methods

Five classes of long hypocotyl mutants of *Arabidopsis thaliana* (*hy*1, *hy*2, *hy*3, *hy*4, and *hy*5) were described previously by Koornneef, Rolff, and Spruit (1980). The alleles used in this study were *hy*1 (21.84N), *hy*2 (To76), *hy*3 (Bo64), *hy*4 (2.23N), and *hy*5 (Ci88). A leaky allele of *hy*1 (d412) that contained about 25% of wild-type photoreversible phytochrome levels (Koornneef, Rolff, and Spruit, 1980) was also used in some experiments. These mutants were isolated in the Landsberg erecta ecotype. The *hy*2-2 allele isolated during the course of these studies looked paler than the *hy*2 (To76) allele and showed more severe phytochrome defects on gene expression.

The M2 seeds used in this study were produced by soaking approximately 50,000 wild-type Columbia seeds in 0.3% (v/v) ethyl methanesulfonate for 16 hr, followed by extensive washing with water over a period of 2 hr to 4 hr. These mutagenized M1 seeds were sown at a density of about one plant/cm^2. The resulting M2 seeds from these M1 plants were harvested in eight batches that were kept separate to minimize the number of mutant plants that were derived from the same M1 plant. This procedure was repeated for a separate batch of 50,000 wild-type seeds.

A total of 50,000 M2 seeds were screened on Petri dishes for the long hypocotyl phenotype. Putative long hypocotyl variants were removed to pots and grown to seed. The long hypocotyl phenotype was verified in M3 seeds collected from individual long hypocotyl M2 mutant plants. A total of 40 long hypocotyl mutants were recovered this way. Ten of these variants were analyzed further. Each of the 10 mutants was crossed to representatives from each of the five mutant classes (*hy*1 to *hy*5) previously described by Koornneef, Rolff, and Spruit (1980). The mutant *hy*6 was further back-crossed three times into the Landsberg background before additional analysis was performed. In the physiological experiments described here, the wild-type was Landsberg carrying the *erecta* mutation.

Plants were grown at 20°C under a mixture of fluorescent and incandescent lights at an intensity of 150 μE/m^2/sec to 200 μE/m^2/sec for the times indicated in the text. Methods for the growth of plants in pots, seed harvesting, and cross-pollination have been described (Somerville and Ogren, 1982). The *hy* mutant classes and the wild-type were always grown together under the same light and humidity conditions. Plants were harvested after 3 weeks of growth, prior to bolting and leaf senescence. For plants that were grown in the dark, seeds were imbibed for several hours in 1 mM gibberellic acid (Sigma) prior to plating on a minimal salts medium supplemented with 2% sucrose (Lloyd et al., 1986). Alternatively, the seeds were germinated in the light for 36 hr and then moved to the dark. The material was harvested after 5 days to 7 days of growth in a darkroom. These treatments had no effect on our experiments (data not shown). The treatment of plants with specific wavelengths of light were as follows: red, eight 40-W Sylvania standard Gro-Lux bulbs with red Plexiglas

filter; far-red, five 150-W incandescent bulbs, the output of which was filtered through water for cooling and through one layer of FR-Perspex (Plexiglas type FRF 700; Westlake Plastics Co., Lenni, PA). The outputs of the various light sources, measured at seedling level with a Biospherical Instruments Inc. QSL-100 light detector were: white, 200 $\mu E/m^2/sec$; red, 55.5 $\mu E/m^2/sec$; and far-red, 26 $\mu E/m^2/sec$. Irradiation times are indicated in the text. A green safelight filter was used during all dark manipulations. The harvested tissue was immediately frozen in liquid nitrogen before exposure to light.

Photoreversibility Assay

Seeds were germinated in the light for 2 days, and then moved into absolute darkness at 25°C for an additional 5 days. Phytochrome photoreversibility was measured at 664 nm and 728 nm in a custom-built, dual-wavelength spectrophotometer, similar to that described in detail by Pratt, Wampler, and Rich (1984).

Extraction and Analysis of Chlorophyll and Anthocyanin Pigments

Wild-type and the three phytochrome-deficient mutants were grown together in a growth chamber as described above. Leaves were harvested after 3 weeks and the fresh weight was determined. For chlorophyll determinations, 0.5 g to 1.0 g of frozen plant material was ground in liquid nitrogen in a mortar and pestle, and chlorophyll was extracted from the powder in 80% acetone in the dark. The pellet was extracted repeatedly in 80% acetone (until it appeared colorless). Chlorophyll *a* and *b* contents were calculated using MacKinney's specific absorption coefficients (MacKinney, 1941), in which chlorophyll $a = 12.7(A_{663}) - 2.69(A_{645})$, and chlorophyll $b = 22.9(A_{645}) - 4.68(A_{663})$. The acetone pellet was then suspended in 8 M urea for protein determinations. The total specific chlorophyll content is expressed as milligrams of chlorophyll per milligram of protein.

For anthocyanin determinations, 0.5 g of frozen plant tissue was ground in a mortar and pestle, and total plant pigments were extracted overnight in 1.5 mL of 1% HCl in methanol. After the addition of 1.0 mL of H_2O, chlorophyll was separated from the anthocyanins by extraction with an equal volume of chloroform. The quantity of anthocyanins was determined by spectrophotometric measurements of the aqueous methanol phase ($A_{530} - A_{657}$) and normalized to the total fresh weight of tissue used in each sample (Rabino and Mancinelli, 1986; Beggs et al., 1987).

Measurements of Chloroplast Copy Number and Mesophyll Cell Size

Protoplasts were prepared from leaves obtained from plants that had been grown on a 12-hr light/dark cycle until just prior to bolting, at which time *A. thaliana* leaves are fully expanded. The protoplasts were obtained after digestion of leaves from which the lower epidermal layer had been peeled (McCourt et al., 1987). Ten microliters of the protoplast-containing solution were placed on a microscope slide and the protoplasts were flattened with a coverslip so that the chloroplasts formed a monolayer that could be easily counted. At least 50 individual protoplasts per mutant were counted. At the same time, light micrographs of mesophyll protoplasts were taken, and the cell diameters measured with a ruler. Therefore, the cell size estimates are only relative to wild-type.

Light and Electron Microscopy

Leaves were processed for morphometry and stereology as described elsewhere (Hirsch, Bang, and Ausubel, 1983). For electron microscopy, tissues were fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.0, for 1 hr, rinsed in buffer, postfixed in 2% osmium tetroxide in buffer for 1.5 hr, dehydrated in acetone, and embedded in Spurr's resin (K. Miller, personal communication). Sections 1 μm thick were stained with toluidine blue, photographed, and printed at a final magnification of ×670. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined in a Philips 410LS transmission electron microscope. Micrographs were taken at ×14,000 and printed at a final magnification of ×37,000.

Stereology

Volume fractions were estimated by point counting using multiple stage sampling (Williams, 1977; Weibel, 1980) estimating chloroplast/cell (v/v), chloroplast/leaf (v/v), and cell/leaf (v/v) from light micrographs and grana/chloroplast (v/v) from electron micrographs. Estimates from grana/cell (v/v) and grana/leaf (v/v) were obtained from these ratios. The parameter grana/leaf (v/v) corresponded to the material that was analyzed for chlorophyll concentrations. A nested sample of eight micrographs per plant was used in the light micrograph estimations. From every plant type, two plants were selected; from each plant, two blocks were sectioned; and from each block, two micrographs were taken (Shay, 1975; Gundersen and Osterby, 1981; Gupta et al., 1982). A grid spacing was selected that corresponded to about 1 point per chloroplast profile (Mathieu et al., 1981). For grana/chloroplast (v/v) estimates, five to nine electron micrographs from one or two plants were used. The electron micrographs were taken to show grana at a particular, nonrandom orientation. When the plane of the membranes was close to perpendicular to the plane of section, the grana were seen as stacked lamellae.

RNA Gel Blot Hybridizations and Quantitation

RNA was isolated by a modification of the phenol-SDS method (Ausubel et al., 1987). RNA was separated in formaldehyde-containing agarose gels (Ausubel et al., 1987), and blotted onto GeneScreen *Plus* (Du Pont-New England Nuclear) using the manufacturer's recommendations. The filters were prehybridized for 0.5 hr to 2 hr at 60°C in a solution containing 1 M NaCl, 1% SDS, 10% dextran sulfate. Hybridizations with specific probes were for 12 hr to 16 hr in the same solution containing 100 μg/mL denatured salmon sperm DNA. For homologous probes, filters were washed several times at 65°C in 0.1 × SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) and 1% SDS; for heterologous probes, the filters were washed several times at 65°C in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 1% SDS. The filters were exposed to Kodak XAR-5 film at −70°C with an intensifying screen. The filters were then stripped of probe and rehybridized with an rRNA probe for normalization of the RNA load in each

lane. Autoradiograms for different exposure times were scanned with a Kratos Model SD3000 spectrodensitometer. Relative amounts of mRNAs were determined by peak-area measurements. Relative mRNA levels quantitated in Table 5 are an average of three separate hybridizations on two different batches of plants.

The DNA probes for nuclear genes used in this study were *cab*, a 1.8-kb EcoRI fragment containing the *A. thaliana* AB180 gene for the light-harvesting chlorophyll protein associated with photosystem II (Leutwiler, Meyerowitz, and Tobin, 1986); *chs*, a 3.8-kb partial HindIII fragment containing the *A. thaliana* chalcone synthase gene (Feinbaum and Ausubel, 1988); *rbc*S, a 1.8-kb EcoRI fragment containing the *A. thaliana rbc*S1A gene for the small subunit of the ribulosebisphosphate carboxylase/oxygenase (Krebbers et al., 1988); and rDNA, a 2.5-kb EcoRI fragment containing an *A. thaliana* rRNA gene (E. Richards and F. Ausubel, unpublished data). The heterologous probes used for chloroplast genes were *psa*A-B, genes for the photosystem I reaction center polypeptides (66 kD); *psb*A, the gene for one of the photosystem II reaction center polypeptides (32 kD); *rbc*L, the gene for the large subunit of the ribulosebisphosphate carboxylase/oxygenase; and chloroplast rDNA, the gene for 16S chloroplast rRNA (Mullet and Klein, 1987). The DNA was labeled to a specific activity of about 10^8 cpm/μg of DNA using either a nick translation or random primer kit from Boehringer-Mannheim.

Electrophoresis and Immunological Detection of Proteins

The leaves and stems of 3-week-old seedlings were ground in liquid nitrogen with a mortar and pestle. The sample was then homogenized for 2 min in a Polytron in a buffer containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.4); 5 mM $MgCl_2$, and 50 mM KCl. The homogenate was filtered through one layer of Miracloth and two layers of cheesecloth to remove the intact tissue and then centrifuged at 10,000*g* for 5 min to remove large tissue fragments. The supernatant fraction was precipitated with 5 volumes of cold acetone at −20°C to concentrate the proteins and then resuspended in lysis buffer (O'Farrell, 1975) for protein determinations and electrophoresis. Those samples that were not analyzed immediately were quickly frozen in liquid N_2.

Protein was fractionated by electrophoresis in discontinuous denaturing 12.5% polyacrylamide gels, using the buffer system of Laemmli (1970). Samples were denatured by a 10-min incubation at 70°C in the presence of 2.0% SDS, 5% 2-mercaptoethanol, 50 mM Tris-HCl (pH 6.8), and 10% glycerol. For separation of LHCPII, a linear 10% to 16% polyacrylamide gradient gel was used.

Electrophoretic transfer of protein to nitrocellulose, reaction with antisera, and anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Immun-blot kit) were performed as described by the manufacturer. For the data presented in Table 6, the relative levels of specific proteins were quantified by scanning photographic negatives with a densitometer and normalizing against three randomly chosen Coomassie-stained polypeptides. For each antibody tested, two different loads of proteins were used to obtain a reaction that was in the linear range of the response. The results in Table 6 are an average of two to four different immunoblotting experiments.

The antibody probes used in these studies were rabbit anti-LHCPII (pea), obtained from Dr. J. Bennett (Bennett, Jenkins, and Hartley, 1984); rabbit anti-oxygen-evolving complex (spinach), obtained from Dr. J. Sheen (Sheen, Sayre, and Bogorad, 1987); rabbit anti-LHCPI (19-kD protein from spinach), obtained from Dr. R. Malkin (Lam et al., 1984); and rabbit anti-Rubisco (tobacco large subunit, pea small subunit), obtained from Dr. S. Rodermel (Rodermel, Abbot, and Bogorad, 1988).

For the data of Figure 2A, 7-day-old, dark-grown *Arabidopsis* seedlings were frozen in liquid nitrogen and transferred to a lyophilization apparatus without being permitted to thaw. For the data of Figure 2B, *Arabidopsis* samples used for dual-wavelength spectral assay were transferred from the cuvette into 1.5-mL microcentrifuge tubes, which were then placed into a lyophilization chamber without prefreezing of the tissue. Subsequent extraction of lyophilized tissue into SDS sample buffer (1 mL of SDS sample buffer per 45 mg, powder, for Figure 2A; 650 μL of SDS sample buffer per 325 mg, fresh weight, for Figure 2B), electrophoresis in 7.5% to 15% linear gradient SDS-polyacrylamide gels, electrotransfer to nitrocellulose, and immunostaining with the monoclonal antibody to pea phytochrome, Pea-25, followed by rabbit antibodies to mouse immunoglobulins and alkaline phosphatase-conjugated goat antibodies to rabbit immunoglobulins were as described elsewhere (Cordonnier et al., 1986). Pea-25 has been shown by immunoblot assay to detect phytochrome from a wide variety of dicotyledonous plants, whether grown in the dark or in the light (Cordonnier et al., 1986).

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