Arabidopsis FHY3 defines a key phytochrome A signaling component directly interacting with its homologous partner FAR1

Haiyang Wang and Xing Wang Deng

Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06520-8104, USA

*Corresponding author
E-mail: xingwang.deng@yale.edu

In Arabidopsis, phytochrome A (phyA) is the primary photoreceptor mediating various plant responses to far-red (FR) light. Here we show that phyA signaling involves a combinatorial action of downstream intermediates, which controls overlapping yet distinctive sets of FR responses. FHY3 is a prominent phyA signaling intermediate sharing structural similarity to FAR1, a previously identified phyA signaling component. The fhy3 and far1 mutants display similar yet distinctive defects in phyA signaling; however, overexpression of either FHY3 or FAR1 suppresses the mutant phenotype of both genes. Moreover, overexpression of partial fragments of FHY3 can cause a dominant-negative interference phenotype on phyA signaling that is stronger than those of the fhy3 or far1 null mutants. Further, we demonstrate that FHY3 and FAR1 are capable of homo- and hetero-interaction. Our data indicate that FHY3, together with FAR1, defines a key module in a signaling network underlying phyA-mediated FR light responses.

Keywords: Arabidopsis/FAR1/FHY3/light signaling/phytochrome

Introduction

Plants adjust their growth and development according to their light environment through a network of photoreceptors. Among them, the phytochromes (phys) are best characterized and exist in two distinct but photocconvertible forms, the red (R)-absorbing Pr and the far-red (FR)-absorbing Pfr (Neft et al., 2000; Wang and Deng, 2002). In Arabidopsis, there are five distinct phytochromes, designated phyA–E. These photoreceptors have unique, sometimes partially redundant, or antagonistic roles in different photomorphogenic responses (Deng and Quail, 1999). phyA is the primary photoreceptor mediating the high irradiance response (HIR) to continuous FR light (FRC), including inhibition of hypocotyl elongation, opening of the apical hook, expansion of cotyledons, accumulation of anthocyanin and FRC preconditioned blocking of greening (Nagatani et al., 1993; Whitelam et al., 1993). In addition, phyA is also the photoreceptor responsible for the very low fluence response (VLFR; Yanovsky et al., 1997) and for the regulation of many light-responsive genes by FR light, such as CAB (chlorophyll a/b binding protein), RBCS (small subunit of ribulose-1,5-bisphosphate carboxylase), CHS (chalcone synthase) and PORA (NADPH:Pchlide oxidoreductase A) (Kuno and Furuya, 2000; Ma et al., 2001).

Recent molecular genetic studies have greatly enhanced our understanding of phyA signaling, particularly towards identifying the molecular components potentially involved in the early steps of the signaling pathway linking phyA to light-responsive gene expression and photomorphogenic development. Both general screenings for phytochrome-interacting partners and targeted protein–protein interaction studies have identified a number of phytochrome-interacting factors. These include PIF3 (a nuclear bHLH protein), PKS1 (a cytoplasmic substrate for the kinase activity of phytochrome), NDKP2 (nucleoside diphosphate kinase 2), cryptochromes (both CRY1 and CRY2) and the AUX/IAA proteins (Colón-Carmona et al., 2000; Quail, 2000). One school of thought suggests that light signals could activate the kinase activity of cryptochromes, which phosphorylate themselves and their interacting partners to initiate a signaling cascade (Fankhauser, 2000). On the other hand, genetic analyses have led to the identification and subsequent molecular characterization of a number of phyA signaling intermediates (Hudson, 2000). Several positive regulators have been defined, including both cytosolic and nuclear proteins. For example, LAF6 is a plastid-localized ATP-binding cassette protein involved in coordinating intercompartmental communication between plastids and the nucleus (Møller et al., 2001). PAT1 and FIN219 are cytoplasmic proteins (Bolle et al., 2000; Hsieh et al., 2000), whereas FHY1, FAR1, HFR1 and LAF1 are nuclear-localized factors (Hudson et al., 1999; Fairchild et al., 2000; Ballesteros et al., 2001; Desnos et al., 2001). LAF1 is a MYB-type transcription activator, whereas HFR1 is a bHLH-type transcription factor capable of heterodimerizing with PIF3. Two negative regulators, SPA1 and EID1, have also been defined and shown to be nuclear-localized factors (Hoecker et al., 1999; Dieterle et al., 2001). EID1 is a novel F-box protein probably involved in ubiquitin-dependent proteolysis. The biochemical functions of other components remain largely unknown.

Here we report a detailed genetic and physiological study to characterize the relationships between various genetically defined phyA signaling intermediates. Our data support the notion that phyA signaling involves multiple early intermediates that control overlapping yet distinctive sets of FRC responses. FHY3 (far-red elongated hypocotyl 3) represents one of the early signal transducers of phyA signaling. Loss-of-function fhy3 mutant retains most VLFR responses but is severely impaired in the FR–HIR responses, including hypocotyl growth, cotyledon unfolding, anthocyanin accumulation and FRC preconditioned block of greening (Yanovsky et al., 2000). Molecular cloning of FHY3 revealed that it encodes a nuclear protein...
Table I. Summary of *fhy3* mutants used in this study

<table>
<thead>
<tr>
<th>Allele no.</th>
<th>Isolate name</th>
<th>Ecotype</th>
<th>Molecular lesion</th>
</tr>
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<tbody>
<tr>
<td>fhy3-1</td>
<td>fhy3</td>
<td>Col</td>
<td>R91*</td>
</tr>
<tr>
<td>fhy3-2</td>
<td>128</td>
<td>Col</td>
<td>ND</td>
</tr>
<tr>
<td>fhy3-3</td>
<td>CS6474</td>
<td>WS</td>
<td>36QYTALPSLACIDEG*</td>
</tr>
<tr>
<td>fhy3-4</td>
<td>8RF4</td>
<td>No-0</td>
<td>W901*</td>
</tr>
<tr>
<td>fhy3-5</td>
<td>17FR2</td>
<td>No-0</td>
<td>W171*</td>
</tr>
<tr>
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<td>19FR9</td>
<td>No-0</td>
<td>Q607*</td>
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<tr>
<td>fhy3-7</td>
<td>20FR1</td>
<td>No-0</td>
<td>W269*</td>
</tr>
<tr>
<td>fhy3-8</td>
<td>25FR14</td>
<td>No-0</td>
<td>ND</td>
</tr>
<tr>
<td>fhy3-9</td>
<td>41FR4</td>
<td>No-0</td>
<td>G305R</td>
</tr>
<tr>
<td>fhy3-10</td>
<td>42FR1</td>
<td>No-0</td>
<td>D283N</td>
</tr>
</tbody>
</table>

ND, not determined. Asterisks designate stop codons.

highly similar to FAR1, a previously identified phyA signaling intermediate. We present genetic and molecular evidence to support the view that FHY3, together with FAR1, defines a key module in the phyA signaling network mediating various FRC responses.

**Results**

Isolation of additional *fhy3* mutant alleles

To identify new components in the phyA signaling pathway, we screened two independent T-DNA mutated *Arabidopsis* populations under FRC to select mutants with elongated hypocotyls (see Materials and methods). A number of mutants were identified and subjected to genetic complementation tests with previously identified mutants of similar phenotype. Two new mutations were found to be allelic to the previously identified *fhy3* mutant (designated *fhy3-1*: Whitelam et al., 1993) and were designated *fhy3-2* and *fhy3-3*. Seven additional new alleles (designated *fhy3-4* to *fhy3-10*) were isolated in a previous screen for the *far1* mutants and kindly provided by Dr Quail’s group (Table I; Hudson et al., 1999).

When compared with wild-type (WT) seedlings, the *fhy3* mutants display a long-hypocotyl phenotype and reduced cotyledon expansion under FRC but no significant phenotypes under continuous red (R) or blue light (B) (Figures 1A–C and 2A). There are no observable defects when the seedlings are grown in the dark or under white light (data not shown), indicating that the *fhy3* mutant phenotype is light dependent and specific to FRC. This FRC phenotype is not due to reduced levels of active phyA or to a deficiency in chromophore biosynthesis (Whitelam et al., 1993). Thus, FHY3 likely represents a signaling intermediate for phyA.

Genetic analyses indicate no simple downstream/upstream relationships among phyA signaling components

Among the previously identified phyA signaling mutants, *fhy1*, *fhy3*, *far1* and *fin219* display elongated hypocotyls under FRC (Whitelam et al., 1993; Hudson et al., 1999; Hsieh et al., 2000), and *fhy3* exhibits the most pronounced long-hypocotyl phenotype under our growth condition. On the other hand, the *spa1* mutants have an increased sensitivity to FRC and shorter hypocotyls (Hoecker et al., 1998; Figures 1D and 2B). To examine the genetic relationships among these loci, selective pair-wise double mutants were constructed, and their light-dependent phenotypes were examined and compared with their respective parental mutants and WT controls.

As shown in Figures 1E–G and 2C, under a high fluence rate of FRC, *fhy3-1/far1-2, far1-2/fhy1-1* and *fhy3-1/fhy1-1* double mutants possess longer hypocotyls and further reduced expansion of cotyledons compared with their respective single parental mutants. This result indicates that these mutations have additive effects in phyA signaling, suggesting that they may act in a parallel fashion. It should be noted that these double mutants have a reduced but not a complete loss of sensitivity to FRC. On the other hand, the *fhy3-1/spa1-3* double mutant displays a hypocotyl of intermediate length under FRC (Figures 1H and 2C), indicating that these two mutations

![Fig. 1. Phenotype of fhy3 and double-mutant analysis of FR specific mutants](image-url)
Fig. 2. Quantitative analysis of the hypocotyl length of Arabidopsis phyA signaling mutants and double mutants. (A) Ten alleles of fhy3 mutants, phyA-1 and their corresponding ecotypes: (1) No-0, (2) WS, (3) RLD, (4) Col, (5) Ler, (6) phyA-1, (7) fhy3-1, (8) fhy3-2, (9) fhy3-3, (10) fhy3-4, (11) fhy3-5, (12) fhy3-6, (13) fhy3-7, (14) fhy3-8, (15) fhy3-9, (16) fhy3-10. The error bars represent the standard deviations. (B) phyA signaling mutants and their corresponding ecotypes: (1) RLD, (2) No-0, (3) Col, (4) Ler, (5) phyA-1, (6) fhy1-1, (7) fhy3-1, (8) far1-2, (9) fin219, (10) spa1-3. The error bars represent the standard deviations. (C) The phyA signaling mutants, double mutants and their respective ecotypes: (1) RLD, (2) No-0, (3) Col, (4) Ler, (5) phyA-1, (6) fhy1-1, (7) fhy3-1, (8) far1-2, (9) spa1-3, (10) fhy3-1/far1-2, (11) fhy3-1/spa1-3, (12) fhy3-1/fhy1-1, (13) far1-2/fhy1-1. The error bars represent the standard deviations.

can compensate each other to some extent. This suggests that there may be no simple downstream/upstream relationship between FHY3 and SPA1. We also examined these double mutants under a wide range of FRc fluence rates, and similar effects were observed to those shown in Figure 1E–H, although the differences become less pronounced under low fluence rate irradiations (data not shown). In addition, these double mutants display essentially normal responses to R and B light conditions (data not shown).

Light-regulated gene expression in fhy3 and other phyA signaling mutants
To explore the molecular basis for the developmental defects, we examined the changes in three representative phyA-dependent gene expression patterns (RBCS, CHS, PORa) in fhy3-4 and the following available phyA signaling mutants: phyA-1, fhy1-1, far1-2, fin219 and spa1-3. Seedlings were grown under darkness for 4 days prior to illumination with 4 h of FRc. As shown in Figure 3A, WT seedlings show a clear induction of RBCS and CHS, while the expression of PORa is significantly suppressed. The induction of both RBCS and CHS is almost completely abolished in phyA-1, fin219 and fhy1-1, and is severely attenuated in fhy3-4. No obvious effect was found in far1-2 (Figure 3A). Notably, RBCS induction is clearly enhanced in spa1-3, while the induction of CHS seems impaired under this condition (Figure 3A). However, CHS expression is increased in FRc-grown spa1-3 seedlings (data not shown), consistent with the observed increasing accumulation of anthocyanin in this mutant (Hoecker et al., 1998). The repressive effect of FRc on PORa expression is severely compromised in phyA-1 and fhy1-1, but apparently normal in all other mutant backgrounds examined (Figure 3A).

Previously it has been shown that phyA is also involved in R-mediated CHS induction (Barnes et al., 1996a), therefore we also examined the effects of fhy3 and various phyA signaling mutants on this response. Similarly, seedlings were grown under darkness for 4 days prior to illumination with 4 h of continuous R. As shown in Figure 3B, CHS induction is dramatically reduced in phyB and slightly impaired in phyA-1, indicating that both phyA and phyB are involved in R-mediated induction of CHS expression, with phyB playing a major role in this.
response. Interestingly, this response is almost completely abolished in fin219 and is slightly increased in spa1-3. The effects of fhy3-4, fhy1-1 and far1-2 on this response are minimal.

**FHY3 encodes a protein related to FAR1**

Although two new fhy3 alleles (fhy3-2 and fhy3-3) were identified from two independent T-DNA mutagenesis populations, co-segregation tests show that neither mutation is linked to the T-DNA insertion (data not shown). Therefore, we generated an F2 mapping population by crossing the fhy3-2 allele (COL ecotype) to WT ecotype Ler. We mapped the FHY3 locus to a region of chromosome III between the SSLP markers nga162 and GAPab (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). Further mapping with several newly developed SSLP, CAP and RFLP markers delimited FHY3 to the region flanked by two RFLP markers, mi142 and mi268. A set of BAC clones covering this genomic region was obtained from the Arabidopsis stock center. New RFLP markers were developed from these BAC clones and used to further locate the FHY3 locus to a single BAC clone, F2F24 (Figure 4A).

The F2F24 BAC clone was used to screen an Arabidopsis cDNA library derived from dark-grown seedlings and four different types of cDNA were isolated. Sequence analysis revealed that one of the cDNAs encodes a protein with similarity to FAR1, a nuclear protein required for phyA signaling (Hudson et al., 1999). Thus, we sequenced the genomic region of this gene for eight fhy3 alleles. In each case, a single mutation in the putative open reading frame was identified (Table 1), providing convincing evidence that this cDNA clone defines the FHY3 gene. Comparison of the cDNA with the genomic sequence revealed that the FHY3 gene is composed of eight exons and seven introns (Figure 4B).

FHY3, together with FAR1, FR51–FR53 (Hudson et al., 1999) and eight new members designated FR54–FR511, comprise a multigene family present in the Arabidopsis genome. Interestingly, FHY3 and FAR1 share the highest homology (50% identity and 75% similarity) to each other and they compose a branch of this gene family (Figure 4C). Moreover, similar proteins have also been identified in other plant species, including monocotyledon plants (Hudson et al., 1999), indicating that this family of genes is conserved throughout the evolution of the plant kingdom.

The FHY3 cDNA encodes a predicted polypeptide of 839 amino acids, identical to that of ATg22170 annotated through the Arabidopsis genome project and with a secondary structure similar to FAR1 (Hudson et al., 1999). The residues between 603 and 699 are predicted to form a coiled-coil motif. In addition, FHY3 contains a basic region (KKKNPT−KKRK, residues 709–718, basic residues in bold), which could act as a nuclear localization signal (NLS) (Figure 4D). However, it should be pointed out that putative NLS motifs are only identified in some of the family members, such as FAR1, FHY3 and FR52, but not in others, such as FR51 and FR53. Furthermore, these proteins may also differ in their secondary structures, as some members (such as FR53) lack the coiled-coil domain identified in the C-terminus of both FHY3 and FAR1, whereas other members possess multiple coiled-coil domains (such as FR51 and FR52). These features suggest that those family members may have overlapping as well as distinct functions.

**FHY3 expression is regulated by phyA signaling**

To determine whether the expression of FHY3 is light dependent, and also its dependence on phyA, we examined FHY3 transcript levels in dark- and FRc-grown WT seedlings as well as in selected FRc-grown phyA signaling mutant seedlings. In WT seedlings, the FHY3 transcript level is clearly reduced by FRc treatment as compared with dark treatment. For FRc-grown seedlings, the FHY3 transcript level is dramatically reduced in fhy3-3, fin219 and spa1-3, but is significantly increased in far1-2 (Figure 5A), indicating that the expression of FHY3 is subject to regulation by phyA and its signaling intermediates FIN219, SPA1 and FAR1.

**Overexpression of either FHY3 or FAR1 suppresses the mutant phenotype of both genes**

The high homology shared by FHY3 and FAR1 and their similar mutant phenotypes suggest that these two genes may have similar functions. To examine this possibility, we introduced a 35S promoter-driven myc epitope-tagged FHY3 cDNA transgene (myc-FHY3) or a myc-flag-HA (MFH) epitope-tagged FAR1 cDNA transgene (MFH-FAR1; Figure 5B) into both fhy3-3 and far1-2. As expected, a functional complementation of each parental mutant by overexpressing its corresponding gene was observed (Figures 5C, a and c, and 6G). We also observed an apparent suppression of the mutant phenotype by overexpressing the homologous gene (Figures 5C, b and d, and 6G). It should be noted that we did not observe a strongly enhanced FRc response when the same transgenes were introduced into a WT background, nor was a suppression effect detected when the same transgenes were introduced into other FRc-specific long-hypocotyl mutants such as fhy1 and fin219 (data not shown).

Moreover, dark-grown seedlings overexpressing FHY3 and FAR1 do not exhibit any detectable phenotypes (data not shown), suggesting that these two proteins share a bona fide functional overlap and that their activation requires activated phyA.

**FHY3 protein is constitutively nuclear localized**

To determine the subcellular localization of FHY3, the full-length FHY3 cDNA was translationally fused to the 3′ end of the GUS reporter gene under the control of the strong 35S promoter (GUS-FHY3; Figure 5B). The transgene successfully rescued the fhy3 mutant phenotype (Figure 5C, e and f). In the rescued transgenic plants, the GUS–FHY3 fusion protein is exclusively found in the nucleus under both dark and FRc treatment (Figure 5D, a and b). This localization pattern is similar to that of a constitutive nuclear protein, GUS–Nla (Figure 5D, c and d), indicating that FHY3 is a constitutive nuclear protein.

**Dominant-negative effect of overexpressing partial fragments of FHY3**

To examine the structure–function relationship of FHY3, we generated transgenic plants overexpressing either the N-terminal (N1–541) or C-terminal (C473–839) portions of FHY3 (Figure 5B) in a WT background (COL ecotype).
As shown in Figure 6A and B, heterozygous transgenic plants harboring a single T-DNA insertion of the transgene (either N1–541 or C473–839) produce offspring segregating in a 1:2:1 ratio of long-, medium- and short-hypocotyl seedlings. These long-hypocotyl plants are homozygous transgenic plants, the medium ones are heterozygous for the transgene and the short ones are non-transgenic WT plants. This result suggests that these transgenes cause a
Fig. 5. *FHY3* expression, overexpression and *FHY3* protein localization. (A) RNA gel blot analysis of *FHY3* expression in dark- and FRc-grown WT seedlings as well as in various FRc-grown mutant seedlings. (B) Diagram of the constructs used in plant transformation experiments. The predicted coiled-coil region and the NLS of *FHY3*, the myc and myc-FLAG-MA (MFH) epitopes and the GUS gene coding region are indicated. (C) Overexpression of myc-*FHY3* and MFH-FAR1. The *fhy3* and *far1* mutant phenotypes are rescued by overexpressing the corresponding gene (a and c) and suppressed by overexpressing the homologous gene (b and d). GUS-*FHY3* also rescues the *fhy3* mutant phenotype (e and f). All panels were taken at the same magnification. Scale bar: ~2 mm. (D) Subcellular localization of the *FHY3* protein in the *Arabidopsis* hypocotyl cells. Each panel is composed of two portions. The upper portions are the GUS staining of GUS-*FHY3* (a and b) and GUS–Nla (c and d). The lower portions are DAPI staining of the corresponding images to show the positions of the nuclei (indicated by arrows). The left panels are from dark-grown seedlings and the right panels from FRc-grown seedlings. All panels were taken at the same magnification. Scale bar: ~50 μm.

dosage-dependent dominant-negative effect on phyA-mediated inhibition of hypocotyl elongation in response to FRc. Notably, homozygous transgenic seedlings overexpressing the C-terminal portion of *FHY3* (the E7 line) display a completely etiolated phenotype under FRc, indistinguishable from the phenotype of the phyA-1 null mutant (Figure 6C, D and G). The same plants have an essentially normal response to R and a slightly reduced response to B light (Figure 6E and F). This result indicates that overexpression of the *FHY3* C-terminal fragment in the homozygous seedlings not only impaired the endogenous *FHY3* function but also completely blocked phyA signaling. It is plausible that the overabundant mutated form of *FHY3* titrated out all normal *FHY3* interactive partners and thus completely blocked phyA signaling. This would be consistent with a critical role of *FHY3* in the phyA signaling network.

**FHY3 and FAR1 interact in a yeast two-hybrid assay and in planta**

The structural similarity and genetic interactions between *FHY3* and FAR1 prompted us to examine their possible protein–protein interaction. We first examined the FAR1 and *FHY3* interaction using a yeast two-hybrid assay. Both genes were cloned into the yeast vectors as fusion proteins with the LexA DNA-binding domain and the GAL4 activation domain (AD; Ausubel et al., 1999). As shown in Figure 7A–C, although both LexA–*FHY3* and LexA–FAR1 can auto-activate the β-galactosidase reporter gene to certain extents, when combined with AD–*FHY3* or AD–FAR1, the activities of the reporter gene are significantly increased (3- to 5-fold). In addition, a C-terminal fragment of *FHY3* (amino acids 541–839) containing the coiled-coil domain retains the ability to interact with FAR1, although at a reduced strength. These data suggest that *FHY3* and FAR1 are capable of forming homocomplexes with themselves or heterocomplexes with one another.

To substantiate the physical interaction between *FHY3* and FAR1, we conducted an *in vivo* co-immunoprecipitation assay with F2 plants from a cross of GUS–*FHY3* and MFH–FAR1 transgenic plants. As shown in Figure 7D, a western blot probed with a GUS antibody detected the GUS–*FHY3* fusion protein from the total extract of the F2 seedlings and F2 seedlings subjected to immunoprecipitation with either a myc- or a flag-epitope antibody.
Fig. 6. Dominant-negative effect caused by overexpressing partial fragments of FYH3. (A and B) The T3 generation of the transgenic lines B5 (a representative myc-FYH3N1–541 line) and E7 (a representative myc-FYH3C473–839 line) segregate homozygotes (homo), heterozygotes (hetero) and non-transgenic seedlings. The genotypes of the seedlings are determined by drug-resistance tests and the phenotypic segregation ratios of their T3 generation seedlings. (C) Comparison of the homozygote seedlings of E7 phyA-1 and phy3-1. Also shown are their respective WT ecotype seedlings. (D) Close-ups of the cotyledons for seedlings shown in (C). (E) E7 homozygote seedlings respond normally to R. (F) E7 homozygote seedlings have marginally elongated hypocotyls under B light. Scale bars: -2 mm. (G) Quantitative analysis of hypocotyl length of various transgenic lines compared with the mutants and wild-type controls: (1) Col, (2) No-0, (3) Ler, (4) phyA-1, (5) phy3-1, (6) phy3-1, myc-FYH3, (7) phy3-1, MFH–FAR1, (8) far1-2, (9) far1-2. MFH–FAR1, (10) far1-2, myc-FYH3, (11) phy3-1/far1-2, (12) E7, heterozygotes, (13) E7, homozygotes, (14) E7, segregated non-transgenic seedlings, (15) N-terminal, B5 homozygote.

However, GUS–FYH3 fusion protein was not detectable from the total extract of wild-type seedlings or from immunoprecipitated F2 seedlings processed without adding the antibodies. The MFH–FAR1 fusion protein was also detected from the same immunoprecipitated samples on a separate western blot probed with a flag antibody, suggesting a physical association of FYH3 and FAR1 in planta. The inability to observe the MFH–FAR1 fusion protein in the total extract of F2 seedlings with the flag antibody at the same exposure is due to the low expression level of the tagged protein in the total extract.

Fig. 7. Direct interaction between FYH3 and FAR1. (A–C) Quantitative analyses of the relative β-galactosidase activities for the yeast two-hybrid assay. The LexA and AD fusion constructs used in the assay are shown at the bottom of each panel. Unless otherwise indicated, full-length proteins were used. The ‘–’ signs represent the empty vector controls. (A) FYH3 interacts with itself and with FAR1. (B) FAR1 interacts with itself and with FYH3. (C) An FYH3 C-terminal fragment (FYH3-C, amino acids 541–839) interacts with FAR1. (D) Co-immunoprecipitation of GUS–FYH3 and MFH–FAR1. Light-grown F2 seedlings harboring both GUS–FYH3 and MFH–FAR1 were subjected to an immunoprecipitation procedure with either myc or flag monoclonal antibodies. The myc antibody recognizes both MFH–FAR1 and GUS–FYH3, thus serving as a positive control, whereas the flag antibody only recognizes MFH–FAR1. The precipitates were subjected to western blot analyses probed with either a GUS antibody (Molecular Probes) for detecting the GUS–FYH3 fusion protein (upper panel) or a flag antibody (Sigma) for detecting the MFH–FAR1 fusion protein (lower panel). The asterisk indicates a possible degradation product of MFH–FAR1. T, total protein extracts; F2, F2 seedlings from a genetic cross between the GUS–FYH3 and MFH–FAR1 transgenic lines; ~Ab, the sample was processed for immunoprecipitation without adding any antibody.

**Discussion**

The genetically identified phytochrome A signaling components in Arabidopsis do not define a simple linear pathway

The FYH3 gene was identified in a genetic screen for mutants displaying specific defects in FRc inhibition of hypocotyl elongation (Whitelam et al., 1993). The phy3 mutant displays an elongated hypocotyl specifically under FRc. This FRc phenotype is not due to reduced levels of active phyA or to a deficiency in chromophore biosynthesis (Whitelam et al., 1993). Thus, FYH3 is likely to represent an authentic signaling transducer for phyA. Interestingly, loss-of-function mutants (presumably null mutant alleles) of FYH3 and several other positively acting phyA signaling components only exhibited partial defects with different spectra and strengths in phyA signaling, suggesting that phyA signaling involves multiple branches or parallel pathways. phy3, phy1, pat1 and fin2 mutants display similar defects in various FRc responses, including inhibition of hypocotyl growth, apical hook and cotyledon opening, anthocyanin accumulation and FRc preconditioning blocking of greening. They also affect the induction of RBCS and CHS by FRc (Barnes et al., 1996a; Soh et al., 1998; Bolle et al., 2000; Yanovsky et al., 2000; Figure 3A),
indicating that these loci act early in phyA signaling. However, although the phy3 mutants are severely impaired in the above listed FR–HIR responses, they largely retain VLFR, which is defective in the phy1-1 and the phyA photoreceptor mutants (Yanovsky et al., 2000). Thus, FHY3 and FHY1 are likely to represent two different branch points in the phyA signaling. Consistent with this notion, the repression of PORA gene expression is obviously defective in phyA-1 and phy1-1, but appears normal in phy3 (Figure 3A). The repression of PORA by FRc has been proposed to be responsible for the loss of FRc preconditioned greening block in the phy1 and phyA mutants (Barnes et al., 1996b). Therefore it seems that phy3 may utilize a distinct mechanism to regulate the PORA protein level which might be responsible for the loss of FRc preconditioned greening block in this mutant. It will be interesting to examine both the transcript and protein levels of PORA in phy3, fin2 and pat1, which may clarify this issue.

fin219 and far1 differ from the above mutants in that cotyledon opening and expansion as well as the FRc preconditioned greening block are not affected, although they are defective in hypocotyl elongation and anthocyanin accumulation (Hudson et al., 1999; Hsieh et al., 2000). The induction of RBCS and CHS by FRc is minimally affected in far1-2 but is severely attenuated in fin219 (Figure 3A), indicating that they represent close but different branches in phyA signaling. Although the phenotype of the laf6 mutant seems most similar to that of fin219 and is defective in both hypocotyl elongation and the induction of CHS (Möller et al., 2001), their different subcellular localizations suggest that they function at different steps in phyA-mediated signaling. HFR1 primarily affects the elongation and gravitropic response of the hypocotyl, whereas other FRc responses, including anthocyanin accumulation, FRc preconditioned block of greening and induction of CHS, are unaffected in this mutant (Fairchild et al., 2000). Also, the laf1 mutant is affected in a distinct subset of phyA-dependent responses, including hypocotyl elongation, FRc preconditioned block of greening, anthocyanin accumulation and induction of CHS, whereas the FR-dependent apical hook opening, cotyledon unfolding and expansion, and gravitropism are not altered (Ballesteros et al., 2001). Taken together, the available data support a view that phyA signaling involves distinct combinations of these phyA signaling intermediates for controlling overlapping yet distinctive sets of FRc responses.

The finding that the double mutants phy3-1/far1-2, phy3-1/far1-1 and far1-2/phy1-1 all display more elongated hypocotyls, whereas the phy3-1/spa1-3 double mutant has a hypocotyl of intermediate length (Figure 1E–H), further indicates that there is no simple downstream/upstream relationship among these phyA signaling components. Instead, it suggests that a complex interactive network of these signaling components mediates phyA signaling. For example, non-allelic non-complementation between fin2 and phy3-1 as well as between fin219 and phy1 has been reported (Soh et al., 1998; Hsieh et al., 2000), indicating that their gene products may interact directly or engage in extensive cross-talk. Furthermore, the observed down-regulation of the FHY3 transcript level in FRc-grown fin219 and spa1-3 seedlings, and the increased accumulation of FHY3 transcript in far1-2 seedlings (Figure 5A), suggest that the accumulation of the FHY3 transcript is subject to both positive and negative feedback regulation by specific phyA signaling components, and that the co-action of these signaling components determines the ultimate FHY3 expression level.

**FHY3 and FAR1 constitute a key module in the phyA signaling process**

The findings that phy3 and far1 mutants display similar yet distinct phenotypes and that FHY3 and FAR1 encode two homologous proteins are particularly interesting. Both mutants display elongated hypocotyls and reduced anthocyanin accumulation. However, phy3 has a much more pleiotropic effect on phyA signaling. For example, apical hook and cotyledon opening, and FRc preconditioned block of greening are affected by phy3 but not by far1. The phy3-1/far1-2 double mutant displays a more etiolated phenotype than its respective single-mutant parents under FR, suggesting an additive effect of these two mutations. Moreover, overexpression of FAR1 or FHY3 can suppress the phenotype of each other’s loss-of-function mutations. Furthermore, overexpression of partial fragments of FHY3 in the WT background causes reduced sensitivity to FRc in a dosage-dependent manner (Figure 6). Most strikingly, Arabidopsis seedlings homozygous for the transgene overexpressing the C-terminal portion of FHY3 (C473–839), which contains a coiled-coil domain, display an apparent complete loss of FRc responses, remarkably similar to phyA null mutants. This result indicates that the C-terminal fragment of FHY3 may interact with other intermediates of phyA signaling and that non-productive binding of this truncated FHY3 protein with its interactive partners could shut off the entire phyA signaling by a dominant-negative interference. This interference is substantially stronger than the effects of a phy3 null mutation and the phy3/far1 double mutant. Direct evidence for such a notion is provided by the demonstration that FHY3 and FAR1 directly interact with each other in a yeast two-hybrid assay and an in vivo co-immunoprecipitation assay. Furthermore, our data suggest that FHY3 and FAR1 are capable of homo- and/or hetero-interactions (Figure 7). The capacity for homo- or heterocomplex formation for both proteins presumably provides a great flexibility to integrate the varying signal imports through interactions with other components of the phyA signaling pathway. Therefore, FHY3, together with FAR1, constitutes a key module in a regulatory network mediating phyA signaling.

Although the exact biochemical functions of FHY3 and FAR1 are currently not known, their nuclear localization implies that they are most likely involved in regulation of gene expression. They could either directly bind to DNA to regulate gene expression similar to transcription factors, or interact with DNA–protein complex in a similar manner to co-activators or co-repressors. This scenario is consistent with the observation that light stimulates the formation of nuclear speckles for the phyA–GFP fusion protein (Kircher et al., 1999; Nagy et al., 2000), which may represent distinct protein complexes where phyA interacts with its partners to regulate gene expression directly on light-regulated promoter sequences. Evidence supporting such a view has been provided by the demonstrations that both the HFR1–FIF3 heterodimer and FIF3 homodimer
can bind preferentially to the Pfr form of phyA (Ni et al., 1999; Fairchild et al., 2000). Furthermore, it has been demonstrated that FHY3 could bind specifically to a G-box DNA sequence motif present in various light-regulated gene promoters (Martinez-Garcia et al., 2000). It is likely that FHY3 and FAR1 could, through their interactions with, or by modulation of, FHY3 and/or FAR1, regulate the FHY3 homodimer- or FAR1–FHY3 heterodimer-mediated FR light-specific gene expression. Determining whether FHY3/FAR1 homo- and heterocomplexes bind DNA directly or interact with phyA and/or other DNA-binding transcription factors (such as PIF3, FAR1 and HY5) to impose their regulatory activities on these proteins will certainly enhance our understanding of the mechanisms of phyA signaling.

Materials and methods

Plant materials and growth conditions

The fhy3-1 mutant has been described previously (Whitelam et al., 1993). fhy3-2 was isolated by screening a T-DNA mutated Arabidopsis population generated by Steve Dellaporta’s laboratory at Yale University (ecotype COL; Galbiati et al., 2000). fhy3-3 was isolated by screening the Dellaporta’s T-DNA population under FRc condition (ecotype WS); fhy3-4 to fhy3-10 were isolated during the far1 mutant screen (Hudson et al., 1999) and kindly provided by Dr Quail’s group (No-0 ecotype).

Allelism of these mutations was determined by standard genetic crossing. Other mutant plants used in this study included phyA-1, fhy1-1, phyB/hy1, phyA/B and hy5-1 (all ecotype Ler; flo219 (ecotype COL); spa1-3 (ecotype RLD); far1-2 (ecotype No-0) (Whitelam et al., 1993; Hoekert et al., 1998; Hsieh et al., 2000). Double mutants were constructed by crossing their respective parental mutants. Putative double mutants were selected from FRc-grown F2 seedlings and backcrossed to their respective parental mutants to confirm their genotypes.

Surface sterilization and cold treatment of the seeds, and seedling growth conditions for different light sources were described previously (Hsieh et al., 2000). Seedlings were grown on GM agar plates containing 0.3% sucrose for mutant screening and phenotypic analysis. For hypocotyl length measurements, 20–30 seedlings for each genotype were measured under a dissecting microscope with a ruler.

RNA gel blot analysis

Total RNA was isolated from 4-day-old seedlings using the Qiagen RNAeasy Plant Mini prep kit. The seedlings were grown under darkness or FRc for 4 days. For the light shift experiment, the seedlings were grown under darkness for 4 days prior to illumination with 4 h of FRc or R. Ten micrometers of total RNA were loaded onto the gel and blotted to nylon membrane. The CHS probe was derived from a 0.9 kb EcoRI fragment containing the Arabidopsis CHS coding region (Hsieh et al., 2000). The RBcS probe has been described previously (Torii et al., 1999). The PORA gene probe was a 580 bp genomic fragment generated by PCR using primers CCGACTTACACTCATCAG and GGTACCACATG-ATG. The FHY3 probe was derived from an EcoRI fragment of the cDNA clone. Equal loading of RNA was verified by ethidium bromide staining as well as by rehybridizing the blots with an 18S cDNA probe (Deng et al., 1991). Probes were labeled by random priming. Hybridization and washing were conducted according to a standard method (Deng et al., 1991).

Positional cloning of FHY3 and sequence analysis

For generating the mapping population, the fhy3-2 allele (ecotype COL) was crossed with the Ler wild type. Long-hypocotyl seedlings under FRc light were selected in the F2 generation and transferred to soil for growth. A total of ~2200 recombinant chromosomes were used for fine-mapping analysis. After the FHY3 gene was narrowed down to a single BAC clone (FP224), this BAC clone was used to screen the cDNA libraries (CD4-13 through CD4-16 combined) obtained from ABRC. A total of 22 cDNA clones representing four different genes were isolated. A full-length cDNA clone (14) for FHY3 was sequenced and verified by sequencing the genomic region of eight different fhy3 alleles and their corresponding ecotypes (Table I).

Recombinant plasmids for plant transformation

To generate a c-myc epitope (MEQKLISEEDL)-tagged FHY3, the N-terminal BamHI–BglII fragment of the FHY3 cDNA clone (14) was replaced by a PCR fragment using primers CACGGATCCATGGGAAACAGATCTACTATTGCGACGAGAGAGATCACAGTTTCAGACGGCAGACGTAGTATGAGATCGACCTACTACATCCGTCAGGTTTGGCAGAGGAGGATGATGAG and CTGATCATCCGCCAGATCTACTAGC to generate a modified full-length FHY3 cDNA clone (designated 14A) with the added c-myc epitope at the N-terminus. Recombinant FHY3/SAII fragment containing the full-length FHY3 coding region was cloned into the same sites of the binary vector pZPY112 (Serino et al., 1999), thus placing the FHY3 gene under the control of the 35S promoter. This clone was designated mcFHY3.

The N-terminal fragment of FHY3 was deleted from mcFHY3 by digesting with SpeI and religating to generate the N-terminal deletion construct (designated mcF-HY3CF73-589). The C-terminal fragment of FHY3 was deleted by digesting mcF-HY3 with AvrII and SalI, filling in both ends and re-ligating to generate the C-terminal deletion construct (designated mcF-HY3N1-541).

For localization of the FHY3 protein, a BamHII–BglII fragment containing the 35S promoter and the GUS coding region was derived from the pPZP222-GUS. Phytochrome analysis was conducted in the transgenic line.

To generate a myc-flag-HA epitope-tagged FHY3 gene construct in the binary vector, two complementary oligos (CTGAATGGGAAAAGGAGCTTTAGGCGACGACGTACTACAAAGAACAGCACAG TTACCTCTAGCCGGAGACGATGGCAGATGGATACCGGAGAC CGCAGATGCCGAAGAAAGCCTGTAGCGACGACGTACTACAAAGAACAGCACAG TTACCTCTAGCCGGAGACGATGGCAGATGGATACCGGAGAC CGCAGATGCCGAAGAAAGCCTGTAGCGACGACGT ACTACAAAGAACAGCACAG TTACCTCTAGCCGGAGACGATGGCAGATGGATACCGGAGAC CGCAGATGCCGAAGAA AGCCTGTAGCGACGACGTACTACAAAGAACAGCACAG TTACCTCTAGCCGGAGACGATGGCAGATGGATACCGGAGACG CTCGTTACGATCCTGTAGGGCACGAGGATCAGTGTTACGATCCTGTAGGGCACGAGGATCAGTGTTACGATCCTGTAGGGCACGAGGATCAGTGTTACGATCCTGTAGGGCACGAGGATCAGTGTTACGATCCTGTAGGGCACGAGGATCAGTGTTACGATCCTGTAGGGCACGAGGATCAGTGTTACGAT.
cloning efforts. An EcoRI fragment containing the N-terminal 250 amino acids of FHY3 was cloned into the EcoRI site of pEG202 and pPG4-5 vectors in the correct orientation to generate LexA-FHY3 and AD-FHY3. Then a XhoI fragment overlapping with the N-terminal portion of FHY3 and containing the remaining cDNA of FHY3 was cloned into XhoI-digested LexA-FHY3 and AD-FHY3 in the correct orientation to generate LexA-FHY3 and AD-FHY3, both of which contain the full-length FHY3 gene. A BamHI–SalI fragment containing the C-terminal amino acids 541–839 of FHY3 was generated via PCR using the FHY3 cDNA clone (14) as a template and the primers GGAGATCCGAGACCAGATTCGATCAACGACCTGACATGT and the T7 primer. This PCR product was cloned into the BamHI–SalI sites of the pEG202 to generate LexA-FHY3C. Then, an EcoRI fragment containing the insert was released from this construct and cloned into the EcoRI site of pPG4-5 to generate AD-FHY3. For the FARI gene, a BamHI–XhoI fragment containing the full-length FARI cDNA was released from the clone Ta-FAR1 and ligated into the BamHI–XhoI sites of pEG202 to generate LexA-FARI. A MfeI–XhoI fragment containing the full-length FARI coding region was released from Ta-FAR1 and cloned into the EcoRI–XhoI sites of pPG4-5 to generate AD-FARI. Yeast transformation, mating and liquid assay were conducted as described in Ausubel et al. (1999).

**Immunoprecipitation**

Light-grown WT and frr2 seedlings of a cross between the GUS–FHY3 and MFH–FAR1 transgenic lines were processed for co-immunoprecipitation assay in a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl2, 0.1% NP-40, 1 mM PMSF and 1× complete protease inhibitors (Roche). Either a myc- or a flag-epitope antibody and protein A-agarose beads (Sigma) were used to precipitate the immunoprotein complex. SDS–PAGE and western blotting analysis were performed according to standard procedures.

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**References**


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