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

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The PHYD gene of the Wassilewskija (Ws) ecotype of Arabidopsis contains a 14-bp deletion (the phyD-7 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-7 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-7 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 photoconvertible 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
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 or a 13-bp deletion if the evolutionary progenitor contains the polymorphic synonymous GAG codon shown. aa, amino acid; ORF, open reading frame.

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, Nosseen (No-0), Ler, 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, Ler, and No-0 phyB mutants, but that band was missing in the Ws phyB-70 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-70 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 erecta (Ler) 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).
PhyD-1 alleles into the Ws and Col genetic backgrounds. Poly(A)-
progeny of backcrosses of the PHYD' and tracts of D* and D" F
tested from protein size standards. Gel, blotted, and hybridized with a
PHYD 3' end gene-specific probe.

(A) Immunoblots of phytochrome-enriched protein extracts of 7-day-
old dark-grown seedlings of the Col, Ler, 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 ), Ler phyB-1 (Ler 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 Ler genetic backgrounds and into the Ws phyB-10 and Ler 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 ex-
tracts 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.

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

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 re-
sidual 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
(phyB-9) and in Ler 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 phyD mutant lines, as shown in Figure
2B, cosegregates with the phyD-1 mutation, several back-
crossing 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 back-
ground to produce sibling Ws B D + and Ws B D - F 3 lines. In
addition, the Ws phyD-1 allele and the Ler PHYD + allele were
backcrossed into both the Ler wild-type and the Ler 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 Ler 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, al-
though somewhat lower levels were detected in the D - as
compared with the D + lines. In the B - backgrounds, defi-
ciency 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 im-
munoreactive band was detected in extracts of lines carry-
ing 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 car-
rying the phyD-1 mutant allele, including the original Ws
ecotype, lack the phyD antigen, as predicted from the ge-
etic lesion in that allele (Figure 1). In addition, we conclude
that the phyb-10 T-DNA allele generates a low level of a sta-
ble ~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 re-
sidual 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 phe-
notypic difference was observed consistently between the

Arabidopsis phyD Mutant 1319
Table 1. Hypocotyl Lengths of PHYD+ and phyD-1 Lines

<table>
<thead>
<tr>
<th>Line</th>
<th>Dark</th>
<th>WLc</th>
<th>Re</th>
<th>FRc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ws D+</td>
<td>9.5</td>
<td>1.6</td>
<td>4.1</td>
<td>1.7</td>
</tr>
<tr>
<td>Ws D-</td>
<td>9.5</td>
<td>1.9</td>
<td>4.9</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>(0.58)e</td>
<td>(10^-11)</td>
<td>(10^-17)</td>
<td>(0.02)</td>
</tr>
<tr>
<td>Ws B-D+</td>
<td>9.9</td>
<td>3.3</td>
<td>9.5</td>
<td>1.8</td>
</tr>
<tr>
<td>Ws B-D-</td>
<td>10.2</td>
<td>4.5</td>
<td>11.6</td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td>(0.03)</td>
<td>(10^-30)</td>
<td>(10^-20)</td>
<td>(0.01)</td>
</tr>
<tr>
<td>Ler D+</td>
<td>9.6</td>
<td>2.5</td>
<td>5.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Ler D-</td>
<td>9.8</td>
<td>2.9</td>
<td>6.1</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>(0.34)</td>
<td>(10^-11)</td>
<td>(0.0003)</td>
<td>(0.14)</td>
</tr>
<tr>
<td>Ler B-D+</td>
<td>10.1</td>
<td>5.8</td>
<td>9.6</td>
<td>1.45</td>
</tr>
<tr>
<td>Ler B-D-</td>
<td>10.6</td>
<td>6.9</td>
<td>11.7</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td>(0.06)</td>
<td>(10^-13)</td>
<td>(10^-15)</td>
<td>(0.07)</td>
</tr>
</tbody>
</table>

aValues represent the average lengths in millimeters of 30 to 50 seedlings of two independent lines grown for 5 days under the indicated light conditions.

bWLc, continuous white light.

cRe, continuous R light.
dFRc, continuous FR light.
eTwo-tailed Student’s t tests assuming unequal variance are shown for each pair of values.

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-7 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. 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).

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 F3 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).

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

Phytochrome B has previously been shown to be the photo-receptor 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](https://via.placeholder.com/150)

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) Seedlings 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).

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−; (●), Ler B*D−; (■), Ler D−; (▲), Ler B−; (▼), Ler B D−.

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 Ler background, the effect of the phyD-1 allele on cotyledon size was much less apparent or absent; areas of the cotyledons of Ler 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...
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

**DISCUSSION**

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 cotyle-

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**Table 2. Adult Plant Phenotypes of PHYD+ and phyD-1 Lines**

<table>
<thead>
<tr>
<th>Line</th>
<th>Petiole Length (cm)</th>
<th>Leaf Area (cm²)</th>
<th>Leaf Number at Flowering</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ws B-D+</td>
<td>1.0 ± 0.2</td>
<td>1.5 ± 0.4</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>Ws B-D-</td>
<td>1.0 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td>Ws B-D+</td>
<td>1.3 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>Ws B-D-</td>
<td>1.6 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>4.2 ± 0.4</td>
</tr>
</tbody>
</table>

*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 sens-
ing 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 pbyB mu-
tations in the Ws and Ler backgrounds. Measurement of hy-
cotyl 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 ef-
fects in the Ler background. Notably, the highly penetrant
and uniform effects of the allele on cotyledon size and an-
thocyanin production in the Ws background (Figures 4 and
5) are not seen in the Ler 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 Ler 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 back-
ground, 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 pre-
vious 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 to-

bacco 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. Further-
more, Wagner and Quail (1995) isolated revertants of an Ar-
abidopsis 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 en-
code proteins longer than the phyB-10 product. These re-
vertant 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; Whiteham 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 divi-
sion of specificity of function or even a significant degree of
redundancy is a feature of the phytochrome family. In a si-
tuation similar to that observed here with phyB and phyD, a
role for phyA in regulating hypocotyl elongation and cotyle-
don 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, Devin et al. (1996)
have shown that the phyA phyB double mutant retains sig-
nificant R/FR sensitivity in the early flowering and internode
elongation shade avoidance responses. The results pre-

sented 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 identi-
fied.

Several light responses, such as hypocotyl elongation,
cotyledon expansion, and the EOD FR light hypocotyl re-
sponse, 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 hypo-
cotyl 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 combina-
tions 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 Ler 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 hyper-elongation (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 photo-reversed 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 activity 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., 1999). 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 (Ler), Nossen (No-0), and Wassilewskija (Ws) were originally obtained from, respectively, A. Bleeker (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/FW 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 F₁ plant from each of these crosses was backcrossed to the appropriate Ws wild-type or Ws phyB-10 parental line to produce BC₁ F₁ lines that were backcrossed again to produce BC₂ F₂ lines, and these were selfed to produce BC₂ F₃ seed lots. The F₂ 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 F₂ 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 Ler genetic background, the Ws (phyD-1) wild type was crossed to the Ler (PHYD⁺) wild type and to Ler phyB-1 (PHYD⁺) (Koornneef et al., 1980), and four consecutive backcrosses of heterozygous (PHYD⁺/PHYD⁻) F₁ plants to the recurrent Ler wild type or Ler phyB-1 parents were performed. The BC₄ F₁ plants were selfed, and F₂ plants were screened by PCR for homozygous PHYD⁺/PHYD⁺ and PHYD⁻/PHYD⁻ lines. At least two sibling BC₄ F₂ 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
pesto in a volume of 2 \times extraction buffer containing protease inhibitors (100 mM Tris-HCl, pH 8.5, 10 mM EDTA, 150 mM [NH₄]₂SO₄, 50% [v/v] ethylene glycol, 2 \mu M apronin, 1 \mu M leupeptin, 1 \mu M pepstatin, 2 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, and 5 \mu g/mL NaHSO₃) equal to the wet weight of tissue. A volume of water equal to that of 2 \times extraction buffer used in the previous step was added. This extract was brought to a volume of 2 \times extraction buffer containing protease inhibitors but without (NH₄)₂SO₄, and a sample was removed for protein determination. Routinely, 6 to 8% of initial extract protein was recovered after the (NH₄)₂SO₄ precipitation step. For loading on gels, the amount of (NH₄)₂SO₄-precipitated protein was corrected by this percentage recovery so that a sample equivalent to 300 \mu g of initial extract protein was loaded in each lane. Proteins were mixed with an equal volume of 2 \times SDS-PAGE sample buffer and were separated on 6% SDS-polyacrylamide gels, according to Laemmli (1970). They were electrophoretically to a Hybond ECL membrane (Amersham), blocked in 5% nonfat dry milk in TBS-T (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).

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