The Plant Cell, Vol. 10, 1479–1487, September 1998, www.plantcell.org

Phytochrome E Influences Internode Elongation and Flowering Time in Arabidopsis

Paul F. Devlin,[1] Samita R. Patel, and Garry C. Whitelam[2]

Department of Biology, University of Leicester, Leicester LE1 7RH, United Kingdom

From a screen of M_2 seedlings derived from γ-mutagenesis of seeds doubly null for phytochromes phyA and phyB, we isolated a mutant lacking phyE. The *PHYE* gene of the selected mutant, *phyE-1*, was found to contain a 1-bp deletion at a position equivalent to codon 726, which is predicted to result in a premature stop at codon 739. Immunoblot analysis showed that the phyE protein was undetectable in the *phyE-1* mutant. In the phyA- and phyB-deficient background, phyE deficiency led to early flowering, elongation of internodes between adjacent rosette leaves, and reduced petiole elongation. This is a phenocopy of the response of *phyA phyB* seedlings to end-of-day far-red light treatments. Furthermore, a phyE deficiency attenuated the responses of *phyA phyB* seedlings to end-of-day far-red light treatments. Monogenic *phyE* mutants were indistinguishable from wild-type seedlings. However, *phyB phyE* double mutants flowered earlier and had longer petioles than did *phyB* mutants. The elongation and flowering responses conferred by phyE deficiency are typical of shade avoidance responses to the low red/far-red ratio. We conclude that in conjunction with phyB and to a lesser extent with phyD, phyE functions in the regulation of the shade avoidance syndrome.

INTRODUCTION

Plants are exquisitely sensitive to alterations in their light environment. Through the action of specialized regulatory photoreceptors, plants monitor the intensity, quality, direction, and duration of light and use this information to modulate all aspects of their development. The red light (R)– and far-red light (FR)–absorbing phytochromes and the blue/UV-A light–absorbing cryptochromes play the predominant role in light signal perception (reviewed in Whitelam and Devlin, 1998). The phytochromes are chromoproteins that are reversibly photochromic and exist in either of two stable forms, the R-absorbing Pr form or the FR-absorbing Pfr form. Both are interconvertible by light. Light signals perceived by the phytochromes are important in the regulation of seed germination, seedling deetiolation, and photoperiodic timing and also in the detection of the proximity of neighboring vegetation (see Smith, 1994; Whitelam and Devlin, 1998).

All higher plants studied, as well as several lower plants, possess a family of discrete phytochromes. The apophytochromes are encoded by a small family of divergent genes (see Mathews and Sharrock, 1997). In the flowering plants, there appear to be three major phytochrome types, phyA, phyB, and phyC, that are encoded by the *PHYA*, *PHYB*, and *PHYC* genes (Mathews and Sharrock, 1997). Phylogenetic analysis indicates that these genes are well separated from one another in the earliest flowering plants, suggesting that the gene duplications from which they arose occurred near the origin of flowering plants (Mathews et al., 1995; Mathews and Sharrock, 1997). In dicots, additional *PHY* genes are found, perhaps the products of more recent gene duplications. In particular, dicots are characterized by the possession of *PHYB*-like pairs of genes that are considered to have arisen independently in different taxa (Mathews et al., 1995). Also, *PHYE*-like sequences have so far not been detected in monocots (see Mathews and Sharrock, 1997).

The phytochrome family of Arabidopsis is the most thoroughly characterized. Arabidopsis has five phytochromes, phyA to phyE (Sharrock and Quail, 1989; Clack et al., 1994). The *PHYB* and *PHYD* genes, which encode proteins that share ~80% amino acid sequence identity, are the product of a gene duplication in a recent progenitor of the Cruciferae. The products of these genes are more related to the product of the *PHYE* gene (~55% identity) than to the products of either the *PHYA* or *PHYC* gene (~47% identity). Thus, the *PHYB*, *PHYD*, and *PHYE* genes are considered to form a subgroup of the Arabidopsis *PHY* gene family (Goosey et al., 1997).

Elucidating the precise regulatory functions of the members of the phytochrome family is a crucial step in understanding their molecular mechanisms of action. Through the identification of null mutations, the functions of phyA, phyB, and phyD are being determined. Mutants deficient in phyA display a characteristic loss of the high-irradiance responses that control seedling deetiolation under FR. These

[1] Current address: Department of Cell Biology, Scripps Research Institute, La Jolla, CA 92037.

[2] To whom correspondence should be addressed. E-mail GCW1@le.ac.uk; fax 44-116-252-2791.

responses include the inhibition of hypocotyl elongation, the opening and expansion of cotyledons, the synthesis of anthocyanin, and the regulation of the expression of several genes (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993; Johnson et al., 1994; Barnes et al., 1996). For seedlings growing in the natural environment, phyA deficiency results in failure of seedlings to deetiolate fully under FR-rich conditions, such as those found under vegetational shade (Yanovsky et al., 1995).

Seedlings of mutants lacking phyB display a marked insensitivity to R for many responses, including the inhibition of hypocotyl elongation and the opening and expansion of cotyledons (Koornneef et al., 1980; Reed et al., 1993). Adult *phyB* plants have elongated petioles and are early flowering (e.g., Nagatani et al., 1991; Halliday et al., 1994). This phenotype is reminiscent of the shade avoidance syndrome of responses that is displayed by wild-type seedlings exposed to low R/FR ratio light (e.g., Robson et al., 1993; Whitelam and Devlin, 1997). Furthermore, *phyB* seedlings display attenuated responses to low R/FR (e.g., Robson et al., 1993; Halliday et al., 1994). In wild-type plants, the shade avoidance syndrome of responses can be effectively induced by pulses of FR given at the end of each photoperiod. These end-of-day (EOD) FR responses are also greatly attenuated in phyB null seedlings (Nagatani et al., 1991; Devlin et al., 1996). Taken together, these findings implicate phyB as a major contributor to shade avoidance. Because seedlings null for phyB are not completely devoid of responses to low R/FR or EOD FR, the action of other phytochromes in these responses is indicated (see Devlin et al., 1996).

The recent identification of a naturally occurring mutation in the *PHYD* gene of the Arabidopsis Wassilewskija (Ws) ecotype (Aukerman et al., 1997) has provided evidence that phyD performs a role similar to phyB. To examine phyD functions, a wild-type *PHYD* gene was introgressed into Ws, and the mutant gene from Ws was introgressed into the Landsberg *erecta* (L*er*) ecotype. In both genetic backgrounds, monogenic *phyD* mutants show a slightly reduced inhibition of hypocotyl elongation, compared with wild-type seedlings, after growth under either R or white light (Aukerman et al., 1997). This effect of phyD deficiency was more evident in a *phyB* mutant background in which the *phyB phyD* double mutant seedlings displayed hypocotyl lengths that were significantly longer than those of monogenic *phyB* mutants. Furthermore, phyD deficiency greatly reduced the small residual hypocotyl elongation response to EOD FR displayed by phyB mutants. In mature plants, phyD deficiency was apparent only in the *phyB* mutant background: *phyB phyD* double mutant seedlings had longer petioles and were earlier flowering than *phyB* seedlings. These data indicate that phyD plays a role in shade avoidance responses and that there is conditional redundancy among the phytochromes (Aukerman et al., 1997).

There is evidence that phytochromes other than phyB and phyD play a role in the perception of R/FR ratio signals. We showed previously that *phyA phyB* double mutants display increased internode elongation and early flowering in response to EOD FR treatments (Devlin et al., 1996). More recently, we have established that *phyA phyB phyD* triple mutants also display the internode elongation response and an attenuated flowering response to EOD FR as well as responding to reductions in the R/FR ratio (P.F. Devlin, P.R.H. Robson, S.R. Patel, L. Wester, R.A. Sharrock, and G.C. Whitelam, manuscript submitted). These observations implicate the actions of phyC and/or phyE in these responses.

We have used the internode elongation and early-flowering responses of *phyA phyB* double mutants to EOD FR treatments as the basis of a screen for new phytochrome mutants. Here, we describe the isolation of a *phyE* mutant and the phenotypic effects of phyE deficiency. As is the case for phyD, the effects of phyE deficiency are most evident in the absence of phyB. However, phyE regulates a discrete subset of responses to low R/FR.

RESULTS

Isolation of the *phyE* Mutant

The *phyE* mutant was isolated after γ-mutagenesis of *phyA phyB* seed and a two-stage screen of the M_2 populations. In the first stage, we screened for individuals displaying early-flowering and/or elongated "rosette" internodes. Candidate mutants were identified and allowed to self, and their seed were collected. In the second stage, the progeny of individuals selected in the first stage were screened for attenuated internode elongation and flowering responses to EOD FR treatments. This screen derived from our observations that in response to EOD FR treatments, the *phyA phyB* double mutant displays a marked acceleration in flowering time and a significant promotion of elongation of the internodes between rosette leaves (Devlin et al., 1996). Before mutagenesis, the *phyA phyB* double mutant was introduced into the late-flowering *co* mutant background (see Devlin et al., 1996). The *co* mutation leads to late flowering under long photoperiods (Koornneef et al., 1991) and so extends the period of vegetative development in the first stage of the screen. In this way, the likelihood of observing both early-flowering mutants and elongated-internode mutants is increased.

The first stage of the screen yielded numerous early-flowering mutants and three elongated-internode mutants that also flowered early. Of these three, one displayed a severely attenuated response to EOD FR treatments in the second stage of the screen (Figure 1).

We determined the nucleotide sequence of the *PHYE* gene from the elongated-internode mutant and from the parental *phyA phyB* line. The sequence of the *PHYE* gene amplified from the parental line (L*er* ecotype) differed from the published *PHYE* sequence (Columbia [Col] ecotype) at one point. Nucleotides 1664 to 1669 read GGAATT in *PHYE* from

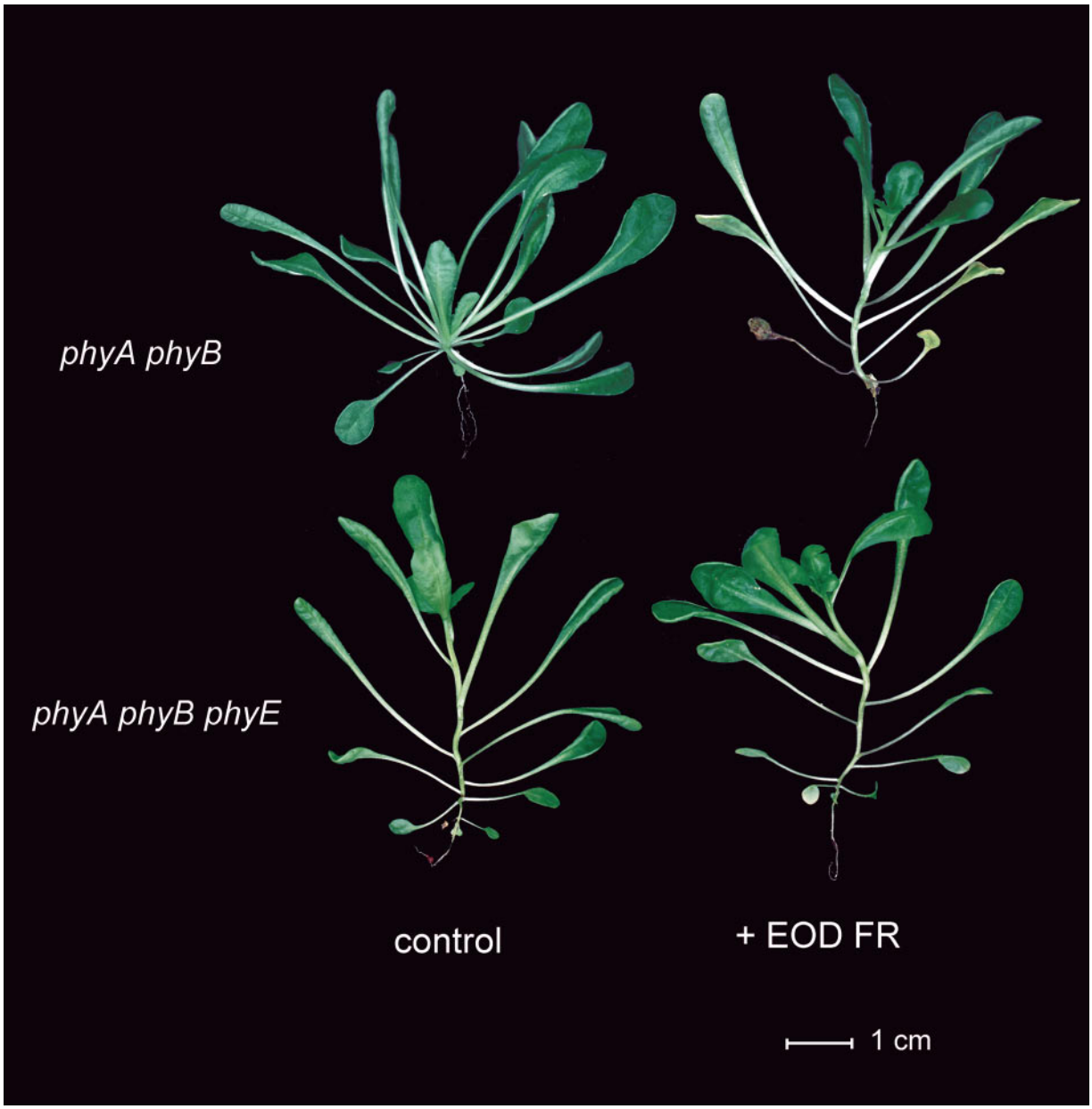

Figure 1. Regulation of Internode Elongation by phyE.

phyA phyB and *phyA phyB phyE* (in the *co* background) seedlings were grown for 60 days under 8-hr-light and 16-hr-dark cycles (control) or under the same conditions with 15-min EOD FR treatments (+EOD FR).

L*er* as opposed to GAATTT in *PHYE* from Col. This polymorphism changes codons 498 and 499 from Glu and Phe in Col to Gly and Ile in L*er.* Sequencing of the *PHYE* gene from the elongated internode mutant revealed a deletion of a single base pair at position 2350 (Figure 2). This deletion causes a frameshift after codon 726 that is predicted to result in a premature stop at codon 739 (Figure 2). We refer to this as the *phyE-1* mutation. The *phyE-1* deletion disrupts a HinfI restriction enzyme site, which has provided a convenient diagnostic test in the isolation of the monogenic *phyE* mutant after a backcross of *phyA phyB phyE* with wild-type L*er.*

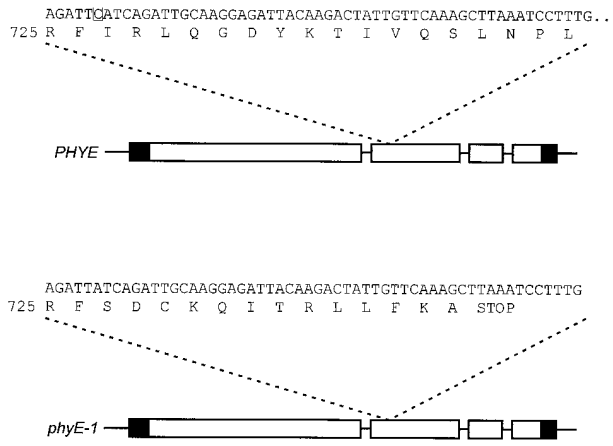

Figure 2. Sequences from the Second Exon of the Wild-Type *PHYE* Gene and the *phyE-1* Deletion Allele.

Exons are represented as boxes. Untranslated regions at the 5′ and 3′ ends of transcripts are shown in black. Hatched lines indicate the position of the displayed sequence. The nucleotide deleted in the *phyE-1* sequence is boxed in the *PHYE* sequence.

Immunochemical Analysis of phyE Protein

Protein extracts from *phyA phyB* and *phyA phyB phyE* seedlings were subjected to immunoblot analysis by using monoclonal antibodies that are selective for phyC, phyD, or phyE (Somers et al., 1991; Hirschfeld et al., 1998). The phyC-selective or phyD-selective monoclonal antibodies detected bands of ~124 kD in samples from either *phyA phyB* or *phyA phyB phyE* (Figure 3). However, whereas the phyE-selective monoclonal antibodies detected a band of ~124 kD in samples from *phyA phyB*, this band was absent in samples from *phyA phyB phyE* seedlings (Figure 3).

Effects of the *phyE* Mutation on Responses of *phyA phyB* to EOD FR

phyA phyB and *phyA phyB phyE* seedlings in the *co* background were grown under control conditions of 8-hr photoperiods or under EOD FR conditions in which each light-to-dark transition was immediately preceded by a 15-min pulse of FR. As previously reported, seedlings of the *phyA phyB* mutant showed a pronounced acceleration in flowering, recorded as the number of rosette leaves at bolting, in response to EOD FR treatment (Table 1 and Figure 1). In contrast, seedlings of the *phyA phyB phyE* mutant displayed a constitutively early-flowering phenotype under control conditions and only a very modest additional response to EOD FR treatments (Table 1 and Figure 1). This result indicates that phyE plays a major role in the flowering response to EOD FR of the *phyA phyB* mutant.

In response to EOD FR treatment, *phyA phyB* mutant seedlings also displayed a marked elongation of internodes between adjacent rosette leaves such that the plants assumed a caulescent habit (Table 2 and Figure 1). Seedlings of the *phyA phyB phyE* mutant displayed a constitutive caulescent growth habit under control conditions (Table 2 and Figure 1). Furthermore, in response to EOD FR treatments, the internodes of *phyA phyB phyE* seedlings displayed a greatly attenuated elongation response (Table 2 and Figure 1). These observations suggest that phyE plays a predominant role in mediating the internode elongation response to EOD FR of *phyA phyB* mutants. The small but significant residual promotion of internode elongation observed in the *phyA phyB phyE* mutant indicates the involvement of other phytochromes in this response.

We showed previously that in response to EOD FR treatments, *phyA phyB* seedlings show a reduction in petiole length (Devlin et al., 1996). This result contrasts with the behavior of wild-type seedlings in which EOD FR treatments led to an increase in petiole elongation. The EOD FR–induced reduction in *phyA phyB* petiole length accompanies the increased elongation of rosette internodes and has been proposed to result from a channeling of resources away from petiole elongation and into internode elongation. Compared with *phyA phyB* seedlings, *phyA phyB phyE* seedlings have short petioles after growth under control conditions (Table 3). Furthermore, the length of *phyA phyB phyE* petioles was not significantly altered in response to EOD FR treatments (Table 3). That *phyA phyB phyE* seedlings have constitutively short petioles is consistent with the observation that these seedlings also produce constitutively elongated inter-

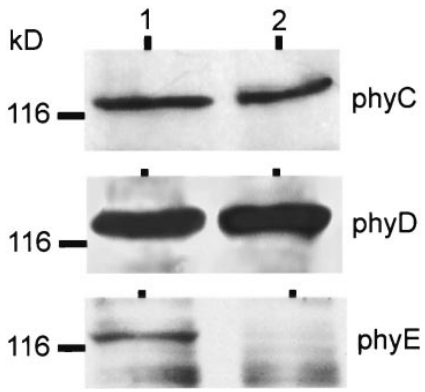

Figure 3. Immunoblot Analyses of Phytochrome Protein Levels in *phyA phyB co* and *phyA phyB phyE co* Mutants.

Protein extracts, enriched by ammonium sulfate precipitation, from *phyA phyB co* plants (lane 1) and *phyA phyB phyE co* plants (lane 2) were resolved on SDS–polyacrylamide gels and electroblotted onto a polyvinylidene difluoride membrane. Blots were probed with monoclonal antibodies selective for phyC, phyD, or phyE. Numbers at left indicate molecular masses estimated from protein size standards.

Table 1. Effect of EOD FR Treatments on Flowering Time in *phyA phyB co* and *phyA phyB phyE co* Seedlings

	Leaf Number[a]	
Genotype	Control[b]	EOD FR[c]
phyA phyB co	22.6 ± 0.9	10.7 ± 0.4
phyA phyB phyE co	11.8 ± 0.3	10.0 ± 0.5

[a] Flowering time was recorded as the number of rosette leaves at bolting.
[b] Eight-hour white light and 16-hr-dark cycles.
[c] Eight-hour white light plus 15-min FR and 15.75-hr-dark cycles.

nodes, supporting the proposal that the reduction in petiole length is a result of a channeling of resources away from petioles and into internode elongation.

Phenotype of the Monogenic *phyE* Mutant

Monogenic *phyE* mutant seedlings were selected after a backcross of *phyA phyB phyE co* with the L*er* wild type. Seedlings that are wild type for *PHYA* were selected from the F_2 population on the basis of a short hypocotyl after growth under FR. These seedlings were allowed to produce seed, and seed color was used as a marker for the presence of the *co* mutation (the *co* mutation is closely linked to the *transparent testa tt4* mutation). Seedlings that were wild type for the *PHYB* gene were then selected from this F_3 population on the basis of a short hypocotyl phenotype after growth under R. Seedlings homozygous for the *phyE-1* mutation were selected by using as diagnostic a polymerase chain reaction (PCR) test, which made use of the disruption of a HinfI site as a result of the *phyE-1* deletion. Finally, seed color was checked for these F_3 plants to select those descended from F_2 plants that were likely to be homozygous for the wild-type *CO* gene. Variations of this strategy were used to select mutants that were doubly homozygous for *phyA* and *phyE* or *phyB* and *phyE.*

After growth under control conditions, monogenic *phyE* plants displayed a wild-type phenotype (data not shown). Similarly, the morphology of *phyA phyE* double mutants was indistinguishable from that of monogenic *phyA* seedlings (data not shown). However, the effects of phyE deficiency were readily detected in the *phyB* mutant background. It is well established that the *phyB* mutation leads to increased petiole and leaf length and decreased flowering time (e.g., Robson et al., 1993; Halliday et al., 1994). The *phyB phyE* double mutant displayed an even earlier flowering time and a further change in leaf shape (Figure 4 and Table 4). The most mature leaves of the *phyB phyE* mutant were less spatulate than those of the *phyB* mutant (or the wild type), having an appearance more similar to juvenile (or early adult) wild-type leaves (Figure 4). The shape of mature rosette leaves in the *phyB phyE* double mutant was similar to that seen in the *phyA phyB* double mutant (see Figure 4). For etiolated seedlings treated with monochromatic R, FR, or white light, phyE deficiency did not lead to any detectable mutant phenotype (data not shown).

DISCUSSION

From a genetic screen for early-flowering, long-internode mutants that was performed in the *phyA phyB* double mutant background, we isolated a *phyE* mutant of Arabidopsis. The data presented here show that the *phyE-1* mutation resulted from the deletion of nucleotide 2350 of the *PHYE* gene. This 1-bp deletion is predicted to cause premature termination of *PHYE* mRNA translation at codon 739, as a consequence of a frameshift. The predicted truncation of the phyE apoprotein, caused by the *phyE-1* deletion, lies within the first part of the C-terminal domain of the polypeptide (see Quail, 1997). Monoclonal antibodies raised to the C terminus of phyE failed to detect any phyE protein in extracts from the *phyE-1* mutant. Although it is possible that a truncated phyE protein could accumulate in the *phyE-1* mutant, such a truncated protein would be expected to be nonfunctional based on previous studies on the biological activity of truncated phyA and phyB proteins (see Quail, 1997). It is proposed, therefore, that the *phyE-1* mutant is null for phyE.

The screen from which the *phyE-1* mutant was isolated derived from the observation that in response to EOD FR treatment, *phyA phyB* double mutants show a pronounced acceleration of flowering and a promotion elongation of the internodes between rosette leaves (Devlin et al., 1996). Compared with the parental line, the *phyA phyB phyE* triple mutant is constitutively early flowering and constitutively produces internodes between rosette leaves. Thus, the phenotype of *phyA phyB phyE* strongly resembles that of *phyA phyB* grown under EOD FR conditions. Therefore, phyE is implicated in the control of the responses. Significantly, the effects of phyE deficiency on flowering time are only detectable in a *phyB* background. In other studies, phyD has also been implicated in the control of flowering time, especially in

Table 2. Effect of EOD FR Treatments on Internode Elongation in *phyA phyB co* and *phyA phyB phyE co* Seedlings

	Internode Length (mm)[a]	
Genotype	Control[b]	EOD FR[c]
phyA phyB co	0	3.5 ± 0.3
phyA phyB phyE co	5.0 ± 0.3	6.6 ± 0.4

[a] Data are for the internode formed between rosette leaves 5 and 6.
[b] Eight-hour white light and 16-hr-dark cycles.
[c] Eight-hour white light plus 15-min FR and 15.75-hr-dark cycles.

Table 3. Effect of EOD FR Treatments on Petiole Elongation in *phyA phyB co* and *phyA phyB phyE co* Seedlings

	Petiole Length (mm)[a]	
Genotype	Control[b]	EOD FR[c]
phyA phyB co	48.2 ± 1.5	29.3 ± 1.1
phyA phyB phyE co	30.0 ± 1.8	28.1 ± 1.0

[a] Data are for the petioles of the largest leaf.
[b] Eight-hour white light and 16-hr-dark cycles.
[c] Eight-hour white light plus 15-min FR and 15.75-hr-dark cycles.

mutants that are deficient in phyB. For plants maintained under continuous white light, the *phyB phyD* double mutant flowers earlier than does the monogenic *phyB* mutant (Aukerman et al., 1997). Furthermore, the *phyA phyB phyD* triple mutant flowers earlier than does *phyA phyB* under control 8-hr photoperiods, and although the response of *phyA phyB phyD* to EOD FR treatment is less than that of the *phyA phyB* mutant, the *phyA phyB phyD* triple mutant shows a clear acceleration in flowering in response to EOD FR treatment (P.F. Devlin, P.R.H. Robson, S.R. Patel, L. Wester, R.A. Sharrock, and G.C. Whitelam, manuscript submitted). It seems very likely that phyE is largely responsible for mediating the EOD FR flowering response of *phyA phyB phyD* seedlings. That phyB, phyD, and phyE all function to inhibit flowering suggests redundancy within the phytochrome family.

It is interesting that although *phyA phyB phyD* mutants retained a significant flowering response to EOD FR treatments, *phyA phyB phyE* mutants did not (Table 1). Thus, *phyA phyB phyE* mutants flowered almost as early under control conditions as they did under EOD FR conditions. This indicates that when phyE is absent, phyD (or phyC) is not able to exert a measurable effect on flowering time in the *phyA phyB* background. Because monogenic *phyB* mutants were early flowering, whereas monogenic *phyE* or *phyD* mutants flowered at the same time as did the wild type, it is clear that phyB plays the major role in the inhibition of flowering. Nevertheless, it appears that when phyB is absent, phyE plays a more dominant role than does phyD in this response.

Loss of phyE phenocopies the effect of EOD FR treatment on internode elongation in the *phyA phyB* double mutant. In contrast, the *phyA phyB phyD* triple mutant maintains a normal rosette habit after growth under control conditions and responds to EOD FR (P.F. Devlin, P.R.H. Robson, S.R. Patel, L. Wester, R.A. Sharrock, and G.C. Whitelam, manuscript submitted). In the *phyA phyB* mutant background, phyD deficiency led to increased petiole elongation. This observation suggests that in the photoregulation of elongation growth, phyE plays a role distinct from that of phyD. This distinction could reflect the differential spatial patterns of activity of the *PHYD* and *PHYE* promoters (Goosey et al., 1997).

For plants grown under control conditions, *phyE* monogenic or *phyB phyE* and *phyA phyE* double mutants displayed a rosette habit, and only *phyA phyB phyE* triple mutants had elongated internodes. This result indicates that the action of any one of phyA, phyB, or phyE is sufficient to suppress internode elongation. This apparent overlap of function suggests a significant degree of redundancy among these members of the phytochrome family. Also, phyD and phyC do not appear to play a significant role in maintaining the rosette habit.

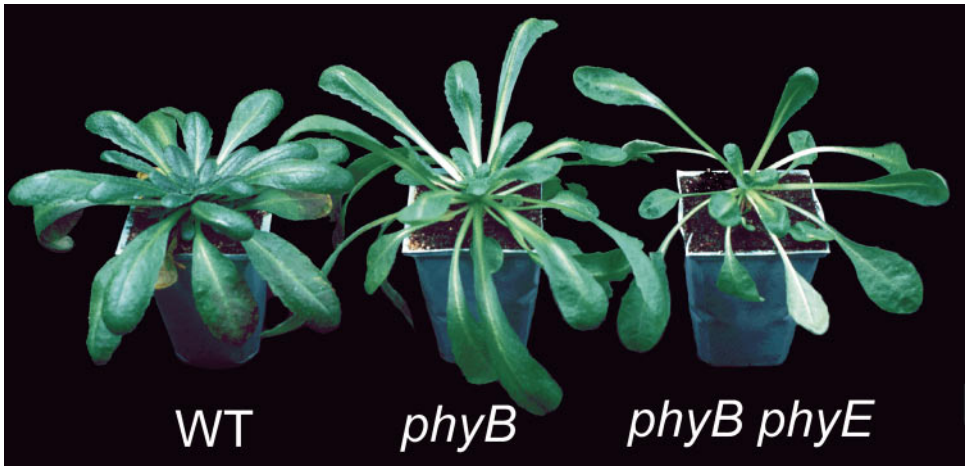

Figure 4. Phenotypes of Wild-Type, *phyB*, and *phyB phyE* Plants.

Seedlings were grown for 60 days under 8-hr-light and 16-hr-dark cycles. WT, wild type.

Table 4. Phenotypes of Wild-Type, *phyB*, and *phyB phyE* Seedlings[a]

Genotype	Petiole Length (cm)	Leaf Number at Bolting
Wild type	1.7 ± 0.2	46.3 ± 4.2
phyB	3.8 ± 0.4	36.8 ± 3.1
phyB phyE	5.7 ± 0.5	23.8 ± 1.0

[a] Plants were grown under 8-hr-light and 16-hr-dark cycles.

The elongation of rosette internodes is always accompanied by a reduction in petiole elongation (see Devlin et al., 1996). This is the case for the *phyA phyB phyE* triple mutant grown under control conditions and for the *phyA phyB* double mutant given EOD FR treatments. This phenomenon is proposed to reflect a reallocation of resources from petioles to internodes.

The early-flowering and elongation growth responses mediated by phyB, phyD, and phyE are typical of the shade avoidance syndrome of responses elicited by low R/FR or by EOD FR treatments (see Smith and Whitelam, 1997). Modeling of the phylogenetic relationships among the different members of the phytochrome family in dicots places phyB, phyD, and phyE within a distinct subgroup. It is tempting to speculate that neighbor detection and shade avoidance provided the selective pressure that led to the evolution of this subgroup of dicot phytochromes. Whether the actions of these three phytochromes alone can account for the whole of the shade avoidance syndrome in Arabidopsis awaits the isolation of phyC mutants as well as the creation of the *phyB phyD phyE* triple mutant and the *phyA phyB phyD phyE* quadruple mutant. Although the *phyA phyB phyE* triple mutant showed no significant flowering response to EOD FR treatment, it did display a small but significant promotion of internode elongation when this treatment was used, indicating the action of another phytochrome. The creation of the *phyA phyB phyD phyE* quadruple mutant will address the question of whether this represents the action of phyD or phyC. Furthermore, the identification of phytochrome-controlled responses remaining in the quadruple mutant will provide information on the role of phyC.

METHODS

Plant Material and Mutagenesis

The *co phyA phyB* triple mutant (*Arabidopsis thaliana*) used as the parental line was described previously (Devlin et al., 1996). γ-Mutagenesis was performed at the University of Nottingham (Nottingham, UK). Ten thousand seeds were γ-irradiated with 90 kR γ-rays from a cesium-137 source. The seeds were planted in batches of 1000 in the greenhouse and allowed to self-pollinate; M_2 seed was bulk harvested from each batch.

Mutant Screening

M_2 seeds were sown in rows at a density of 1600 seed m^{-2} on a 3:1 mixture of peat compost–horticultural silver sand. Trays of seeds were chilled at 4°C for 4 days and then transferred to conditions of 16-hr-light and 8-hr-dark cycles at 22°C. Light was provided by Osram (Osram Ltd., St. Helens, UK) L65/80W/30 warm-white fluorescent tubes (photon irradiance, 400 to 700 nm, 100 μmol m^{-2} sec^{-1}). Seedlings displaying elongated internodes and/or early flowering were labeled and allowed to self-pollinate. M_3 seed collected from these individuals was then subjected to a second round of screening. The M_3 seeds were allowed to germinate under 16-hr-light and 8-hr-dark cycles. After 4 days, seedlings were divided into two batches: half were transferred to (control) 8-hr-light and 16-hr-dark cycles and half were transferred to the same conditions supplemented with daily 15-min end-of-day far-red light (EOD FR) treatments. Flowering time and internode production were recorded in control versus EOD FR–treated plants, and those M_3 batches showing attenuated responses to EOD FR treatment were marked for further investigation.

Sequencing of the *PHYE* Gene

Genomic DNA was isolated from 4-week-old light-grown plants by using the method described by Edwards et al. (1991). Primers were designed from the published *A. thaliana PHYE* cDNA sequence to amplify nine overlapping DNA fragments (~500 bp each), covering the entire coding region of the *PHYE* gene. For each primer pair, four independent polymerase chain reactions (PCRs) were performed, and the amplified fragments were pooled and purified using a Quiaquick gel purification kit (Quiagen Ltd., Crawley, UK). Purified fragments were sequenced directly using primers from the original amplification step and dye-labeled terminators on an ABI 377 automated sequencer (ABI, Warrington, Cheshire, UK).

Backcrossing and Selection of Mutant Lines

Before physiological analyses were performed, the *phyA phyB phyE co* mutant was backcrossed to the parental *phyA phyB co* line. The *phyE* mutant was selected from the F_2 generation, as described below. The *phyA phyB phyE co* mutant was also backcrossed to the Landsberg *erecta* (L*er*) wild type to select the monogenic *phyE* mutant and the various double mutants. Seeds from the F_2 generation were sown on mineral salts agar (Lehle Seeds, Round Rock, TX) and stratified for 4 days in darkness at 4°C. The plates were exposed to a 2-hr pulse of white fluorescent light (photon irradiance, 400 to 700 nm, 100 μmol m^{-2} sec^{-1}) at 22°C, then transferred to darkness for 24 hr, and then to continuous FR (photon irradiance, 700 to 800 nm, 10 μmol m^{-2} sec^{-1}) for an additional 60 hr. Seedlings homozygous for the wild-type *PHYA* gene, selected on the basis of their extremely short hypocotyls and open cotyledons, were transferred to fresh plates and allowed to green for 2 days under continuous white fluorescent light. These seedlings were then transferred to soil and allowed to set seed. Seed from each of these F_2 plants were collected separately. Seed color was scored for absence of the *transparent testa tt4* mutation, which is closely linked to the *co* mutation, and each F_3 population was then screened under R light (as above) for presence of the wild-type *PHYB* gene. Seedlings from F_3 populations homozygous for wild-type *PHYB* were transferred to soil, and rosette

leaf tissue was collected from each plant and used to make extracts of DNA, which were assayed using the cleaved amplified polymorphic sequences technique for the polymorphism associated with the *phyE* mutation.

Briefly, a 543-bp fragment of the *PHYE* gene was amplified by PCR, using the upstream primer 5′-GTCACTTGCCGATGAGATTG-3′ and the downstream primer 5′-CTCCAAAGACTTCACCGGG-3′. The amplified fragment was restriction digested with HinfI to yield fragments of 252, 127, 80, 55, and 29 bp and from the wild-type *PHYE* allele and fragments of 252, 134, 127, and 29 bp from the mutant *phyE-1* allele. Of the F_3 populations analyzed, those in which all plants were homozygous for the *phyE-1* mutation were allowed to set seed, and seed color was scored for each plant within that population for absence of the *tt4* mutation.

The *phyA phyE*, *phyB phyE*, and *phyA phyB phyE* mutant combinations were also selected from this backcross by using a long hypocotyl in FR and R as markers for the *phyA* and *phyB* mutations, respectively.

Phytochrome Extraction and Immunoblotting

Protein extraction and immunoblotting were performed as described previously (Devlin et al., 1992), with the following modifications. After resolution on SDS–polyacrylamide gels, protein samples were electroblotted onto an Imobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Membranes were then probed with the following monoclonal antibodies: C11 and C13, which are selective for phyC (Somers et al., 1991); 2C1, which is selective for phyD; and 7B3, which is selective for phyE (Hirschfeld et al., 1998). Secondary incubations were performed with anti–mouse IgG antibodies conjugated to horseradish peroxidase. Bands were visualized using chemiluminescent reagents according to the procedures recommended by the manufacturer (Boehringer Mannheim).

Light Sources

Control conditions used in the EOD FR experiments comprised 8-hr warm white fluorescent light (photon irradiance, 400 to 700 nm, 102 μmol m^{-2} sec^{-1}). When plants were given EOD FR pulses, FR light (photon irradiance, 700 to 800 nm, 57 μmol m^{-2} sec^{-1}) was obtained by filtering the output of Osram (Osram Ltd.) Haloline 500W tungsten halogen lamps through 10 mm of flowing water and one layer (3 mm) of black Plexiglas (type FRF 700; West Lakes Plastics, Lenni, PA). All light measurements were made using a PS-II spectroradiometer (LI-COR, Lincoln, NE).

Measurements of Growth and Flowering

Internode and petiole lengths were measured using a ruler. Measurements were made after 75 days of treatment, by which time all plants had completed bolting. Data represent the means ±SE of at least 10 plants. Petiole lengths were determined for the largest fully grown leaf, and internode lengths were measured for the internode between rosette leaves 5 and 6.

Flowering time was recorded as the number of rosette leaves at inflorescence production. As described previously, rosette leaves were readily distinguished from cauline leaves on the basis of their morphologies (Devlin et al., 1996).

ACKNOWLEDGMENTS

This work was supported by Grant No. P02394 from the Biotechnology and Biological Sciences Research Council (UK). We thank Malcolm Pratt for technical assistance with experimental growth conditions. Sincere thanks are also extended to Drs. Peter Quail and Bob Sharrock for providing phytochrome-selective monoclonal antibodies.

Received May 15, 1998; accepted July 13, 1998.

REFERENCES

Aukerman, M.J., Hirschfeld, M., Wester, L., Weaver, M., Clack, T., Amasino, R.M., and Sharrock, R.A. (1997). A deletion in the *PHYD* gene of the Arabidopsis Wassilewskija ecotype defines a role for phytochrome D in red/far-red light sensing. Plant Cell **9,** 1317–1326.

Barnes, S.A., Quaggio, R.B., Whitelam, G.C., and Chua, N.-H. (1996). *fhy1* defines a branchpoint in phytochrome A signal transduction pathways for gene expression. Plant J. **10,** 1155–1161.

Clack, T., Matthews, 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 protein. 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. Nucleic Acids Res. **19,** 1349.

Goosey, L., Palecanda, L., and Sharrock, R.A. (1997). Differential patterns of expression of the Arabidopsis *PHYB*, *PHYD*, and *PHYE* phytochrome genes. Plant Physiol. **115,** 959–969.

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.

Hirschfeld, M., Tepperman, J.M., Clack, T., Quail, P.H., and Sharrock, R.A. (1998). Coordination of phytochrome levels in *phyB* mutants of Arabidopsis as revealed by apoprotein-specific monoclonal antibodies. Genetics **149,** 523–535.

Johnson, E., Bradley, M., Harberd, N.P., and Whitelam, G.C. (1994). Photoresponses of light-grown *phyA* mutants of Arabidopsis. Plant Physiol. **105,** 141–149.

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.

Koornneef, M., Hanhart, C.J., and van der Veen, J.H. (1991). A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana.* Mol. Gen. Genet. **229,** 57–66.

Mathews, S., and Sharrock, R.A. (1997). Phytochrome gene diversity. Plant Cell Environ. **20,** 666–671.

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

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.

Quail, P.H. (1997). An emerging molecular map of the phytochromes. Plant Cell Environ. **20,** 657–665.

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

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.

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). Physiological and ecological function within the phytochrome family. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46,** 289–315.

Smith, H., and Whitelam, G.C. (1997). The shade avoidance syndrome: Multiple responses mediated by multiple phytochromes. Plant Cell Environ. **20,** 840–844.

Somers, D.E., Sharrock, R.A., Tepperman, J.M., and Quail, P.H. (1991). The *hy3* long hypocotyl mutant of Arabidopsis is deficient in phytochrome B. Plant Cell **3,** 1263–1274.

Whitelam, G.C., and Devlin, P.F. (1997). Roles of different phytochromes in *Arabidopsis* photomorphogenesis. Plant Cell Environ. **20,** 752–758.

Whitelam, G.C., and Devlin, P.F. (1998). Light signalling in Arabidopsis. Plant Physiol. Biochem. **36,** 125–133.

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.

Yanovsky, M.J., Casal, J.J., and Whitelam, G.C. (1995). Phytochrome A, phytochrome B and HY4 are involved in hypocotyl growth responses to natural radiation in *Arabidopsis*: Weak de-etiolation of the *phyA* mutant under dense canopies. Plant Cell Environ. **18,** 788–794.