The Plant Cell, Vol. 9, 1317–1326, August 1997 © 1997 American Society of Plant Physiologists

A Deletion in the *PHYD* Gene of the Arabidopsis Wassilewskija Ecotype Defines a Role for Phytochrome D in Red/Far-Red Light Sensing

Milo J. Aukerman,[a] Matthew Hirschfeld,[b,1] Lynn Wester,[b] Michael Weaver,[a] Ted Clack,[b] Richard M. Amasino,[a] and Robert A. Sharrock[b,2]

[a]Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706
[b]Department of Biology, Montana State University, Bozeman, Montana 59717

The *PHYD* gene of the Wassilewskija (Ws) ecotype of Arabidopsis contains a 14-bp deletion (the *phyD-1* mutation) beginning at amino acid 29 of the reading frame, resulting in translation termination at a nonsense codon 138 nucleotides downstream of the deletion end point. Immunoblot analyses showed that Ws lacks phyD but contains normal levels of phyA, phyB, and phyC. By backcrossing into the Ws and Landsberg *erecta* genetic backgrounds, we constructed sibling pairs of *PHYD*$^+$ and *phyD-1* lines and of *phyB*$^-$ *PHYD*$^+$ and *phyB*$^-$ *phyD*$^-$ lines. Hypocotyl lengths after growth under white or red light increased sequentially in strains that were B$^+$D$^+$, B$^+$D$^-$, B$^-$D$^+$, and B$^-$D$^-$. In the Ws genetic background, an increase in petiole length, a reduction in cotyledon area and in anthocyanin accumulation in seedling stems, a diminished effect of an end-of-day pulse of far-red light on hypocotyl elongation, and a decrease in the number of rosette leaves at the onset of flowering were also seen sequentially in these lines. Thus, phyD, which is ~80% identical in amino acid sequence to phyB, acts in conjunction with phyB in regulating many shade avoidance responses. The existence of the apparently naturally occurring *phyD-1* mutation indicates that phyD is not essential in some natural environments.

INTRODUCTION

Determining the structures and functions of plant receptor families are important steps toward understanding the molecular mechanisms of plant responses to both external environmental cues and internal developmental signals. Red (R) and far-red (FR) light play important roles as environmental signals in deetiolation responses of dark-grown seedlings or dark-adapted plants, in signaling the proximity of neighboring or canopy vegetation via the R/FR ratio of light (the shade avoidance response), and in influencing photoperiodic timing (reviewed in Smith, 1994). The plant R/FR photoreceptors, members of the phytochrome family, are chromoproteins with photoreversible activation and limited protein sequence similarity to the sensor domains of two-component signal transducers (Schneider-Poetsch, 1992; Kehoe and Grossman, 1996), but they have no well-defined biochemical mode of action (Millar et al., 1994; Pratt, 1995; Quail et al., 1995). In flowering plants, the family comprises at least three major types, designated phyA, phyB, and phyC. These are encoded by the *PHYA*, *PHYB*, and *PHYC* genes, which are found in both monocots and dicots (Mathews et al., 1995).

In many dicot plants, additional *PHY* genes, which are most likely the products of recent gene duplications, are present (Mathews et al., 1995; Mathews and Sharrock, 1996). Examples include the independent evolution of *PHYB*-like pairs of genes in at least three highly divergent plant families, the Cruciferae, Solanaceae, and Umbelliferae (Mathews et al., 1995; Pratt et al., 1995), and expansion of *PHYA*-like groups of genes in the Fabaceae, Solanaceae, and Caryophyllaceae (Adam et al., 1993; Mathews et al., 1995). The most highly characterized phytochrome family is that of Arabidopsis, which contains five forms, designated phyA to phyE (Sharrock and Quail, 1989; Clack et al., 1994). Among these, phyB and phyD share ~80% amino acid sequence identity, whereas the other forms are 48 to 55% identical. Phylogenetic analysis indicates that the genes that encode phyB and phyD are representative of recently diverged *PHY* genes (Mathews et al., 1995).

The functions of Arabidopsis phyA and phyB in photomorphogenesis have been defined by the identification of null mutations in the *PHYA* and *PHYB* genes. Mutants lacking phyA exhibit loss of FR high-irradiance control of hypocotyl elongation, cotyledon expansion, and seed germination (Nagatani et

[1]Current address: University of Utah School of Medicine, Salt Lake City, UT 84132.
[2]To whom correspondence should be addressed. E-mail ubisr@montana.edu; fax 406-994-3190.

al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). Mutants lacking phyB have altered regulation of hypocotyl elongation, flowering time, and leaf morphology in response to low fluence R light or the ratio of R/FR light (Reed et al., 1993; Robson et al., 1993; Halliday et al., 1994). Double mutants lacking both phyA and phyB have phenotypes that indicate both additive and synergistic interactions of these photoreceptors (Reed et al., 1994). The functions of phyC, phyD, and phyE have not been determined previously. We describe the identification of a naturally occurring mutation in the Arabidopsis *PHYD* gene and the phenotypic effects of deficiency for phytochrome D and deficiency for both phyB and phyD. These experiments further define the functions of the phytochrome family members in Arabidopsis and illustrate the role of recent evolutionary expansion of photoreceptor diversity in plant light signaling.

RESULTS

Identification of the *phyD-1* Mutation

We determined the nucleotide sequence of the 5′ coding region of the *PHYD* gene from the Arabidopsis Wassilewskija (Ws) ecotype. Figure 1 shows that compared with the *PHYD* sequences from other ecotypes (Clack et al., 1994), the Ws *PHYD* gene contains a 15-bp deletion with an insertion of a single G nucleotide. Alternatively, the sequence alteration may be a simple 14-bp deletion if the evolutionary progenitor to the Ws line had a silent third position A-to-G substitution polymorphism relative to the Columbia (Col) and Landsberg *erecta* (L*er*) ecotypes in the glutamate codon at the upstream deletion end point (Figure 1). This mutation results in a frameshift and is predicted to cause translation termination at a stop codon 138 nucleotides downstream of the deletion and production of a 75–amino acid truncated phyD protein consisting of 29 amino acids of the phyD polypeptide and 46 frameshift amino acids. This observation is particularly notable because a large number of T-DNA insertion mutations have been generated in the Ws background (Feldmann, 1991), and for any mutation potentially influenced by light regulatory pathways, it may be significant that these strains are also *phyD* mutants. Among the strains in the Arabidopsis Biological Resource Center (Columbus, OH) stock list, those listed as Ws, Ws-1, Ws-2, and Ws-3 contain the *phyD-1* deletion but strain Ws-0 does not. An analysis of a collection of simple sequence length polymorphism markers (Bell and Ecker, 1994) indicates that Ws, Ws-1, and Ws-2 are indistinguishable, whereas the Ws-0 line is a genetically distinct ecotype (data not shown).

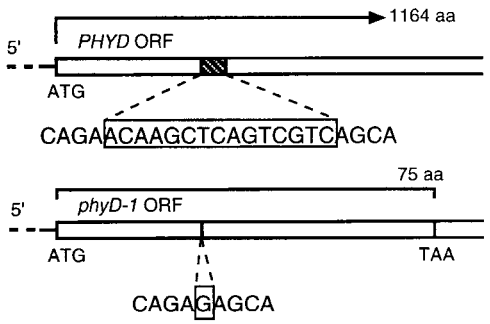

Figure 1. Sequences from the 5′ Coding Regions of the Arabidopsis Wild-Type *PHYD* Gene and the Deletion *phyD-1* Allele.

Protein coding regions of the *PHYD* gene from genomic DNA of the Columbia (Col) and Ws ecotypes were polymerase chain reaction (PCR) amplified, and the sequences of the regions around the deletion found in Ws were determined. The deletion in Ws may be either a 15-bp deletion with the insertion of a G residue (boxed) or a 14-bp deletion if the progenitor of the Ws ecotype contains the polymorphic synonymous GAG codon shown. aa, amino acid; ORF, open reading frame.

The *phyD-1* Mutation Causes Deficiency for phyD Protein

Using *Escherichia coli*–expressed apoproteins, Somers et al. (1991) generated monoclonal antibodies (MAbs) that selectively recognized Arabidopsis phyA, phyB, and phyC on immunoblots. However, the phyB-selective antibody pool (MAbs B1, B7, and B8) detected a low level of antigen in protein extracts from *phyB* mutants, and it was proposed that this might be the result of cross-reactivity with another phytochrome apoprotein (Somers et al., 1991). To test whether this second antigen is phyD, immunoblot analyses of several ecotypes, including Ws, which contained either a wild-type *PHYB*$^+$ allele or a *phyB* mutant allele, were performed with the antibodies. Figure 2A shows that the phyA-, phyB-, and phyC-selective antibodies detected equivalent levels of their respective antigens in *PHYB*$^+$ lines of the Col, Nossen (No-0), L*er*, and Ws ecotypes. However, when extracts of *phyB* mutant lines of each of these ecotypes were probed with the phyB-selective MAb pool, a band of the appropriate molecular mass (~130 kD) for phyD was detected in the Col, L*er*, and No-0 *phyB* mutants, but that band was missing in the Ws *phyB-10* strain (Figure 2B). This result is as predicted if the B1, B7, and B8 MAb pool is cross-reacting with phyD and the *phyD-1* mutation causes loss of that protein. Low levels of degradation products of both phyB and phyD were often detected on blots probed with the B1, B7, and B8 pool (Figures 2A and 2B), but an additional ~120-kD band not present in any of the other lines is visible in the Ws *phyB-10* line, as shown in Figure 2B. The size of this ~120-kD protein is consistent with its being the product of the *phyB-10* mutation, which is the result of a T-DNA insertion in the 3′ end of the *PHYB* gene (Reed et al., 1993). That this is in fact the case is demonstrated in the backcross lines described below. The *phyB* mutant alleles in Col

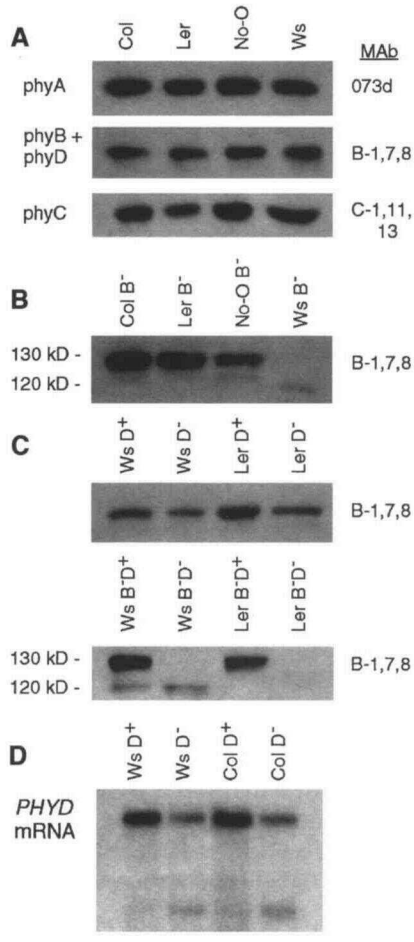

Figure 2. Phytochrome Protein and RNA Levels in Arabidopsis Lines Carrying the *PHYD*$^+$ Wild-Type Allele and the *phyD-1* Deletion Allele.

(A) Immunoblots of phytochrome-enriched protein extracts of 7-day-old dark-grown seedlings of the Col, L*er*, No-0, and Ws ecotypes. Lanes were loaded with ammonium sulfate–precipitated protein equivalent to 300 μg of crude extract protein and probed with MAb 073d (phyA), the MAb B1, B7, and B8 pool (phyB + phyD), or the MAb C1, C11, and C13 pool (phyC).

(B) Immunoblot of extracts of dark-grown seedlings of the Col *phyB-9* (Col B$^-$), L*er phyB-1* (L*er* B$^-$), No-0 *phyB-1* (No-0 B$^-$), and Ws *phyB-10* (Ws B$^-$) mutants, loaded as given in **(A)**, were probed with the MAb B1, B7, and B8 pool. Numbers at left indicate molecular masses estimated from protein size standards.

(C) Immunoblots of dark-grown seedlings of D$^+$ and D$^-$ F_3 progeny of backcrosses of the *PHYD*$^+$ and *phyD-1* alleles into the Ws and L*er* genetic backgrounds and into the Ws *phyB-10* and L*er phyB-1* backgrounds. Lanes were loaded as given in **(A)** and were probed with the MAb B1, B7, and B8 pool. Numbers at left indicate molecular masses estimated from protein size standards.

(D) RNA gel blot of *PHYD* mRNA levels in 7-day-old dark-grown extracts of D$^+$ and D$^-$ F_3 progeny of backcrosses of the *PHYD*$^+$ and *phyD-1* alleles into the Ws and Col genetic backgrounds. Poly(A)-selected RNA (0.8 μg) was fractionated on an agarose–formaldehyde gel, blotted, and hybridized with a *PHYD* 3′ end gene-specific probe.

(*phyB-9*) and in L*er* and No-0 (*phyB-1*) are nonsense codons in the first coding exon of the *PHYB* gene and are predicted to generate much shorter truncated polypeptides (Reed et al., 1993).

To determine the phenotypic consequences of loss of phyD, it is necessary to compare genetically similar strains containing and lacking phyD. To construct such lines and at the same time to demonstrate that the absence of the ~130-kD protein that cross-reacts with the MAb B1, B7, and B8 antibody pool in the *phyB* mutant lines, as shown in Figure 2B, cosegregates with the *phyD-1* mutation, several backcrossing regimens were performed. The Col *PHYD*$^+$ allele and the Ws *phyD-1* allele were backcrossed into the Ws and Col wild-type genetic backgrounds, and sibling homozygous D$^+$ and D$^-$ F_3 lines were isolated (see Methods). These alleles were also backcrossed into the Ws *phyB-10* mutant background to produce sibling Ws B$^-$D$^+$ and Ws B$^-$D$^-$ F_3 lines. In addition, the Ws *phyD-1* allele and the L*er PHYD*$^+$ allele were backcrossed into both the L*er* wild-type and the L*er phyB-1* genetic backgrounds to generate homozygous D$^+$, D$^-$, B$^-$D$^+$, and B$^-$D$^-$ F_3 lines.

Immunoblots run on extracts of the Ws and L*er* F_3 lines are shown in Figure 2C. In the B$^+$ backgrounds, the MAb B1, B7, and B8 pool detected antigen in all of the lines, although somewhat lower levels were detected in the D$^-$ as compared with the D$^+$ lines. In the B$^-$ backgrounds, deficiency for the ~130-kD protein that cross-reacts with the MAb B1, B7, and B8 antibody pool segregated with the *phyD-1* mutation in the crosses. The smaller ~120-kD immunoreactive band was detected in extracts of lines carrying the *phyB-10* allele but not in those carrying the *phyB-1* allele. These results provide strong evidence that the B1, B7, and B8 MAb pool of Somers et al. (1991) recognizes an epitope present on both phyB and phyD and that lines carrying the *phyD-1* mutant allele, including the original Ws ecotype, lack the phyD antigen, as predicted from the genetic lesion in that allele (Figure 1). In addition, we conclude that the *phyB-10* T-DNA allele generates a low level of a stable ~120-kD truncated phyB protein. Gel blot analysis of RNA from D$^+$ and D$^-$ lines in the Ws and Col backgrounds showed that a two- to threefold reduction in the abundance of the *PHYD* mRNA and the appearance of a presumed mRNA degradation product are also caused by the *phyD-1* mutation (Figure 2D).

Effects of the *phyD-1* Mutation on Hypocotyl Elongation under Continuous Light

The Ws D$^+$, D$^-$, B$^-$D$^+$, and B$^-$D$^-$ lines produced in the backcrosses described above are predicted to contain a significant fraction (~12% in the absence of selection) of residual genotype of the Col parental strain. Hence, for all of the physiology experiments described below, three sibling pairs of F_3 D$^+$ and D$^-$ lines were characterized. When a phenotypic difference was observed consistently between the

Table 1. Hypocotyl Lengths of *PHYD*$^+$ and *phyD-1* Lines[a]

Line	Dark	WL_c[b]	R_c[c]	FR_c[d]
Ws D^+	9.5	1.6	4.1	1.7
Ws D^-	9.5	1.9	4.9	1.75
	(0.58)[e]	(10^{-11})	(10^{-17})	(0.02)
Ws B^-D^+	9.9	3.3	9.5	1.8
Ws B^-D^-	10.2	4.5	11.6	1.85
	(0.03)	(10^{-34})	(10^{-29})	(0.01)
Ler D^+	9.6	2.5	5.5	1.3
Ler D^-	9.8	2.9	6.1	1.3
	(0.34)	(10^{-11})	(0.0003)	(0.14)
Ler B^-D^+	10.1	5.8	9.6	1.45
Ler B^-D^+	10.6	6.9	11.7	1.55
	(0.06)	(10^{-10})	(10^{-10})	(0.07)

[a] Values represent the average lengths in millimeters of 30 to 50 seedlings of two independent lines grown for 5 days under the indicated light conditions.
[b] WL_c, continuous white light.
[c] R_c, continuous R light.
[d] FR_c, continuous FR light.
[e] Two-tailed Student's *t* tests assuming unequal variance are shown for each pair of values.

independent D^+ and D^- lines, we concluded that it was due to the allele at the *PHYD* locus. Two additional backcrosses were performed in generating the *Ler* D^+, D^-, B^-D^+, and B^-D^- lines (see Methods); therefore, these are predicted to contain ~3% residual genotype of the Ws parent, and only two sibling F_3 D^+ and D^- lines were characterized. Additional backcrosses of the D^+ and D^- alleles into the Ws and *Ler* genetic backgrounds have confirmed the observations described here in more highly introgressed lines (L. Wester and R.A. Sharrock, unpublished data).

Table 1 shows hypocotyl lengths of 5-day-old seedlings grown under various light sources. In the dark and under continuous FR light, no difference was observed between lines carrying the *phyD-1* and the *PHYD*$^+$ alleles. A small but highly reproducible increase in the length of hypocotyls under continuous white or R light was seen in the D^- strains compared with the D^+ strains in both the Ws and *Ler* genetic backgrounds. These results indicate that phyD plays a minor but detectable role in R light control of stem growth. The most pronounced effects of the *phyD-1* mutation on hypocotyl growth were seen when comparing the B^-D^+ and B^-D^- lines (Table 1). In these lines, the increase in hypocotyl length under white and R light associated with deficiency for phyB was enhanced by a simultaneous deficiency for phyD. Hence, although phyB appears to have the predominant role in controlling this response, a significant activity of phyD in controlling hypocotyl elongation is revealed in *phyB* mutants. Both the Ws and *Ler* B^-D^- double mutants were taller when grown under R light than when grown in complete darkness (Table 1). The same phenomenon has been observed for the *phyA phyB* double mutant in the *Ler* genetic background (Reed et al., 1994).

A Role for phyD in the End-of-Day FR Response

Phytochrome B has previously been shown to be the photoreceptor responsible for most but not all of the increase in seedling elongation that accompanies a pulse of end-of-day (EOD) FR light in Arabidopsis (Nagatani et al., 1991; Wester et al., 1994). Figure 3 shows that although D^- seedlings were a little taller than were D^+ seedlings under all light conditions in the experiment, the *phyD-1* mutation had a negligible effect by itself on the EOD FR light response. The *phyB-10* mutation, as expected, strongly reduced the effect of an EOD FR pulse, but the B^-D^- double mutant was reduced even further in its response and approached insensitivity to the FR pulse (Figure 3). This indicates that photoconversion of both phyB and phyD to the Pr form accounts for almost the complete EOD FR light response.

Effects of the *phyD-1* Mutation on Other Seedling Phenotypes

Figure 4A shows that in the Ws genetic background, the area of the cotyledons of 5-day-old white light–grown seedlings was affected by the presence or absence of both phyB and phyD. Cotyledon area decreased sequentially in the B^+D^+, D^-, B^-, and B^-D^- lines, indicating that although phyB plays a somewhat larger role in controlling this trait,

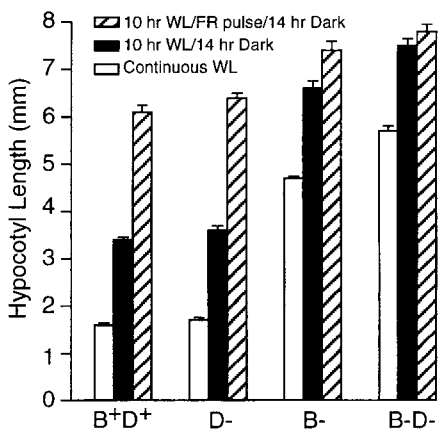

Figure 3. Effect of EOD FR Light on Hypocotyl Elongation in the Ws B^+D^+,D^-, B^-, and B^-D^- Lines.

Seedlings were grown for 2 days in continuous white light (WL) before growth for 4 days under the indicated dark and light cycles. The FR pulse consisted of 10 min of FR light, and hypocotyls were measured at the end of the dark period of the sixth day. Values are the mean of 25 to 30 seedlings, and bars represent one standard error of the mean.

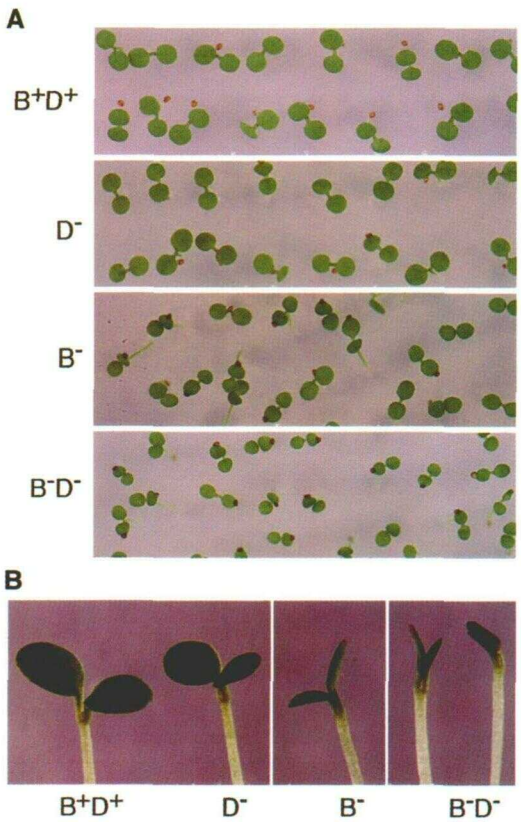

Figure 4. Light-Induced Cotyledon Expansion in the Ws B^+D^+, D^-, B^-, and B^-D^- Lines.

(A) Seedings were grown for 5 days on GM agar under continuous white light and photographed from above. Between 25 and 30 cotyledons from each line were excised and measured, and the areas relative to the B^+D^+ line were B^+D^+ (1.0), D^- (0.80), B^- (0.71), and B^-D^- (0.54).

(B) Seedlings were grown for 5 days on GM agar under continuous R light. Two seedlings representative of the variation for cotyledon opening observed in the B^-D^- line are shown. In the seedling populations, the areas of the cotyledons relative to the B^+D^+ line were B^+D^+ (1.0), D^- (0.74), B^- (0.41), and B^-D^- (0.24).

the two receptor forms are both required in an almost additive fashion for wild-type cotyledon size. Figure 4B shows that for dark-imbibed seeds placed directly under continuous R light for 5 days, a progressive decrease in the extent of cotyledon expansion could be seen in seedlings of the *phyD-1* mutant, the *phyB-10* mutant, and the B^-D^- double mutant as compared with the B^+D^+ seedling; however, the cotyledons of all four lines were green. Germination, as determined by radicle protrusion, and the appearance of the first true leaf occurred at approximately the same time in all four lines (data not shown). Together, Table 1 and Figure 4 show that phyB and phyD have parallel effects on R light–induced cell shortening in the seedling stem and cell expansion in the cotyledons. In the L*er* background, the effect of the *phyD-1* allele on cotyledon size was much less apparent or absent; areas of the cotyledons of L*er phyB* mutant lines were 64 to 66% those of *PHYB*$^+$ lines, irrespective of the *PHYD* genotype (data not shown).

In the Ws background, growth under continuous white light led to a visible accumulation of anthocyanin pigment in the hypocotyl, crown, and petioles of 8-day-old B^+D^+, D^-, and B^- seedlings but not B^-D^- seedlings, as shown in Figure 5A. At 12 days, the amount of extractable anthocyanin was progressively lower in D^-, B^-, and B^-D^- plants compared with that in B^+D^+ plants (Figure 5B), indicating an additive contribution of phytochromes B and D in this response. Again, although the effect of the *phyD-1* mutation

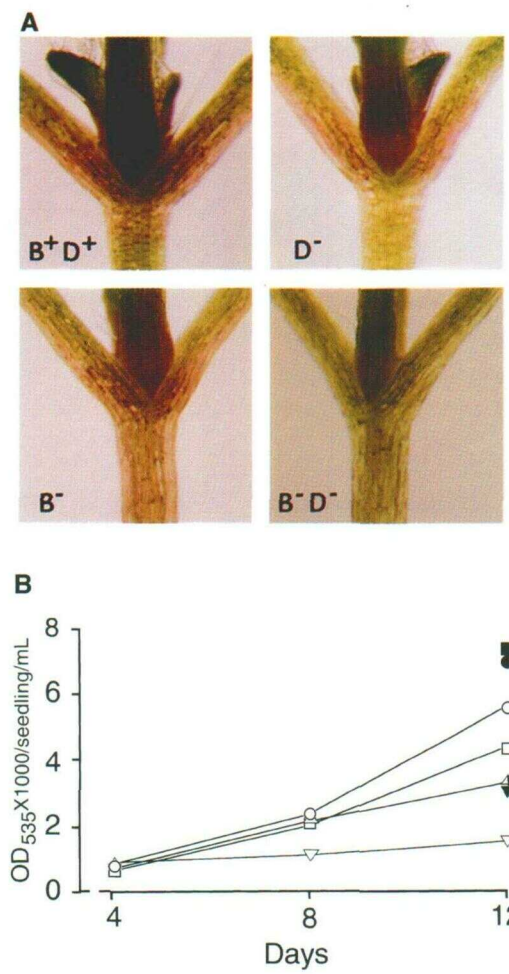

Figure 5. Anthocyanin Levels in Ws B^+D^+, D^-, B^-, and B^-D^- Lines.

(A) Anthocyanin accumulation in the crown region of 8-day-old seedlings grown under continuous white light.

(B) Time course of anthocyanin accumulation in seedlings grown under continuous white light. (○), Ws B^+D^+; (□), Ws D^-; (△), Ws B^-; (▽), Ws B^-D^-; (●), L*er* B^+D^+; (■), L*er* D^-; (▲), L*er* B^-; (▼), L*er* B^-D^-.

Figure 6. Phenotype of Mature Ws B^+D^+, D^-, B^-, and B^-D^- Plants.

Plants of the four indicated genotypes were grown in continuous white light for 3 weeks. Four representative plants from each line are shown.

on this trait is pronounced and highly penetrant in the Ws background, the trait appears to be exclusively mediated by phyB in the L*er* genotype, as illustrated by the data points shown in Figure 5B for anthocyanin accumulation in the L*er* lines at 12 days.

Effects of the *phyD-1* Mutation on Mature Plant Phenotypes

In mature plants at the rosette stage, leaf size and shape and leaf number at the time of flowering were influenced by a deficiency of phyB and phyD. Figure 6 and Table 2 show the effects of the *phyD-1* and *phyB-10* mutations individually and together at 3 weeks of growth in the Ws background. The *phyB-10* mutation caused elongation of petioles and leaves and a reduction in the number of leaves at the onset of flowering, as described previously for other *phyB* alleles (Robson et al., 1993; Halliday et al., 1994; Reed et al., 1994). In contrast, the *phyD-1* mutation itself did not significantly affect rosette morphology or development. However, the B^-D^- double mutant exhibited an exaggerated leaf shape phenotype relative to the *phyB* mutant, paralleling the small cotyledon phenotype in seedlings of the double mutant (Figure 4), and a further reduction in the number of leaves at flowering.

DISCUSSION

The data presented here show that the Arabidopsis Ws ecotype is homozygous for a 14-bp deletion in its *PHYD* gene. This mutation is predicted to cause early truncation of the phyD apoprotein and hence a phyD null phenotype. Immunoblot analysis indicates that Ws indeed lacks the phyD apoprotein. The occurrence of a *phyD* null allele in a natural population of Arabidopsis suggests that absence of this form of phytochrome does not confer a strong selective disadvantage in the wild, at least in some geographic regions, and that the *PHYD* gene in the Ws ecotype has become a pseudogene. One of the five accessions designated Ws in the Arabidopsis Biological Resource Center, Ws-0, does not contain the *phyD-1* allele. The Ws-0 accession also shows variation from the other Ws lines for a number of simple sequence length polymorphisms, indicating that it is a distinct ecotype.

We have shown that phyD has a R/FR–reversible biological activity that extensively overlaps with the activity of phyB. Phytochrome gene phylogenies indicate that the Arabidopsis *PHYB* and *PHYD* genes are the products of a relatively recent gene duplication within the Brassicaceae (Mathews et al., 1995); therefore, it is perhaps not surprising that the two photoreceptors have similar functions. All of the phenotypic effects of the *phyD-1* mutation that we have identified, including morphological traits such as leaf shape in mature plants and the influence of R light on hypocotyl elongation and cotyledon expansion in seedlings, regulation of biochemical pathways such as anthocyanin accumulation, and the timing of developmental events such as the transition from vegetative growth to flowering, have been observed in *phyB* mutants. These R light–modulated effects are components of the shade avoidance response and involve detection of the ratio of R/FR light incident upon the plant rather than simply the presence and intensity of R irradiation (Smith, 1994). The low fluence R light responses that we have examined, such as induction of seed germination and of chlorophyll *a/b* binding protein mRNA expression, are

Table 2. Adult Plant Phenotypes of $PHYD^+$ and *phyD-1* Lines[a]

Line	Petiole Length (cm)	Leaf Area (cm^2)	Leaf Number at Flowering
Ws B^+D^+	1.0 ± 0.2	1.5 ± 0.4	5.9 ± 0.2
Ws B^+D^-	1.0 ± 0.1	1.4 ± 0.2	6.2 ± 0.7
Ws B^-D^+	1.3 ± 0.1	1.5 ± 0.2	5.1 ± 0.3
Ws B^-D^-	1.6 ± 0.1	1.1 ± 0.1	4.2 ± 0.4

[a] Plants were grown under continuous white light at 24°C. Values are the averages of nine plants, and the standard deviations are shown.

not significantly altered in the *phyD-1* lines compared with the *PHYD*$^+$ lines (T. Clack and R.A. Sharrock, unpublished data). The parallel functions of phyB and phyD in R/FR sensing suggest that the evolutionary divergence of the *PHYB* and *PHYD* genes divided the control of a set of responses previously regulated by a single progenitor B-type receptor.

The role of phyD and its interaction with phyB have been characterized by monitoring the phenotypic effects of the *phyD-1* mutation by itself and in combination with *phyB* mutations in the Ws and L*er* backgrounds. Measurement of hypocotyl length after growth under continuous light reveals a minor role for phyD in R light sensing, which is additive with the much larger role of phyB in this response. This function of phyD is readily demonstrated in both ecotypes. However, for several other responses, loss of phyD has a significant effect in the Ws background but has weak or negligible effects in the L*er* background. Notably, the highly penetrant and uniform effects of the allele on cotyledon size and anthocyanin production in the Ws background (Figures 4 and 5) are not seen in the L*er* strains. This presumably reflects genetic differences in the ecotypes, and it is striking that the mutation is found naturally in an ecotype in which loss of phyD has relatively strong phenotypic consequences. It is possible that in the L*er* background, phyD plays a major role in regulating responses other than those described in this study; however, our current results indicate that phyB is highly dominant to phyD in this ecotype. In the Ws background, no consistent pattern of dominance of phyB over phyD is seen. Phytochrome B has a more pronounced role than does phyD in regulating the hypocotyl elongation and flowering time responses, but for the control of cotyledon area and anthocyanin level, the individual contributions of the two receptors are similar in this ecotype.

Some of the differences in the interaction of *phyD-1* with *phyB* mutations in the two ecotypes could also be due to the different *phyB* alleles present in these lines. It is possible that the Ws *phyB-10* allele has residual, perhaps altered, phyB activity and that under this circumstance, the activity of phyD is somehow magnified. We consider this unlikely for the following reasons. The T-DNA insertion in the Ws *phyB-10* allele is located very close to or within the third intron of the *PHYB* gene (Reed et al., 1993); therefore, the product of that allele is predicted to consist of the first 1087 to 1088 amino acids of the phyB sequence fused to a C-terminal frameshift sequence. We have shown that this truncated phyB protein is present at low levels in the *phyB-10* lines (Figure 2). A previous study by Cherry et al. (1993) indicates that such a truncated phytochrome is probably inactive. By using the "light-exaggerated" short growth phenotype induced by overexpression of the oat phyA protein in transgenic tobacco plants, these authors showed that a Δ1094 to 1129 C-terminal deletion, which is 65 homologous amino acids longer than is the predicted *phyB-10* product, resulted in a stable photochemically active pigment that completely lacked activity in the overexpression assay. This indicates that deletion of only 35 amino acids from the C terminus of a phytochrome can very strongly diminish its activity. Furthermore, Wagner and Quail (1995) isolated revertants of an Arabidopsis phyB overexpresser line on the basis of loss of the light-exaggerated phenotype and recovered two lines with nonsense mutations in the *PHYB* transgene 3′ end (R-1105 and R-1136), both of which are predicted to encode proteins longer than the *phyB-10* product. These revertant overexpresser lines failed to accumulate high levels of the truncated phyB proteins, much as *phyB-10* produces only a low level of product, and no significant phenotypic difference from the wild type was described for these lines. Hence, although we cannot eliminate the possibility that the *phyB-10* product specifically influences the activity of phyD, all available evidence suggests this is unlikely.

Previously, mutations in the Arabidopsis *PHYA* and *PHYB* genes have allowed definition of their individual functions (Somers et al., 1991; Nagatani et al., 1993; Parks and Quail, 1993; Reed et al., 1993, 1994; Whitelam et al., 1993). The contrasting roles of phyA and phyB in sensing, respectively, continuous FR and R light indicate that these two members of the phytochrome family have for the most part discrete functions (reviewed in Chory et al., 1995; Quail et al., 1995). From the results presented here, this appears to be much less the case for phyB and phyD. The extensive overlap in the functions of phyB and phyD indicates that a subtle division of specificity of function or even a significant degree of redundancy is a feature of the phytochrome family. In a situation similar to that observed here with phyB and phyD, a role for phyA in regulating hypocotyl elongation and cotyledon development in response to R light was revealed when the *phyA* mutation was combined with a *phyB* mutation (Reed et al., 1994). Hence, for R/FR sensing, phyB appears to be the predominant receptor, with both phyA and phyD acting as accessory pigments. Recently, Devlin et al. (1996) have shown that the *phyA phyB* double mutant retains significant R/FR sensitivity in the early flowering and internode elongation shade avoidance responses. The results presented here indicate that some of this residual sensitivity is mediated by phyD. Whether phyC and phyE also contribute subtly to the control of the same responses or whether they have completely different roles in photomorphogenesis will remain unclear until mutations in these receptors are identified.

Several light responses, such as hypocotyl elongation, cotyledon expansion, and the EOD FR light hypocotyl response, have at least conceptual end points, so it should be possible to ascertain whether all of the receptors that have input into these responses have been eliminated. In the case of promotion of cotyledon expansion and suppression of hypocotyl elongation, removal of all R light receptors involved in mediating these responses should yield seedlings that, when grown under R light, resemble dark-grown seedlings. For cotyledon expansion, there is an additive inhibitory effect of combining the *phyB* and *phyD* mutations (Figure 4), and there is a similar additive effect of the *phyA* and *phyB* mutations (Reed et al., 1994); however, both double mutant combinations retain some R light–mediated cotyledon development.

Hence, it is likely that all three of these forms are involved and that the triple mutant may completely lack the response. For hypocotyl elongation, the situation is more complex. In both the Ws and L*er* backgrounds, B^-D^- lines reproducibly elongate further under R light than in the dark (Table 1). An A^-B^- double mutant is also reported to show this hyperelongation (Reed et al., 1994). These observations challenge the concept of a dark-grown end point for this response and suggest that in B^-D^- and A^-B^- double mutants, hypocotyl growth is R light stimulated, perhaps by an activity of phyC or phyE. For the EOD FR response, mutational loss of all of the phytochromes that ordinarily suppress hypocotyl elongation during the dark period but can be photoreversed by an EOD FR pulse should result in seedlings that elongate to the same height with or without a FR pulse. Both phyB and phyD are involved in sensing EOD FR light (Figure 3), but the B^-D^- double mutant retains a small responsiveness to EOD FR, suggesting that at least one more FR-reversible receptor is active.

In addition to the *phyB* and *phyD* mutations in Arabidopsis, mutations in phyB-related phytochromes have been identified in Brassica (Devlin et al., 1992), cucumber (Lopez-Juez et al., 1992), tomato (Van Tuinen et al., 1995), and pea (Weller et al., 1995). The phenotypes of the Brassica, cucumber, and pea mutants are quite strong and similar to the Arabidopsis *phyB* mutants; thus, the lesions in these mutants are likely to be in *PHYB* orthologs. In contrast, the tomato *tri* (for temporary R light–insensitive) mutant exhibits a relatively mild effect on stem elongation and only during early seedling development (Van Tuinen et al., 1995). This mutation, which is presumably located in either the *PHYB1* or *PHYB2* gene (Pratt et al., 1995), may inactivate a phytochrome more functionally similar to Arabidopsis phyD. Because of the complexity of duplication of the *PHYB/D*–related *PHY* genes in several groups of plants (Mathews et al., 1995), it will be difficult to determine the orthology of these genes to the Arabidopsis *PHYB* and *PHYD* genes or to draw comprehensive conclusions about the roles of various phyB-related phytochromes in controlling plant response to light. Nevertheless, by identifying a mutation in the Arabidopsis *PHYD* gene and by comparing and combining this mutation with a *phyB* mutation, we have been able to advance our understanding of the roles of the five phytochromes in this plant and to initiate an analysis of the relative functions of recently diverged plant photoreceptor genes.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotypes Columbia (Col), Landsberg *erecta* (L*er*), Nossen (No-0), and Wassilewskija (Ws) were originally obtained from, respectively, A. Bleecker (University of Wisconsin, Madison), M. Koornneef (Wageningen Agricultural University, Wageningen, The Netherlands), B. Baker (U.S. Department of Agriculture Plant Gene Expression Center, Albany, CA), and K. Feldmann (University of Arizona, Tucson). Seeds of ecotypes Ws (CS 915), Ws-0 (CS 1602), Ws-1 (CS 2223), Ws-2 (CS 2360), and Ws-3 (CS 1638) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). For all hypocotyl length and cotyledon area experiments, except for those done under far-red (FR) light, seeds were sterilized and plated on GM medium (Valvekens et al., 1988), as described in Wester et al. (1994). For hypocotyl lengths under FR light and for anthocyanin accumulation, seeds were sterilized and plated on GM plates lacking sucrose. Seedlings for FR hypocotyl length measurements were incubated for 1 day in the dark and then transferred to continuous FR for 4 days. Light sources were the following: white light from a bank of 40-W cool-white fluorescent bulbs (11 W m^{-2}), red (R) light from Sylvania 20WT12/232 fluorescent bulbs filtered through Roscolene No. 823 plastic (Musson Theatrical, Santa Clara, CA) (2.0 W m^{-2}), and FR light from Sylvania 20WT12/2364 fluorescent bulbs filtered through FRF700 plastic (Westlake Plastics, Lenni, PA) (3.0 W m^{-2}). Radiant output was determined using an IL1400A radiometer equipped with an SEL033/F/W detector (International Light, Newburyport, MA). Mature rosette-stage plants were grown in soil under continuous white light (120 μmol m^{-2} sec^{-1}) at 24°C. Anthocyanin levels were determined as described by Schmidt and Mohr (1981).

Detection of the *phyD-1* Allele and Construction of Mutant Lines

DNA was prepared from plant tissue by the method of Edwards et al. (1991) or Klimyuk et al. (1993). The *PHYD*$^+$ or mutant *phyD-1* alleles were distinguished by polymerase chain reaction (PCR) amplification with upstream primer 5′-GGCCATCGCCGAAGTCGTC-3′ and downstream primer 5′-GATTCTGTGACCTTAGGGC-3′, which generates a 78-bp product from the wild-type allele and a 64-bp product from the deletion allele of the *PHYD* gene (Clack et al., 1994). To generate D^+, D^-, B^-D^+, and B^-D^- lines in the Ws genetic background, the Col (*PHYD*$^+$) wild type was crossed to both the Ws (*phyD-1*) wild type and the Ws *phyB-10* (*phyD-1*) mutant (Reed et al., 1993). An F_1 plant from each of these crosses was backcrossed to the appropriate Ws wild-type or Ws *phyB-10* parental line to produce BC1 F_1 lines that were backcrossed again to produce BC2 F_1 lines, and these were selfed to produce BC2 F_2 seed lots. The F_2 plants were screened by PCR, and homozygous *PHYD*$^+$/*PHYD*$^+$ and *phyD-1*/*phyD-1* lines were identified. Three sibling lines each of the B^+D^+, B^+D^-, B^-D^+, and B^-D^- genotypes were identified and grown to produce F_3 seed used in molecular and physiological experiments. The Col *PHYD*$^+$ and Ws *phyD-1* alleles were similarly backcrossed twice into the Col background, starting from the same initial cross of the two ecotypes. To generate the B^+D^+, B^+D^-, B^-D^+, and B^-D^- lines in the L*er* genetic background, the Ws (*phyD-1*) wild type was crossed to the L*er* (*PHYD*$^+$) wild type and to L*er phyB-1* (*PHYD*$^+$) (Koornneef et al., 1980), and four consecutive backcrosses of heterozygous (*PHYD*$^+$/*phyD-1*) F_1 plants to the recurrent L*er* wild type or L*er phyB-1* parents were performed. The BC4 F_1 plants were selfed, and F_2 plants were screened by PCR for homozygous *PHYD*$^+$/*PHYD*$^+$ and *phyD-1*/*phyD-1* lines. At least two sibling BC4 F_3 seed lots of each of the B^+D^+, B^+D^-, B^-D^+, and B^-D^- genotypes were used in subsequent experiments.

Protein Extraction and Immunoblotting

Proteins were extracted by a modification of the method of Tokuhisa and Quail (1987). Frozen tissue was ground in a chilled mortar and

pestle in a volume of 2 × extraction buffer containing protease inhibitors (100 mM Tris-HCl, pH 8.5, 10 mM EDTA, 150 mM $[NH_4]_2SO_4$, 50% [v/v] ethylene glycol, 2 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 2 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, and 5 μg/mL $NaHSO_3$) equal to the wet weight of tissue. A volume of water equal to that of 2 × extraction buffer used in the previous step was added. This extract was brought to 0.1% polyethyleneimine from a 10% (w/v) stock solution and was centrifuged at 20,000*g* for 20 min. A sample of the supernatant, referred to as the initial extract, was removed, and the protein concentration was determined by the method of Bradford (1976). The supernatant was mixed with 0.25 g of powdered $(NH_4)_2SO_4$ per mL of supernatant, stirred for 30 min on ice, and centrifuged as before. The pellet was suspended in 1 × extraction buffer containing protease inhibitors but without $(NH_4)_2SO_4$, and a sample was removed for protein determination. Routinely, 6 to 8% of initial extract protein was recovered after the $(NH_4)_2SO_4$ precipitation step. For loading on gels, the amount of $(NH_4)_2SO_4$-precipitated protein was corrected by this percentage recovery so that a sample equivalent to 300 μg of initial extract protein was loaded in each lane. Proteins were mixed with an equal volume of 2 × SDS-PAGE sample buffer and were separated on 6% SDS–polyacrylamide gels, according to Laemmli (1970). They were electroblotted to a Hybond ECL membrane (Amersham), blocked in 5% nonfat dry milk in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20), incubated with the primary antibody and horseradish peroxidase–conjugated secondary antibody, and detected with ECL reagents as recommended by the manufacturer (Amersham). Monoclonal antibodies were MAb 073d for phyA, the MAb (B1, B7, and B8) pool for phyB and phyD, and the MAb (C1, C11, and C13) pool for phyC (Somers et al., 1991).

RNA Gel Blot Analysis

RNA was isolated, enriched for poly(A) RNA by oligo(dT) chromatography, fractionated on an agarose–formaldehyde gel, blotted, hybridized with the D3′-600 probe, and washed as described by Clack et al. (1994).

ACKNOWLEDGMENTS

We thank Todd Richmond for assistance in mapping the *PHYD* gene and the Arabidopsis Biological Resource Center and Joanne Chory for providing genetic stocks. This work was supported by grants No. IBN-9407864 (to R.A.S.) and No. 9318481 (to R.M.A.) from the National Science Foundation and the MONTS/EPSCoR Program (to R.A.S.).

Received January 14, 1997; accepted May 2, 1997.

REFERENCES

Adam, E., Deak, M., Kay, S., Chua, N.-H., and Nagy, F. (1993). Sequence of a tobacco (*Nicotiana tabacum*) gene coding for type A phytochrome. Plant Physiol. **101,** 1407–1408.

Bell, C.J., and Ecker, J.R. (1994). Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis.* Genomics **19,** 137–144.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. **72,** 248–254.

Cherry, J.R., Hondred, D., Walker, J.M., Keller, J.M., Hershey, H.P., and Vierstra, R.D. (1993). Carboxy-terminal deletion analysis of oat phytochrome A reveals the presence of separate domains required for structure and biological activity. Plant Cell **5,** 565–575.

Chory, J., Cook, R.K., Dixon, R., Elich, T., Li, H.M., Lopez, E., Mochizuki, N., Nagpal, P., Pepper, A., Poole, D., and Reed, J. (1995). Signal transduction pathways controlling light-regulated development in Arabidopsis. Philos. Trans. R. Soc. Lond. Biol. Sci. **350,** 59–65.

Clack, T., Mathews, S., and Sharrock, R.A. (1994). The phytochrome apoprotein family in Arabidopsis is encoded by five genes: The sequences and expression of *PHYD* and *PHYE*. Plant Mol. Biol. **25,** 413–427.

Devlin, P.F., Rood, S.B., Somers, D.E., Quail, P.H., and Whitelam, G.C. (1992). Photophysiology of the elongated internode (*ein*) mutant of *Brassica rapa*. *ein* mutant lacks a detectable phytochrome B–like polypeptide. Plant Physiol. **100,** 1442–1447.

Devlin, P.F., Halliday, K.J., Harberd, N.P., and Whitelam, G.C. (1996). The rosette habit of *Arabidopsis thaliana* is dependent upon phytochrome action: Novel phytochromes control internode elongation and flowering time. Plant J. **10,** 1127–1134.

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. **19,** 1349.

Feldmann, K.A. (1991). T-DNA insertion mutagenesis in *Arabidopsis*: Mutational spectrum. Plant J. **1,** 71–82.

Halliday, K.J., Koornneef, M., and Whitelam, G.C. (1994). Phytochrome B and at least one other phytochrome mediate the accelerated flowering response of *Arabidopsis thaliana* L. to low red/far-red ratio. Plant Physiol. **104,** 1311–1315.

Kehoe, D.M., and Grossman, A.R. (1996). Similarity of a chromatic adaptation sensor to phytochrome and ethylene receptors. Science **273,** 1409–1412.

Klimyuk, V.I., Carroll, B.J., Thomas, C.M., and Jones, J.D. (1993). Alkali treatment for rapid preparation of plant material for reliable PCR analysis. Plant J. **3,** 493–494.

Koornneef, M., Rolff, E., and Spruit, C.J.P. (1980). Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana* (L.) Heynh. Z. Pflanzenphysiol. **100,** 147–160.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Lopez-Juez, E., Nagatani, A., Tomizawa, K.-I., Deak, M., Kern, R., Kendrick, R.E., and Furuya, M. (1992). The cucumber long hypocotyl mutant lacks a light-stable phyB-like phytochrome. Plant Cell **4,** 241–251.

Mathews, S., and Sharrock, R.A. (1996). The phytochrome gene family in grasses (Poaceae): A phylogeny and evidence that grasses have a subset of the loci found in dicot angiosperms. Mol. Biol. Evol. **13,** 1141–1150.

Mathews, S., Lavin, M., and Sharrock, R.A. (1995). Evolution of the phytochrome gene family and its utility for phylogenetic analysis of angiosperms. Ann. Mo. Bot. Gard. **82,** 296–321.

Millar, A.J., McGrath, R.B., and Chua, N.-H. (1994). Phytochrome phototransduction pathways. Annu. Rev. Genet. **28,** 325–350.

Nagatani, A., Chory, J., and Furuya, M. (1991). Phytochrome B is not detectable in the *hy3* mutant of *Arabidopsis*, which is deficient in responding to end-of-day far-red light treatments. Plant Cell Physiol. **32,** 1119–1122.

Nagatani, A., Reed, J.W., and Chory, J. (1993). Isolation and initial characterization of *Arabidopsis* mutants that are deficient in phytochrome A. Plant Physiol. **102,** 269–277.

Parks, B.M., and Quail, P.H. (1993). *hy*8, a new class of Arabidopsis long hypocotyl mutants deficient in functional phytochrome A. Plant Cell **5,** 39–48.

Pratt, L.H. (1995). Phytochromes: Differential properties, expression patterns, and molecular evolution. Photochem. Photobiol. **61,** 10–21.

Pratt, L.H., Cordonnier-Pratt, M.-M., Hauser, B., and Caboche, M. (1995). Tomato contains two differentially expressed genes encoding B-type phytochromes, neither of which can be considered an ortholog of *Arabidopsis* phytochrome B. Planta **197,** 203–206.

Quail, P.H., Boylan, M.T., Parks, B.M., Short, T.W., Xu, Y., and Wagner, D. (1995). Phytochromes: Photosensory perception and signal transduction. Science **268,** 675–680.

Reed, J.W., Nagpal, P., Poole, D.S., Furuya, M., and Chory, J. (1993). Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout Arabidopsis development. Plant Cell **5,** 147–157.

Reed, J.W., Nagatani, A., Elich, T.D., Fagan, M., and Chory, J. (1994). Phytochrome A and phytochrome B have overlapping but distinct functions in *Arabidopsis* development. Plant Physiol. **104,** 1139–1149.

Robson, P.R.H., Whitelam, G.C., and Smith, H. (1993). Selected components of the shade-avoidance syndrome are displayed in a normal manner in mutants of *Arabidopsis thaliana* and *Brassica rapa* deficient in phytochrome B. Plant Physiol. **102,** 1179–1184.

Schmidt, R., and Mohr, H. (1981). Time-dependent changes in the responsiveness to light of phytochrome-mediated anthocyanin synthesis. Plant Cell Environ. **4,** 433–437.

Schneider-Poetsch, H.A.W. (1992). Signal transduction by phytochrome: Phytochromes have a module related to the transmitter modules of bacterial sensor proteins. Photochem. Photobiol. **56,** 839–846.

Sharrock, R.A., and Quail, P.H. (1989). Novel phytochrome sequences in *Arabidopsis thaliana*: Structure, evolution, and differential expression of a plant regulatory photoreceptor family. Genes Dev. **3,** 1745–1757.

Smith, H. (1994). Sensing the light environment: The functions of the phytochrome family. In Photomorphogenesis in Plants, 2nd ed, R.E. Kendrick and G.H.M. Kronenberg, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 377–416.

Somers, D.E., Sharrock, R.A., Tepperman, J.M., and Quail, P.H. (1991). The *hy*3 long hypocotyl mutant of Arabidopsis is deficient in phytochrome B. Plant Cell **3,** 1263–1274.

Tokuhisa, J.G., and Quail, P.H. (1987). The levels of two distinct species of phytochrome are regulated differently during germination in *Avena sativa* L. Planta **172,** 371–377.

Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. (1988). *Agrobacterium tumefaciens*–mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA **85,** 5536–5540.

Van Tuinen, A., Kerckhoffs, L.H.J., Nagatani, A., Kendrick, R.E., and Koornneef, M. (1995). A temporarily red light–insensitive mutant of tomato lacks a light-stable, B-like phytochrome. Plant Physiol. **108,** 939–947.

Wagner, D., and Quail, P.H. (1995). Mutational analysis of phytochrome B identifies a small COOH-terminal-domain region critical for regulatory activity. Proc. Natl. Acad. Sci. USA **92,** 8596–8600.

Weller, J.L., Nagatani, A., Kendrick, R.E., Murfet, I.C., and Reid, J.B. (1995). New *lv* mutants of *Pisum* are deficient in phytochrome B. Plant Physiol. **108,** 525–532.

Wester, L., Somers, D.E., Clack, T., and Sharrock, R.A. (1994). Transgenic complementation of the *hy3* phytochrome B mutation and response to *PHYB* gene copy number in *Arabidopsis*. Plant J. **5,** 261–272.

Whitelam, G.C., Johnson, E., Peng, J., Carol, P., Anderson, M.L., Cowl, J.S., and Harberd, N.P. (1993). Phytochrome A null mutants of Arabidopsis display a wild-type phenotype in white light. Plant Cell **5,** 757–768.