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FHY1: a phytochrome A-specific signal transducer

Thierry Desnos,[1,3] Pilar Puente,[1] Garry C. Whitelam,[2] and Nicholas P. Harberd[1,4]

[1]John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK; [2]Department of Biology, University of Leicester, Leicester LE1 7RH, UK

Phytochromes are plant photoreceptors that regulate plant growth and development with respect to the light environment. Following the initial light-perception event, the phytochromes initiate a signal-transduction process that eventually results in alterations in cellular behavior, including gene expression. Here we describe the molecular cloning and functional characterization of *Arabidopsis FHY1*. *FHY1* encodes a product (FHY1) that specifically transduces signals downstream of the far-red (FR) light-responsive phytochrome A (PHYA) photoreceptor. We show that FHY1 is a novel light-regulated protein that accumulates in dark (D)-grown but not in FR-grown hypocotyl cells. In addition, *FHY1* transcript levels are regulated by light, and by the product of *FHY3*, another gene implicated in FR signaling. These observations indicate that FHY1 function is both FR-signal transducing and FR-signal regulated, suggesting a negative feedback regulation of *FHY1* function. Seedlings homozygous for loss-of-function *fhy1* alleles are partially blind to FR, whereas seedlings overexpressing *FHY1* exhibit increased responses to FR, but not to white (WL) or red (R) light. The increased FR-responses conferred by overexpression of *FHY1* are abolished in a PHYA-deficient mutant background, showing that FHY1 requires a signal from PHYA for function, and cannot modulate growth independently of PHYA.

[*Key Words*: *Arabidopsis*; phytochrome; FHY1; FHY3; far-red light; signal transduction]

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Light controls the growth of plants through a network of photoreceptors (Kendrick and Kronenberg 1994). These photoreceptors display distinct photosensory and physiological properties, with growth responses to different light wavelengths being attributable to different individual photoreceptors (Kendrick and Kronenberg 1994; Quail et al. 1995). The phytochromes are a small family of red (R)/far-red (FR)-absorbing plant photoreceptors, the best understood of which are phytochrome A (PHYA) and phytochrome B (PHYB). The phytochromes exist in two distinct but interconvertible forms, R-absorbing P_R and FR-absorbing P_{FR}, and conversion from P_R to P_{FR} initiates phytochrome signaling (Quail et al. 1995).

Recent studies have resulted in several important advances in our understanding of phytochrome function. First, it has become clear that photoconversion of cytoplasmic P_R to P_{FR} causes translocation of phytochrome into the nucleus (Sakamoto and Nagatani 1996; Kircher et al. 1999; Yamaguchi et al. 1999). Thus, activation of phytochrome signaling brings phytochrome into the vicinity of the genes that it regulates. Second, P_{FR} interacts with transcriptional regulators such as PIF3 (Ni et al. 1998, 1999; Halliday et al. 1999; Martínez-Garcia et al. 2000; Zhu et al. 2000). Together, these advances have resulted in an emerging general model for phytochrome action, whereby phytochromes perceive light, enter the nucleus, interact with transcriptional regulators, and thus activate gene transcription (Martínez-Garcia et al. 2000). Other studies have also identified important cytoplasmic events in phytochrome signaling, events that possibly influence the nuclear localization of phytochrome (Choi et al. 1999; Fankhauser et al. 1999).

The phytochromes are differentially responsive to light of different wavelengths. For example, in laboratory conditions, *Arabidopsis* PHYA is uniquely responsible for the response of seedlings to continuous FR (cFR) (Dehesh et al. 1993; Whitelam et al. 1993; Quail et al. 1995). PHYA-deficient mutant seedlings are completely blind to cFR, and exhibit the etiolated phenotypes characteristic of wild-type seedlings grown in continuous dark (cD) (Dehesh et al. 1993; Whitelam et al. 1993). Mutations in *FHY1*, *FHY3*, and other loci confer cFR-specific, altered PHYA-signaling phenotypes (Whitelam et al. 1993; Hoecker et al. 1998, 1999; Soh et al. 1998, 2000; Hudson et al. 1999; Bolle et al. 2000; Büche et al. 2000; Fairchild et al. 2000; Fankhauser and Chory 2000; Hsieh et al. 2000). Although many of these loci are cloned, the functional relationships between the proteins that they encode and PHYA are largely unknown.

[3]Present address: DEVM/LMC, Bat.178, CEA cadarache, 13108 St-Paul-lez-Durance cedex, France.
[4]Corresponding author.
E-MAIL nicholas.harberd@bbsrc.ac.uk; FAX 44-1603-450025.
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Here we describe the cloning and characterization of *FHY1*. We show that *FHY1* transcript levels are regulated by light, and by the product of *FHY3*. This is the first demonstration of a functional relationship between two independent FR-signaling loci. Studies using FHY1–GFP fusion proteins show that FHY1 is found in both nucleus and cytoplasm and that its abundance is influenced by light. We also show that overexpression of FHY1 specifically enhances PHYA signaling. Plants overexpressing FHY1 exhibit enhanced responses to cFR, but not to continuous red (cR) or continuous white (cWL) light. Furthermore, the enhanced FR responses exhibited by FHY1-overexpressing plants are abolished in the absence of functional PHYA, thus showing that FHY1 does not itself have intrinsic growth-modulating activity but is involved in the transduction of the PHYA signal in the regulation of downstream FR responses.

Results

Map-based cloning of FHY1

We cloned *FHY1* using a map-based approach (Fig. 1A; Materials and Methods). *FHY1* was mapped to the bottom of chromosome 2, between markers ve017 and *DET2* (Li et al. 1996; Zachgo et al. 1996). Further recombinant mapping using markers from yeast and bacterial artificial chromosome (YAC and BAC) clones located *FHY1* on BAC F16C5 (see Materials and Methods). The *FHY1* genomic region was sequenced (GenBank accession no. AC004684) and contains several putative open reading frames (ORFs) (Fig. 1A). A contig of cosmids covering ORFs 9–16 did not complement the *fhy1-1* mutant (data not shown). In addition, a single telomeric chromosome breakpoint was detected using an ORF-21-derived RFLP marker, thus excluding ORFs 22–24 from being *FHY1*. We therefore sequenced ORFs 17–21 from the

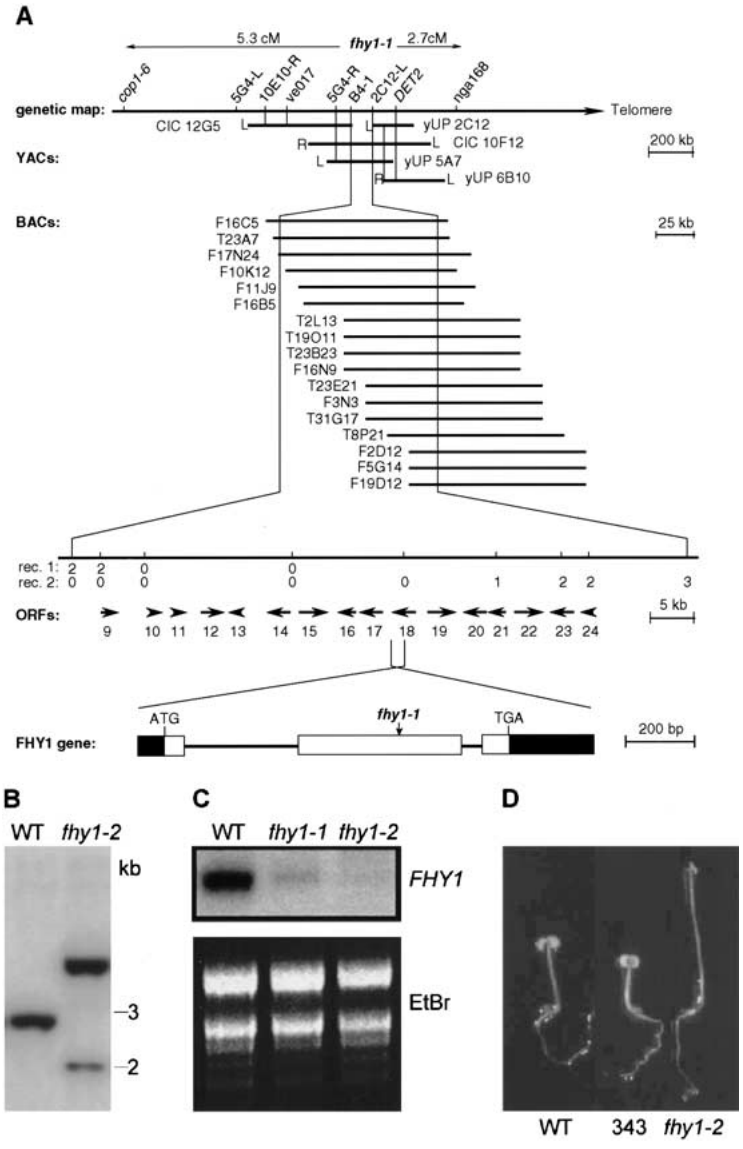

Figure 1. Molecular cloning of *FHY1*. (*A*) Genetic and physical map of part of chromosome 2 containing *FHY1*. *FHY1* was mapped using 714 (rec. 1) and 1708 (rec. 2) chromosomes that were recombinant between *FHY1* and the markers *cop1-6* and *nga168*, respectively. We positioned *FHY1* on a contig of YACs and BACs (see Materials and Methods) and identified a mutation in the sequence of ORF 18 in the *fhy1-1* mutant. The structure of *FHY1* is shown (thin line, introns; thick line, ORF; filled thick line, noncoding 5′ and 3′ transcribed sequence) with the position of the *fhy1-1* mutation. *FHY1* corresponds roughly to, but differs substantially from, the last part of the ORF 18 originally identified by the genefinder program. (*B*) Genomic DNA gel-blot analysis showing the rearrangement in *fhy1-2*. DNA was digested with *Bgl*II and probed with a radiolabeled *FHY1* probe. (*C*) An *FHY1* probe reveals *FHY1* transcripts in *FHY1*, *fhy1-1* but not *fhy1-2* plants. (*Upper* panel) RNA gel-blot hybridization of RNA from wild type (WT), *fhy1-1*, and *fhy1-2*. (*Lower* panel) UV fluorescence of RNA blotted and hybridized in panel above. (*D*) A genomic DNA fragment containing *FHY1* complements the *fhy1-2* phenotype. Seedlings grown 5 d in cFR are shown; 343 is an *fhy1-2* homozygote that is also homozygous for an *FHY1*-containing transgene.

fhy1-1 allele and from the WT progenitor and identified a single base pair deletion in ORF 18 of *fhy1-1* (Fig. 1A), thus permitting the tentative identification of *FHY1*.

Further screens for mutants exhibiting a FR-specific elongated hypocotyl phenotype had resulted in the identification of the fast-neutron-generated *fhy1-2* allele (Materials and Methods). Molecular analysis using an *FHY1* probe detected an altered restriction fragment pattern in *fhy1-2* genomic DNA (Fig. 1B). Further analysis of *fhy1-2* revealed that this mutation is caused by an inversion of an ~550-kb DNA fragment from chromosome 2. This inversion interrupts *FHY1* and disrupts the *FHY1* ORF (data not shown). In addition, *FHY1* transcripts were detected in *FHY1*, at reduced level in *fhy1-1* plants, but not in *fhy1-2* plants (Fig. 1C). These observations suggest that *fhy1-2* is a loss-of-function allele of *FHY1*. Furthermore, because the FR-response phenotypes conferred by *fhy1-1* and *fhy1-2* are indistinguishable (data not shown), it is likely that *fhy1-1* (which potentially encodes a prematurely truncated protein; see below) is also a loss-of-function allele of *FHY1*. Final proof that the gene tentatively identified as *FHY1* is indeed *FHY1* was obtained via genetic complementation. When grown in cFR, the *fhy1-2* mutant displays an elongated hypocotyl and unexpanded cotyledons (Fig. 1D). A 2.1-kb genomic fragment, spanning 795 bp upstream to 304 bp downstream of the *FHY1* coding sequence, fully complemented *fhy1-2*, conferring a phenotype resembling that of the wild-type control (short hypocotyl and expanded cotyledons in cFR; Fig. 1D).

FHY1 encodes a predicted polypeptide (FHY1) of 23 kD (202 amino acids) that is rich in serine and glutamic acid residues (Fig. 2A). FHY1 has no obvious overall similarity to any protein of known function, and contains putative nuclear localization sequence (NLS) and nuclear export sequence (NES) domains (Fig. 2A; Nigg 1997). *fhy1-1* lacks a single adenine residue 671 bp downstream of the *FHY1* start codon, resulting in a premature STOP codon (Fig. 2A). Database searches revealed the existence of a second *Arabidopsis* ORF that encodes a predicted protein that is related in sequence to FHY1, together with *FHY1*-related ESTs from soybean and tomato (data not shown). Interestingly, FHY1 contains a region of shared homology with part of the PHYA PAS-A domain (Fig. 2B; Quail et al. 1995). The biological significance of this shared homology is unknown, because it does not overlap with the region of the PAS domain that is conserved in PAS-domain proteins involved in a variety of roles from photoperception to circadian clock function (Heintzen et al. 2001).

FHY1 *expression is regulated by light and by* FHY3

It seemed likely that seedling FHY1 levels are related to morphogenesis in cFR, for the *fhy1-1* loss-of-function allele confers a cFR-specific elongated hypocotyl phenotype (Whitelam et al. 1993). We addressed this possibility in two ways. First, we investigated the environmental and genetic factors that regulate *FHY1* gene expression. Second, we studied the effect of increased expression of *FHY1* on morphogenesis in cFR (see below).

We examined *FHY1* mRNA levels in seedlings grown in cD and in various light conditions: cWL, cR, and cFR (Fig. 3A). In wild-type seedlings, *FHY1* mRNA levels accumulated to higher levels in cD than in any light condition, suggesting that light negatively regulates *FHY1* gene expression. In cFR, but not in cWL or cR, *FHY1* transcript levels were higher in PHYA-deficient *phyA-201* seedlings than in wild-type seedlings (Fig. 3A).

Figure 2. FHY1 sequence. (*A*) Predicted amino acid sequence of FHY1. Arrowheads indicate intron position; the putative NLS is single-underlined; the putative NES is underlined with a dotted line (for consensus NLS and NES sequences, see Nigg 1997). A region of homology with the phytochrome PAS-A domain (Quail et al. 1995) is double-underlined (see *B*). (*B*) Sequence alignment of a region conserved between FHY1, PHYA, and phytochrome C (PHYC). Numbers refer to the N-terminal residue position for each respective sequence. Identical residues are shown on a black background, similar residues are shown on a gray background. The SwissProt accession nos. are P14712: *Arabidopsis thaliana* PHYA [PhyA (A. t.)]; P06593: *Avena sativa* PHYA [PhyA (A. s.)], and P14714: *Arabidopsis thaliana* PHYC [PhyC (A. t.)].

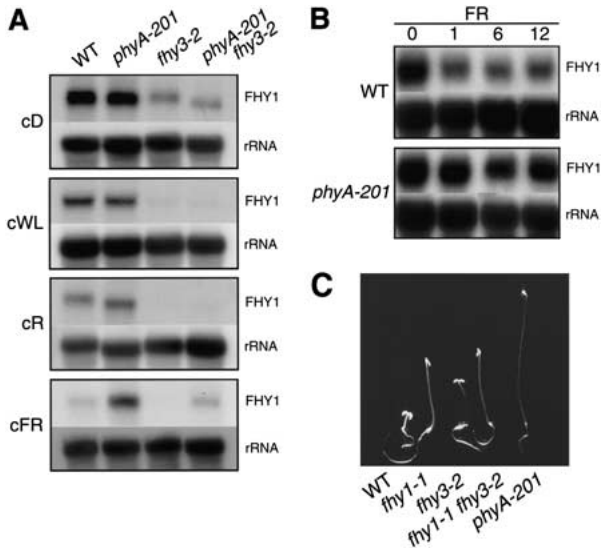

Figure 3. *FHY1* mRNA level is regulated by light and by *FHY3*. (*A*) RNA gel-blot analysis of *FHY1* transcripts in seedlings grown for 5 d in cD, cWL, cR, or cFR. (*B*) RNA gel-blot analysis of *FHY1* transcripts in wild-type (WT) seedlings grown for 3 d in cD and then exposed to cFR for 0–12 h. In *A* and *B*, loading control hybridization with rDNA probe is shown. (*C*) Phenotype of seedlings grown for 5 d in cFR.

Therefore, PHYA reduces *FHY1* mRNA levels in cFR, and other photoreceptors do the same in cWL and cR.

When etiolated wild-type seedlings were moved into cFR, *FHY1* transcript levels decreased within an hour of exposure, and then remained unchanged (Fig. 3B). This response was not observed in *phyA-201* seedlings (Fig. 3B), and is therefore PHYA-dependent. This rapid PHYA-dependent down-regulation of *FHY1* mRNA levels indicates the existence of a negative feedback loop, whereby PHYA influences the abundance of *FHY1* transcripts, and possibly of FHY1 itself (see below).

Like the *fhy1-1* and *fhy3-2* single mutants, the *fhy1-1 fhy3-2* double mutant displayed a reduced cFR response (Fig. 3C). However, the cFR-grown *fhy1-1 fhy3-2* hypocotyl was only slightly longer than that of *fhy1-1* (in both standard and nonsaturating cFR conditions; Fig. 3C; data not shown), and a strongly synergistic, cFR-blind phenotype was not observed. This observation suggests that FHY1 and FHY3 may act in the same pathway, and that, in addition to the FHY1–FHY3 pathway, additional pathways may mediate the cFR response. Alternatively, FHY1 and FHY3 may act in separate pathways. We investigated *FHY1* mRNA levels in *fhy3-2* seedlings (Fig. 3A). In all conditions, *fhy3-2* seedlings accumulated less *FHY1* transcript than did wild-type seedlings, showing that *FHY3* positively regulates *FHY1* mRNA levels, and suggesting that FHY1 acts downstream of FHY3. Furthermore, *fhy3-2* seedlings still exhibited light-dependent reductions in *FHY1* mRNA levels (Fig. 3A), suggesting that these reductions are not *FHY3*-dependent.

Because cFR and *FHY3* have opposite effects on *FHY1* transcript levels, *FHY1* expression in a *phyA-201 fhy3-2* double mutant was investigated. In cFR, *FHY1* mRNA levels in *phyA-201 fhy3-2* were intermediate between those of *phyA-201* and *fhy3-2*, and were similar to those of wild type (Fig. 3A). This suggests that PHYA and FHY3 regulate *FHY1* transcript levels independently of each other.

Increased FHY1 *expression specifically enhances the cFR response*

The above observations on *FHY1* expression present a paradox: reductions in *FHY1* function (in *fhy1* loss-of-function mutants) reduce the cFR response, but apparent reductions in *FHY1* function (via reduced *FHY1* transcript levels) are also part of the cFR response. To resolve this paradox we investigated the consequences of increasing *FHY1* expression (in a way that was not subject to down-regulation by light). *fhy1-2* plants that expressed *FHY1* under the control of the *CaMV 35S* promoter had elevated *FHY1* transcript levels in cD, cWL, and cFR (Fig. 4A). These plants exhibited an enhanced cFR response, being more deetiolated in cFR than wild-type plants (Fig. 4B,C). Therefore, overexpression of *FHY1* enhances the FR response above that of wild-type plants. To determine if this effect was FR-specific, plants overexpressing *FHY1* were also grown in cD, cR, and cWL (Fig. 4C,D,E, respectively). In all cases, *FHY1*-overexpressing plants were indistinguishable from wild-type controls, thereby showing that the effect of *FHY1* overexpression is light-dependent and FR-specific. Furthermore, these observations suggest that the paradox outlined above can be resolved if *FHY1* function is (in normal plants) subject to negative feedback regulation via PHYA at the transcriptional level (as shown above).

FHY1 requires a signal from PHYA to effect a cFR response

We investigated the effect of *FHY1* overexpression in the PHYA-deficient *phyA-201* mutant. As shown in Figure 5A, *phyA-201* plants containing the *35S::FHY1* construct are blind to cFR, and indistinguishable from control *phyA-201* plants. To confirm that these plants were expressing the *35S::FHY1* construct, RNA gel-blot analysis showed that the *phyA-201 35S::FHY1* line accumulated *FHY1* transcripts to levels comparable with that seen in the control *35S::FHY1* line (Fig. 5B). Therefore, overexpression of *FHY1* causes enhanced cFR responses in the presence (Fig. 4B,C), but not in the absence (Fig. 5A), of PHYA. FHY1 cannot influence growth in the absence of a signal from PHYA, suggesting that the enhanced FR responses of plants overexpressing *FHY1* (in the presence of PHYA) are attributable to a *FHY1* overexpression-dependent enhancement of the PHYA signal.

In further experiments, we investigated the consequences of *FHY1* overexpression in mutant plants containing a partially defective PHYA (Fig. 5C). The *phyA-3* and *phyA-4* alleles encode mutant PHYA polypeptides whose function is reduced, but not completely abol-

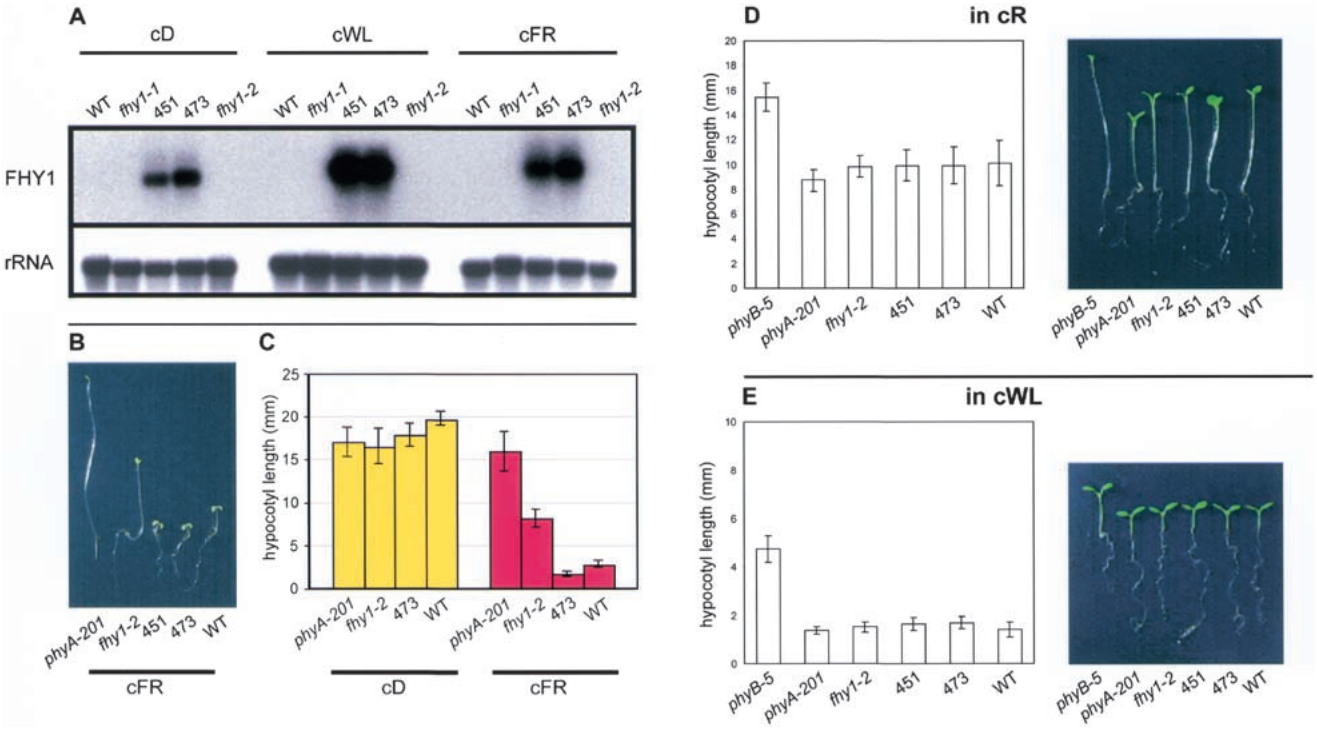

Figure 4. Seedlings overexpressing FHY1 exhibit enhanced cFR responses. (*A*) Plants containing the *35S::FHY1* construct have elevated levels of *FHY1* transcript. (*Upper* panel) *FHY1* transcripts are detectable in 451 and 473 (*fhy1-2* lines containing a *35S::FHY1* transgene) but not detectable in wild type (WT), *fhy1-1*, or *fhy1-2* in cD, cWL, or cFR. This was a shorter exposure than in Figure 3A, thus explaining why *FHY1* transcripts are visible in cD-grown WT in Figure 3A but not in this figure. (*Lower* panel) rRNA loading control for hybridization in *upper* panel. (*B*) *35S::FHY1;fhy1-2* hypocotyls exhibit enhanced responses to cFR. Seedlings from lines 451 and 473 (*35S::FHY1;fhy1-2*) have shorter hypocotyls in cFR than WT, *fhy1-2*, or *phyA-201*. (*C*) Histogram shows mean and standard error of hypocotyl lengths of seedlings grown in cD and cFR (n = 25–38). Seedlings grown in cD are indistinguishable from one another. 473 (*35S:FHY1;fhy1-2*) hypocotyls are significantly shorter than WT hypocotyls in cFR. (*D*) *35S::FHY1;fhy1-2* hypocotyls do not exhibit enhanced responses to cR. Photograph and histogram of seedlings of various genotypes. Histogram shows mean and standard error of hypocotyl lengths (n = 24–28). For reference, the *phyB-5* mutant, which exhibits a long hypocotyl in cR, is shown. The 451 and 473 lines are indistinguishable from WT. (*E*) *35S::FHY1;fhy1-2* hypocotyls do not exhibit enhanced responses to cWL. Photograph and histogram of seedlings of various genotypes. Histogram shows mean and standard error of hypocotyl lengths (n = 18–23). The 451 and 473 lines are indistinguishable from WT.

ished. For example, *phyA-3* confers a hypocotyl that is taller than wild type, but shorter than that conferred by the *phyA-201* null allele in high-intensity cFR (Hi-cFR), indicating that PHYA-3 function is reduced compared with that of PHYA (Fig. 5C). In low-intensity cFR (Lo-cFR), the *phyA-3* and *phyA-201* hypocotyls are of a more similar length (Fig. 5C). When the *35S:FHY1* construct was introduced into *phyA-3*, it partially suppressed the cFR phenotype of *phyA-3*. In Hi-cFR conditions, the *phyA-3 35S::FHY1* line (043) had shorter hypocotyls than the *phyA-3* controls, an effect that was less apparent in Lo-cFR (Fig. 5C). These effects are cFR-dependent, because, when grown in cD, these lines were fully etiolated (data not shown). Furthermore, the *35S::FHY1* construct also suppressed the phenotype of *phyA-4* (data not shown), indicating that *FHY1* overexpression suppresses the general effects of a reduced PHYA signal, rather than any specific effects of the individual mutant *PHYA* alleles. These observations suggest that overexpression of *FHY1* enhances responses to a reduced PHYA signal.

FHY1–GFP fusion proteins can be detected in the hypocotyl cells of etiolated plants

The intracellular localization of FHY1 was investigated using plants containing a transgene construct that expressed a FHY1–GFP fusion protein under the control of the *FHY1* promoter. Preliminary experiments showed that this construct complemented the *fhy1-2* phenotype, thus showing that the FHY1–GFP fusion protein has FHY1 function (data not shown).

Fluorescence microscopy was used to determine the intracellular location of the FHY1–GFP fusion protein. As shown in Figure 6, strong fluorescence was observed in the nuclei of the hypocotyl cells of cD-grown FHY1–GFP-expressing plants, but not in hypocotyl nuclei of cFR-grown FHY1–GFP-expressing plants, or of cD- or cFR-grown control (nontransgenic) plants. In addition, as shown in Figure 7A, weak fluorescence was also observed in the cytoplasm of the hypocotyl cells of cD-grown FHY1–GFP-expressing plants. This fluorescence

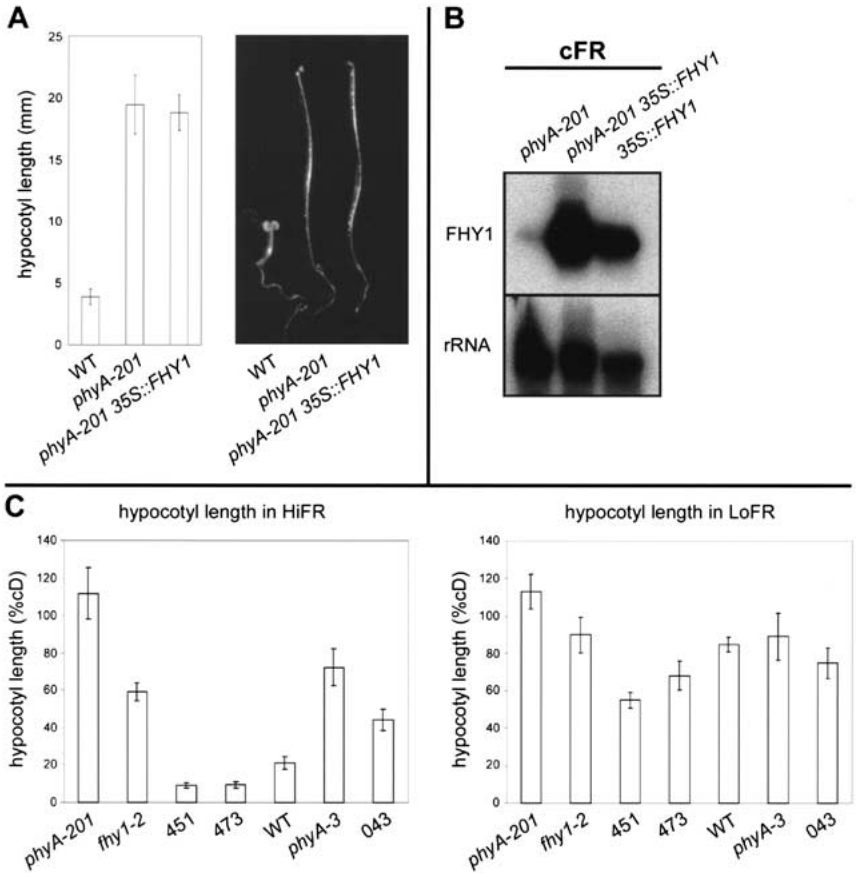

Figure 5. The enhanced FR responses of seedlings overexpressing FHY1 are PHYA-dependent. (*A*) Photograph and histogram of seedlings of various genotypes grown in cFR. Histogram shows mean and standard error of hypocotyl lengths ($n = 29$). *phyA-201* and *phyA-201 35S::FHY1* seedlings have indistinguishable phenotypes in FR. (*B*) RNA gel-blot hybridization showing that *phyA-201 35S::FHY1* seedlings have similar levels of *FHY1* transcripts to *35S::FHY1* seedlings. (*Upper* panel) *FHY1* transcripts; (*lower* panel) ribosomal RNA (loading control). (*C*) Hypocotyl lengths of various genotypes grown in high-intensity (HiFR) and low-intensity (LoFR) cFR. Histograms show mean and standard error of hypocotyl lengths ($n = 23$–32). For comparison, seedlings from lines 451 and 473 (*35S::FHY1 fhy1-2* lines) are also shown.

was clearly brighter than the background fluorescence seen in nontransgenic controls (Fig. 7A). Therefore, FHY1–GFP is distributed (in a manner similar to that of GFP itself; Fig. 7A) between the nucleus and the cytoplasm of cD-grown hypocotyls, although the nuclear fluorescence is stronger than that of the cytoplasm. In further experiments, we looked to see if the nuclear localization of FHY1–GFP would change rapidly following

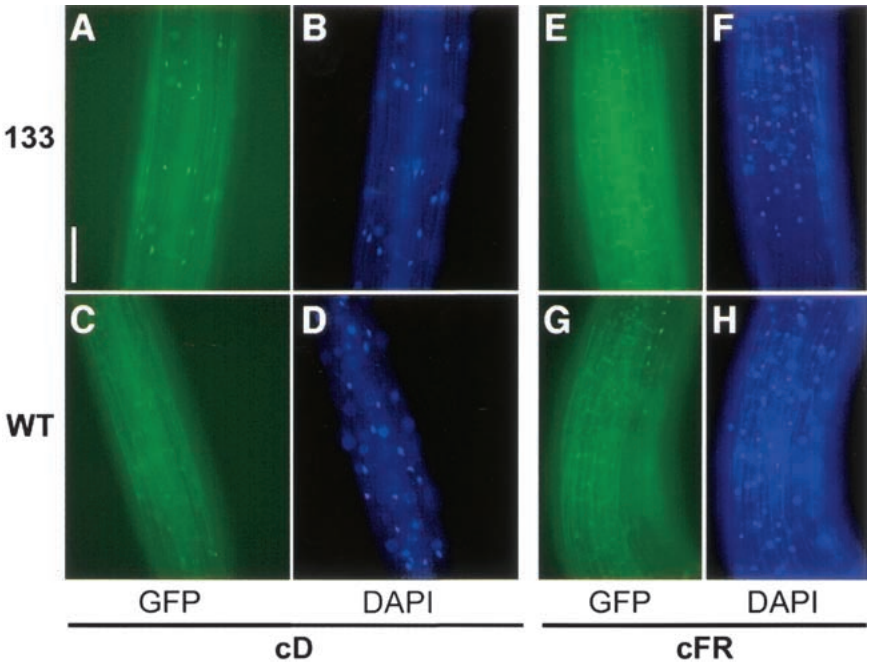

Figure 6. FHY1–GFP is detected in cD-grown hypocotyls. Line 133 expresses FHY1–GFP in an *fhy1-2* genetic background. GFP is detected in nuclei and cytoplasm of cD-grown 133 hypocotyls (cf. same hypocotyl in *A* [GFP fluorescence] and *B* [DAPI-stained nuclei]), but not in WT hypocotyls (that lack the FHY1–GFP expressing transgene, cf. *C* and *D*). GFP fluorescence is not detected in cells of cFR-grown 133 or WT hypocotyls (cf. *E* with *F*, *G* with *H*). All GFP fluorescence images were obtained using the same camera set-up parameters. Therefore, FHY1–GFP accumulates in the cells of cD-grown hypocotyls but not in the cells of cFR-grown hypocotyls. Bar, 100 μm. Seedlings were 3 d old.

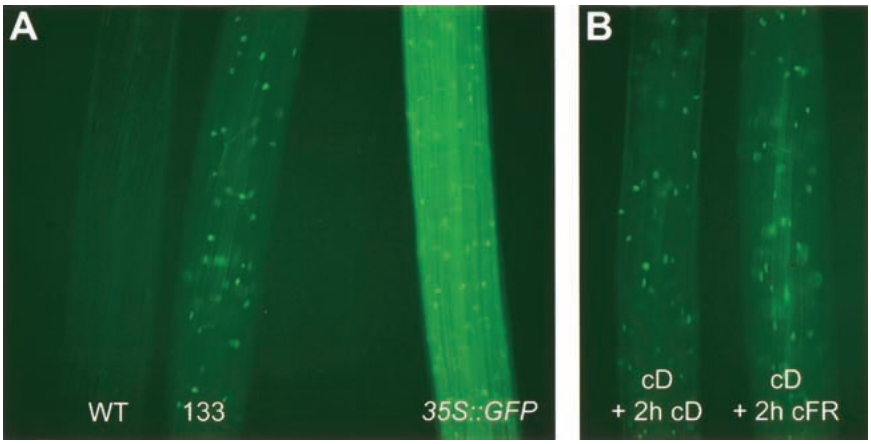

Figure 7. Nuclear FHY1–GFP is not rapidly relocalized or destroyed following transfer of hypocotyls from cD to light. (*A*) GFP fluorescence is clearly detectable in hypocotyl nuclei and cytoplasm of cD-grown 3-day-old 133 and *35S::GFP* seedlings, but not in WT seedlings. (*B*) Compared with 3-day-old cD-grown 133 seedlings (cD + 2 h cD), GFP fluorescence is still detectable in the hypocotyl nuclei of cD-grown 133 seedlings transferred into cFR for 2 h (cD + 2 h cFR). Bar, 100 µm.

transfer of seedlings from cD to cFR (Fig. 7B). These experiments showed that the nuclear fluorescence attributed to FHY1–GFP was still detectable 2 h after transfer to cFR. In addition, there appeared to be a slight increase in cytoplasmic fluorescence following 2 h of cFR. However, the fact that clear nuclear fluorescence was retained following the 2-h cFR treatment suggests that nuclear FHY1–GFP is not rapidly relocalized or destroyed following transfer of hypocotyls from cD to cFR. Additional experiments showed that fluorescence due to FHY1–GFP in seedlings first grown in cD is reduced by subsequent light treatments of longer duration. We observed that 24 h of cFR was needed to reduce the fluorescence to almost undetectable levels (data not shown). Interestingly, treatments with cB, cR, or cWL resulted in a more rapid loss, with fluorescence essentially disappearing between 4 and 12 h of the onset of exposure (data not shown).

Discussion

Phytochrome action involves light-dependent nuclear targeting, light-dependent phosphorylation of one or more signaling component substrates, and the regulation of nuclear gene expression via transcription factors (Quail et al. 1995; Ni et al. 1998, 1999; Yeh and Lagarias 1998; Choi et al. 1999; Fankhauser et al. 1999; Halliday et al. 1999; Kircher et al. 1999; Yamaguchi et al. 1999; Martínez-Garcia et al. 2000). In addition, there is evidence suggesting the involvement of GTP-binding proteins, cGMP, and Ca^{2+}/calmodulin (CaM) in phytochrome signaling (Neuhaus et al. 1993; Bowler et al. 1994). Although PHYA likely operates via these general mechanisms, there is clear evidence for the existence of additional components, some of them nuclear, that appear to be more specific for PHYA signaling (Hoecker et al. 1999; Hudson et al. 1999; Bolle et al. 2000; Fairchild et al. 2000; Hsieh et al. 2000; Soh et al. 2000). In this paper we describe the isolation and characterization of *FHY1*, a gene encoding the PHYA-specific signaling component FHY1. Previous experiments indicated that FHY1 links PHYA to gene activation via a cGMP-dependent pathway (Barnes et al. 1996), or that FHY1 functions within a PHYA signaling pathway that involves a Gα subunit (Okamoto et al. 2001). Here we have shown that *FHY1* transcript levels are regulated by light and by the product of *FHY3*, another gene involved in FR-signaling (Whitelam et al. 1993). We have also shown that overexpression of FHY1 confers an exaggerated response to cFR, and unaltered responses to cD, cR, and cWL. This, together with the observation that loss-of-function *fhy1* alleles confer a FR-specific phenotype (Whitelam et al. 1993), shows that FHY1 function is specifically related to FR-signaling. Furthermore, we have shown that overexpression of FHY1 confers increased responses to cFR only in the presence of functional PHYA, thus confirming that FHY1 acts in PHYA-signaling and has no intrinsic effect in promoting FR responses.

FHY1 presumably operates within the context of the action of other FR-specific signaling components, such as those encoded by the *FHY3*, *SPA1*, *PAT1*, *FIN2*, *FAR1*, *FIN219*, *HFR1*, and *REP1* loci (Whitelam et al. 1993; Soh et al. 1998; Hoecker et al. 1998, 1999; Hudson et al. 1999; Bolle et al. 2000; Fairchild et al. 2000; Hsieh et al. 2000; Soh et al. 2000). Some of these components have been identified and partially characterized, and it is instructive to attempt to draw together these various observations to provide an overall view of how PHYA-signaling works. As outlined above, PHYA exists in the P_R form in the cytoplasm and, upon conversion to P_{FR}, becomes localized in the nucleus. Two of the above loci, *PAT1* and *FIN219*, encode products that appear to be cytoplasmically localized (Bolle et al. 2000; Hsieh et al. 2000). Perhaps PAT1 and FIN219 are involved with cytoplasmic stages of PHYA-signaling, or with the targeting of the P_{FR} form of PHYA to the nucleus. All of the other FR-signaling loci cloned to date appear to encode nuclear factors: SPA1 is a nuclear WD-repeat protein that acts as a negative regulator of FR-signaling (Hoecker et al. 1999), FAR1 is a novel nuclear protein of unknown function that appears to be a positive regulator of FR-signaling (Hudson et al. 1999), and HFR1 (also known as REP1) is a positive regulator of FR-signaling and a member of the basic helix–loop–helix (bHLH) family of DNA-binding

proteins (Fairchild et al. 2000; Soh et al. 2000). FHY1, as shown here, is a novel protein that can exist in both nucleus and cytoplasm and that acts as a positive regulator of FR-signaling. Presumably the various nuclear factors act together with PHYA within complexes that regulate the transcription of PHYA-regulated genes. Because some mutants, such as the *hfr1* and *fhy1* mutants (Whitelam et al. 1993; Johnson et al. 1994; Barnes et al. 1996; Fairchild et al. 2000), affect subsets of the full FR response, it seems possible that individual components may target the regulation of defined subsets of PHYA-regulated genes. In addition, it might be that *FHY1* function partially overlaps with that of the *FHY1*-related gene also found in the *Arabidopsis* genome, and that loss-of-function alleles at both loci are required to completely abolish FR responses.

We have shown that FHY1–GFP accumulates predominantly in the nucleus (but is also detectable in the cytoplasm) in cD-grown seedlings, is not found in light-grown seedlings, and disappears with relatively slow kinetics from seedlings moved from cD into the light. At present we do not know how these different nucleocytoplasmic distributions relate to FHY1 function. However, FHY1 behavior follows a pattern observed with several light-signaling proteins including phytochrome A, COP1, and HY5 (von Arnim and Deng 1994; Kircher et al. 1999; Osterlund et al. 2000), whereby light-dependent differential stability and/or light-dependent differential subcellular localization are important components of function.

Although several different factors have been identified as FR-signaling components, there is little information concerning how these factors interact with each other, with PHYA, or within the context of information from photoreceptors other than PHYA itself. Here, we have described the results of initial experiments that investigate the effects of some of these factors on the expression of *FHY1*. First, we have shown that *FHY1* expression (at the level of transcript accumulation) is down-regulated by light. PHYA is clearly involved in this process, suggesting negative-feedback regulation of FR-signaling by cFR via PHYA. However, additional photoreceptors are also involved. Regulation of *FHY1* mRNA levels by photoreceptors other than PHYA may reflect a point of interaction between the signal-transduction pathways associated with these different photoreceptors and the PHYA signal-transduction pathway. Intriguingly, the *FHY1* promoter contains a G-box consensus sequence similar to that bound by PIF3 (Martínez-Garcia et al. 2000; data not shown), perhaps identifying a site of regulation by PHYB, PHYA, or other phytochromes.

The effect of light on the expression of other FR-signaling genes has also been investigated. For example, *SPA1* mRNA levels were increased when dark-grown (D) seedlings were transferred to cFR or cR. The effect of cFR on *SPA1* transcript levels was PHYA-mediated, whereas the effect of cR was mediated by PHYA, PHYB, and additional phytochromes (Hoecker et al. 1999). Therefore, there are clear parallels between the regulation of *FHY1* and *SPA1* expression. Both genes display PHYA-mediated cFR regulation of their respective transcript levels, and cR regulates the transcript levels of both genes via phytochromes additional to PHYA. Furthermore, it is interesting to note that cFR promotes the accumulation of *SPA1* transcripts (which encode a negative regulator of FR-signaling) and reduces the accumulation of *FHY1* transcripts (which encode a positive regulator of FR-signaling). However, this pattern does not hold for all FR-signaling genes, because the levels of *FAR1* transcripts (which encode a positive regulator of FR-signaling) are unaffected by light (Hudson et al. 1999) and levels of *HFR1* transcripts (which also encode a positive regulator of FR-signaling) are actually increased by cFR (Fairchild et al. 2000).

FHY1 expression is also regulated, in a light-independent fashion, by the *FHY3* gene product. This result is significant because it shows, for the first time, a clear relationship between the function of different genes involved in regulating FR-signaling. Further work is needed to determine the precise relationships among PHYA, FHY1, and FHY3. It is possible that FHY1 and FHY3 act in the same pathway. However, the FR phenotype conferred by *fhy3* mutants is different from that conferred by *fhy1* mutants (Fig. 3C). Therefore, it is unlikely that regulation of *FHY1* transcript levels is the sole FR-related function of FHY3. Alternatively, FHY3 could regulate *FHY1* transcript levels independently of PHYA-signaling, but additionally be part of a PHYA-signaling pathway that involves PHYA, FHY1, and FHY3. In addition, recent evidence suggests that FHY1 and FHY3 may operate within separate signaling pathways (Okamoto et al. 2001).

We have shown that FHY1 is functional only if PHYA is also functional. Plants overexpressing FHY1 in the absence of functional PHYA do not exhibit the enhanced FR responses exhibited by FHY1-overexpressing plants possessing normal PHYA function. Therefore, the function of FHY1 is clearly associated with the transduction of the PHYA signal, and in the absence of a PHYA signal, FHY1 has no obvious function. In this respect, FHY1 function is similar to that of SPA1, because the phenotype of *spa1* mutants is dependent on the presence of functional PHYA (Hoecker et al. 1999). Furthermore, it has been suggested that the *spa1* mutations specifically amplify PHYA-signaling and that the function of SPA1 is therefore to diminish PHYA-signaling (Hoecker et al. 1999). Here we have shown that overexpression of FHY1 in partially defective PHYA mutants results in enhancement of the response of plants to the reduced PHYA signals generated by the defective PHYA proteins. Perhaps FHY1 and SPA1 act as counterbalancing amplifying and dampening controls on the transmission of the PHYA signal in the modulation of FR responses. Intriguingly, *spa1* mutant seedlings are hyperresponsive to cR, in a PHYA-dependent fashion (Hoecker et al. 1999), but *35S::FHY1* seedlings are not (this study; Fig. 4D). These observations indicate that SPA1 does not inhibit PHYA-signaling via FHY1. Alternatively, overexpression of FHY1 alone is not sufficient to enhance cR-responsiveness.

In conclusion, our observations suggest that FHY1 accumulates in the cells of cD-grown seedlings in preparation for light-activated nuclear import of PHYA, and then acts as part of a specific PHYA signal-transduction mechanism. FHY1 may achieve this by imposing specificity/selectivity on the general transcription factor-mediated phytochrome gene-activation mechanism, and appears to act as a specific amplifier of PHYA signaling.

Materials and methods

Mutant lines

Throughout this paper, the wild type is *Arabidopsis thaliana* ecotype Landsberg *erecta*. *phyA-201*, *fhy1-1*, and *fhy3-1* are as described (Whitelam et al. 1993; Quail et al. 1994). *fhy1-2* and *fhy3-2* were identified in a fast-neutron mutagenized wild-type population (Lehle Seeds, Round Rock, TX). *phyA-3* and *phyA-4* (named according to Quail et al. 1994) were identified in an ethyl methane sulfonate-mutagenized wild-type population, and contain missense alleles that confer a substantial reduction in, but not complete loss of, PHYA function (data not shown). All putative double-mutant homozygotes were test-crossed to both parents to confirm their genotypes.

Molecular cloning of FHY1

Mapping populations were generated by crossing *fhy1-1* (Landsberg *erecta* background) with wild type or *cop1-6* (Columbia background). DNA from *fhy1-1* homozygote F_2 or F_3 plants was analyzed with cleaved amplified polymorphic sequence (CAPS; Konieczny and Ausubel 1993) and simple sequence length polymorphism (SSLP; Bell and Ecker 1994) markers. *FHY1* (*FHY1* sequence has been deposited in GenBank, accession no. AF432142).was mapped to the bottom of chromosome 2, with 714 and 1708 recombinant chromosomes identified between *FHY1* and the markers *cop1-6* and *nga168*, respectively. We designed a CAPS marker with the gene *DET2* (Li et al. 1996; GenBank accession no. U53860) (*Dde*I cleavage of a PCR product amplified with primers 5′-AAATCCAATACCGGCCCAA GACCA-3′ and 5′-AAATCCAATACCGGCCCAAGACCA-3′). Analysis of the recombinant chromosomes enabled us to place *FHY1* between the markers ve017 and *DET2*. With these two markers and published data (Li et al. 1996; Zachgo et al. 1996), we constructed a new yeast artificial chromosome (YAC) map of the region. Total yeast DNA from the selected CIC YACs (Creusot et al. 1995) and yUP (Ecker 1990) clones was extracted according to Gibson and Somerville (1992). The YAC-ends CIC10E10-R, CIC5G4-L, CIC5G4-R, and yUP2C12-L were isolated by inverse PCR for the right end or by plasmid rescue for the left end (Gibson and Somerville 1992) and converted into RFLP markers. Nylon filters spotted with the IGF (Mozo et al. 1998a,b) and TAMU (Choi et al. 1996) bacterial artificial chromosome (BAC) libraries of *Arabidopsis* Col0 genomic DNA were screened with the ^{32}P-labeled yUP2C12-L marker. The relative positions of the BACs were determined by using BAC-end derived markers isolated as described (http://www.tigr.org/tdb/at/atgenome/atgenome.html), a *Bam*HI subclone of the BAC F16C5 (B4-1), a PCR product of the *ACT1* gene (which corresponds to ORF 12 in Fig. 1; amplified with primers 5′-TGTAGCGCTTTTGTGTCCTTATGG-3′ and 5′-CGGCTT GAGAAATGGTCGGA-3′), and the yUP6B10-R fragment. Analysis of the *cop1-6/fhy1-1* recombinants showed that B4-1, an RFLP marker derived from BAC F16C5, mapped centromeric to *FHY1*, thus showing that F16C5 covers *FHY1*.

The CD4-14 λZapII cDNA library (Kieber et al. 1993) was screened with a fragment of the second exon of *FHY1* (PCR-amplified with primers 18-9R, 5′-TCACATGATCATAAG TAGTAGTAAA-3′; and 18-5F, 5′-CCAGAGGACAGAACAAA CTTAGCA-3′). Fourteen positive clones were isolated from ~8.5×10^5 pfu, of which 11 were sequenced. These cDNAs represented a single gene (*FHY1*). Total RNA was extracted from cD-grown wild-type seedlings, and 5′-RACE (Frohman et al. 1988) was performed using the Marathon kit (Stratagene) and primer 18-5F. PCR products were cloned into the pGEM-T easy vector (Promega), and two clones were sequenced. The *FHY1* transcript contains an in-frame STOP codon upstream of the first ATG, suggesting that the *FHY1* ORF (Fig. 1) represents the complete FHY1 protein.

For molecular complementation with constructs containing *FHY1*, plant transformations were as described (Clough and Bent 1998).

DNA sequencing was performed using the Big Dye terminator cycle sequencing kit (Perkin Elmer).

Transcript analysis

Total RNA was separated on denaturing gels and transferred onto Hybond-NX (Amersham). An 18-5F/18-9R PCR fragment was cloned into the pGEM-T easy vector (Promega), and used to make an [α-^{32}P]UTP-labeled RNA probe (Riboprobe systems, Promega). The ribosomal control was as in Deng et al. (1991).

Plant growth and light conditions

In vitro seedling growth medium was as described in Estelle and Somerville (1987), but without sugar. For FR, light sources were FL20S.FR74 bulbs (Toshiba), output filtered through black plexiglass (A.S.H Filters Ltd, UK); for R, FL20SS.BRN/18 bulbs (Toshiba, Japan), output filtered through Fire-red filter (Lee Filters, UK), intensity 3 W/m^2; for WL, as described in Whitelam et al. (1993). Light intensities were measured with a Li-Cor model LI-185-B radiometer with a Li-Cor pyranometer probe.

Transgenic lines overexpressing FHY1

A PCR product, amplified from a subclone of BAC F16C5 using primers 18-19R (5′-AAGATCTATGCCTGAAGTGGAAGTG-3′; containing a *Bgl*II site) and 18-8F (5′-CAGGGATACTCTT GAACA-3′), was cloned in the pGEM-T easy vector (Promega), isolated as a *Bgl*II/*Sac*I fragment, and inserted behind the *CaMV 35S* promoter in a *Bam*HI/*Sac*I opened pBIN121 derivative vector. The construct was introduced into *fhy1-2*, *phyA-3*, and *phyA-4* mutants. Transformants were kanamycin-selected, and the progeny were tested in cFR. Transformants grown in cD were indistinguishable from untransformed controls. A *phyA-4* transformant was crossed with *phyA-201*; F_2 and F_3 progeny of this cross were screened for plants homozygous for both the *phyA-201* mutation and the transgene.

Transgenic lines expressing FHY1–GFP

The *FHY1* gene and 795 bp of upstream sequence were amplified by PCR from a genomic DNA clone, inserted upstream of, and in frame with, the GFP4 coding sequence in a pBIN121 derivative vector, and then transformed into *fhy1-2*. Seedlings (progeny of primary transformants) were examined under UV light with a Leica DMRXA microscope equipped with filter A (Leica) and filter XF115 (Omega Optical) for GFP and DAPI (4′,6-diamidino-2-phenylindole) fluorescence, respectively. For DAPI staining of the nuclei, seedlings were dipped in isopropanol for

a few seconds, rinsed in water, stained in DAPI (1 µg/mL) for 5 min, washed 2 min in water, and mounted on a microscope slide. Images were acquired using a SPOT camera (Diagnostic Instruments). For the photographs in Figure 7, A and B, the transgenic and control seedlings were mounted side by side on the same slide, and both photographs were taken with the same microscope and camera settings. The *35S::GFP* line is in the WS background.

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