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Phytochrome A and Phytochrome B Have Overlapping but Distinct Functions in *Arabidopsis* Development[1]

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Plant responses to red and far-red light are mediated by a family of photoreceptors called phytochromes. In *Arabidopsis thaliana*, there are genes encoding at least five phytochromes, and it is of interest to learn if the different phytochromes have overlapping or distinct functions. To address this question for two of the phytochromes in *Arabidopsis*, we have compared light responses of the wild type with those of a *phyA* null mutant, a *phyB* null mutant, and a *phyA phyB* double mutant. We have found that both *phyA* and *phyB* mutants have a deficiency in germination, the *phyA* mutant in far-red light and the *phyB* mutant in the dark. Furthermore, the germination defect caused by the *phyA* mutation in far-red light could be suppressed by a *phyB* mutation, suggesting that phytochrome B (PHYB) can have an inhibitory as well as a stimulatory effect on germination. In red light, the *phyA phyB* double mutant, but neither single mutant, had poorly developed cotyledons, as well as reduced red-light induction of *CAB* gene expression and potentiation of chlorophyll induction. The *phyA* mutant was deficient in sensing a flowering response inductive photoperiod, suggesting that PHYA participates in sensing daylength. In contrast, the *phyB* mutant flowered earlier than the wild type (and the *phyA* mutant) under all photoperiods tested, but responded to an inductive photoperiod. Thus, PHYA and PHYB appear to have complementary functions in controlling germination, seedling development, and flowering. We discuss the implications of these results for possible mechanisms of PHYA and PHYB signal transduction.

Phytochromes are a family of light-sensing proteins required for plant developmental responses to red and far-red light (reviewed by Furuya, 1989, 1993; Quail, 1991). They consist of an apoprotein with a covalently attached linear tetrapyrrole pigment, and are interconverted by light between red- and far-red-light-absorbing forms. In the dark, they are synthesized in the red-light-absorbing form, Pr, and are thought to mediate light-induced responses after conversion by red light to the far-red-light-absorbing form, Pfr. Thus, phytochromes can serve either to sense the onset of light conditions after growth in the dark or as monitors of the light quality environment (that is, the ratio of red to far-red incident light). Light responses that are known to be mediated by phytochromes include germination, chloroplast development, leaf expansion, regulation of gene expression, inhibition of cell elongation, and photoperiodic control of flowering (Cosgrove, 1986; Mullet, 1988; Chory, 1991; Thompson and White, 1991).

Phytochromes are encoded by a multigene family in numerous higher and lower plants (Furuya, 1989, 1993; Quail, 1991). Within a single plant, different phytochromes have amino acid sequences diverging by up to 50% (Sharrock and Quail, 1989). Biochemical and physiological studies have distinguished two classes of phytochromes, light-labile and light-stable (or type I and type II), which differ in their stability in vivo when converted to the far-red-light-absorbing form (Furuya, 1989, 1993; Quail, 1991). In dark-grown plants, light-labile phytochrome predominates, although light-stable phytochromes are also present. In light-grown plants, the level of light-labile phytochrome decreases dramatically, whereas light-stable phytochromes persist at the same level as in the dark (Somers et al., 1991). Presumably, the differential stabilities of these two classes of phytochrome allow them to perform different functions in development, although it is not known if they normally interact with the same signal-transduction chain components.

In *Arabidopsis*, light-stable phytochromes are encoded by at least two genes, *PHYB* and *PHYC* (Sharrock and Quail, 1989; Somers et al., 1991). *Arabidopsis* lines carrying mutations in the gene for phytochrome B, one of the stable phytochromes, have been described. They were originally called *hy3*, for the long hypocotyl phenotype by which they were recognized (Koornneef et al., 1980). These mutants have elongated hypocotyls, stems, petioles, and root hairs; they accumulate less Chl; and they flower earlier than the wild type (Koornneef et al., 1980; Goto et al., 1991; Reed et al., 1993). They also elongate to a lesser extent than the wild type in response to supplementary far-red light, implicating PHYB in control of the so-called shade-avoidance response (Nagatani et al., 1991; Whitelam and Smith, 1991). Similar mutants deficient in light-stable phytochromes have also been isolated in cucumber, sorghum, and *Brassica* (Childs et

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Abbreviations: PHYA, PHYB, phytochrome proteins encoded by the *PHYA* and *PHYB* genes; *phyA*, *phyB*, mutations in the *PHYA* and *PHYB* genes.

al., 1992; Devlin et al., 1992; López-Juez et al., 1992). Although these have not been shown to have mutations in a *PHYB* cognate gene, each of them is missing a light-stable phytochrome species and has morphological and physiological phenotypes analogous to those of the *Arabidopsis phyB* mutants.

The principal (and perhaps the only) light-labile phytochrome in *Arabidopsis* is phytochrome A, encoded by the *PHYA* gene. We and others have previously described mutants deficient in phytochrome A, identified by their long hypocotyls in continuous far-red light (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). These mutants have been called *hy8* (for long hypocotyl) (Parks and Quail, 1993), *fre1* (for far-red elongated) (Nagatani et al., 1993), or *fhy2* (for far-red long hypocotyl) (Whitelam et al., 1993). These mutants carry mutations in the *PHYA* gene (Dehesh et al., 1993; Whitelam et al., 1993; see below). In contrast to the fairly dramatic effect of *phyB* mutations on plant growth at various developmental stages, none of these PHYA-deficient mutants had any obvious phenotype in white light, suggesting that phytochrome A has a subtle or specialized function in development.

Studies with transgenic *Arabidopsis* lines overexpressing phytochrome genes have shown that overexpression of PHYA confers light responses distinct from those caused by overexpression of PHYB. In particular, overexpression of PHYA caused increased sensitivity to inhibition of hypocotyl elongation by far-red light, whereas overexpression of PHYB caused increased sensitivity to red (but not far-red) light (Whitelam et al., 1992; McCormac et al., 1993). These results suggest that PHYA and PHYB have different functions, although the results are subject to the caveat that some aspects of the observed phenotypes might have been caused by the ectopic overexpression of the phytochromes in question. In this work, we have examined several developmental phenotypes of a *phyA* (*fre1*) mutant, a *phyB* mutant, and a *phyA phyB* double mutant. The data suggest that both PHYA and PHYB control germination, seedling development, and flowering time. However, their actions are at times complementary and at times opposing. Presumably, the differences between the functions of PHYA and PHYB arise at least in part from their differential stabilities in light.

MATERIALS AND METHODS

Plant Material and General Growth Conditions

phyA mutants were isolated by their long hypocotyl and etiolated appearance under far-red-rich light, as described previously (Nagatani et al., 1993). Mutants were identified among M2 progeny of EMS- or γ-irradiation-treated *Arabidopsis thaliana* Landsberg *erecta* Seed (Lehle Seeds, Tuscon, AZ), or among M2 progeny of γ-irradiated pOCA107–2 seed (pOCA107–2 is a Columbia ecotype carrying a transgenic *cab*-promoter construct [Chory et al., 1993]). Seeds were surface sterilized and plated on Murashige-Skoog (MS) plates (1× MS salts [Gibco, Grand Island, NY], 0.8% phytagar [Gibco], 1× Gamborg's B5 vitamin mix [Sigma]), stored overnight at 4°C, and grown in light chambers (16-h day/8-h night) kept at 22°C. In some cases, the plates were supplemented with 2% Suc. Except for studies of germination frequency, seedlings were induced to germinate by treatment with white light for 16 to 24 h. Seedlings were transferred to soil 10 d to 2 weeks after sowing and grown in a greenhouse. Crosses were by standard methods. The *phyA phyB* double mutant was identified as a tall seedling in white light in an F2 population of a cross between the *fre1-1* (*phyA-201*) mutant (recipient) and mutant *phyB-8-36* (Reed et al., 1993) (pollen donor), and was tested for its genotype by assessing the F1 phenotype of seeds from test crosses with each of the two starting mutants. From this same cross we also identified F2 plants homozygous for the *phyA* mutation and heterozygous for the *phyB* mutation. We used F3 seed from these plants for the germination studies shown in Table III.

Sequencing of *phyA* Mutant Alleles

A series of oligodeoxynucleotide primers corresponding to the published cDNA sequence of *PHYA* (Sharrock and Quail, 1989) were synthesized. These were used to amplify segments of the *PHYA* gene from the wild type and the different mutants by PCR amplification from genomic DNA. PCR products were either sequenced directly as described previously (Reed et al., 1993) or cloned into Bluescript SK⁻ (Stratagene) and then sequenced. While sequencing mutant alleles, we discovered a DNA sequence polymorphism that we used for the linkage analysis described in "Results." Nucleotide position 600 of the published cDNA sequence (Sharrock and Quail, 1989) is a G in the Columbia ecotype and a C in the Landsberg ecotype, and this difference results in the elimination of an *Hga*I restriction site present in the Columbia sequence.

RNA Analysis

RNA was purified as described previously (Chory et al., 1991). RNA blot hybridizations and *CAB* and rDNA probes were described previously (Chory et al., 1989). As a *PHYA* probe, we used a gel-purified insert fragment from a *PHYA* cDNA clone (M. Fagan, unpublished result).

Light Conditions

For studies of germination and hypocotyl length in continuous light, white light was from six cool-white fluorescent bulbs (F24T12/CW/HO, Sylvania) and two incandescent bulbs (T10481C, Sylvania), red light was from the same source but filtered through a red glass cutoff filter, and far-red light was provided by fluorescent tubes that emit a high proportion of far-red light (FL20S.FR-74, Toshiba, Tokyo, Japan) filtered through red plastic. For fluence rate experiments, red light was as described previously (Nagatani et al., 1993) and was attenuated with combinations of gray plastics (Takiron plates S-802 and S-909, Takiron, Tokyo, Japan). Light-pulse experiments employed either far-red fluorescent tubes wrapped with red and blue plastic (Nagatani et al., 1993) or two 300-W incandescent lamps filtered through red or far-red plastic filters. For the *CAB* mRNA induction experiment, we used red pulses of 2 min (5×10^2 μmol m^{-2} s^{-1}) and far-red pulses of 4 min. Conditions for the Chl accu-

mulation experiment and measurement of Chl were as described previously (Nagatani et al., 1993).

Flowering

Seeds were sown on MS plates, incubated overnight at 4°C, and then incubated in growth chambers at 20°C under six cool-white fluorescent bulbs (F24T12/CW/HO, Sylvania) and two incandescent bulbs (T10481C, Sylvania). Total fluence rate was 3×10^2 μmol m^{-2} s^{-1} to 5×10^2 μmol m^{-2} s^{-1}. Plants were transferred to soil after 14 d of growth in the light and were judged to have flowered when the first flower buds became visible.

RESULTS

Mutations in the *phyA* Gene

fre mutants were identified as those that appeared tall when grown under far-red-enriched light (Nagatani et al., 1993). We initially isolated two such mutants (*fre1-1* and *fre1-2*) in the Landsberg *erecta* background (Nagatani et al., 1993). Subsequently, we have isolated six more (m10, m20, m26, m34, m35, and m36) in the Landsberg *erecta* background and three more (γ11, γ12, and γ18) in the Columbia pOCA107–2 background (see "Materials and Methods"). Backcrosses to corresponding wild-type strains revealed that all 11 mutations were recessive, and complementation tests among these mutants showed that all 11 carry mutations in the same complementation group, *fre1* (data not shown). We noticed some variation among the mutants in their phenotypes under far-red-rich light. Mutants *fre1-1*, *fre1-2*, m20, m35, m36, γ11, and γ18 were very tall and also had unexpanded cotyledons. By contrast, mutants m10, m26, m34, and γ12 were not as tall as the other mutants and had more expanded cotyledons. In complementation tests, F1 seedlings from crosses between two of these weak mutants, or between a weak mutant and a strong mutant, had the weak phenotype. In addition, we tested the segregation of F2 populations of representative complementation crosses. In all cases 100% of these F2 progeny exhibited the strong or weak far-red elongated phenotype (data not shown). Thus, it appears that the strong and weak alleles map to the same location.

Three lines of evidence indicate that the *fre1* mutations are *phyA* mutations. First, they map to *PHYA*. We previously mapped the *fre1* mutations to the top arm of chromosome 1, in the vicinity of the *PHYA* gene (Nagatani et al., 1993). We refined our mapping of the *fre1-1* mutation by testing segregation of an *Hga*I restriction fragment length polymorphism within a PCR-amplified fragment of the *PHYA* gene (see "Materials and Methods"). We analyzed 57 *fre1*$^-$ F2 progeny of a cross between the *fre1-1* mutant (in the Landsberg *erecta* ecotype) and the Columbia ecotype for this polymorphism found that all 57 had the Landsberg pattern. This result established that the *fre1-1* mutation maps very close to the *PHYA* gene, probably within 1 cM.

Second, we have found that the *fre1-1* mutant fails to complement the *fhy2-2* mutant, which falls in a complementation group that includes an allele with a rearrangement in the *PHYA* gene (Whitelam et al., 1993). Finally, sequence analysis of the *PHYA* genes from mutants *fre1-1* and m26 revealed that these strains had mutations in the *PHYA* gene. The *fre1-1* mutant has a C-to-T transition mutation that changes a Gln codon to a stop codon at amino acid 980. The m26 mutant has a G-to-A transition mutation that changes a Val codon to a Met codon at amino acid 631. These data confirm that the *fre1* mutations fall in the *PHYA* gene encoding phytochrome A. Therefore, we rename the *fre1* mutations *phyA*, in accordance with the suggestion of Whitelam et al. (1993). As summarized in Table I, we now refer to the *fre1-1* mutation as *phyA-201*, the *fre1-2* mutation as *phyA-202*, and the m26 mutation as *phyA-205*.

The available molecular data suggest that the *phyA-201* (*fre1-1*) mutation is a null mutation. First, the mutation creates a stop codon that would cause the predicted protein product to lack 243 amino acids at the C-terminal end. It has been found previously that deletion derivatives of oat PHYA lacking just 35 amino acids at the C-terminal end confer no phenotype when overexpressed in transgenic tobacco plants (Cherry et al., 1993), suggesting that a truncation of 243 amino acids would be more than sufficient to inactivate PHYA. Second, we previously failed to detect any PHYA protein or spectral activity in this mutant (Nagatani et al., 1993), suggesting that the PHYA protein was in fact entirely absent. Finally, mutant *phyA-201* had a very low level of *PHYA* mRNA (T.D. Elich, unpublished result). Previously, we found that several stop codon alleles of *phyB* have decreased levels of *PHYB* mRNA (Reed et al., 1993). We presume that premature termination of translation in the *phyA-201* mutant causes the decreased level of *PHYA* mRNA (see Pulak and Anderson, 1993, and refs. therein), and that this decreased mRNA level could in turn cause the observed decrease in protein level. Our finding that mutant *phyA-205* (m26) carries a missense mutation is consistent with the less-severe phenotype exhibited by this mutant.

Phenotypes Caused by Loss of PHYA

Phytochromes contribute to control of germination, early seedling development, and flowering, so it was of interest to examine the behavior of the *phyA* mutants in these responses under various light conditions. In parallel, we have analyzed

Table I. *Summary of phyA mutant alleles*

Isolation No.	New Name	Phenotype[a]
Landsberg *erecta* (wild type)		Wild type
fre1-1	*phyA-201*	Strong
fre1-2	*phyA-202*	Strong
m10	*phyA-203*	Weak
m20	*phyA-204*	Strong
m26	*phyA-205*	Weak
m34	*phyA-206*	Weak
m35	*phyA-207*	Strong
m36	*phyA-208*	Strong
γ11	*phyA-209*	Strong
γ12	*phyA-210*	Weak
γ18	*phyA-211*	Strong

[a] Appearance after 5 d in far-red-rich light. Wild type, Short hypocotyl and open cotyledons; strong, long hypocotyl and closed cotyledons; weak, medium hypocotyl length and open cotyledons.

Table II. *Germination frequencies under different light conditions*
Seeds were sown on MS/Suc plates and given 19 h of cold before transferring them to light chambers. Between 26 and 46 seeds were assessed for each entry. Comparable results were obtained on MS plates without Suc. W, White light; R, red light; FR, far-red-rich light.

Light	Ler (w.t.)	*phyA*	*phyB*	*phyA phyB*
Dark	0.97	0.98	0.83	0.98
W	0.87	0.93	0.97	1.0
R	0.97	0.97	1.0	0.97
FR	0.61	0.00	0.97	0.33

the phenotypes of a *phyB* mutant and a *phyA phyB* double mutant. For this analysis, we used the presumed null mutants *phyA-201* (Nagatani et al., 1993; see above) and *phyB-8-36* (Reed et al., 1993) and the corresponding double mutant combination.

Germination

Table II shows a comparison of germination frequencies of wild-type, *phyA*, *phyB*, and *phyA phyB* strains under different light conditions. All of the strains germinated well in continuous white and red light. In far-red light, however, the *phyA* mutant failed to germinate, and the wild type germinated less than in white or red light. In contrast, the *phyB* mutant germinated well in far-red light, and in addition the *phyA phyB* mutant germinated better in far-red light than the *phyA* single mutant. Thus, it appears that PHYA promotes, whereas PHYB inhibits, germination in far-red light. In the dark, all strains germinated well except that the *phyB* mutant appeared to have a slight germination deficiency (Table II).

In addition to the light environment, germination is known to depend on multiple variables including temperature, length of seed dormancy, cold treatment during dormancy, and light conditions during seed maturation (Hayes and Klein, 1974; Derkx and Karssen, 1993). Therefore, we confirmed these germination results by studying germination in populations segregating for the *phyA* or *phyB* mutations (see "Materials and Methods"). Because PHY^+ and phy^- seeds came from the same parent plants, factors such as seed age, dormancy, and light conditions during seed maturation were internally controlled in these experiments. In each case we expected 75% of the seeds in the population to carry a dominant (wild-type) allele at the relevant *PHY* locus, and therefore to be phenotypically PHY^+, and 25% of the seeds to be homozygous for the mutant allele and to be phenotypically phy^-. These experiments were performed multiple times, and representative results from one set of experiments are presented in Table III.

We confirmed that a *phyA* mutation decreases germination

Table III. *Germination of segregating populations*

Progeny of a *PHYA/phyA* Heterozygote						
Light[a]	Germinated					Not Germinated
	Total	*PHYA*	*phyA*	Not scored	χ^{2b}	
W	120			100%		9
R	120			100%		10
Dark	213			100%		30
Dark then FR	306	74%	26%	0	0.11	3
W then FR	222	66%	31%	3%	5.6	14
FR	358	89%	5%	6%	280	152

Progeny of *phyA/phyA PHYB/phyB* Heterozygote										
Light[a]	Germinated[c]					Not Germinated[d]				
	Total	*PHYB*	*phyB*	Not scored	χ^{2b}	Total	*PHYB*	*phyB*	Not scored	χ^{2b}
W	348	77%	23%	0	0.98	0				
FR	37			100%		333	81%	10%	9%	59
Dark	211	80%	14%	7%	17	55	35%	64%	2%	59

[a] W, White light; R, red light; FR, far-red-rich light. [b] χ^2 values are for 3:1 ratio of *PHY:phy*. Seedlings not scored for *PHY* genotype were omitted from the calculation. [c] The *PHYB* genotype of seeds that germinated in the dark was determined by transferring seedlings to soil and assessing their flowering time. [d] The *PHYB* genotype of seeds that did not germinate was determined by transferring seeds to white light and assessing hypocotyl length after 1 week.

in far-red light by measuring germination frequencies of selfed progeny of a *PHYA/phyA* heterozygote. When germinated in white light, red light, or dark, between 87 and 99% of the seeds germinated. Between 19 and 32% of the scorable germinated seeds had an etiolated appearance after growth in far-red light and therefore had a *phyA/phyA* genotype, consistent with the expected proportion of *phyA/phyA* seeds in the population of 25% (Table III, Dark then FR, W then FR; data not shown). By contrast, when germinated in far-red light, in different experiments the seeds germinated at a frequency of between 43 and 74%. Between 2 and 12% of the scorable germinated seeds were *phyA/phyA*, significantly fewer than the expected proportion of 25% (Table III; data not shown). Apparently, most of the *phyA/phyA* seeds failed to germinate. The ability of a small number of *phyA/phyA* seeds to germinate in far-red light in this experiment, in contrast to the failure of *phyA* seeds to germinate in other experiments (Table II), probably reflects variability of germination frequencies from seed batch to seed batch (see above). This variability underscores the utility of these internally controlled experiments. These data confirm the above conclusion that in far-red light, PHYA promotes germination.

In a *PHYA*$^+$ background, a *phyB* mutation had no significant effect on far-red germination in a segregating population (data not shown). However, in a *phyA/phyA* background, we observed an enhancement of germination by the *phyB* mutation. In white light, all of the progeny of a plant heterozygous for *phyB* (i.e. *PHYB/phyB*) and homozygous for *phyA* germinated. Twenty-three percent of these seedlings had long hypocotyls and were therefore *phyB/phyB*, close to the expected proportion of 25% (Table III). In far-red light, just 7 to 15% of these seeds germinated (Table III; data not shown). Most of the germinated seedlings failed to deetiolate upon transfer to white light (data not shown), precluding accurate assessment of the proportion that were *phyB/phyB*. (We have previously found that seedlings grown in far-red light sometimes fail to deetiolate effectively [Nagatani et al., 1993].) However, it was possible to assess the *PHYB* genotype of the seeds that did not germinate by transferring them to white light to induce germination. Of seeds that failed to germinate in far-red light and subsequently scored in white light, between 2 and 11% were tall (*phyB/phyB*), significantly fewer than the expected 25% (Table III; data not shown). Therefore, a preponderance of the seeds that failed to germinate in far-red light were *PHYB/PHYB* or *PHYB/phyB*. This result confirms the above conclusion that PHYB inhibits germination in far-red light.

Finally, analysis of a population segregating for a *phyB* mutation confirmed the promotive effect of PHYB on germination in the dark. Between 79 and 82% of seeds from populations segregating *PHYB/phyB* in a *phyA/phyA* background germinated in the dark (Table III; data not shown). The *PHYB* genotype of seeds that had germinated in the dark was assessed by transferring the seedlings to soil and observing their flowering time. *phyB* mutant plants flower substantially earlier than *PHYB* plants (see below). Between 9 and 15% of plants that we could score in this experiment flowered early and were therefore *phyB/phyB*, fewer than the expected proportion of 25% (Table III). Thus, a greater than expected proportion of germinated seeds were *PHYB/PHYB* or *PHYB/phyB*. Conversely, when seeds that had failed to germinate in the dark were induced to germinate by transfer to white light, 65% grew into tall (*phyB/phyB*) seedlings, significantly more than the expected proportion of 25% (Table III). These data confirm that seeds wild type for *PHYB* germinated better in the dark than *phyB* mutant seeds. Similar data were obtained for germination of progeny of a *PHYB/phyB* plant in a wild-type *PHYA* background (data not shown).

Seedling Development

Light influences several aspects of seedling development, including inhibition of hypocotyl elongation, cotyledon expansion, and induction of expression of genes involved in photosynthesis. We have examined the ability of the *phyA*, *phyB*, and *phyA phyB* mutant seedlings to carry out these developmental steps. We have found that the phenotypes of the *phyA phyB* double mutant reveal activities of these phytochromes not readily apparent from examination of the *phyA* or *phyB* single mutants.

Hypocotyl lengths of wild-type, *phyA*, *phyB*, and *phyA phyB* seedlings under different conditions are presented in Table IV. All four strains had similar hypocotyl lengths when grown in the dark (Table IV). In white light, as previously described, the *phyA* mutant had a hypocotyl length very similar to that of the wild type, whereas the *phyB* mutant had an elongated hypocotyl (Table IV). The *phyA phyB* double mutant had a hypocotyl slightly longer than the *phyB* single mutant in white light. In red light, we found that the *phyA* mutant had virtually the same hypocotyl length as the wild type (Table

Table IV. *Hypocotyl lengths of mutants under different light conditions.*
Seedlings were grown on MS plates for 6 d after a 1-d cold treatment. Values are means of between 21 and 30 seedlings ± SD. For far-red measurements, seedlings were induced to germinate with white light before transferring to far-red-rich light. W, White light; R, red light; FR, far-red-rich light.

Light	Ler (wild type)	Hypocotyl Length		*phyA phyB*
		phyA	*phyB*	
		mm		
Dark	10.7 ± 2.0	10.1 ± 2.3	9.9 ± 3.0	11.9 ± 1.1
W	1.0 ± 0.2	1.3 ± 0.3	4.3 ± 1.4	6.7 ± 1.8
R	4.0 ± 1.7	5.5 ± 2.3	10.8 ± 3.0	15.2 ± 1.9
FR	1.6 ± 0.3	5.8 ± 1.4	1.5 ± 0.3	6.6 ± 0.9

IV). However, in the *phyB* background, the *phyA* mutation caused an increase in hypocotyl length. Thus, the *phyA phyB* double mutant was taller than the *phyB* single mutant (Table IV). Finally, in far-red light, the *phyA* mutant was taller than the wild type, and the *phyB* mutant had a wild-type hypocotyl length. The *phyA phyB* double mutant had a hypocotyl about as long as that of the *phyA* mutant in far-red light. Thus, the *phyB* mutation does not appear to affect hypocotyl elongation in far-red light.

Notwithstanding previous observations that *phyA* mutations did not affect hypocotyl elongation in white light or red light (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993), the hypocotyl length data from the *phyA phyB* double mutant suggest that the *phyA* mutation may influence hypocotyl elongation slightly under both of these light conditions. To investigate this phenomenon in more detail, we examined the hypocotyl elongation behavior of the mutants under different fluence rates of red light. As shown in Figure 1, we found that at low, red-light fluence rates the *phyA* mutant had a slightly longer hypocotyl than the wild-type or the *phyB* mutant (Fig. 1). However, this effect was very small and may not be significant. By contrast, at high fluence rates the *phyB* mutant appeared abnormally tall, whereas the *phyA* mutant showed a wild-type degree of inhibition of elongation (Fig. 1). We obtained similar results for hypocotyl elongation in white light (data not shown). Thus, it appears that PHYB is more important than PHYA for control of hypocotyl growth in white light and red light. The effect of the *phyA* mutation on hypocotyl length under these conditions was clearly visible only in the *phyB* mutant background.

We have also examined responses of the wild-type, *phyA*, and *phyB* strains to short pulses of red and far-red light. We measured the effect of six daily pulses of red light or red followed by far-red light on hypocotyl elongation of otherwise dark-grown seedlings. As shown in Figure 2, short pulses of red light inhibited hypocotyl elongation of both wild-type and *phyA* seedlings to almost the same extent as

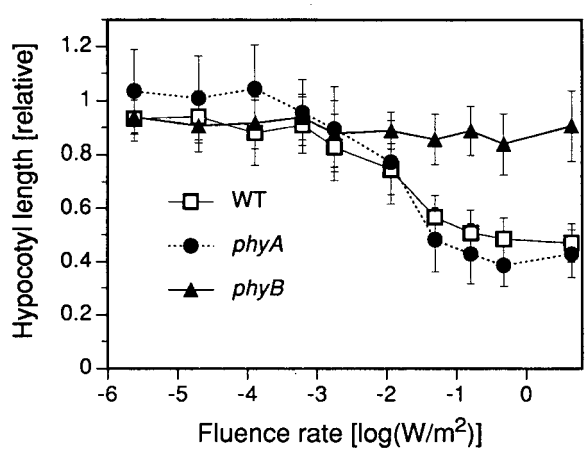

Figure 1. Hypocotyl lengths of wild-type, *phyA*, and *phyB* mutant seedlings after growth in different fluence rates of continuous red light. Hypocotyl lengths are normalized to hypocotyl lengths of dark-grown seedlings and are averages of about 90 seedlings. Error bars indicate SD of measurements.

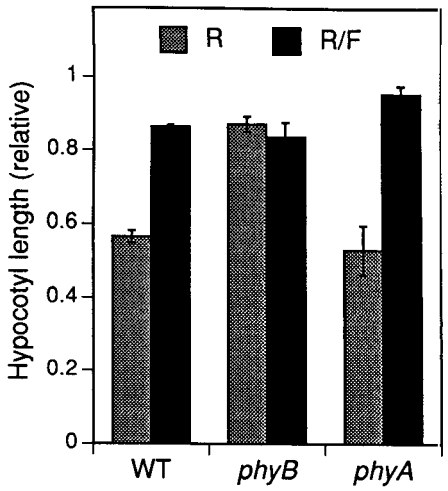

Figure 2. Effect of pulses of red and far-red light on hypocotyl elongation of etiolated wild-type, *phyA*, and *phyB* seedlings. Etiolated seedlings were given pulses of red light (R; 5 min of red light, 2.2 W/m²) or red light followed by far-red light (R/FR; 15 min of far-red light, 1.4 W/m²), at 4-h intervals, and hypocotyl lengths were measured after 5 d. Data are normalized to hypocotyl lengths in the absence of light pulses and are means of three independent experiments. Error bars indicate SD of measurements.

continuous irradiation with red light (Fig. 1). This inhibition was reversible by pulses of far-red light. In contrast, the same light regimen inhibited hypocotyl elongation in the *phyB* mutant only slightly (Fig. 2). Therefore, PHYB appears to mediate inhibition of hypocotyl elongation through a low-fluence response (Briggs et al., 1985). Pulses of far-red light alone had no significant effect on hypocotyl length (data not shown).

In addition to effects of the phytochrome mutations on hypocotyl elongation, we observed effects on greening. In particular, in red light the cotyledons of the *phyA phyB* double mutant were smaller than the cotyledons of either single mutant, and the apical hook was also less open (Fig. 3). The apical hook and cotyledons of red-light-grown *phyA phyB* seedlings appeared very similar to those of the *hy1*, *hy2*, and *hy6* mutants (Liscum and Hangarter, 1993b; data not shown), which are deficient in multiple phytochromes as a consequence of presumed defects in phytochrome chromophore synthesis or attachment (Chory et al., 1989; Parks et al., 1989; Parks and Quail, 1991). Thus, it appears that, as for inhibition of hypocotyl elongation, PHYA and PHYB act together to promote hook opening and cotyledon development in red light. In far-red light, cotyledon expansion and unfolding depended on PHYA alone, since cotyledons of the *phyB* mutant looked like those of the wild type (Fig. 3). Other workers have also observed this defect in *phyA* single mutants (Parks and Quail, 1993). In white light, cotyledons of all four strains expanded and unfolded, which is consistent with the finding that a blue-light system also contributes to this response (Liscum and Hangarter, 1993a, 1993b). These results show that both PHYA and PHYB contribute to deetiolation in constant red light, whereas only PHYA contributes to deetiolation in constant far-red light.

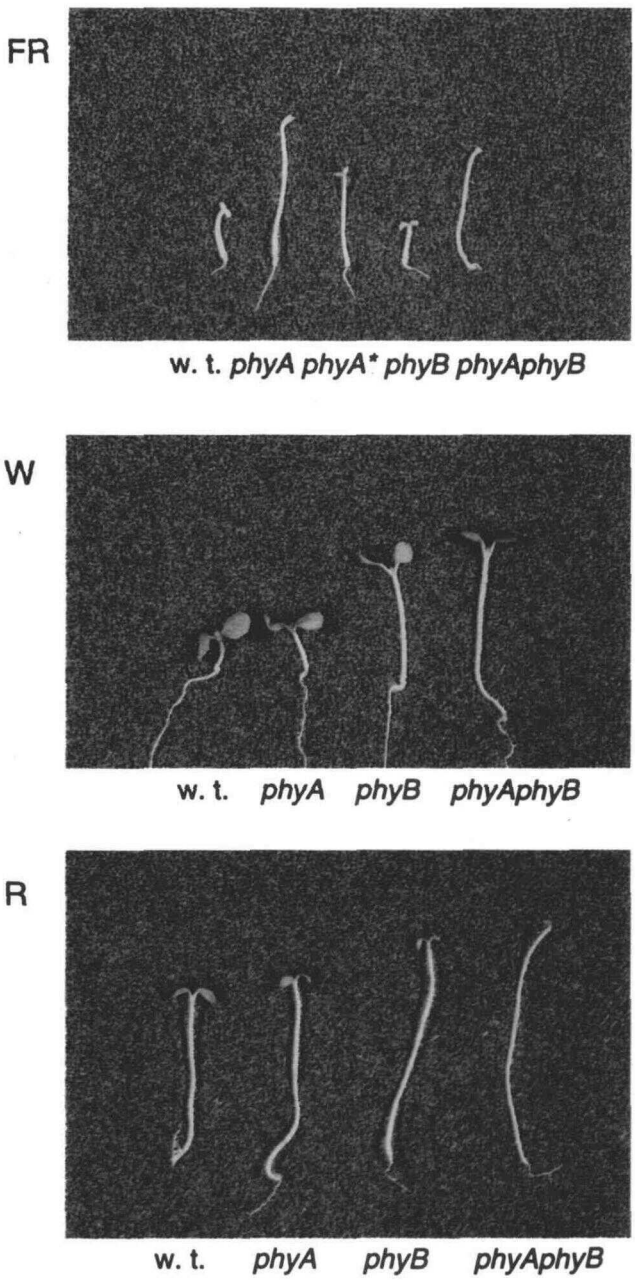

Figure 3. Appearance of cotyledons of wild-type, *phyA*, *phyB*, and *phyA phyB* mutant seedlings after growth in far-red-rich light (FR), white light (W), and red light (R). *phyA**, A seedling with the weak *phyA* mutation *phyA-205*. Seedlings were grown on MS plates for 1 week under the different light conditions.

Phytochromes are known to mediate induction of expression of genes required for photosynthesis. In particular, it is known that the *CAB* genes, encoding light-harvesting Chl *a*/*b*-binding proteins, are induced by phytochrome (Silverthorne and Tobin, 1987; Karlin-Neumann et al., 1988; Sun and Tobin, 1990). However, it is not known which phytochrome(s) mediate this gene expression response. Therefore, we have examined the ability of a pulse of red light to induce the *CAB* genes of etiolated wild-type, *phyA*, *phyB*, and *phyA phyB* seedlings. As shown in Figure 4, whereas both the *phyA* and *phyB* single mutants accumulated *CAB* mRNA to approximately the same degree as the wild type, the *phyA phyB* double mutant accumulated substantially less. In time-course experiments, we found that the double mutant did accumulate *CAB* mRNA, but that the peak of accumulation occurred after at least 12 h, compared to within 4 h for the wild-type, *phyA*, and *phyB* strains (data not shown). When the inducing red-light pulse was followed by a far-red-light pulse, the amount of induction was reduced, showing that the response was indeed attributable to phytochrome (Fig. 4). Interestingly, this reversal by a far-red-light pulse appeared most complete in the *phyA* mutant (Fig. 4), suggesting that PHYA might be able to mediate *CAB* induction by far-red light, just as it mediates germination and inhibition of hypocotyl elongation by far-red light. Consistent with this notion, we have found that a *phyA* mutant expresses the *CAB* genes at a lower level than the wild type in continuous far-red light (T.D. Elich, unpublished result). These results show that under the conditions employed, either PHYA or PHYB can mediate induction of *CAB* gene expression by red light. Moreover, the eventual induction of *CAB* in the *phyA phyB* double mutant suggests that an additional phytochrome(s) may also contribute to this response.

Consistent with these *CAB* induction results, the *phyA phyB* double mutant, but not the *phyA* or *phyB* single mutants, had a defect in potentiation of Chl accumulation by a pulse of red light. We treated etiolated seedlings with a pulse of red light 4 h before transfer to white light, and then measured the amount of Chl accumulation after 2 h of white light. As shown in Figure 5, whereas all four strains accumulated Chl upon transfer to white light, the *phyA phyB* double mutant showed only a weak potentiation by the red-light pulse. Thus, for both *CAB* induction and potentiation of Chl accumulation, it appears that either PHYA or PHYB is sufficient for full responsiveness to pulses of red light.

Flowering

The Landsberg ecotype of *Arabidopsis* is a LDP, which flowers earlier when grown under long days and short nights than when grown under short days (Brown and Klein, 1971;

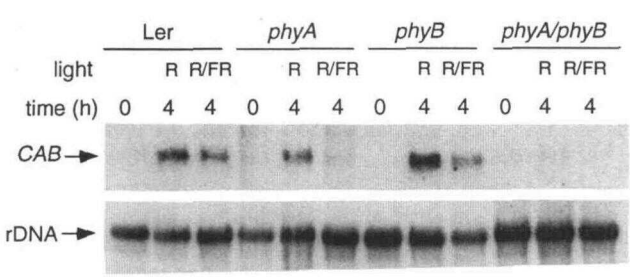

Figure 4. *CAB* mRNA induction in etiolated wild-type, *phyA*, *phyB*, and *phyA phyB* mutant seedlings by a flash of red light. Seedlings were grown for 5 d in the dark, given a pulse of red light (R) or red followed by far-red light (R/FR; see "Materials and Methods"), and then returned to the dark for 4 h until RNA was extracted. *CAB*, Hybridization signals using a *CAB* gene probe; rDNA, hybridization signals using a ribosomal DNA probe.

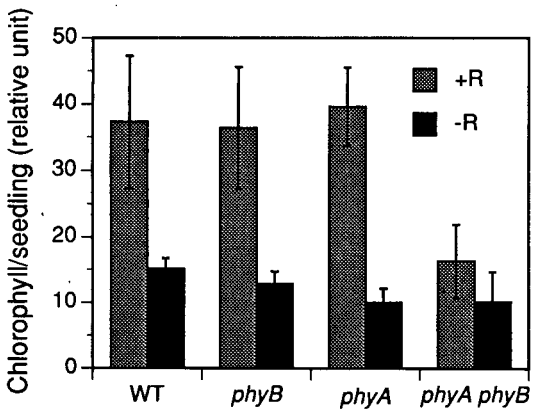

Figure 5. Potentiation of Chl accumulation in etiolated wild-type, *phyA*, *phyB*, and *phyA phyB* seedlings by a pulse of red light. A 10-min red-light pulse (4.5 W/m^2) was given 4 h before transfer to white light (+R), or no such pulse was given (−R), and Chl was measured 2 h after transfer to white light. Values are means of four independent samples. Error bars indicate SD of measurements.

Goto et al., 1991). One way to probe the photoperiodic behavior of plants is to subject them to short days with a "night break" of light in the middle of the long night. This light treatment mimics the effect of long days and allows investigation of informational light sensing in the absence of differential effects caused by differing amounts of total PAR. For several plants, it has been established that night breaks are most effective when given in the middle of a long night (Lane et al., 1965; Hanke et al., 1969; Ishiguri and Oda, 1972; Saji et al., 1982; Vince-Prue, 1983). We have tested the behavior of the *phyA*, *phyB*, and *phyA phyB* mutants in such an experiment.

As shown in Figure 6, the *phyA* mutant responded significantly less than the wild type to night breaks, and the *phyA phyB* double mutant responded slightly less than the *phyB* mutant. Without night breaks, the wild-type and *phyA* plants flowered at the same time and with the same number of rosette leaves (Fig. 6; Student's *t* test, $P > 0.5$ for both measurements). However, whereas night breaks accelerated flowering in the wild type by about 6 d, they only accelerated flowering in the *phyA* mutant plants by about 2 d (Fig. 6A; $P < 0.05$ for wild type versus *phyA* when given night breaks). Similarly, the wild-type plants flowered with about eight fewer rosette leaves when given night breaks, but the *phyA* mutant flowered with just four fewer leaves (Fig. 6B; $P < 0.005$ for wild-type versus *phyA* with night breaks). This experiment was performed three times with comparable results (data not shown). As reported previously (Goto et al., 1991; Reed et al., 1993), the *phyB* mutant flowered substantially earlier than the wild type both with and without a night break. The *phyA* mutation also reduced the night break response in the *phyB* mutant background, although the effect was smaller than in a wild-type (*PHYB*) background. Thus, the flowering time of *phyB* and *phyA phyB* strains was the same in short days ($P > 0.5$ for both days to flowering and leaf number). With night breaks, flowering of the *phyB* strain was accelerated by about 3 d, whereas flowering of the *phyA phyB* strain was accelerated by just 2 d (Fig. 6A; $P < 0.025$ for *phyB* versus *phyA phyB* with night breaks). The *phyA phyB* strain also had more rosette leaves at flowering than the *phyB* single mutant (Fig. 6B; $P < 0.05$).

DISCUSSION

The *phyA* mutations described here have allowed us to assess the physiological role of PHYA in *Arabidopsis* growth and development with a specificity heretofore impossible. Analogous study of a *phyB* mutant and a *phyA phyB* double mutant has permitted us to determine the relative contributions of PHYA and PHYB to various light-regulated processes. We have found that PHYA promotes germination in far-red light, whereas PHYB controls germination in the dark. PHYA inhibits hypocotyl elongation in far-red light and may also contribute to inhibition of hypocotyl elongation in red and white light. However, this effect in red or white light is easily visible only in the *phyB* mutant background, and PHYB therefore appears to be more important than PHYA in red and white light. PHYA and PHYB together promote cotyledon development in red light and induce expression of light-regulated genes in response to a brief red-light treatment.

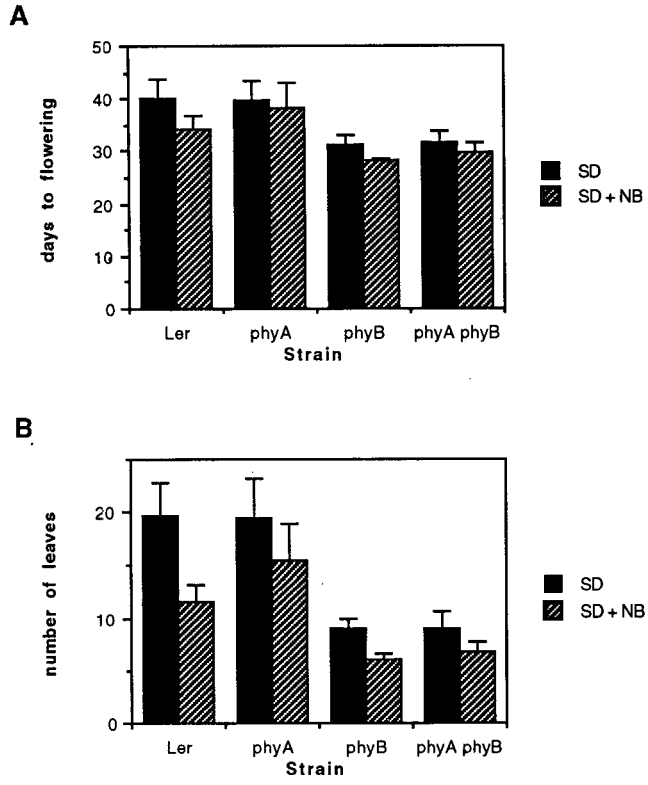

Figure 6. Flowering response of wild-type and mutant plants grown in short days with or without a night break. Flowering was assessed as the point at which flower buds were first visible and is presented as number of days after sowing (A) and as number of rosette leaves at flowering (B). Plants were grown under either 9-h days (SD, short days) or 8-h days with a 1-h white-light treatment (the night break) in the middle of the night (SD + NB). Each value represents the mean of 11 or 12 plants. Error bars indicate SD of measurements.

Finally, PHYA contributes to induction of flowering by long days, whereas PHYB inhibits flowering without obviously affecting sensing of daylength.

These and other studies (Dehesh et al., 1993; Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993; Shinomura et al., 1994) reveal that the *phyA* mutant shows its most drastic physiological defects in germination and inhibition of hypocotyl elongation in plants grown under far-red-rich light. Moreover, other workers have reported a significant effect of far-red light in night-break experiments in *Arabidopsis* (Goto et al., 1991), which would be consistent with our observation that the *phyA* mutant has a defect in this response. It is at first glance curious that a phytochrome should apparently exhibit its primary effect in far-red light because phytochromes are presumed to act upon sensing of red light and consequent conversion to the Pfr. However, the far-red component of such "high-irradiance responses" is generally considered to be mediated by a phytochrome (Beggs et al., 1980; Holmes and Schäfer, 1981; Wall and Johnson, 1983; Carr-Smith et al., 1989; Smith and Whitelam, 1990). The best available explanation is that far-red light increases the rate of conversion from the far-red-absorbing form of PHYA to the red-absorbing form, which is more stable. This shift of the PHYA photoequilibrium results in higher steady-state levels of both forms of phytochrome and of putative intermediates that might have regulatory activity. Theoretical models based on known phytochrome photoconversion rates (Wall and Johnson, 1983) and actual measurements of phytochrome levels under light of different fluences (Smith et al., 1988) both support this type of model. In this model the increase in Pfr under incident light of low compared with high red to far-red (R:FR) ratio would depend on the greater lability of the Pfr relative to the Pr form of the phytochrome in question. Therefore, light-stable phytochromes would not be expected to mediate this type of response. The evolution of a light-labile phytochrome may thus be an elegant solution to the problem of how to use a phytochrome, whose action would normally be inhibited by far-red light, to mediate responses to far-red light. The ability to respond to far-red light might be particularly useful to plants germinating under leaves or thin layers of soil, environments potentially enriched in far-red frequencies (Smith, 1982).

Our finding that PHYB inhibits germination in far-red light contradicts the accepted orthodoxy that phytochromes have no activity in the red-light-absorbing form. Far-red light pushes the phytochrome photoequilibrium toward the red-light-absorbing form, and therefore (for stable phytochromes) far-red light should mimic the effect of a loss-of-function phytochrome mutation. Earlier data on reversibility (inhibition) of germination by far-red light were interpreted as indicating inactivation of phytochrome by conversion to Pr (Shropshire et al., 1961; McCullough and Shropshire, 1970; Hayes and Klein, 1974; Cone and Kendrick, 1985). However, we have found that for germination of a *phyA* mutant, and to a lesser extent the wild type, the *phyB* null mutation actually compensates for the apparent inhibitory effect of far-red light on germination. Therefore, PHYB must be inhibiting germination under these conditions rather than failing to promote germination. Since it has been shown that the Pfr form of PHYB promotes germination (Shinomura et al., 1994), and since in far-red-rich light PHYB would be expected to exist primarily in the red-light absorbing form Pr, this form of PHYB probably carries out the inhibition of germination in this case. Using pure seed stocks only, Shinomura et al. (1994) also reached this conclusion. Moreover, Liscum and Hangarter (1993c) have also concluded that the red-light-absorbing form of PHYB actively promotes shoot gravitropism. There is precedent for alternate regulatory states of sensory molecules having opposite activities, as opposed to only one of two forms having an activity. For example, the NtrB protein of *Escherichia coli* acts as a kinase under conditions of nitrogen starvation and as a phosphatase under conditions of nitrogen excess (or, possibly, facilitates autophosphorylation and autodephosphorylation of its substrate, NtrC). NtrB is a member of the sensor class of bacterial two-component regulatory systems (Parkinson and Kofoid, 1992), a family with limited homology to phytochromes (Schneider-Poetsch, 1992).

In SDP physiological experiments have suggested that two phytochrome pools might play roles in regulating flowering time (Saji et al., 1982; Takimoto and Saji, 1984). The results of our flowering time experiments suggest that this is also true in *Arabidopsis*, a LDP. PHYA appears to interact with the circadian rhythm involved in sensing daylength, whereas PHYB appears to act more generally to inhibit flowering, perhaps functioning to sense the degree of shade rather than the daylength. This idea is consistent with studies of other LDP, which have suggested control of flowering both by circadian rhythm and by a high-fluence response (Lane et al., 1965; Schneider et al., 1967; Ishiguri and Oda, 1972; Deitzer et al., 1979). The *phyB* mutation affects flowering time more drastically than the *phyA* mutation, suggesting that in this plant, general growth environment is more important than change in daylength for determining flowering time. In addition, in this and other experiments it appeared that the *phyA* and *phyA phyB* mutants retained a residual night-break response (Fig. 6; J.W. Reed, unpublished observation). This residual night-break response may indicate that another photoreceptor participates in this response. Such a photoreceptor could be another phytochrome or a blue-light photoreceptor. It has previously been reported that blue light can promote flowering in *Arabidopsis* (Brown and Klein, 1971; Eskins, 1992), and that both blue and far-red light can mediate the night-break response (Goto et al., 1991).

One intriguing conclusion to emerge from these physiological studies is that in responses in which PHYA and PHYB both play roles, they act either synergistically or in opposite directions. These data have implications for the mechanisms of phytochrome signal transduction. For example, in red light, both PHYA and PHYB inhibit hypocotyl elongation. Similarly, PHYA and PHYB both contribute to cotyledon development in red light and to Chl accumulation and induction of gene expression by a pulse of red light. For both inhibition of hypocotyl elongation and greening of cotyledons, then, it seems likely that the two phytochromes activate the same signal-transduction pathway. This idea is consistent with results from studies of overexpression of PHYA and PHYB in transgenic *Arabidopsis* plants, in which either PHYA or PHYB could cause a decrease in hypocotyl length (Boylan

and Quail, 1991; Wagner et al., 1991; Whitelam et al., 1992; McCormac et al., 1993).

For other responses, PHYA and PHYB appear to act in opposite directions. For example, PHYA promotes and PHYB inhibits germination in far-red light. In this case, the promotion of germination in far-red light by PHYA can be explained as a high-irradiance response, in which the active form of PHYA might still be Pfr. As explained above, we attribute the inhibition of germination by PHYB to the Pr form. In fact, both PHYA and PHYB promote germination in red light, presumably through action of the Pfr form (Shinomura et al., 1994). For germination, then, PHYA and PHYB might also activate the same signal-transduction pathway, but only PHYA promotes germination in far-red light, through the "high-irradiance response."

The flowering data are more difficult to reconcile in this manner. In our night-break experiments, we observed a delay of flowering compared with the wild type in the *phyA* mutant and an acceleration of flowering in the *phyB* mutant. If the effects on flowering of PHYA and PHYB are both mediated by Pfr, then the Pfr forms of PHYA and PHYB must actually act in opposite directions. This conclusion suggests that PHYA and PHYB might act through separable signaling pathways. This could be accomplished by physical separation of the two phytochromes, for example by expression in different tissues or at different times, a mechanism suggested by the differential levels of PHYA in etiolated versus light-grown seedlings (Somers et al., 1991), or through interaction of PHYA and PHYB with distinct signal-transduction components. Alternatively, it is possible, as in the case of germination in far-red light, that PHYB exerts an effect on flowering through the Pr form. Thus, under the light conditions employed in our experiments, the inhibitory effect of the Pr form of PHYB may be more visible than a putative promoting effect of the Pfr form. It will be interesting to discover whether the two phytochromes affect these processes through different effects on the same signal-transduction systems or through distinct signal-transduction systems.

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