# *EARLY FLOWERING 4* Functions in Phytochrome B-Regulated Seedling De-Etiolation[1]

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To define the functions of genes previously identified by expression profiling as being rapidly light induced under phytochrome (phy) control, we are investigating the seedling de-etiolation phenotypes of mutants carrying T-DNA insertional disruptions at these loci. Mutants at one such locus displayed reduced responsiveness to continuous red, but not continuous far-red light, suggesting a role in phyB signaling but not phyA signaling. Consistent with such a role, expression of this gene is induced by continuous red light in wild-type seedlings, but the level of induction is strongly reduced in *phyB*-null mutants. The locus encodes a novel protein that we show localizes to the nucleus, thus suggesting a function in light-regulated gene expression. Recently, this locus was identified as *EARLY FLOWERING 4*, a gene implicated in floral induction and regulating the expression of the gene *CIRCADIAN CLOCK-ASSOCIATED 1*. Together with these previous data, our findings suggest that *EARLY FLOWERING 4* functions as a signaling intermediate in phy-regulated gene expression involved in promotion of seedling de-etiolation, circadian clock function, and photoperiod perception.

Arabidopsis has developed elaborate photosensory systems to optimize its growth and development to daily and seasonal changes in the light environment. The five phytochromes (phyA–E), the two cryptochromes (cry1 and cry2), and the two phototropins (phot1 and phot2) confer sensitivity to light quality, quantity, duration, and direction (for review, see Quail, 2002). Seedlings deficient in phyA-mediated-signaling pathways are tall in continuous far-red light (FRc), and ones deficient in phyB pathways are tall in continuous red light (Rc). In addition to these influences on cell elongation and shoot morphogenesis, light also controls the transition to flowering. phy and cry have been implicated in the entrainment of the Arabidopsis circadian clock (Somers et al., 1998; also see Fankhauser and Staiger, 2002), triggering flowering under the appropriate photoperiods. For example, Arabidopsis flowers earlier under long-day than short-day conditions. This relationship between light input and clock output is important for synchronization to the environmental changes.

Microarray-based analysis has revealed that phyA regulates the expression of a master set of transcription factor genes early on during the de-etiolation process (Tepperman et al., 2001). Several of these early-response transcription regulators have G-box (CACGTG) sequences in their promoters (Hudson and Quail, 2003). The phy-interacting factor 3 (PIF3), a basic helix-loop-helix type transcription factor is capable of binding the G-box sequences in these promoters. Furthermore, light induces nuclear translocation of the biologically active (Pfr) form of phyB, which can bind to the DNA-bound PIF3 (Ni et al., 1998, 1999; Martinéz-García et al., 2000), suggesting regulation of the expression of a set of specific target genes (see Quail, 2000). Among others, the group of early light-induced genes, with G-box sequences in their promoters, includes *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), *CONSTANS* (*CO*), and *TIMING OF CHLOROPHYLL A/B BINDING PROTEIN 1* (*TOC1*)-*LIKE* genes (Tepperman et al., 2001). CCA1 and LHY are two closely related MYB-like transcription factors that when overexpressed, cause photoperiod-insensitive early flowering and circadian arhythmicity (Schaffer et al., 1998; Wang and Tobin, 1998; Green and Tobin, 1999). CCA1, LHY, and TOC1, which is a pseudoresponse regulator, function in an autoregulatory feedback loop and are integral components of the central oscillator in Arabidopsis (Harmer et al., 2001; Devlin, 2002). The evening element (EE; AAATATCT) is a known binding site for the CCA1 and LHY proteins in the *TOC1* promoter, repressing its expression (Harmer et al., 2000; Mizoguchi et al., 2002). The transcriptional feedback loop is completed by TOC1 activating the expression of CCA1 and LHY late in the day (Alabadi et al., 2001). Mutations in the *TOC1* gene result in long

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hypocotyl phenotypes in Rc and FRc (Mas et al., 2003), indicating reduced sensitivity to Rc and FRc.

Several other mutations that affect light-dependent circadian processes in Arabidopsis also exhibit altered light sensitivities in various developmental processes. *EARLY FLOWERING 3* (*ELF3*) function is required for the light sensitivity of the central oscillator in the circadian cycle (McWatters et al., 2000). This gene encodes a nuclear-localized putative transcriptional regulator that interacts with phyB (Hicks et al., 2001; Liu et al., 2001), and loss-of-function *elf3* mutant plants have a long hypocotyl phenotype (Zagotta et al., 1996). *PSEUDO RESPONSE REGULATOR 7*, related to *TOC1*, functions as a signaling intermediate in phy-regulated phasing of the circadian clock, as well as in seedling de-etiolation (Kaczorowski and Quail, 2003). *ZEITLUPE* encodes a clock-associated F-box containing protein, and the *zeitlupe* seedlings are hypersensitive to Rc (Somers et al., 2000). GIGANTEA (GI) and CO promote flowering in long-day conditions without any effect in short-day conditions (see Simpson et al., 1999). GI is a novel protein (Fowler et al., 1999) that is localized to the nucleus and is involved in seedling Rc sensitivity (Huq et al., 2000). *CO* encodes a putative zinc finger transcription factor (Putterill et al., 1995) that is induced early in a phyA-dependent manner (Tepperman et al., 2001). It promotes flowering by activating the expression of *FLOWERING LOCUS T* and *SUPPRESSOR OF OVER EXPRESSION OF CO1* (Kabayashi et al., 1999; Kardailsky et al., 1999; Samach et al., 2000). Hence, *CO* expression is light sensitive, and it underlies the photoperiodic control of flowering (Suarez-Lopez et al., 2001).

To examine the global patterns of gene expression early on during the de-etiolation process and to identify new genes involved in the early phy-signaling pathways, we performed oligonucleotide microarray analysis of the wild-type and *phyA*-null mutant Arabidopsis seedlings grown in FRc (Tepperman et al., 2001). To determine the functional relevance of these rapidly regulated genes to phy signaling, we have initiated a systematic analysis using reverse-genetic approaches to disrupt their activities. One of the genes identified as 5293 (protein accession number AAB95293) belonging to the hypothetical and unknown group (see Tepperman et al., 2001) was selected for further analysis of its potential role in early phyA-regulated transcriptional events. We identified insertional mutants at this locus and evaluated their photomorphogenic phenotypes. Toward the completion of this study, a report appeared identifying this locus as *EARLY FLOWERING 4* (*ELF4*), involved in photoperiod perception and circadian regulation (Doyle et al., 2002). Mutations in *elf4* were shown to affect the expression patterns of *CCA1*, *COLD-CIRCADIAN RHYTHM-RNA BINDING 2*, and *CHLOROPHYLL A/B BINDING PROTEIN* and to cause early flowering under short-day conditions, which was attributed to the observed elevated expression of *CO* (Doyle et al., 2002). Here, we report that ELF4 is involved in controlling phyB-mediated seedling de-etiolation and is localized to the nucleus, suggesting an intermediary signaling function between the photoreceptor and downstream light-dependent pathways, including the circadian clock.

## RESULTS

### ELF4 Is a phyA-Regulated Early-Induced Gene Belonging to a Small Arabidopsis Gene Family

A group of phyA-regulated early response genes of yet unknown function were identified by comparative oligonucleotide microarray analysis of expression in wild-type and *phyA*-null Arabidopsis 4-d-old seedlings grown in FRc for up to 24 h (Tepperman et al., 2001). One of the genes in this group, designated as *5293* by the last four digits of its protein accession number (AAB95293), was induced rapidly in FRc, exhibiting robust responsiveness within 1 h, only in the wild-type seedlings, and relatively insignificant expression levels in the *phyA* seedlings. This gene *5293* was recently identified in a screen for early flowering mutations by Doyle et al. (2002) and is therefore designated hereafter by its given name, *ELF4*.

The *ELF4* gene is predicted to encode a novel 111-amino acid protein with no significant homology to proteins of known function. *ELF4* belongs to a small but highly conserved Arabidopsis gene family (Fig. 1A; also see Doyle et al., 2002). Comparison of the predicted amino acid sequences of the family members showed that they share a high percentage of identity with ELF4 and are designated here as ELF4-Like (ELF4-L). Our database searches here have identified a new member of this family, ELF4-L3 (At2g06255) not reported previously (see Fig. 1, A and B). The ELF4 family now consists of seven members, including one from rice (ELF4Os), one from sorghum (ELF4Sb), and five from Arabidopsis (percent identities to ELF4: ELF4-L1, 53%; ELF4-L2, 47%; and ELF4-L3 and ELF4-L4, 44%). Furthermore, 26 of the 111 amino acid residues of ELF4 (about 23%) are invariant (D22, F33, Q37, L40, D41, N43, R44, L46, I47, N51, N53, H54, S56, D60, N65, V66, L68, I69, E71, N73, N75, V79, Y83, D85, L86, and F90) within the entire ELF4 protein family (Fig. 1A). Phylogenetic tree analysis of the family members (Fig. 1B) shows that ELF4 and ELF4-L1 form a separate phylogenetic subgroup. ELF4-L2 and ELF4-L4 are very closely related to each other, and they are more closely related to the other family members than to the ELF4 subgroup (Fig. 1, A and B). There are an additional 27 amino acid residues that are invariant among ELF4-L2, ELF4-L3, ELF4-L4, ELF4OS, and ELF4SB (Fig. 1A). Hence, within these other family members, a total of 53 amino acid residues are invariant. Our analysis of the *ELF4* gene structure revealed three

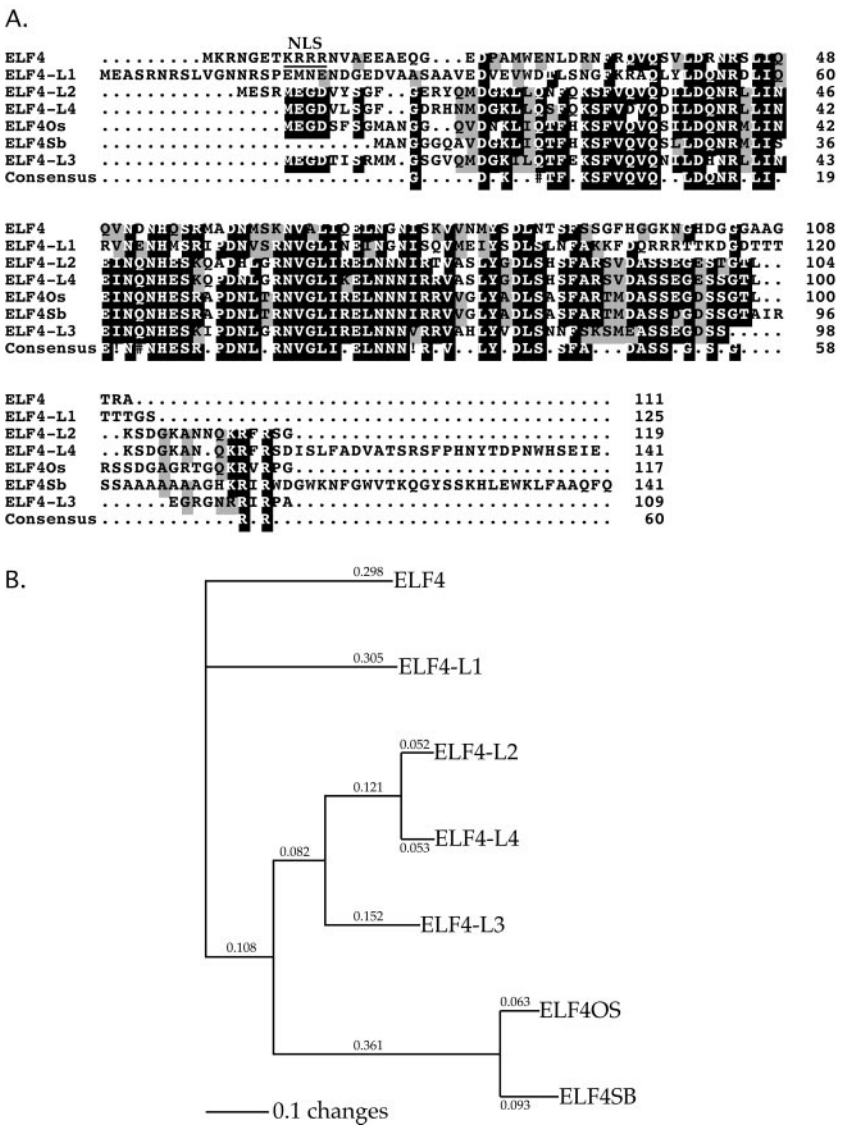



**Figure 1.** Comparison of predicted amino acid sequences of the ELF4 family. A, Amino acid sequence alignments of ELF4 protein family members, including ELF4 (At2g40080), ELF4-L1 (At2g29950), ELF4-L2 (At1g72630), ELF4-L3 (At2g06255), ELF4-L4 (At1g17455) from Arabidopsis, ELF4Os (AAD27669) from rice (*Oryza sativa*) and ELF4Sb (AAD27564) from sorghum (*Sorghum bicolor*). Reverse font, Identical residues; gray boxes, similar residues. Numbers at the right indicate amino acid residues. All sequences shown are full length, except for ELF4Sb (1–141 of the 438 amino acids shown). The alignments were performed using MultiAlign (Corpet, 1988). The putative nuclear localization signal (NLS) in ELF4 is underlined. B, Phylogenetic neighbor-joining tree of the aligned sequences. The unrooted tree was constructed using PAUP 4.0 software, showing the putative evolutionary relationships of the ELF4 family members. The branch lengths are proportional to the indicated distance values (changes) between sequences.

EEs in its promoter within 600 nucleotides upstream of the ATG start site (Fig. 2A), consistent with circadian regulation of its expression (Doyle et al., 2002).

### elf4 Mutant Seedlings Have Reduced Sensitivity to Rc during De-Etiolation

We obtained two independent T-DNA insertion lines from Syngenta Biotechnology, Inc. (Research Triangle Park, NC). *elf4-101* is predicted to have a T-DNA insertion 183 nucleotides upstream of the ATG start codon, whereas *elf4-102* is predicted to have a T-DNA insertion at 323 bases downstream of the start codon, near the C terminus (Fig. 2A). Homozygous T-DNA lines of *elf4-101* and *elf4-102* were selected and back-crossed (Columbia [Col] ecotype). A wild-type sibling (lacking the *elf4-102* insertion, designated as Col-sib) was selected as a control wild-type line for phenotypic comparisons. As shown in Figure 2B, the *elf4-101* and *elf4-102* seedlings have longer hypocotyls than the Col-sib seedlings when grown in Rc for 4 d. There was no difference in the hypocotyl lengths between the mutant and wild-type seedlings when grown in darkness or FRc for 4 d (Fig. 2B).

The *elf4* mutant seedlings were subjected to Rc and FRc fluence rate response analysis to quantitatively characterize their sensitivities to the two wavelengths. *elf4-101* and *elf4-102* seedlings are taller than the wild-type (Col-sib) seedlings at all fluence rates tested in Rc (Fig. 3A), indicating reduced sensitivity to Rc. In contrast, the *elf4* mutant seedlings are indistinguishable from the Col-sib seedlings in the FRc fluence rates tested (Fig. 3B). A second index of reduced Rc sensitivity in the de-etiolation process is cotyledon expansion. The *elf4-101* and *elf4-102* mutant seedlings have reduced expansion of cotyledon area, in comparison with the Col-sib and the *phyA-211* (Fig. 3C). In addition, the adult *elf4* mutant plants have slightly elongated petioles (data not shown).

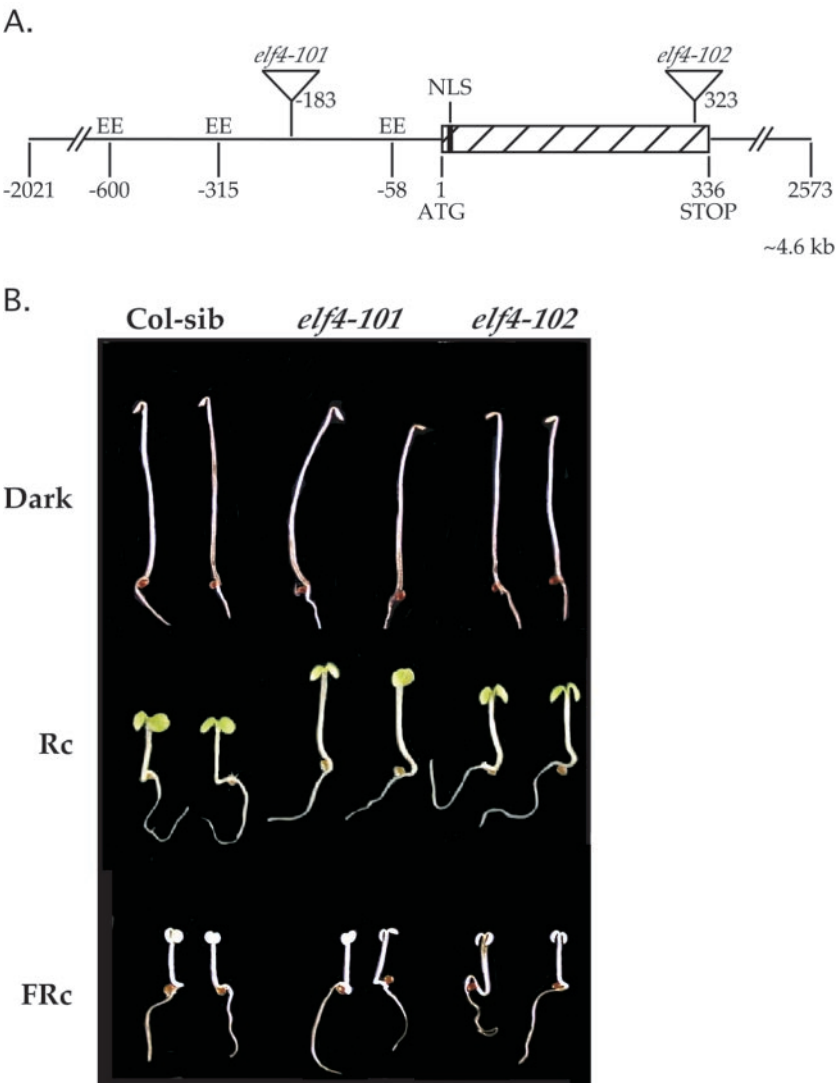



**Figure 2.** *elf4* mutant seedlings have reduced responsiveness to Rc. A, Structure of the *ELF4* gene, showing the positions of the T-DNA insertion sites in the *elf4-101* and the *elf4-102* mutants, three EEs in the promoter, and the putative NLS (black rectangle). The numbers indicate nucleotide positions relative to the first nucleotide of the ATG start codon. B, Four-day-old wild-type sibling (Col-sib), *elf4-101*, and *elf4-102* mutant seedlings grown in the dark, Rc (9.5 $\mu$mol $m^{-2}$ $s^{-1}$), or FRc (2.7 $\mu$mol $m^{-2}$ $s^{-1}$).

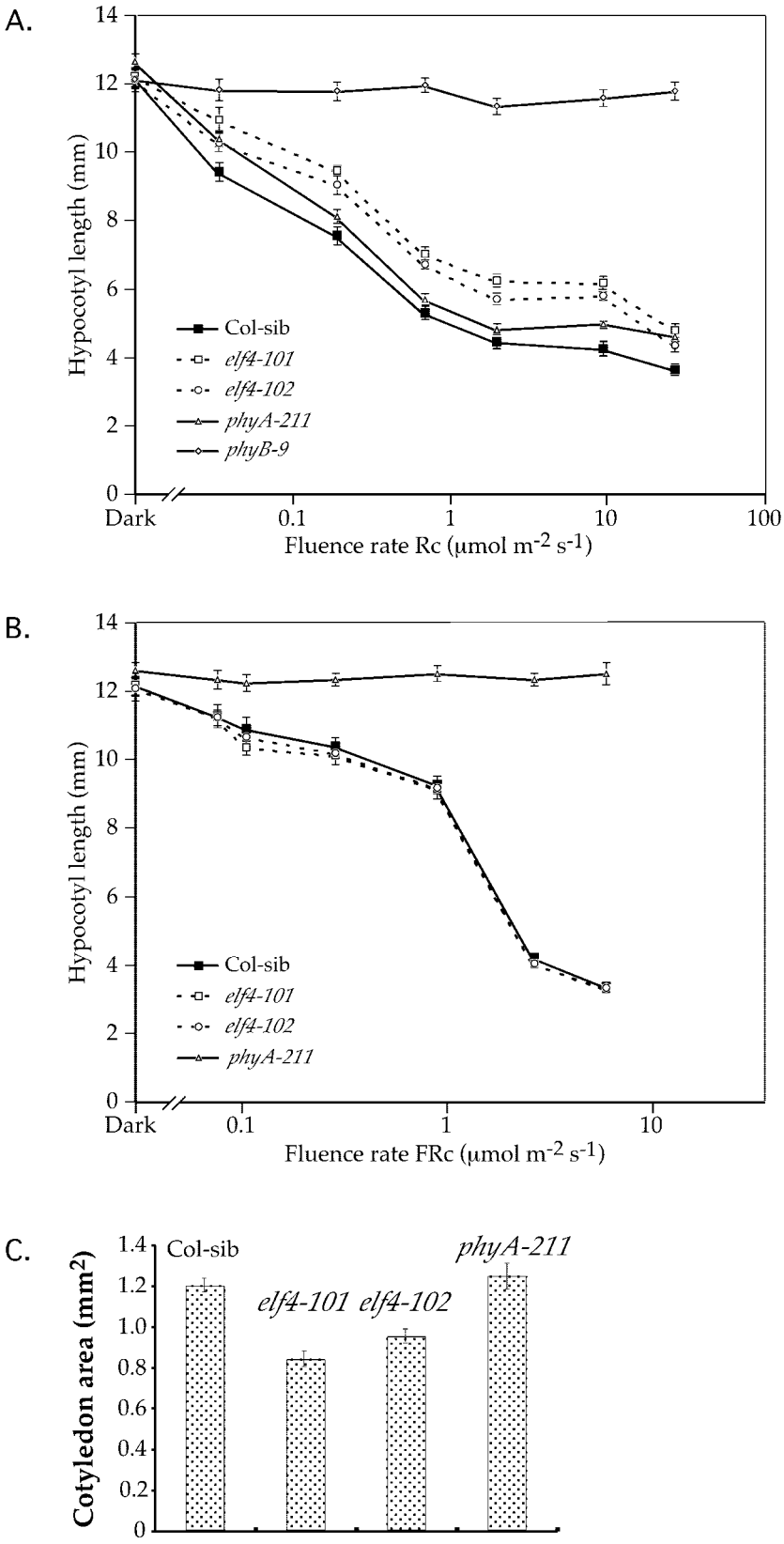
Overall, these results indicate that *elf4* mutant seedlings are hyposensitive to Rc and are impaired in phyB-mediated seedling de-etiolation.

### PhyB Is Required to Induce Normal Levels of *ELF4* Gene Expression in Rc

The *ELF4* transcript levels peak at 12 h in FRc (Tepperman et al., 2001), so we used this time point to assay the *ELF4* transcript levels in the Col-sib and the *elf4* mutant seedlings. As seen in Figure 4A, the *ELF4* transcript is present in Col-sib seedlings grown in FRc for 12 h, but the transcript was undetectable in the *elf4-101* mutant (T-DNA insertion in the promoter) seedlings (Fig. 4A). The *ELF4* transcript was also undetectable in all of the *elf4-101* mutant seedlings exposed to any of the Rc or other FRc treatments (data not shown). Hence, *elf4-101* appears to be a null allele. The *elf4-102* mutant (T-DNA insertion near the C terminus) seedlings express a smaller sized transcript at very low levels (Fig. 4A).

*ELF4* gene expression is regulated by phyA (Tepperman et al., 2001), but the *elf4* mutant seedlings show reduced Rc sensitivity (Figs. 2 and 3). To test the involvement of phyB in *ELF4* gene expression, we examined the *ELF4* transcript levels in 4-d-old Col-sib and *phyB-9* mutant seedlings grown in darkness or exposed to 6, 12, or 24 h of Rc and compared them with those observed in FRc. The pattern of expression in FRc was similar to that previously reported (Tepperman et al., 2001), with expression peaking at 12 h of FRc exposure (data not shown). Rc also induced expression in wild-type, Col-sib seedlings with a temporal pattern very similar to FRc (Fig. 4, B and C). By contrast, the *phyB-9* mutation has a considerable affect on *ELF4* expression levels in Rc (Fig. 4, B and C). Although the temporal pattern of *ELF4* expression remains similar in Col-sib and *phyB-9* mutant seedlings, *ELF4* expression is reduced in the *phyB-9* null seedlings by 3- to 4-fold (Fig. 4C). This indicates that phyB is necessary for normal induction of *ELF4* expression in response to the Rc signal. This obser-

**Figure 3.** *elf4* mutants have reduced sensitivity to Rc but not to FRc. Fluence rate response curves for Col-sib, *elf4-101*, and *elf4-102* mutants under Rc (A) and FRc (B). The *phyB-9* mutant in Rc and *phyA-211* mutant in Rc and FRc, are included for hypocotyl length comparisons. C, *elf4* mutant seedlings show reduced cotyledon expansion. Four-day-old seedlings grown in Rc (9.5 $\mu$mol $m^{-2}$ $s^{-1}$).



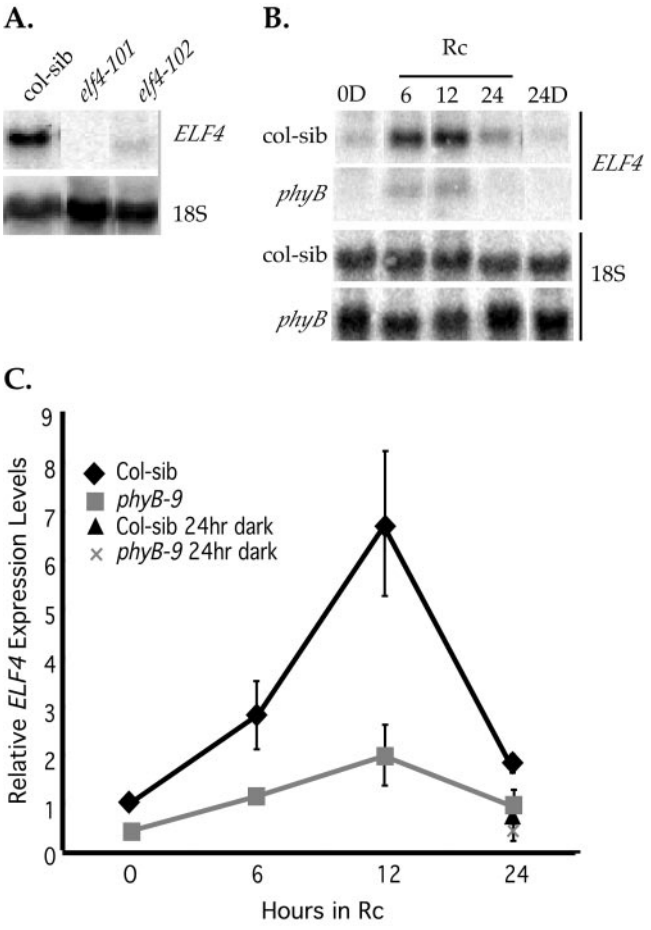



**Figure 4.** Rc-induced expression of the *ELF4* gene is phyB dependent. Representative northern blots probed with *ELF4* riboprobes. A, *ELF4* transcript levels in Col-sib, *elf4-101*, and *elf4-102* mutant seedlings grown in FRc for 12 h. B, *ELF4* expression in wild-type, Col-sib, and *phyB-9* mutant seedlings grown in Rc (7 $\mu$mol $m^{-2}$ $s^{-1}$) for 6, 12, or 24 h. RNA from dark controls (0D and 24D) are also included. C, Quantitation of the *ELF4* transcript levels from four independent replicates. The values were normalized to the 18S rRNA signal, and the mean values for each time point were plotted with SES.

vation is consistent with the *ELF4* expression profile observed in microarray-based analysis of wild-type and *phyB* mutant seedlings grown in Rc (J.M. Tepperman and P.H. Quail, unpublished data).

### ELF4 Is Localized to the Nucleus

The predicted amino acid sequence of ELF4 contains a cluster of basic residues (amino acids 8–11, KRRR) that can function as a potential NLS (marked in Figs. 1A and 2A). To investigate the subcellular localization of ELF4, an enhanced green fluorescent protein (EGFP)-ELF4 fusion driven by a 35S promoter was transiently expressed in onion epidermal cells. The green fluorescent protein (GFP) fluorescence in the transformed onion epidermal cells expressing this fusion protein is observed exclusively in the nucleus (Fig. 5, A and B). In contrast, a parallel assay with EGFP alone showed distribution throughout the cell in addition to the nucleus (Fig. 5, C and D). The passive distribution of the EGFP-alone control into the nucleus in addition to the cytoplasm is expected, due to its small size. The EGFP-ELF4 fusion, however, is present only in the nucleus and is not detectable in the cytoplasm. These results provide evidence that the ELF4 protein is likely to be targeted to the nucleus, consistent with a potential function in gene regulation.

## DISCUSSION

The expression of several genes of as yet unknown function is rapidly regulated by phyA in response to FRc light signals, as reported in our previous microarray-based analysis of wild-type and *phyA* mutant seedlings (Tepperman et al., 2001). We speculated that these genes might function in phy-regulated networks. The results presented here indicate that one of the phyA-regulated early-induced "unknown" genes, recently identified as *ELF4* (Doyle et al., 2002), is involved in the phyB-mediated Rc-induced seedling de-etiolation process (Fig. 6). The reason for the absence of a phenotype in *elf4* mutant seedlings under FRc is unknown, but the data suggest a bifurcation between phyA- and phyB-signaling pathways.

The *elf4* mutant phenotype observed in Rc appears to be attributable to the lack of normal phyB-induced expression of *ELF4* (Fig. 4). *phyB*-null mutant seedlings have a 3- to 4-fold reduction in *ELF4* expression levels in Rc compared with wild type (Fig. 4E). These data indicate that phyB is required for the normal high levels of expression in Rc. Evidently, this phyB-induced expression in Rc is important for ELF4 function, because *elf4* mutant seedlings lacking expression exhibit a Rc-specific phenotype. The Rc specificity of the phenotype suggests that ELF4 functions downstream in a phyB-regulated pathway.

There is compelling evidence that upon photoconversion to the biologically active form, the phy molecule is translocated into the nucleus (Nagy and Schäfer, 2000a, 2000b). PIF3, a basic helix-loop-helix-class transcriptional regulator, interacts specifically with the Pfr form of phy while bound to its DNA target site and appears to be involved in Rc-regulated gene expression (for review, see Quail, 2002). There is a growing list of other proteins that are also nuclear localized and implicated in phy-mediated signaling, including some that are involved in the circadian clock and photoperiod sensitivity, like ELF3, GI, TOC1, and PSEUDO RESPONSE REGULATOR 7 (Huq et al., 2000; Strayer et al., 2000; Liu et al., 2001; Kaczorowski and Quail, 2003). As we show here, ELF4 is also localized to the nucleus (Fig. 5) and may function to coregulate the expression of a subset of phyB-regulated genes. Our preliminary attempts using in vitro immunoprecipitation assays do not show

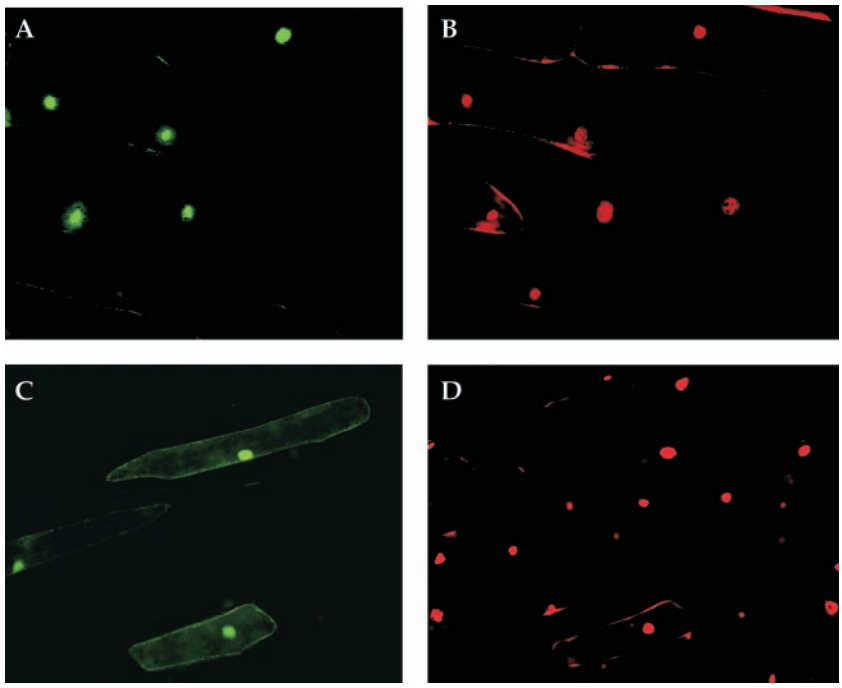



**Figure 5.** ELF4 localizes to the nucleus. Transient transfection assays in onion epidermal cells using EGFP-ELF4 constructs. A and B, Cells expressing the EGFP-ELF4 fusion protein; C and D, cells expressing the EGFP-protein control. GFP florescence (A) and propidium iodide (PI)-stained (B) nuclei show that all of the detectable EGFP-ELF4 fusion protein is nuclear localized. GFP fluorescence (C) and PI-stained (D) nuclei show that the EGFP protein control is distributed throughout the cell, including the cytoplasm and nucleus.

any evidence for direct interactions between ELF4 and the phys (data not shown).

We isolated two T-DNA insertion lines, *elf4-101* and *elf4-102*. The insertion in *elf4-101* is in the promoter region, and the *ELF4* mRNA was not detected in the mutant seedlings, indicating that this is likely to be an *elf4*-null allele. The *elf4-102* insertion is near the C terminus, and a smaller sized transcript was observed in the *elf4-102* seedlings. *elf4-101* and *elf4-102* are defective in phyB-mediated Rc responses in the hypocotyl and cotyledons. The inhibition of these Rc responses is more subtle in *elf4-102* compared with the inhibition in *elf4-101* seedlings. This difference may be attributed to reduced ELF4 functionality due to the short, lower abundance transcript and potentially truncated protein present in the *elf4-102* mutant seedlings. On the basis of these findings, it appears that ELF4 functions in a positive manner downstream in the phyB-signaling pathway, regulating Rc-induced seedling photomorphogenesis (Fig. 6). *elf4* mutants flower earlier than the wild type under short-day, noninductive conditions (Doyle et al., 2002). The *elf4-101* and *elf4-102* mutants also flower early (data not shown) and are evidently photoperiod insensitive. It has been proposed that the early-flowering phenotype is a result of the elevated levels of *CO* in the *elf4* mutants (Doyle et al., 2002). As depicted in our proposed simplified model shown in Figure 6, ELF4 might function to repress the expression of *CO*. ELF4 plays a crucial role in photoperiod perception and the control of flowering time by regulating the expression of this key gene in the flowering pathway (Doyle et al., 2002).

Arabidopsis plants lacking *ELF4* function have lower levels of *CCA1* transcripts (Doyle et al., 2002), indicating that ELF4 controls *CCA1* expression (Fig. 6). *CCA1* and *LHY* are also proposed direct targets of the phy-dependent light-induction pathway (Fig. 6), possibly via PIF3 bound to their G-box promoter elements (Martinéz-García et al., 2000; Tepperman et al., 2001; Quail, 2002). CCA1 and LHY bind to an EE in the *TOC1* promoter, repressing its expression as part of the mechanism by which the circadian oscillator functions (Fig. 6; Harmer et al., 2000; Mizoguchi et al., 2002). Our searches for promoter sequences revealed three EE in the *ELF4* promoter (Fig. 2A), suggesting that it may also be a target for repression by CCA1 and LHY (Fig. 6). Lower levels of *CCA1* and *LHY* in *elf4* mutants suggests that like TOC1, ELF4 may function to activate the transcription of these genes, thereby perhaps being closely linked to the

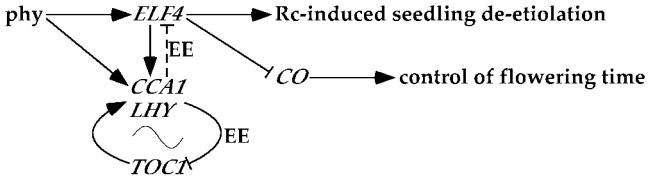



**Figure 6.** Simplified model of ELF4 function in phy-regulated seedling de-etiolation, circadian rhythms, and flowering time. *ELF4* expression is regulated by phy in response to light signals, as is the expression of *CCA1* and *LHY*. ELF4 functions as a positive regulator of phyB-mediated seedling de-etiolation. ELF4 function is closely linked to the central oscillator, thereby functioning in clock maintenance and regulating circadian rhythmicity. Like *TOC1*, *ELF4* has EE in its promoter, and it induces the expression of *CCA1*. We propose that the *ELF4* expression is regulated in a negative manner, possibly by CCA1/LHY binding to the EE in its promoter, similar to the regulation of *TOC1*. ELF4 represses *CO* expression exerting control on flowering time.

feedback loop, which is essential for the free-running clock and consistent with the view that ELF4 function is closely associated with the central oscillator (Eriksson and Miller, 2003). Together, these and previous data suggest that ELF4 functions in a phyB-mediated signaling pathway, possibly by regulating gene expression, and that ELF4 is closely associated with the central oscillator and is essential in clock maintenance and photoperiod perception.

## MATERIALS AND METHODS

### Plant Material, Growth Conditions, and Measurements

The Arabidopsis T-DNA insertion lines in Col ecotype background were identified by searching the database of the Syngenta Arabidopsis Insertion Library Project (formerly GARLIC), through an Academic collaboration with Syngenta Biotechnology. Homozygous T-DNA insertion lines were isolated using PCR with T-DNA- and gene-specific primers flanking the T-DNA insertion sites. The homozygous lines were crossed back once to Col and then isolated as *elf4-101* and *elf4-102*. One of the wild-type siblings (Col-sib), lacking the *elf4-102* T-DNA insertion was used thereon as a control.

Sterilized seeds were plated on growth medium plates without Suc (Hoecker et al., 1999), stratified for 3 d at 4°C, synchronized by a 3-h white-light (WL) treatment followed by a 21-h dark treatment at 21°C and then transferred to various light conditions as specified. The light sources used were as described (Wagner et al., 1991) and the fluence rates were monitored by using a spectroradiometer (model L1–1800, LI-COR, Lincoln, NE). Hypocotyl and cotyledon measurements were performed 96 h after the WL treatment using a digital camera (Coolpix 990, Nikon, Tokyo) and NIH Image software (National Institutes of Health, Bethesda, MD).

### RNA Isolation and Hybridization

Seeds were plated as described above. After the synchronization with WL, seeds were transferred back to the dark at 21°C for 4 d. The 4-d-old seedlings were irradiated with Rc (7 $\mu$mol $m^{-2}$ $s^{-1}$) or FRc (2 $\mu$mol $m^{-2}$ $s^{-1}$) for 6, 12, or 24 h. Dark control seedlings were harvested before (0 D) or after (24 D) the light treatments. Tissue was harvested in the dark and frozen immediately in liquid nitrogen. RNA was isolated using the Plant RNeasy kit according to the manufacturer's instructions (Qiagen,Valencia, CA). Ten micrograms of total RNA was separated on 1.2% (w/v) agarose gels containing 0.67% (w/v) formaldehyde and blotted on to Magna nylon transfer membranes (Osmonics, Westborough, MA) by capillary action in 20× SSC. Membranes were cross-linked using a UV-Crosslinker 1800 (Stratagene, La Jolla, CA). A full-length cDNA clone (expressed sequence tag, 118M24) was obtained (Arabidopsis Biological Resource Center, Columbus, OH), was sequenced to confirm the *ELF4* sequence, and was linearized at the 5′ end before being used as a template for riboprobes. The *ELF4* antisense-RNA probe was synthesized in vitro in the presence of [$\alpha$-$^{32}$P]UTP (3,000 Ci $mmol^{-1}$; Amersham Biosciences, Piscataway, NJ) using the Riboprobe Transcription System (Promega, Madison, WI). Blots were prehybridized for 2 h at 60°C, hybridized overnight, and then washed, as described (Khanna et al., 1999). Blots were then exposed overnight and visualized using a PhosphorImager (Storm 860, Molecular Dynamics, Sunnyvale, CA). The 18S probe was labeled using the Redi-Prime II kit (Amersham Biosciences) and hybridized according to Church and Gilbert (1984). The ELF4 expression levels were quantified using ImageQuant for Mac v.1.2 (Molecular Dynamics) and were normalized to the 18S rRNA signal. Four independent replicates were performed, and the mean values for each time point were plotted with SES.

### Subcellular Localization

The expressed sequence tag clone (118M24) was used as a template for PCR, using PFU Turbo polymerase (Stratagene) and primers containing internal restriction sites (*Eco*RI and *Xba*I) to amplify the full-length open reading frame of *ELF4*, which was inserted into the pEZS-CL vector (S. Cutler and D. Ehrhardt, Carnegie Institute of Washington, Stanford, CA) to create a 35S:EGFP-ELF4 construct. The construct was sequenced and used for bombarding onion epidermal peels for transient transfection assays as described (Huq et al., 2000). The nuclei were stained with 0.1 $\mu$g $mL^{-1}$ PI. A 35S:EGFP construct was used as control.

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