Independent action of ELF3 and phyB to control hypocotyl elongation and flowering time

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Independent Action of ELF3 and phyB to Control Hypocotyl Elongation and Flowering Time[1]

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Light regulates various aspects of plant growth, and the photoreceptor phytochrome B (phyB) mediates many responses to red light. In a screen for Arabidopsis mutants with phenotypes similar to those of *phyB* mutants, we isolated two new *elf3* mutants. One has weaker morphological phenotypes than previously identified *elf3* alleles, but still abolishes circadian rhythms under continuous light. Like *phyB* mutants, *elf3* mutants have elongated hypocotyls and petioles, flower early, and have defects in the red light response. However, we found that *elf3* mutations have an additive interaction with a *phyB* null mutation, with *phyA* or *hy4* null mutations, or with a PHYB overexpression construct, and that an *elf3* mutation does not prevent nuclear localization of phyB. These results suggest that either there is substantial redundancy in phyB and elf3 function, or the two genes regulate distinct signaling pathways.

Plants adjust their growth and development according to diurnal, seasonal, and local variations in their light environment. Light can induce leaf formation, leaf expansion, and chloroplast differentiation; inhibit stem elongation; induce bending toward or away from light; and induce or repress flowering. Light can also phase the circadian rhythm. Several photoreceptors sense light, including red/far-red light receptors called phytochromes, blue light receptors called cryptochromes, the NPH1 photoreceptor required for phototropism, and hypothesized UV light receptors (Fankhauser and Chory, 1997; Deng and Quail, 1999).

Genetic analyses in Arabidopsis have been particularly helpful in dissecting the roles of the various photoreceptors. Arabidopsis has five phytochromes, phyA to phyE, and two cryptochromes, cry1 (also known as HY4) and cry2. Analyses of the effects of mutations in genes encoding PHYA, PHYB, PHYD, PHYE, CRY1, CRY2, and NPH1 and transgenic plants overexpressing PHYA, PHYB, PHYC, CRY1, or CRY2 have revealed the developmental functions and capabilities of each of these photoreceptors (Fankhauser and Chory, 1997; Deng and Quail, 1999). The various phytochromes and cryptochromes share some functions, but are also specialized to some degree. For example, different photoreceptors contribute to inhibition of hypocotyl elongation under different light conditions. In white light, phyB and cry1 play the largest roles and phyA, phyD, and cry2 play lesser roles (Reed et al., 1994; Aukerman et al., 1997; Smith et al., 1997; Lin et al., 1998). Signal transduction pathways downstream of these photoreceptors probably interact. For example, under some light conditions phyB and cry1 require each other's activity for maximum inhibition of hypocotyl elongation (Casal and Boccalandro, 1995; Casal and Mazzella, 1998). Conversely, whereas phyB normally inhibits flowering, phyA and cry2 each promote flowering under certain light conditions (Johnson et al., 1994; Reed et al., 1994; Guo et al., 1998). *cry2* mutant plants flower later than wild-type plants in light containing both red and blue frequencies, and a *phyB* mutation suppresses this effect, indicating that cry2 antagonizes phyB-mediated inhibition of flowering (Guo et al., 1998; Mockler et al., 1999). Thus, signal transduction pathways downstream of different photoreceptors may reinforce or antagonize each other, depending on the response.

Phytochromes exist in two photointerconvertible forms called Pr and Pfr. Red light converts Pr to Pfr, which absorbs far-red light. Far-red light reconverts Pfr to Pr. For most responses it is thought that Pfr is the active form, because most phytochrome-mediated responses are induced by red light (Furuya, 1993; Quail et al., 1995). However, phyA mediates far-red light responses, and therefore it is possible that the Pr form of phyA is active (Shinomura et al., 2000). Recent biochemical results have shown that phytochromes act as kinases (Yeh et al., 1997; Yeh and Lagarias, 1998; for review, see Reed, 1999). Both phyA and phyB proteins localize to the nucleus under light conditions when they mediate light responses, suggesting that nuclear localization may be important for phytochrome signaling (Sakamoto and Nagatani, 1996; Kircher et al., 1999; Yamaguchi et al., 1999).

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Other recent work has aimed to identify downstream targets of phytochromes. Several mutations cause phenotypes similar to those caused by mutations in phytochrome genes (Whitelam et al., 1993; Ahmad and Cashmore, 1996; Barnes et al., 1996; Lin and Cheng, 1997; Wagner et al., 1997; Soh et al., 1998; Hudson et al., 1999) or confer hypersensitive red and/or far-red light responses (Genoud et al., 1998; Hoecker et al., 1998). These mutations may affect genes encoding immediate targets of phytochrome action or downstream regulators of phytochrome signaling. Other potential phytochrome signaling components have been identified in yeast two-hybrid screens. The PIF3 and PKS1 proteins can interact with both phyA and phyB, and NDPK2 interacts with phyA (Ni et al., 1998; Choi et al., 1999; Fankhauser et al., 1999). Studies with plants that overexpress or underexpress these genes suggest that PIF3 and NDPK2 activate phytochrome responses, whereas PKS1 may repress phytochrome responses (Ni et al., 1998; Choi et al., 1999; Fankhauser et al., 1999; Halliday et al., 1999). Given the complexity of light responses and the relatively subtle phenotypes of these transgenic plants, these proteins are probably just a subset of actual phytochrome signaling components.

The circadian system controls biological rhythms with a period of roughly 24 h (Lumsden and Millar, 1998). Circadian-regulated outputs in Arabidopsis include expression of many genes, leaf movements, and hypocotyl growth (Millar and Kay, 1991; Hicks et al., 1996; Dowson-Day and Millar, 1999). Both red and blue light signals control the phase, period, and amplitude of circadian rhythms in higher plants (Lumsden and Millar, 1998). In Arabidopsis, phyA, phyB, and cry1 have all been shown to participate in these responses of the circadian system (Millar et al., 1995; Anderson et al., 1997; Somers et al., 1998). Light regulation and circadian control may allow more flexible responses together than either does alone; the two modes of regulation are frequently associated, sometimes in a complex manner. The processes that are directly regulated by phyB, for example, overlap with those controlled by circadian rhythms. For *CAB* gene activation, the amplitude of the light response is modulated by the circadian clock (Millar and Kay, 1996; Anderson et al., 1997).

Arabidopsis phyB mutants have several defects in red light responses, including reduced seed germination, reduced induction of *CAB* gene expression, elongated hypocotyls and stems, and a longer circadian rhythm period, and they also flower early (Koornneef et al., 1980; Reed et al., 1993, 1994; Halliday et al., 1994; Shinomura et al., 1994; Somers et al., 1998). In a screen for mutants with phenotypes similar to those of the *phyB* mutants, we have discovered two alleles of a previously known locus called *ELF3*. *elf3* mutants were first identified based on their early flowering phenotype, but also have elongated hypocotyls and lack circadian rhythms in constant light (Hicks et al., 1996; Zagotta et al., 1996; Dowson-Day and Millar, 1999). In constant darkness, *elf3-1* plants retained a circadian rhythm, and it was proposed that ELF3 mediates an interaction between light and the circadian clock, rather than being a component of the clock itself (Hicks et al., 1996). We report genetic and physiological experiments that explore the relationship between ELF3 and phyB. We have compared several phenotypes of *elf3* and *phyB* mutants, and have assayed light responsiveness of hypocotyl elongation in *elf3* mutants and several double mutants between *elf3* and photoreceptor mutations. These phenotypic analyses showed that *elf3* mutants resemble *phyB* mutants in several respects, and we have therefore tested whether the *elf3-1* null mutation affects either the ability of overexpressed phyB to confer a phenotype or phyB nuclear localization. Finally, we have examined the effects of a weak *elf3* allele on circadian rhythms. We find that ELF3 and phyB can act independently to control a common set of phenotypes.

MATERIALS AND METHODS

Mutant Alleles and Double Mutants

The *phyB-1* mutation has a stop codon in the *PHYB* gene and is in the Arabidopsis Landsberg *erecta* ecotype (Reed et al., 1993). All other mutants used in this study were in the Columbia ecotypic background. The *elf3-1* mutation (Zagotta et al., 1996) and the *elf3-9* mutation each create a stop codon and behave like null alleles of *ELF3* (K. Hicks and D.R. Wagner, personal communication). *elf3-1* also carries the *gl1* mutation. *elf3-7* has a splice site mutation (K. Hicks and D.R. Wagner, personal communication). *elf3-9* was isolated in the *phyA-211* background. *phyA-211* has a rearrangement in the *PHYA* gene and is a presumed null mutation (Reed et al., 1994; C. Fankhauser, personal communication). The *phyB-9* mutation has a stop codon and is also a presumed null mutation (Reed et al., 1993). *hy4-101* was originally called *blu1* and has not been sequenced, but lacks CRY1 protein (Liscum and Hangarter, 1991; Bagnall et al., 1996; Mockler et al., 1999). To construct double mutants between the linked *elf3-1* (or *elf3-7*) and *phyB-9* mutations, single mutants were crossed, and the resulting F_1 double heterozygotes were crossed with the corresponding *elf3* single mutant. Multiple tall F_1 individuals (*elf3/elf3*) from this second cross were test-crossed with *phyB-9* to identify individuals with a recombinant *elf3 phyB-9* chromosome. Double mutants were then identified among the self progeny of plants carrying such a recombinant chromosome. Double mutants involving unlinked mutations were identified by phenotypic criteria and then confirmed by test crosses.

Growth Conditions and Light Response Experiments

Growth conditions and light response experiments were performed as described previously (Reed et al., 1998): Seeds were surface-sterilized and plated on Murashige and Skoog (MS)/agar plates containing 1× MS salts (Gibco, Grand Island, NY), 0.8% (w/v) Phytagar (Gibco), and 1× Gamborg's B5 vitamin mix (Sigma, St. Louis) with or without 2% (w/v) Suc, stored overnight at 4°C, and moved to the appropriate light condition. Red, far-red, and blue light sources were as described previously (Reed et al., 1998). For far-red and blue light experiments, results for only the highest fluence rates (approximately 50 μmol m^{-2} s^{-1} for far-red light and 10 μmol m^{-2} s^{-1} for blue light) are

shown. For flowering time determinations, seedlings were grown on MS/agar/2% (w/v) Suc plates for 10 to 14 d, and then transplanted to soil (Pro-Mix BX, Hummert, St. Louis). Plants were grown in a Conviron (Winnipeg, Manitoba, Canada) growth chamber at 21°C. Light was provided on a 9:15 h day:night cycle from 12 fluorescent (F72T12/CW/VHO, 160 W) and six incandescent (60 W) bulbs, and had an intensity at plant height of 170 to 220 μmol m^{-2} s^{-1}.

Circadian Rhythm Assays

Seeds for circadian rhythm experiments were grown in MS/agar medium with 3% (w/v) Suc, as described previously (Millar et al., 1995; Dowson-Day and Millar, 1999). Seed containing the *CAB2::LUC* reporter gene in the *elf3-1* background have also been described previously (Hicks et al., 1996). The same reporter gene was introduced into the *elf3-7* background in a similar manner, following a cross between a homozygous *elf3-7* mutant and a line in which the *CAB2::LUC* reporter had been introgressed into the Columbia background. Rhythmic cotyledon movement and hypocotyl elongation were analyzed (Dowson-Day and Millar, 1999), and the percentage of arrhythmic plants was calculated (Hicks et al., 1996). Studies of circadian-regulated *CAB* gene expression were performed (Millar et al., 1995) and analyzed as for the morphological data (Dowson-Day and Millar, 1999). Unless indicated in the text, the data presented are representative of at least two experiments (*CAB2::LUC* rhythms).

Immunodetection of PHYB

Protein extracts were prepared from 10-d-old seedlings grown in constant light on MS/agar/2% (w/v) Suc plates or from young leaves of greenhouse-grown plants. To prepare the extracts, 100 mg of tissue was added to a microfuge tube containing about 80 to 100 mg of sand and ground with a pestle for 2 min in 75 μL of extraction buffer containing 100 mM Tris, pH 6.8, 4% (w/v) SDS, 20% (w/v) glycerol, 200 mM dithiothreitol (DTT), 5 mM phenylmethylsulfonyl fluoride (PMSF), and 100 μg/mL leupeptin at 95°C. The extracts were incubated at 95°C for 3 more min, then centrifuged at 14,000 rpm for 2 min. The supernatant was transferred to a fresh tube and the protein concentration was determined using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA). The desired volume of total protein extract was mixed with one-tenth volume of bromphenol blue (0.05%, w/v), heated again at 95°C for 2 min, and fractionated on 12% (w/v) SDS-PAGE (Laemmli, 1970).

Proteins were transferred to nitrocellulose membrane (no. BA85, Schleicher & Schuell, Keene, NH) using transblot buffer (containing 3.02 g of Tris, 14.42 g of Gly, and 200 mL of methanol per liter) at 100 V for 30 min at 4°C. The membrane was blocked in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% [w/v] Tween 20) containing 1% (w/v) nonfat dry milk at room temperature for at least 1 h. The antibodies were diluted in PBS (135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 15 mM Na_2HPO_4, pH 7.5) containing 3% (w/v) bovine serum albumin. The primary antibody, anti-phyB monoclonal mBA2 (a gift from Akira Nagatani, Kyoto University, Kyoto) or anti-c-myc monoclonal from hybridoma line 9E10 (obtained from the Tissue Culture Facility at the University of North Carolina at Chapel Hill Lineberger Comprehensive Cancer Center), was used at a final concentration of 5 μg/mL at room temperature for 2 h. The membrane was washed with TBST three times for 10 min each. The secondary antibody was alkaline-phosphatase-conjugated anti-mouse IgG (Sigma) and was diluted 1:2,000. Proteins were visualized using the chromogenic reagents nitroblue tetrazolium chloride (Jersey Lab Supply, Livingston, NJ) and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Jersey Lab Supply) as described in Sambrook et al. (1989).

Construction of Transgenic Plants

T-DNA constructs were introduced into plants by vacuum infiltration (Bechtold et al., 1993). The 35S::*PHYB* construct was a gift from R. Sharrock (Hirschfeld et al., 1998). An *elf3-1* transformant that overexpressed PHYB was identified by western blot, crossed to wild-type Columbia, and *ELF3/ELF3* and *elf3-1/elf3-1* progeny of this cross used for the phenotypic analyses. The overexpressing line selected had more than one insertion element (data not shown), and therefore we performed western blots on the same seed analyzed for phenotype to confirm that the *ELF3/ELF3* and *elf3-1/elf3-1* lines contained similar amounts of PHYB protein. Deduced *elf3* genotypes of these lines were confirmed by a cleaved-amplified polymorphic sequence assay for *elf3-1* generously performed by Karen Hicks (Kenyon College, Gambier, OH).

For nuclear localization experiments, we engineered a c-myc epitope at the C-terminal end of a *PHYB* cDNA expressed behind its own promoter. We amplified the 3′ end of *PHYB* by PCR using the oligonucleotides PB7J (5′-TCTGTTTCTTGCAAATCCCGAGC-3′) and PB41 (5′CCT-CCCGGGACCATATGGCATCATCAGCATC-3′, *Sma*I site underlined), cut the product with *Eco*RI and *Sma*I, and ligated the resulting fragment into the corresponding sites of a *PHYB* cDNA clone in pBluescript (Elich and Chory, 1998). This step eliminated the stop codon and introduced a *Sma*I restriction site at the C-terminal end of the coding sequence. We then ligated a c-myc cassette derived from a *Dra*I/*Sac*I digestion of plasmid CD3-128 (Arabidopsis Biological Resource Center, Ohio State University, Columbus) into this *Sma*I site. We sequenced the resulting plasmid to confirm that the c-myc epitope tag was fused in-frame with *PHYB* at the C-terminal end. Finally, this *PHYB*-c-myc cDNA construct was fused at an internal *Sac*I site to a genomic *PHYB* gene containing 2.3 kb of genomic DNA 5′ of the *PHYB* start codon (corresponding to the upstream sequences determined by Reed et al. [1993]), in a derivative of T-DNA binary vector pBI121 (CLONTECH, Palo Alto, CA) lacking the *35S::GUS* gene. Columbia, *phyB-1*, and *elf3-1* transformants carrying this construct and segregating approximately 3:1 for kanamycin resistance were used to

generate homozygous lines for the immunolocalization experiments.

Immunolocalization

Tissue for immunolocalization experiments was obtained from leaves of greenhouse-grown (photoperiod artificially extended in winter to 16 h of light/8 h of dark) plants. Protoplasts were obtained as described in Staub et al. (1996), except that a 90-μm nylon mesh was used to filter the protoplasts. Ten to 25 μL of protoplasts were placed per chamber in several chambers of a 16-well glass slide (Lab-Tek Chamber slide system, Nunc, Napierville, IL), allowed to bind to the slide for 2 to 3 min, and extra wash solution was removed with a pipette. The protoplasts were fixed immediately with 50 μL of fixative (3.5% [w/v] paraformaldehyde, 50 mM potassium phosphate, pH 7.0, and 1 mM EGTA) per chamber at room temperature for 30 min. The fixative was discarded, and the protoplasts were allowed to air-dry for 45 min and then left at 4°C overnight.

Fixed protoplasts were treated with cold methanol for 10 min once followed by PBST (PBS + 0.5% [w/v] Tween 20) for 10 min twice to permeabilize the cells. For immunoreactions, the antibodies were diluted in PBST containing 200 μg bovine serum albumin/mL. Both the primary antibody (monoclonal from hybridoma line 9E10) and the secondary antibody fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cappell, Durham, NC) were used at a dilution of 1:100. The protoplasts were incubated with the primary antibody for 2 h and with the secondary antibody for 1 h at room temperature. Washes were done with PBS (once for 5 min) followed by PBS plus 0.1% (w/v) Tween 20 (once for 5 min), and then PBS (twice for 5 min each). Nuclei were stained with 4′,6-diamidino-2-phenyl-indole (DAPI) at 10 μg/mL at room temperature for 20 min, followed by two washes of 5 min each with PBS. The samples were mounted in Mowiol (Calbiochem, San Diego) containing the antifade reagent *p*-phenylene diamine dihydrochloride (Polysciences, Warrington, PA), and the slides were left in the cold room overnight to harden. Immunofluorescence was observed using a fluorescence microscope (Nikon, Tokyo) equipped with an FITC filter (excitation 450–490, barrier 520) and a UV filter (excitation 360–370, barrier 400). For photographs, Kodak Select 400 films (Eastman Kodak, Rochester, NY) were used.

RESULTS

Isolation and Morphology of *elf3* Mutants

In screens for long hypocotyl mutants, we isolated two mutants that had a strong resemblance to *phyB* mutants in having elongated hypocotyls and petioles and in flowering early. Mapping and complementation analyses established that the mutations in both of these were alleles of *ELF3* (data not shown), and we have called them *elf3-7* and *elf3-9*. The *elf3-7* mutation alters a splice site junction (K. Hicks and D.R. Wagner, personal communication), and conferred slightly less extreme phenotypes than the *elf3-1* null mutation (Zagotta et al., 1996; see below). The *elf3-9* mutant had a stop codon in the coding sequence and is likely a null mutant (K. Hicks and D.R. Wagner, personal communication). For our phenotypic studies we have used the previously described *elf3-1* null mutant and our weaker *elf3-7* mutant.

When grown under short-day conditions, *elf3-1* and *elf3-7* mutants each had long hypocotyls, as did the *phyB-9* mutant (Fig. 1A; Table I). In addition, all three mutants had elongated petioles (Fig. 1B; Table I) and flowered earlier and with fewer leaves than wild type (Table I). For each of these phenotypes, the *elf3-1* and *elf3-7* mutants had more extreme phenotypes than the *phyB-9* mutant. The relative effect of the two *elf3* alleles depended on the assay. *elf3-1* and *elf3-7* mutants had similar petiole lengths, but the *elf3-1* mutant had a slightly longer hypocotyl and flowered earlier than the *elf3-7* mutant. As noted previously for *elf3-1* (Zagotta et al., 1996), the *elf3-7* long hypocotyl phenotype was less severe when the plants were grown under constant white light (data not shown).

Effects of *elf3-7* on Circadian Rhythms

The *elf3-1* mutant is aperiodic in constant light for several circadian rhythm outputs, including *CAB::LUC* expression,

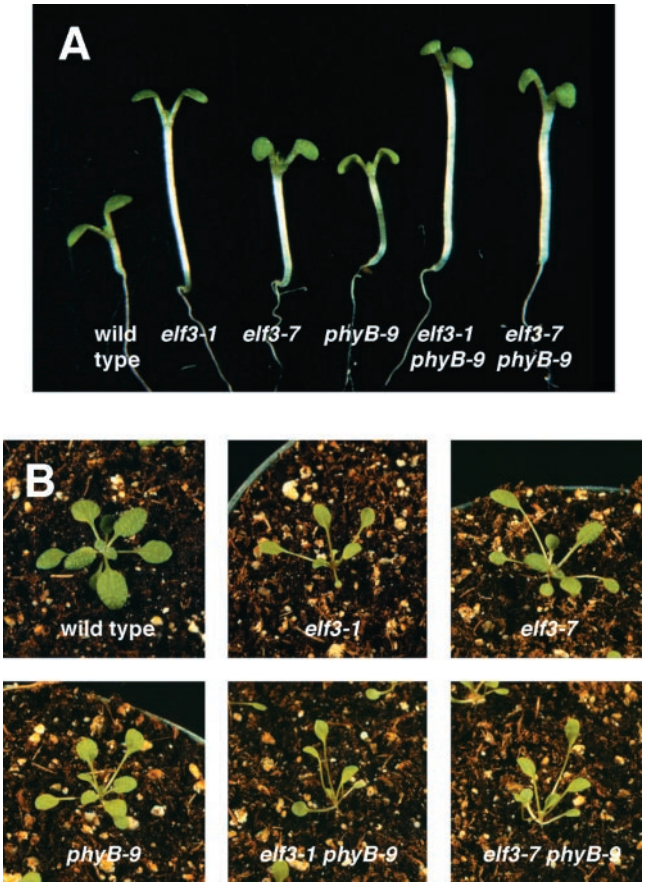

Figure 1. Morphology of *elf3*, *phyB*, and *elf3 phyB* double mutant plants grown under short-day conditions. A, Appearance of seedlings after 8 d of growth under short-day conditions; B, appearance after 3 weeks of growth under short-day conditions.

Table I. *Measurements of wild type, elf3, phyB, and elf3 phyB double mutant plants grown under short-day conditions ± SD*

Hypocotyl lengths are means of between 12 and 20 measurements of 8-d-old seedlings. Other data are means of 12 to 14 measurements. Leaf dimensions are for the first pair of true leaves of 3-week-old plants.

Phenotype	Genotype					
	Wild type	elf3-1	elf3-7	phyB-9	elf3-1 phyB-9	elf3-7 phyB-9
Hypocotyl length, mm	1.8 ± 0.1	6.8 ± 0.6	4.9 ± 0.3	4.0 ± 0.3	8.2 ± 0.5	
Petiole length, mm	5.6 ± 0.7	9.3 ± 1.4	9.1 ± 0.7	7.4 ± 0.9	12.9 ± 0.7	12.4 ± 1.4
Leaf blade length, mm	5.6 ± 0.6	4.3 ± 0.5	4.1 ± 0.8	3.8 ± 0.6	4.2 ± 0.5	4.1 ± 0.6
Leaf blade width, mm	4.3 ± 0.5	3.1 ± 0.2	3.0 ± 0.3	3.2 ± 0.4	3.0 ± 0.2	2.8 ± 0.2
Days to flower	38.1 ± 4.2	24.6 ± 0.6	30.6 ± 2.2	34.4 ± 6.7	23.3 ± 0.5	25.0 ± 0.8
Leaf number upon flowering	31.9 ± 6.3	8.4 ± 0.7	16.8 ± 2.9	20.2 ± 8.6	6.9 ± 0.7	7.6 ± 1.1

hypocotyl elongation, and cotyledon movements (Hicks et al., 1996; Dowson-Day and Millar, 1999). We tested the *elf3-7* mutant for each of these circadian rhythm outputs. As shown in Figure 2, in constant light the *elf3-7* mutant was arrhythmic for all three outputs (Fig. 2, A and C), just as has been found previously for the *elf3-1* mutant (Hicks et al., 1996). For example, when the criteria of Hicks et al. (1996) are applied to our data, only 12% of *elf3-7* plants (n = 26 from five experiments) showed a robust circadian rhythm of cotyledon movement, compared with 93% of wild-type plants (n = 27 from 10 experiments) and 8% of *elf3-1* plants (n = 24 from nine experiments). The pattern of *CAB::LUC* expression was also similar in *elf3-1* and *elf3-7* plants tested under a light-dark cycle followed by constant darkness (Fig. 2B). The phase advance of the first peak in darkness is less pronounced in *elf3-7* (approximately 2 h before the wild type) than in *elf3-1* (approximately 4 h before the wild type). The rapid damping of *CAB::LUC* gene expression in prolonged darkness prevented accurate scoring of later peaks.

Effects of *elf3* Mutations on Light Responses

The similar morphological phenotypes of *elf3* and *phyB* mutants suggested that ELF3 may regulate signaling by phyB or other photoreceptors. To determine whether *elf3* mutations might preferentially affect responses to any particular photoreceptor, we tested the effect of *elf3* mutations on inhibition of hypocotyl elongation by continuous red, far-red, or blue light. *phyB* mutants have a decreased hypocotyl inhibition response to red light compared with wild type, but a normal response to far-red light and only a subtle decrease in response to blue light. As shown in Figure 3, *elf3* mutants also had a decreased response to red light. This decrease was most obvious at high fluence rates, just as for the *phyB* mutant (Fig. 3A). The magnitude of the effect was similar for *elf3-1* and *elf3-7* seedlings, each of which showed a slightly greater responsiveness than *phyB-9* seedlings.

We performed similar experiments under constant far-red light and under constant blue light. In Arabidopsis, phyA mediates far-red light inhibition of hypocotyl elongation (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993), and multiple photoreceptors including cry1, cry2, phyA, phyB, and possibly other phytochromes mediate blue light inhibition of hypocotyl elongation (Koornneef et al., 1980; Young et al., 1992; Ahmad and Cashmore, 1993; Casal and Mazzella, 1998; Lin et al., 1998; Neff and Chory, 1998). As shown in Figure 3B, *elf3* seedlings had similar hypocotyl lengths as wild-type seedlings under far-red light. These data suggest that ELF3 plays little role in phyA signaling. Under blue light, *elf3-1* and *elf3-7* mutant seedlings had slightly longer hypocotyls than wild-type seedlings (Fig. 3C). However, this phenotype was far less dramatic than that of *hy4* mutant seedlings (Fig. 3C), suggesting that ELF3 plays a lesser role than cry1 does in this response.

The *elf3* seedlings also resembled *phyB* seedlings in their response to end-of-day far-red light. When given far-red light at the end of each day, hypocotyls of wild-type plants elongate more than if not given far-red light. This response is attributed to persistence in wild-type plants of phyB (and possibly other stable phytochromes) in the active Pfr form at night. When this population is converted to the inactive Pr form by a pulse of far-red light at the end of the day, the hypocotyls elongate more. Since *phyB* mutant plants lack phyB, they elongate constitutively and thus show only a slight response to end-of-day far-red treatments (Robson et al., 1993). As shown in Figure 4, *elf3-1* and *elf3-7* mutants had a reduced response, very similar to that of the *phyB-9* mutant.

Genetic Interactions between *elf3* Mutations and Photoreceptor Mutations

These hypocotyl elongation results suggested that ELF3 may participate in light responses. To explore the relations among ELF3 and photoreceptors in more detail, we constructed double mutants between *elf3-1* and *elf3-7* and each of the photoreceptor mutations *hy4-101*, *phyA-211*, and *phyB-9*. Each of these photoreceptor mutations is a putative null allele based on phenotype and biochemical data (see "Materials and Methods").

In constant red light, the *elf3-1 phyB-9* and *elf3-7 phyB-9* double mutants had longer hypocotyls than the corresponding single mutants (Fig. 3A). The *elf3-1 phyB-9* and *elf3-7 phyB-9* double mutants also had more elongated petioles than the corresponding single mutants, and flowered earlier (Fig. 1). These additive phenotypes show that, to some degree, ELF3 and phyB act independently of each other. The *elf3-1 phyB-9* double mutant retained a small end-of-day far-red response, as did each of the single mu-

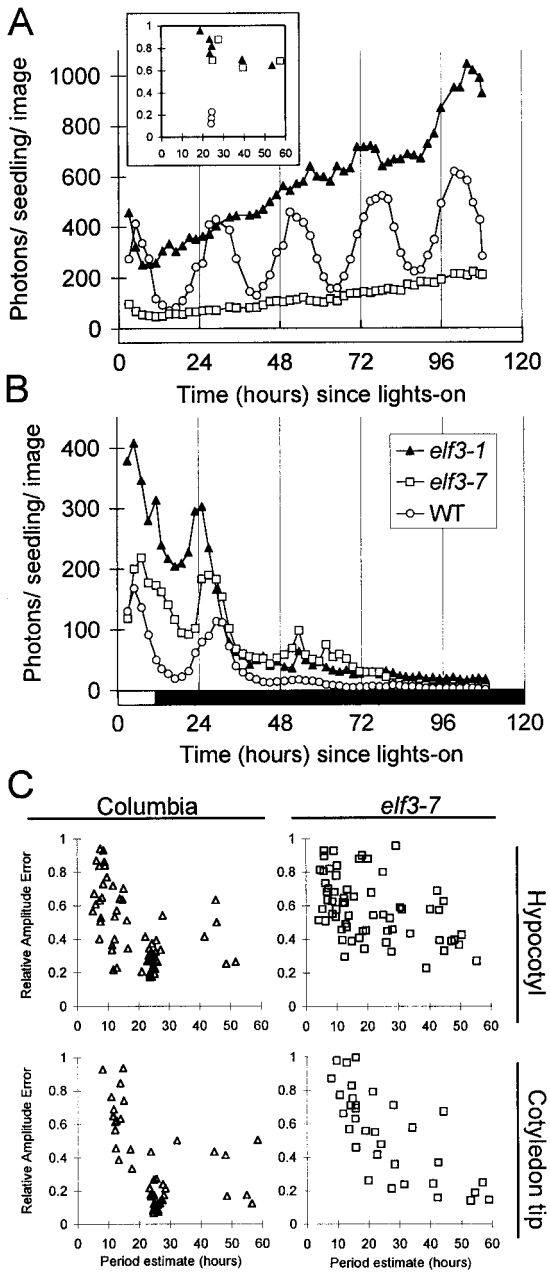

Figure 2. Circadian rhythm defects in *elf3-7*. The activity of the *CAB::LUC* reporter transgene was assayed in seedlings grown under light-dark cycles for 7 d and transferred to constant light at time 0 (A) or constant darkness at time 12 (B). Inset, Rhythmicity of wild-type *CAB2::LUC* (○; $n = 3$), *elf3-1* (▲; $n = 6$), and *elf3-7* (□; $n = 5$) plants in the experiment depicted in A was assessed by FFT-NLLS analysis, as in C. Mean expression levels in *elf3-7* were variable among experiments, falling within approximately a 4-fold range on either side of the wild-type mean (compare A and B). Black box on time axis, Dark interval; white box, light interval. C, Circadian rhythms of hypocotyl elongation (upper panels) and cotyledon movement (lower panels) were assayed in seeds germinated under two light-dark cycles and transferred to constant dim light. Rhythmicity was assessed by FFT-NLLS analysis (Dowson-Day and Millar, 1999). The clustering of data points with approximately 24-h period and low relative amplitude error (indicating robust rhythmicity) reflect the strong circadian rhythms of wild-type seedlings. *elf3-7* seedlings show a uniform scatter of periods, almost all of which have high relative amplitude error (indicating weak rhythms or noise).

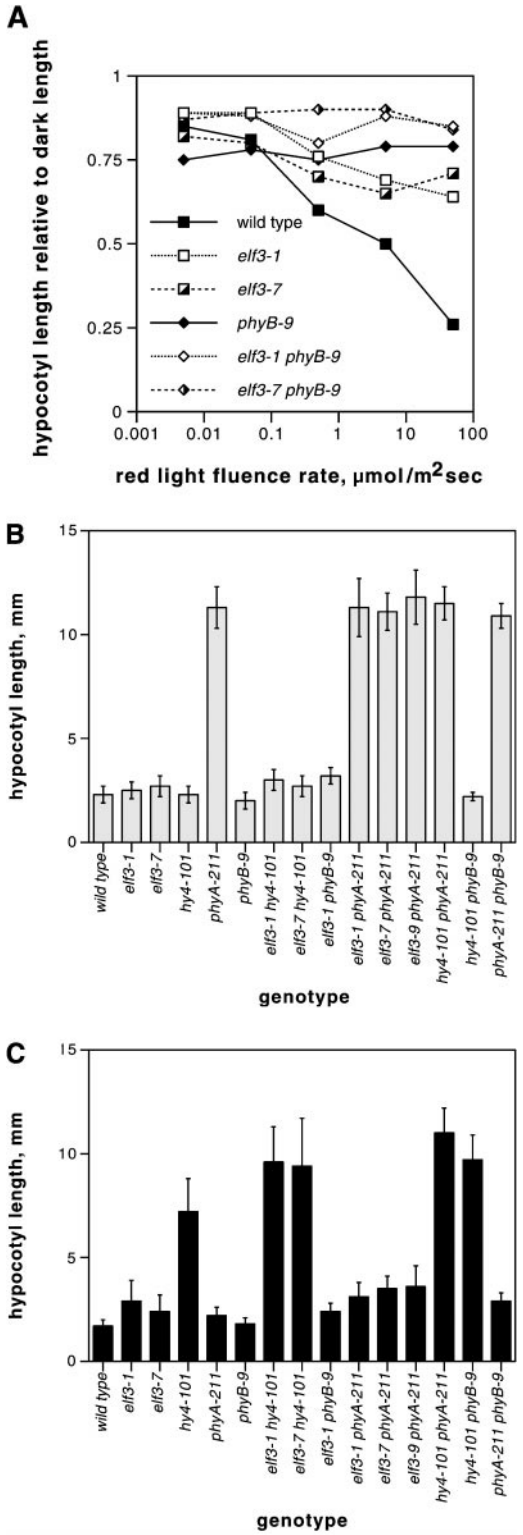

Figure 3. Hypocotyl lengths of *elf3* mutants and double mutants in different constant light conditions. A, Hypocotyl lengths under various fluence rates of constant red light, normalized to hypocotyl length in the dark. B and C, Hypocotyl lengths under constant far-red (B) and blue light (C). Between 14 and 25 seedlings were measured for each data point. SDs in A are omitted for clarity, but were generally about 20% of the mean or less, with higher relative SD at higher fluence rates.

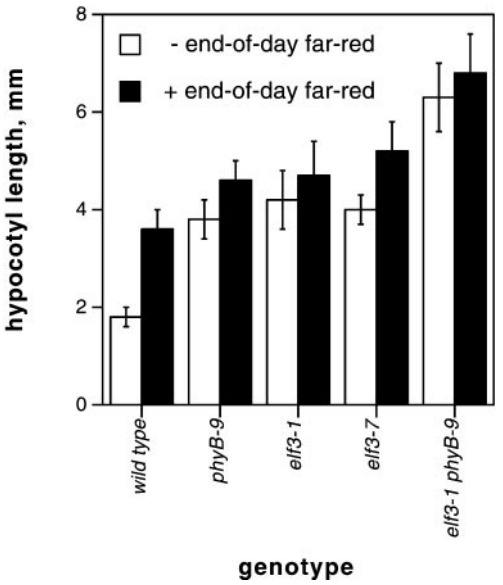

Figure 4. End-of-day far-red hypocotyl elongation response. Seedlings were grown under short-day conditions, given either no extra light or a saturating pulse of far-red light at the end of each of the 3rd, 4th, 5th, and 6th d, and the hypocotyl lengths measured on the 7th d (±SD). Between 19 and 27 seedlings were measured for each data point.

tants (Fig. 4). In constant blue light, the *elf3-1 phyB-9* double mutant had a hypocotyl length similar to that of the *elf3-1* single mutant (Fig. 3C).

The *elf3-1 phyA-211* and *elf3-7 phyA-211* double mutants had the same hypocotyl lengths as the *phyA-211* single mutant under constant far-red light (Fig. 3B), which is consistent with phyA mediating the entire hypocotyl inhibition response to far-red light (Whitelam et al., 1993) and with the lack of phenotype of *elf3* single mutants in far-red light. Under constant blue light, the *elf3-1 phyA-211* and *elf3-7 phyA-211* double mutants were as tall or slightly taller than the corresponding single mutants (Fig. 3C; data not shown).

The *elf3-1 hy4-101* and *elf3-7 hy4-101* double mutants were taller than the corresponding *elf3* and *hy4-101* single mutants in constant blue light (Fig. 3C). The *hy4-101 phyB-9* double mutant was also significantly taller than the *hy4-101* single mutant. These results show that both ELF3 and phyB can affect blue light responses in a manner that does not depend on cry1. None of these double mutants was as tall in blue light as the *hy4-101 phyA-211* double mutant (Fig. 3C). Consistent with these data, previous studies have concluded that phyA and cry1 contribute more to the blue light response than does phyB (Whitelam et al., 1993; Neff and Chory, 1998).

An *elf3* Null Mutation Does Not Eliminate *phyB*-Mediated Hypocotyl Elongation Control

The additive phenotypes in the *elf3 phyA*, *elf3 phyB*, and *elf3 hy4* double mutants reveal no functional interaction between ELF3 and these photoreceptors. However, phytochromes and cryptochromes act redundantly with each other, and this redundancy may have masked such functional interactions. As the morphological and light response phenotypes of *elf3* mutants most closely resembled those of *phyB* mutants, we performed two experiments to test more directly whether ELF3 is required for phyB function. First, we examined whether an *elf3* null mutation could suppress the effect of overexpression of phyB. Second, we tested whether an *elf3* mutation affected nuclear localization of phyB.

To test the effect of *elf3* on phenotypes caused by overexpression of PHYB, we introduced a construct that drives overexpression of PHYB (35S::*PHYB*; Hirschfeld et al., 1998) into *elf3-1* plants (see "Materials and Methods"). We

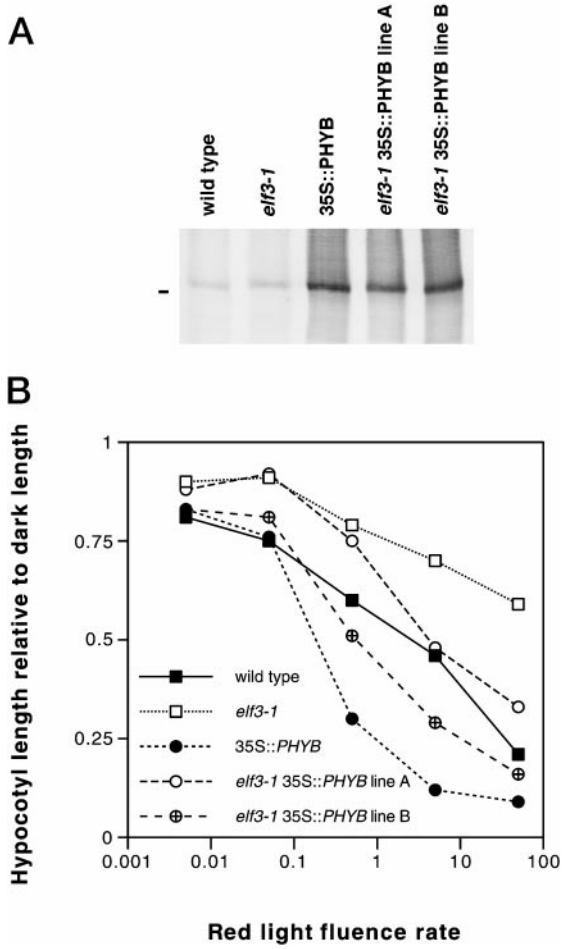

Figure 5. Effect of overexpressed PHYB protein on red light inhibition of hypocotyl elongation in wild-type and *elf3-1* seedlings. A, PHYB protein levels in 1-week-old seedlings of wild-type Columbia, *elf3-1*, Columbia 35S::*PHYB*, and two different *elf3-1* 35S::*PHYB* populations. Forty micrograms of total protein was loaded for wild-type and *elf3-1* lanes, and 10 μg of total protein was loaded for each of the 35S::*PHYB* lanes. The bar to the left of the blot marks the location of the 118-kD molecular mass marker. B, Hypocotyl length after growth of the same populations under different fluence rates of constant red light for 4 d. Between 11 and 24 seedlings were measured for each data point. SDs are omitted for clarity, but were generally about 20% of the mean or less, with higher relative SD at higher fluence rates.

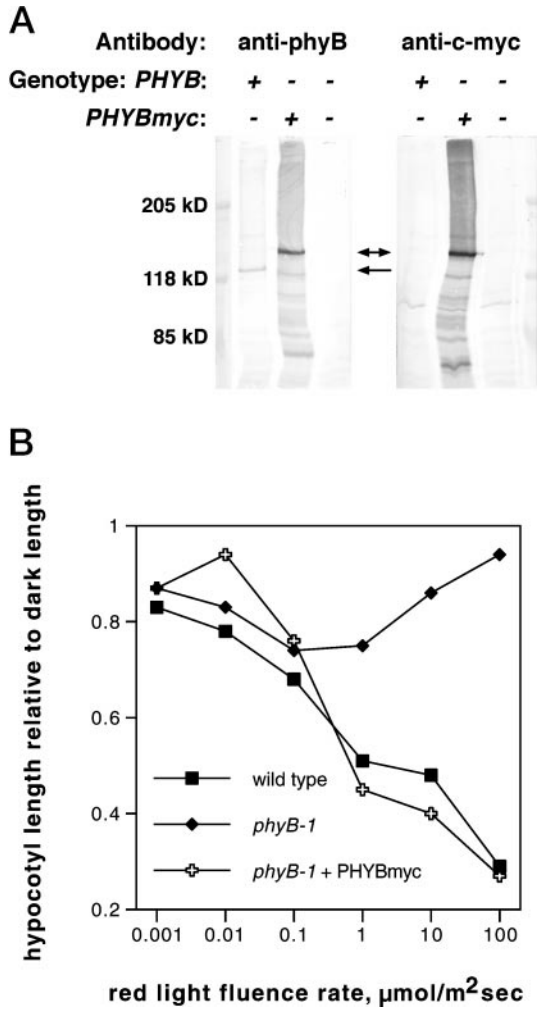

Figure 6. Rescue of a *phyB-1* mutant by PHYBmyc. A, Western blots of total proteins from wild-type, *phyB-1 PHYBmyc* transgenic, and *phyB-1* mutant plants. Duplicate blots were probed with antibody against phyB (left panel) or c-myc (right panel). Arrows indicate PHYBmyc protein (double headed arrow) or phyB protein (single-headed arrow). Equal volumes of protein extracts were loaded in each lane. B, Hypocotyl lengths relative to dark lengths of plants of the same genotypes as in A under different fluence rates of continuous red light. Between 18 and 29 seedlings were measured for each data point. SDs were generally about 20% of the mean or less, with higher relative SD at higher fluence rates, and are omitted for clarity.

crossed an *elf3-1* transgenic plant carrying this construct with a wild-type (*ELF3*) plant, and from among the progeny selected cousin *ELF3* and *elf3-1* lines expressing similar levels of PHYB protein for phenotypic analysis (Fig. 5A). PHYB overexpression in a wild-type *ELF3* background markedly enhanced the red light response (Fig. 5B), as described previously (Wagner et al., 1991; Wester et al., 1994; Hirschfeld et al., 1998). Similarly, overexpression of PHYB in the *elf3-1* background increased the red light response, although these seedlings were still taller than *ELF3* 35S::*PHYB* seedlings (Fig. 5B). Thus, the *elf3-1* mutation and the overexpressed PHYB appeared to interact additively.

Effect of ELF3 on Localization of PHYB

Western blots showed that wild-type and *elf3-1* mutant plants had similar steady-state levels of PHYB protein (Fig. 5A). Moreover, the *elf3-1* mutation still caused a decreased red light response in transgenic lines having similar levels of overexpressed PHYB (Fig. 5). These results suggest that ELF3 does not affect light responses by regulating expression or stability of PHYB, but rather affects either some downstream aspect of phyB signaling or some phyB-independent regulatory pathway.

phyB has been shown to localize to the nucleus in light-grown plants (Sakamoto and Nagatani, 1996; Kircher et al., 1999; Yamaguchi et al., 1999). To test whether ELF3 is required for phyB nuclear localization, we constructed a c-myc epitope-tagged version of PHYB and introduced this into *ELF3* and *elf3-1* transgenic plants. This PHYBmyc construct directed synthesis of a protein slightly larger than native phyB (Figs. 6A and 7) and complemented the elongated hypocotyl phenotype of a *phyB-1* mutant (Fig. 6B), indicating that the protein was biologically active.

We localized PHYBmyc protein in fixed protoplasts of light-grown transgenic plants by indirect immunofluorescence. As shown in Figure 8, PHYBmyc localized to the nucleus in protoplasts of *phyB-1* or wild-type plants carrying the PHYBmyc construct (Fig. 8, A, B, E, and F). In the *elf3-1* plants, PHYBmyc also localized to the nucleus (Fig. 8, G and H). Control experiments with wild-type (non-transgenic) plants showed only a very faint background band in western blots probed with anti-myc antibody (Fig. 6A), and no nuclear fluorescence in immunolocalization experiments (Fig. 8, C and D), indicating that the anti-c-myc antibody specifically recognized nuclear PHYBmyc in the transgenic plants. The nuclear fluorescence also depended on the presence of the anti-c-myc antibody (Fig. 8, I and J). Therefore, these experiments revealed no effect of the *elf3-1* mutation on nuclear localization of phyB.

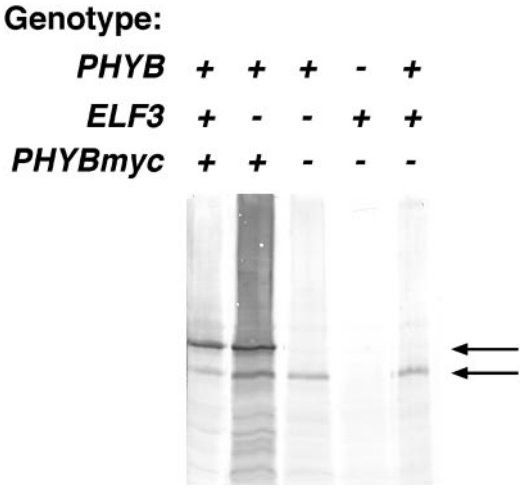

Figure 7. PHYBmyc protein in transgenic lines. Extracts of transgenic and non-transgenic lines of the indicated genotypes were probed with anti-phyB monoclonal antibody BA2. Arrows indicate positions of PHYBmyc (top arrow) and native phyB (bottom arrow). Equal volumes of protein extracts were loaded in each lane.

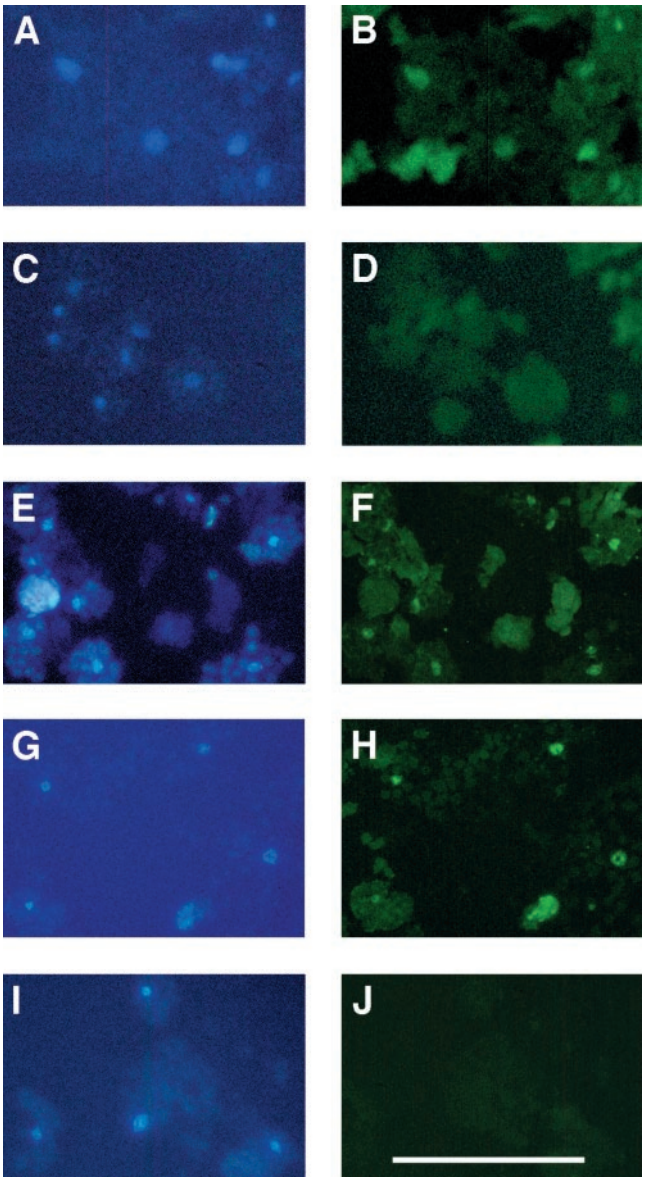

Figure 8. Immunolocalization of PHYBmyc. Protoplasts from different plant lines were fixed and stained with anti-myc primary antibody and FITC-conjugated anti-mouse secondary antibody, and stained with DAPI. A, DAPI fluorescence of *phyB-1* PHYBmyc protoplasts. B, FITC fluorescence of *phyB-1* PHYBmyc protoplasts stained with primary and secondary antibodies. C, DAPI fluorescence of wild-type (non-transgenic) protoplasts. D, FITC fluorescence of wild-type protoplasts. E, DAPI fluorescence of Columbia PHYBmyc protoplasts. F, FITC fluorescence of Columbia PHYBmyc protoplasts. G, DAPI fluorescence of *elf3-1* PHYBmyc protoplasts. H, FITC fluorescence of *elf3-1* PHYBmyc protoplasts. I, DAPI fluorescence of *elf3-1* PHYBmyc protoplasts. J, FITC fluorescence of *elf3-1* PHYBmyc protoplasts stained with secondary antibody only. Scale bar in J = 100 μm.

DISCUSSION

The similar phenotypes of *elf3* and *phyB* mutants show that ELF3 and phyB control an overlapping set of responses. However, they have a different relative importance for each response. Thus, *phyB* mutations decreased red-light-induced inhibition of hypocotyl elongation, as well as the "acute" induction of *CAB* in dark-grown seedlings, more than *elf3* mutations (Anderson et al., 1997). In contrast, *elf3* mutant plants had longer hypocotyls in blue light, and under short-day conditions had longer petioles and flowered earlier than *phyB* mutant plants. Lastly, *elf3* mutations eliminate circadian rhythm outputs in constant light (Hicks et al., 1996; Dowson-Day and Millar, 1999), whereas *phyB* mutations have quantitative effects on amplitude and period without eliminating rhythmicity (Anderson et al., 1997; Somers et al., 1998).

Our analyses of inhibition of *elf3* mutant hypocotyl elongation by different light qualities agree broadly with those of Zagotta et al. (1996), except that those investigators found a larger effect of the *elf3-1* mutation on blue light response than we did. They also found that the *elf3-1* deficiency in blue light was as severe as that of the *hy4-101* mutant, whereas in our study the *hy4-101* mutant was substantially taller than the *elf3* mutants. Different light conditions or the different ages of the seedlings measured (2 d in the previous work versus 5 d in this work) may explain this quantitative discrepancy. As discussed below, our results suggest that ELF3 cannot be considered simply as a unique phytochrome or cryptochrome signaling component. ELF3 may affect red and blue light responses by changing phytochrome or cryptochrome signaling, or by altering circadian rhythms. Altered phytochrome signaling might account for both red and blue light phenotypes, as several groups have reported effects of *phyB* mutations on blue light responses, and have seen synergistic effects between phyB and cry1 (Casal and Boccalandro, 1995; Casal and Mazzella, 1998; Neff and Chory, 1998; see also the double mutant data in Fig. 3).

Under short-day conditions, the hypocotyl length and flowering time phenotypes of *elf3-7* seedlings were intermediate between those of the wild type and the *elf3-1* null mutant. The phase of *CAB::LUC* gene expression was also intermediate in the *elf3-7* mutant grown in photoperiods of 12 h or less. While we do not know the precise effect of the *elf3-7* mutation on ELF3 protein amount or structure, the mutation probably causes a partial loss of function. Therefore, these results suggest that the absolute level of ELF3 activity may determine the severity of phenotype, at least under short-day conditions. Consistent with this idea, we have observed partial dominance of both *elf3-1* and *elf3-7* mutations for flowering time under short-day conditions (K.S. Solomon and J.W. Reed, unpublished results). It appears paradoxical that these phenotypes are more severe under short-day conditions, whereas the circadian rhythms of *elf3* plants are more aberrant in constant light. However, a photoperiodic response rhythm might also have an altered phase in the *elf3* mutants under short photoperiods, and the phase change, rather than arrhythmia, might affect flowering time and hypocotyl elongation. Further work may resolve this question.

Experiments to determine whether the phenotypic similarities between *elf3* and *phyB-9* mutants reflect ELF3 participation in phyB signaling revealed no functional inter-

action. Thus, *elf3* and *phyB-9* mutations had additive effects on hypocotyl elongation, petiole length, and flowering time; and the *elf3-1* mutation also had an additive effect on hypocotyl length in combination with the 35S::*PHYB* construct. Moreover, the *elf3-1* mutation did not prevent phyB from moving to the nucleus. Although it remains possible that ELF3 and phyB signal in a common pathway, if this were the case, then both would have to act redundantly with other proteins. phyB acts redundantly with other phytochromes to control hypocotyl elongation (phyA, phyD), petiole elongation (phyD, phyE), and flowering (phyD, phyE) (Reed et al., 1994; Aukerman et al., 1997; Devlin et al., 1998), suggesting that ELF3 could mediate signals from multiple phytochromes. The multiplicity of putative direct phytochrome signaling targets recently identified also suggests that there may be considerable redundancy downstream of phyB (Ni et al., 1998; Choi et al., 1999; Fankhauser et al., 1999).

An alternative model is that the circadian rhythm regulates many of the same outputs as phyB does, and the phenotypes of *elf3* mutants arise from a defective circadian rhythm. This would be consistent with the additive effects of the mutations and with the distinct effects of *elf3* and *phyB* mutations on circadian rhythm outputs. Red light and the circadian rhythm each control hypocotyl elongation (Dowson-Day and Millar, 1999), *CAB* gene expression (Karlin-Neumann et al., 1988; Millar and Kay, 1991), and flowering time (Smith, 1994; Lumsden and Millar, 1998).

Evaluation of this model is complicated by interactions between light and circadian signaling (Lumsden and Millar, 1998). Although phyB contributes light input signals to the circadian system, a single photoreceptor mutation has little or no effect on circadian rhythms in white light (Millar et al., 1995; Somers et al., 1998), so the phenotypes of *phyB* mutants are unlikely to arise as secondary effects of a circadian defect. However, the circadian system may rhythmically regulate (or "gate") phyB activity, or a downstream component of phyB signaling. The effectiveness of light to induce *CAB* depends on the phase of the circadian rhythm (Millar and Kay, 1996), indicating that such a model is plausible. As the acute activation of *CAB* by phytochrome is intact in *elf3-1* plants (Anderson et al., 1997), it is unlikely that *elf3* mutations uniformly suppress all phyB functions and phenocopy the *phyB* mutation as a secondary consequence of their circadian defect. These results also suggest that ELF3 and phyB act independently.

Molecular regulation at two levels may provide further insight into the mechanisms of this signaling network. First, rhythmic expression patterns have recently been discovered for the *PHYB* (Kozma-Bognar et al., 1999) and *ELF3* genes (K. Hicks and D.R. Wagner, personal communication). Second, phyB moves to the nucleus in the light. Although the *elf3-1* mutation did not prevent phyB nuclear localization, it remains possible that this localization is normally circadian. Experiments are currently under way to determine the effect of *elf3* mutations on the temporal pattern of *PHYB* gene expression and phyB nuclear localization.

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