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FHY1 and FHL Act Together to Mediate Nuclear Accumulation of the Phytochrome A Photoreceptor

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The phytochrome family of red/far-red photoreceptors is involved in the regulation of a wide range of developmental responses in plants. The Arabidopsis genome contains five phytochromes (phyA–E), among which phyA and phyB play the most important roles. Phytochromes localize to the cytosol in the dark and accumulate in the nucleus under light conditions, inducing specific phytochrome-mediated responses. Light-regulated nuclear accumulation of the phytochrome photoreceptors is therefore considered a key regulatory step of these pathways. In fact, one of the most severe phyA signaling mutants, fhy1 (far red elongated hypocotyl 1), is strongly affected in nuclear accumulation of phyA. The fhy1 fhl (fhy1 like) double mutant, lacking both FHY1 and its only close homolog FHL, is virtually blind to far-red light like phyA null seedlings. Here we show that FHL accounts for residual amounts of phyA in the nucleus in a fhy1 background and that nuclear accumulation of phyA is completely inhibited in an fhy1 FHL RNAi knock-down line. Moreover, we demonstrate that FHL and phyA interact with each other in a light-dependent manner and that they co-localize in light-induced nuclear speckles. We also identify a phyA-binding site at the C-terminus of FHY1 and FHL, and show that the N-terminal 406 amino acids of phyA are sufficient for the interaction with FHY1/FHL.

Keywords: Arabidopsis — FHL — FHY1 — Nuclear accumulation — Nuclear speckles — Phytochrome.

Introduction

Plants are sessile organisms and therefore have to adapt their growth and development to the environmental conditions at the place of germination. One of the most important factors directing plant development is light. To detect the intensity, the quality (wavelength) and the direction of incident light, plants have evolved a set of different photoreceptors (Chen et al. 2004). The phytochromes, which mainly track the red and far-red light wavelengths of the spectrum, are important in the regulation of a wide range of developmental responses in plants. These include, for instance, seed germination, circadian rhythms, shade avoidance, growth and flowering, as well as chloroplast development (Casal et al. 2003, Chen et al. 2004, Franklin and Whitelam 2005). Microarray analysis has shown that roughly 17% of the genes in Arabidopsis are subject to regulation by red and/or far-red light, which suggests a key role for phytochromes in transcriptional control of gene expression (Tepperman et al. 2004).

The Arabidopsis genome contains five phytochromes (phyA–E) (Sharrock and Quail 1989, Clack et al. 1994), among which phyA and phyB are the most important. PhyB, which belongs to the light-stable type II phytochromes, is the major red light receptor in Arabidopsis and mediates the red/far-red reversible low fluence response (LFR). In contrast, phyA, a light-labile type I phytochrome, is primarily involved in far-red-light sensing (high irradiance response, HIR) and in responses to very weak light (very low fluence response, VLFR) (Nagy and Schäfer 2002, Sharrock and Clack 2002).

Upon light perception, phytochromes translocate from the cytosol, where they reside in the dark, into the nucleus (Kircher et al. 1999, Yamaguchi et al. 1999, Hisada et al. 2000, Kircher et al. 2002). It has been shown that the active phyB photoreceptor trapped in the cytosol is unable to inhibit hypocotyl elongation in red light, suggesting that nuclear translocation is an essential step in phyB signaling.
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(Huq et al. 2003, Matsushita et al. 2003). Despite the lack of experimental proof, it is reasonable to assume that phyA signaling also depends on nuclear accumulation of the photoreceptor. The fhy1 and fhy3 mutants have the most severe hyposensitive phenotype among all phyA signaling mutants (Whitelam et al. 1993), and microarray analysis has shown that almost as many genes in Arabidopsis are regulated by FHY1 and/or FHY3 as by phyA (Wang et al. 2002). Together with the finding that both HIR and VLFR are affected in the fhy1 mutant (Cerdan et al. 1999), this suggests that FHY1 plays a fundamental role in phyA signaling. Our recent finding that nuclear accumulation of phyA is vastly reduced in the fhy1 mutant strongly supports (although it does not strictly prove) that nuclear accumulation of the photoreceptor is a key step in phyA signaling (Hiltbrunner et al. 2005).

FHY1 and FHL, the only close FHY1 homolog in Arabidopsis, are small (23 and 20 kDa) plant-specific proteins which contain both functional NLS (nuclear localization signal) and NES (nuclear export signal) sequences (Zeidler et al. 2004, Zhou et al. 2005). Complementation analysis with FHY1 mutant versions containing either a disrupted NLS or NES has shown that only the NLS but not the NES is required for protein functionality (Zeidler et al. 2004). Moreover, the C-terminal 19 amino acids, which are almost identical in FHY1 and FHL, are also essential for proper function of FHY1, and expression of a C-terminally truncated FHY1 protein in the fhy1 background results in a dominant-negative phenotype (Zeidler et al. 2004).

The homology between FHY1 and FHL is restricted to the NLS/NES region in their N-terminal half and to the extreme C-terminus. Although the overall similarity at the amino acid level is quite low (<30% identical amino acids), there is good evidence that FHY1 and FHL are nevertheless functional homologs. First, expression of FHL in the fhy1 mutant background reinstates a wild-type phenotype, and secondly, the functional homologs. First, expression of FHL in the fhy1 mutant-background results in a dominant-negative phenotype (Zeidler et al. 2004).

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In both light- and dark-grown seedlings, FHY1 and FHL localize predominantly to the nucleus and to a minor degree to the cytosol (Desnos et al. 2001, Zeidler et al. 2001, Zeidler et al. 2004, Zhou et al. 2005). Previously we have shown that phyA and FHY1 co-localize in light-induced nuclear speckles and that phyA binds to FHY1, predominantly in its biologically active Pfr form (Hiltbrunner et al. 2005). Here we extend these studies to FHL, and furthermore map the domains of FHY1/FHL and phyA essential for their interaction.

Results

Expression of FHL in the fhy1 background complements the mutant phenotype and confers phyA-dependent hypersensitivity to far-red light (Zhou et al. 2005). Moreover, the fhy1 fhl double mutant and fhy1 FHL RNAi knock-down lines exhibit a phyA null phenotype, and FHY1 and FHL act redundantly to control phyA-regulated gene expression (Fig. 1A and Supplementary Fig. 1) (Zhou et al. 2005). Previously we have shown that FHY1 plays a key role in nuclear accumulation of phyA, and that fhy1 mutant seedlings are unable to accumulate wild-type levels of phyA in the nucleus in response to VLFR and HIR treatments (Hiltbrunner et al. 2005). PhyA nuclear accumulation is, however, not completely inhibited in the fhy1 background, suggesting that FHL may be sufficient to allow accumulation of residual amounts of phyA in the nucleus in the absence of FHY1. Thus, FHY1 and FHL may act together to mediate phyA nuclear accumulation.

To test this hypothesis, we transformed an FHL RNAi construct, similar to that described by Zhou et al. (2005), into phyA-211 fhy1-1 plants expressing the PHYA promoter-driven phyA-green fluorescent protein (GFP). Irrespective of the light treatment, phyA-GFP is restricted to the cytosol in fhy1-1 FHL RNAi knock-down lines and does not accumulate to detectable levels in the nucleus (Fig. 1B), confirming that residual nuclear accumulation of phyA in the fhy1-1 background is due to FHL. Immunoblot analysis using a GFP-specific antibody shows that the levels of phyA-GFP are identical or at least very similar in a FHY1, fhy1-1 and fhy1-1 FHL RNAi background (Supplementary Fig. 2).

Previously it has been reported that the fhy1-1 mutant contains wild-type levels of photoreversible phytochrome (Whiteham et al. 1993), suggesting that reduced hypocotyl growth inhibition in the fhy1-1 and probably also in the fhy1-1 FHL RNAi background is not due to reduced amounts of hyA. Western blot analysis clearly shows that the fhy1-1 mutant as well as two independent fhy1-1 FHL RNAi lines contain phyA levels similar to the wild type (Fig. 1C). As the long hypocotyl phenotype of fhy1-1 and fhy1-1 FHL RNAi seedlings is specific to far-red light, it can hardly be attributed to diminished availability of phytochromobilin, the chromophore of plant phototropes. Reduced phytochromobilin levels would also affect responses mediated by phyB–E such as in the chromophore biosynthesis mutants hy1 and hy2 (Koornneef et al. 1980, Parks and Quail 1991). We nevertheless used fhy1-1 and fhy1-1 FHL RNAi seedlings for in vivo spectroscopy, which


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**Fig. 1** PhyA nuclear accumulation depends on FHY1 and FHL. (A) *fhy1-1 FHL RNAi* seedlings exhibit a phyA null phenotype. Wild-type (*Ler*), *fhy1-1, fhy1-1 FHL RNAi* (lines 417 and 423), *phyA-201* and *phyB-5* seedlings were grown for 5 d in the dark (D), red (R, 12 μmol m⁻² s⁻¹) far-red (FR, 13 μmol m⁻² s⁻¹) or blue (B, 21 μmol m⁻² s⁻¹) light. (B) PhyA nuclear accumulation is inhibited in *fhy1-1 FHL RNAi* seedlings. Three-day-old dark-grown wild-type (*FHY1*), *fhy1-1* and *fhy1-1 FHL RNAi* seedlings expressing *PHYA* promoter-driven phyA–GFP were irradiated for 6 h with far-red light followed by a 2 min white light pulse. A specific GFP filter set was used for microscopic analysis. The scale bar represents 5 μm. (C) *fhy1-1 FHL RNAi* seedlings contain wild-type levels of phyA. Total protein was extracted from 4-day-old dark-grown seedlings (*Ler, fhy1-1, phyA-201, fhy1-1 FHL RNAi* lines 417 and 423) and used for immunoblot analysis with an antibody specific to the N-terminus of Arabidopsis phyA (top panel). The lower panel shows a Coomassie blue-stained SDS–polyacrylamide gel confirming equal amounts of total protein in the extracts. A 30 μg aliquot of total protein was loaded per lane.

Confirms that the photoreversibility of phyA does not require FHY1 and FHL (data not shown). Therefore, we conclude that the lack of hypocotyl growth inhibition in *fhy1-1 FHL RNAi* lines is caused by the mislocalization of phyA, and that nuclear accumulation of phyA is essential to control hypocotyl elongation in far-red light.

*fhy1* and *fhy1 FHL RNAi* seedlings grown in red light do not differ from the wild type regarding hypocotyl length (Fig. 1A and Supplementary Fig. 1) (Whitelam et al. 1993, Desnos et al. 2001, Zeidler et al. 2001, Zhou et al. 2005). Together with the findings that phyB nuclear accumulation is not affected in a *fhy1-1* mutant background (Hiltbrunner et al. 2005) and that nuclear transport of the photoreceptor is an essential step in phyB-mediated hypocotyl growth inhibition (Huq et al. 2003, Matsushita et al. 2003), this suggests that FHY1 and FHL may be specifically required for nuclear accumulation of phyA but not phyB. To test this hypothesis, we crossed *fhy1-1* plants expressing the cauliflower mosaic virus 35S promoter (35S promoter)-driven phyB–GFP into the *fhy1-1 FHL RNAi* background. PhyB nuclear accumulation and speckle formation in response to 6 h irradiation with red light is indistinguishable in wild-type and *fhy1-1 FHL RNAi* seedlings (Fig. 2). Also the early phyB speckles, which depend on PIF3 and form within a few minutes after a short light pulse (Bauer et al. 2004), are detectable in the *fhy1-1 FHL RNAi* background, suggesting that phyB signaling is not affected in *fhy1-1 FHL RNAi* seedlings. Thus it seems very unlikely that FHY1 and FHL play a significant role in phyB nuclear accumulation.

The physiological redundancy as well as the homology between FHY1 and FHL at the amino acid level indicate that they most probably use similar, if not identical, mechanisms to control phyA nuclear accumulation, and in turn hypocotyl growth inhibition. To verify this prediction, we extended our previous studies done for FHY1 (Hiltbrunner et al. 2005) to FHL and analyzed the co-localization and light-dependent interaction of phyA and FHL.

In Arabidopsis plants transformed with a P₃₅S:YFP-FHL construct, FHL forms light-induced nuclear speckles reminiscent of those described for FHY1 and phyA (Fig. 3A) (Hiltbrunner et al. 2005). Transient expression of the same P₃₅S:YFP-FHL construct and phyA-cyan fluorescent protein (CFP) in etiolated mustard seedlings confirms that not only FHY1 but also FHL co-localizes with phyA in these light-dependent speckles (Fig. 3B). In dark-grown phyA-201 and *fhy1-1* seedlings complemented with P₃₅S:PHYA-YFP and P₃₅S:YFP-FHY1, respectively, as well as in wild-type plants expressing yellow fluorescent protein (YFP)–FHL, a short white light pulse given after 6 h irradiation with far-red light induces rapid formation of nuclear speckles of the YFP fusion...
proteins (Fig. 3A). Irradiation with red light for 6 h followed by a short white light pulse, however, does not induce formation of nuclear FHY1 and FHL speckles and results in complete degradation of phyA (Fig. 3A), suggesting that the FHY1 and FHL speckles depend on the presence of phyA. In fact, in phyA-201 seedlings expressing 35S promoter-driven FHY1–GFP or YFP–FHL, no speckles are detectable after 6 h exposure to far-red light followed by a white light pulse (Fig. 3C). The finding that FHY1 and FHL depend on phyA for assembly into light-induced nuclear speckles is consistent with the co-localization of FHY1/FHL and phyA in these speckles (Fig. 3B).

Although co-localization is a prerequisite for a physical interaction, it does not necessarily mean that FHL and phyA directly interact with each other as previously shown for FHY1 (Hiltbrunner et al. 2005). We therefore used glutathione S-transferase (GST) pull-down and yeast two-hybrid analysis to test for a physical interaction of FHL and phyA. For photoactivity, phytochromes strictly depend on their chromophore, which is autocatalytically attached to a conserved cysteine residue. However, phycocyanobilin (PCB), the chromophore of cyanobacterial phytochromes, can substitute for phytochromobilin in plant phytochromes (Kami et al. 2004). This allows reconstitution of photoactive phyA by supplementing the growth medium in the yeast two-hybrid assay with PCB purified from cyanobacteria or by adding it to in vitro synthesized phyA (Fairchild et al. 2000, Shimizu-Sato et al. 2002). In vitro pull-down analysis confirms that 35S-labeled photoactive phyA specifically binds to GST-tagged FHL and FHY1 but not to GST alone (Fig. 4A). Moreover, both FHL and FHY1 interact predominantly with the biologically active Pfr form of phyA. To corroborate further the light-regulated interaction of FHL and phyA, we employed a yeast two-hybrid-based growth assay. A 5 µl aliquot of yeast culture expressing FHL and phyA fused to the GAL4 activation and DNA-binding domains, respectively, was spotted onto selective plates supplemented with PCB. Growth was strongly dependent on a light treatment resulting in active phyA (Pfr), confirming that FHL and phyA interact with each other in a light-regulated fashion (Fig. 4B).

It seems to be contradictory that far-red light induces nuclear accumulation of phyA in plants, whereas the pull-down and yeast two-hybrid assays rather suggest a red light-specific interaction of phyA and FHY1/FHL. Exposure of phyA to far-red light results in about 3% of active phyA (PfrA) that accumulates in the nucleus (Kim et al. 2000, Nagatani 2004). This relatively low amount of active phyA is, however, optimal to induce, under continuous irradiation, a saturating phyA response in planta (Mancinelli 1994, Nagy and Schäfer 2002). Irradiation with red light results in >20 times higher levels of active phyA (Mancinelli 1994, Nagy and Schäfer 2002), suggesting that the in vitro assays are simply not sensitive enough to detect the interaction of FHY1/FHL with the comparably low amount of PfrA obtained after a far-red light treatment. Consistent with this idea, red light-induced
nuclear accumulation of phyA is also dependent on FHY1/FHL (data not shown).

FHL and FHY1 behave identically regarding co-localization and light-dependent interaction with phyA. This supports the notion that FHY1 and FHL employ the same molecular mechanism to achieve nuclear accumulation of phyA, and that amino acid residues indispensable for FHY1/FHL function may be conserved. At the sequence level, the homology between FHY1 and FHL is limited to the NLS/NES region and the extreme C-terminus (Fig. 5A). Amino acids 186-201 of FHY1 correspond exactly to residues 165-180 of FHL and, among the C-terminal 36 amino acids, 73% are identical in FHY1 and FHL. FHY1 lacking the C-terminal 19 amino acids (FHY1 1–183) does not complement the fhy1 mutant phenotype (Zeidler et al. 2004), and C-terminal deletions of FHY1 and FHL (FHY1 1–166 and FHL 1–146) do not form light-induced nuclear speckles in transgenic

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**Fig. 3** FHY1/FHL and phyA co-localize in light-induced nuclear speckles. (A) FHL forms light-induced nuclear speckles. Three-day-old dark-grown phyA-201 and fhy1-1 seedlings complemented with PPHYA:PHYA-YFP and P35S:YFP-FHY1, respectively, as well as wild-type (Ler) seedlings expressing P35S:YFP-FHL were analyzed directly (D) or subjected to different light treatments prior to microscopy (6 h FR, 6 h FR + W, 8 h R). A specific YFP filter set was used for microscopic analysis. The scale bar represents 10 μm. 6 h FR, 6 h far-red light; 6 h FR + W, 6 h far-red light followed by a 2 min white light pulse; 8 h R, 8 h red light. (B) FHL and phyA co-localize in nuclear speckles. P35S:PHYA-CFP was co-bombarded with either P35S:YFP-FHY1 or P35S:YFP-FHL into 3-day-old etiolated mustard seedlings. A 2 min white light pulse was applied prior to microscopy to induce formation of phyA, FHY1 and FHL speckles. Specific YFP and CFP filter sets were used for microscopic analysis. The third column shows the overlay of the respective CFP and YFP images. The scale bar represents 10 μm. (C) FHY1 and FHL speckles depend on phyA. fhy1-1 seedlings complemented with P35S:FHY1-GFP, phyA-201 seedlings expressing the same construct, as well as Ler and phyA-201 seedlings transformed with P35S:YFP-FHL were grown for 3 d in the dark and analyzed directly (D) or irradiated for 6 h with far-red light followed by a 2 min white light pulse (6 h FR + W) prior to microscopy. GFP- and YFP-specific filter sets were used for microscopic analysis. The scale bar represents 10 μm.
Arabidopsis lines (Fig. 5B), indicating that binding to phyA may depend on the C-terminal 36 amino acids. To test for such a function of the C-terminus of FHY1/FHL, we engineered various FHY1 and FHL deletions, truncated either at the N- or C-terminus (Fig. 5A), and subcloned them into a GAL4 activation domain vector for yeast two-hybrid analysis with phyA. None of the FHY1 and FHL deletions lacking the C-terminus interacts with phyA, whereas a C-terminal 17 amino acids fragment of FHY1 (FHY1 167–202) and all other FHY1/FHL versions containing the C-terminal 17 amino acids bind to phyA in a light-regulated fashion (Fig. 5C). The interaction between phyA and FHY1/FHL and some of the FHY1 deletions was also tested in a yeast two-hybrid β-galactosidase activity assay which confirms that the C-terminal 36 amino acids of FHY1 are essential and sufficient for light-regulated binding to phyA (Fig. 5D).

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Fig. 4 Light-regulated interaction of FHL and phyA in vitro and in yeast. (A) FHL interacts predominantly with the Pfr form of phyA. In vitro synthesized 35S-labeled phyA was incubated with PCB to allow the covalent conjugation of PCB to phyA. PhyA was then exposed to red light for 5 min, either followed by a 5 min far-red pulse (IVT phyA Pr) or not (IVT phyA Pfr), and incubated with recombinant GST–FHY1-H6, GST–FHL-H6 or GST–H6 (non-binding control) bound to glutathione–Sepharose. After washing, the Sepharose beads were incubated with SDS–PAGE sample buffer for elution. The samples were separated by SDS–PAGE and transferred onto a PVDF membrane. A phosphorimager was used for signal detection. Both the autoradiogram (upper panel) and the amido black-stained membrane (middle panel) are shown. To confirm equal amounts of input, 1.6% of the non-binding fractions were run on an SDS–polyacrylamide gel and blotted onto a PVDF membrane. The signals were detected using a phosphorimager. (B) Light-dependent interaction of FHL and phyA in yeast cells. Yeast (strain AH109) was transformed with the indicated plasmids. A 5 μl aliquot of overnight cultures was spotted onto selective synthetic dropout plates (L-W-H-, containing 1 mM 3-aminotriazole) supplemented with 20 μM PCB. The plates were incubated for 3 d in 1 μmol m−2 s−1 red light (Pfr) or 13 μmol m−2 s−1 far-red light (Pr). As a control, equal amounts of overnight cultures were spotted onto non-selective (L-W-) plates without PCB. AD, GAL4 activation domain; BD, GAL4 DNA-binding domain.
lyase domain of phyA (phyA 71–398) (Mathews et al. 1995, Quail 1997). As the C-terminal half of phyA (phyA 563–1122) is strongly transactivating in our system, we cannot rule out the possibility that—although not essential for binding to FHY1/FHL—it may have a regulatory function and stabilize or destabilize the FHY1/FHL–phyA interaction.

**Discussion**

Phytochromes partition between the cytosol and the nucleus in a light-regulated fashion (Kircher et al. 2002, Nagatani 2004). However, even under light conditions most favorable for nuclear accumulation, a pool of phyA and phyB is retained in the cytosol and does not translocate to the nucleus (Nagy and Schäfer 2002). Currently it is not known how nucleo-/cytoplasmic partitioning of phytochromes is regulated and if the active Pfr form of phytochromes in the cytosol is able to induce physiological responses. So far, phyB-mediated regulation of cotyledon size and hypocotyl elongation are the only phytochrome responses which have been proven to depend strictly on nuclear accumulation of the photoreceptor (Huq et al. 2003, Matsushita et al. 2003). Both phyA and phyB interact with various transcription factors (Fairchild et al. 2000, Martinez-Garcia et al. 2000, Huq et al. 2004, Khamma et al. 2004, Oh et al. 2004), and roughly 17% of the Arabidopsis genes are subject to regulation by red and/or far-red light (Tepperman et al. 2004). Thus it seems likely that many more—if not all—phytochrome-induced responses require nuclear accumulation of the photoreceptor.

Far-red light-grown fhy1-1 FHL RNAi seedlings are indistinguishable from the phyA null mutant regarding hypocotyl growth inhibition, cotyledon expansion and apical hook opening (Fig. 1A and Supplementary Fig. 1), which is consistent with data reported by Zhou et al. (2005). Here we show that phyA is restricted to the cytosol in fhy1-1 FHL RNAi seedlings, and that they are unable to accumulate detectable levels of phyA in the nucleus (Fig. 1B). Thus, control of hypocotyl elongation, cotyledon expansion and apical hook opening by far-red light depends on nuclear accumulation of phyA. In contrast, phyB nuclear accumulation and signal transduction are not affected in fhy1-1 and fhy1-1 FHL RNAi seedlings (Figs. 1A and 2, Supplementary Fig. 1), suggesting that FHY1 and FHL are specifically required for nuclear accumulation of phyA but not phyB. Despite the high similarity at the amino acid level (roughly 50% identical residues), phyA and phyB behave differently regarding nuclear transport and accumulation. PhyB nuclear translocation requires red light and is red/far-red reversible (LFR) whereas phyA nuclear accumulation is induced by short far-red light pulses (VLFR) or by continuous far-red light (HIR) (Kircher et al. 2005). In addition, nuclear accumulation of phyA is one order of magnitude faster as compared with phyB (Kircher et al. 2002). Therefore, it is likely that phyA and phyB employ at least partially different mechanisms for nuclear accumulation.

Over the past few years, a whole suite of factors involved in phytochrome signaling has been reported to form nuclear speckles (i.e. LAF1, HFR1, COP1, HY5, PIF3, SPA1, EID1, PAPPS and FHY1) (Ang et al. 1998, Ballesteros et al. 2001, Seo et al. 2003, Bauer et al. 2004, Hiltbrunner et al. 2005, Jang et al. 2005, Ryu et al. 2005, Marrocco et al. 2006), and our present study adds one more component, FHL, to this ever growing list (Fig. 3). For many of these proteins—including FHY1 and FHL—the physiological relevance and the exact function of the speckles is unknown. FHY1 and FHL lacking the C-terminal phyA-binding domain are not recruited into nuclear speckles, suggesting that light-induced co-localization of FHY1/FHL and phyA in such speckles depends on their physical interaction (Figs. 3–5). However, this does not necessarily mean that binding of FHY1/FHL to phyA is restricted to the speckles. Whether FHY1 and FHL are recruited to pre-existing phyA speckles or whether they assemble into nuclear speckles as pre-formed phyA–FHY1/FHL complexes is one of the most important questions. Given the list of speckle-forming components, another question is if any of these proteins co-localize with phyA and FHY1/FHL. As COP1 is essential for FHY1 accumulation in the dark (Shen et al. 2005), it may also be a component of the speckles containing phyA and FHY1/FHL. Answering these key questions will help to understand the role of FHY1 and FHL in nuclear accumulation of phyA.

Currently we can think of three models to explain FHY1/FHL-controlled nuclear accumulation of phyA. So far, no NLS motif has been identified in phyA, suggesting that it may rely on transport facilitators for nuclear translocation. A mutant version of FHY1 containing a disrupted NLS does not complement the fhy1-1 phenotype, which confirms that the NLS is essential for proper FHY1 function (Zeidler et al. 2004). Thus, FHY1 and FHL may simply provide an NLS for nuclear transport of phyA. As a result of the light-dependent interaction of phyA and FHY1/FHL, nuclear translocation and accumulation of phyA would be regulated by light. Alternatively, phyA nuclear transport may be independent of FHY1/FHL, but FHY1/FHL action may be essential to protect phyA from rapid degradation after import into the nucleus or to trap phyA in the nucleus and prevent it from being exported to the cytosol. The light-dependent interaction of phyA and FHY1/FHL would therefore result in light-regulated nuclear accumulation of phyA either due to increased protein stability or due to reduced export...
to the cytosol. However, the three models are mutually not exclusive, and phyA nuclear accumulation may be controlled at the same time at the level of import, export and protein stability.

We have shown that nuclear accumulation of the photoreceptor is an essential step in phyA-mediated hypocotyl growth inhibition. However, other phyA-induced responses—such as inhibition of gravitropism in far-red and blue light or responsiveness amplification by far-red light (Poppe et al. 1996, Hennig et al. 1999, Lariguet and Fankhauser 2004)—may be mediated by cytosolic phyA and therefore work normally in *fhy1-1 FHL RNAi* knockdown plants. PKS1, a protein localizing to the cytosol, interacts with phyA and plays a role in the phyA-mediated VLFR response (Fankhauser et al. 1999, Lariguet et al. 2003). Another protein involved in red and far-red light signaling, NDPK2, binds preferentially to the Pfr form of phyA and localizes to the cytosol and the nucleus (Choi et al. 1999). Thus it seems possible that there are two main signaling pathways for phyA, one in the cytosol which...
The FHL cDNA was obtained as follows. An FHL (At5g02200) cDNA fragment was amplified by reverse transcription-PCR (RT-PCR) from Col total RNA using 5′ (5′-CGC GGA TTC AAA AAT GAT AGT TGC TGT GGA A-3′) and 3′ (5′-GGA CTA GTC ATC ATG GTA GAA AAG-3′) primers including BamHI and SpeI sites, and ligated into pBS II KS cut with BamHI-SpeI.

pGEX6p1H₄-FHY1 and pGEX6p1H₆ are pGEX6p1 (Amersham Biosciences, Sunnyvale, CA, USA) derivatives and have been described previously (Hiltbrunner et al. 2005). To obtain pGEX6p1H₄-FHL, the BamHI-SpeI fragment of pBS II KS-FHL encoding FHL was ligated into pGEX6p1H₄-FHY1 cut with BamHI and SpeI to replace FHY1.

The construct used for coupled in vitro transcription/translation of phyA has been described previously (Fairchild et al. 2000).

FHY1 and FHL fragments were obtained as follows. FHY1 1–166, FHY1 1–185, FHY1 117–202, FHL 1–146 and FHL 1–164 were PCR amplified from pBS II KS-FHY1 and pBS II KS-FHL using 5′ and 3′ primers including BamHI and SpeI sites (FHY1 1–166, 5′-CGC GGA TTC AAA AAT GAT GCC TGA AGT GGA AGT G-3′ and 5′-GGA CTA GTC TTT CCA AAA AAT GCT GTC CTC TGG GCC TGA AGT GGA AGT G-3′; FHY1 1–185, 5′-CGC GGA TTC AAA AAT GAT GCC TGA AGT GGA AGT G-3′ and 5′-GGA CTA GTC TTT CCA AAA AAT GCT GTC CTC TGG GCC TGA AGT GGA AGT G-3′; FHY1 117–202, 5′-CGC GGA TTC AAA AAT GCT GTC CTC TGG GCC TGA AGT GGA AGT G-3′ and 5′-GGA CTA GTC TTT CCA AAA AAT GCT GTC CTC TGG GCC TGA AGT GGA AGT G-3′; FHL 1–146, 5′-CGC GGA TTC AAA AAT GAT GCC TGA AGT GGA AGT G-3′ and 5′-GGA CTA GTC TTT CCA AAA AAT GCT GTC CTC TGG GCC TGA AGT GGA AGT G-3′; FHL 1–164, 5′-CGC GGA TTC AAA AAT GAT GCC TGA AGT GGA AGT G-3′ and 5′-GGA CTA GTC TTT CCA AAA AAT GCT GTC CTC TGG GCC TGA AGT GGA AGT G-3′). The construct used for coupled in vitro transcription/translation of phyA has been described previously (Fairchild et al. 2000).

**Materials and Methods**

**Cloning of constructs**

pBS II KS-FHY1 is a derivative of pBS II KS (Stratagene, La Jolla, CA, USA) containing the FHY1 cDNA (Hiltbrunner et al. 2005).
ACC TTG G-3' and 5'-CAT GGA GCT CTT AAC TAG TCT TGT TG CAG CAA G-3' and ligated into D153ah-phyA digested with BamHI and SpeI to replace full-length phyA.

pCHF20 and pCHF30 are T-DNA vectors containing P35S::BamHI-XbaI-GFP-TerRbcS and P35S::BamHI-XbaI-YFP-TerRbcS cassettes, respectively. They were obtained as follows. GFP and YFP were PCR amplified from pEGFP and pEYFP (Clontech) using a 5'-CCG GGT ACC TCC TGG TCA ATG AGC AAG GCC GAG G-3' including BamHI and XbaI sites and a 3'-primer (5'-CCG GGT ACC TCT AGC TTA ACT AGT CTT GTA CAG CTC GTC CAT G-3') containing Nhel and SpeI sites. The GFP and YFP fragments were digested with BamHI and Nhel, and ligated into pCHF5 (Hiltbrunner et al. 2005) cut with BamHI–XbaI to obtain pCHF20 and pCHF30.

PCHF70 is a T-DNA vector containing a P35S::YFP-BamHI-XbaI-TerRbcS cassette. pUC1940 is a pUC19 (Fermentas, Burlington, Canada) derivative and contains a P35S::BamHI-XbaI-CFP-TerRbcS cassette. pCHF70, pCHF70-FHY1, pUC1940 and pUC1940-phyA have been described previously (Hiltbrunner et al. 2005).

Wild-type and truncated versions of FYH1 and FHL fused to the N-terminus of GFP were obtained as follows. FYH1, FYH1 1-166, FHL and FHL 1-146 were cut with BamHI and Nhel sites (5'-CCG GGT ACC GCT AGC G-3') containing Nhel and SpeI sites. The GFP and YFP fragments were digested with BamHI and Nhel, and ligated into pCHF5 (Hiltbrunner et al. 2005) cut with BamHI–XbaI to obtain pCHF20 and pCHF30.

Plant material

The phyA-201 and fhy1-1 mutants as well as phyA-211 FYH1 PPHYA::PHYA-GFP, phyA-211 fhy1-1 PPHYA::PHYA-GFP, Ws P35S::PHYB-GFP, fhy1-1 P35S::PHYB-GFP and fhy1-1 P35S::YFP-FHY1 plants have been described previously (Nagatani et al. 1993, Whitelam et al. 1993, Reed et al. 1994, Desnos et al. 2001, Kircher et al. 2002, Hiltbrunner et al. 2005). phyA-211 fhy1-1 PPHYA::PHYA-GFP P35S::FHL RNAi, fhy1-1 P35S::FHL RNAi, phyA-211 PPHYA::PHYA-YFP, Ler P35S::YFP-FHY1, fhy1-1 P35S::FYH1-GFP, phyA-201 P35S::PHYB-GFP, phyA-201 P35S::YFP-FHL, fhy1-1 P35S::FYH1 fhy1-1 P35S::FYH1 RNAi, Ler P35S::FHL and Ler P35S::FHL 1-146-GFP have been obtained by Agrobacterium-mediated transformation (Clough and Bent 1998). The fhy1-1 P35S::PHYB-GFP P35S::FHL RNAi line was obtained by crossing fhy1-1 P35S::PHYB-GFP plants into the fhy1-1 P35S::FYH1 RNAi background.

Transient expression assay

Transient transformation of mustard seedlings has been described previously (Hiltbrunner et al. 2005, Stolpe et al. 2005).

Protein purification and in vitro pull-down assay

For protein expression, pGEX6P1H6-FHY1, pGEX6P1H6-FHL and pGEX6P1H6 were transformed into Escherichia coli DH5a. Expression and purification using Ni-NTA agarose (Qiagen, Valencia, CA, USA) was done according to the supplier’s instructions. The in vitro GST pull-down assay was done as described previously (Hiltbrunner et al. 2005).

Yeast two-hybrid assay

The yeast two-hybrid assays were done as described previously (Shimizu-Sato et al. 2002, Hiltbrunner et al. 2005).

Western blot analysis

Protein extraction and immunoblot analysis was done as described previously (Kircher et al. 2002). For immunodetection of phyA, an antisera raised against the N-terminal half of Arabidopsis phyA was used. The GFP-specific antibody has been described previously (Kircher et al. 1999).

Plant growth, light sources and microscopy

Seedlings for microscopy, hypocotyl length measurement and protein extraction were grown at 25 °C in Petri dishes on four layers of wet filter paper. The seeds were imbibed for 2 d at 8 °C in the dark. Germination was induced by 6 h irradiation with red light. For induction of germination and growth in red light, a standard red light field was used (12 μmol m-2 s-1, 656 nm, 24 nm full width at half-maximum). Standard far-red (13 μmol m-2 s-1, 730 nm, 128 nm full width at half-maximum) and blue light fields (21 μmol m-2 s-1, 436 nm, 43 nm full width at half-maximum) were used for all far-red and blue light treatments. The microscope lamp was used for white light pulses prior to microscopy. To confirm that white light and red light pulses have the same effect on speckle formation, we also did the experiments with seedlings irradiated in the red light field. The results were identical irrespective of whether white light or red light was used to induce speckle formation (data not shown). For microscopic analysis, an Axioskop microscope (Zeiss, Oberkochen, Germany) with an AxioCam camera system (Zeiss) and GFP-, YFP- and CFP-specific filter sets (AHF Analysetechnik, Tübingen, Germany) were used.

The FYH1–SpeI fragment of pBS II KS-phyA (Hiltbrunner et al. 2005) encoding PHYA cDNA was ligated into pCHF30 cut with BamHI–XbaI to obtain pCHF30-phyA. Secondly, a 2290 bp phyA promoter fragment containing EcoRI and BamHI sites at the 5' and 3' ends (provided by F. Nagy, Plant Biology Institute, Biological Research Center, Szeged, Hungary) was ligated into the EcoRI–BamHI site of pSV-SPORT I (Gibco BRL, Gaithersburg, MD, USA), resulting in pSV-SPORT I-phyA. The 25S promoter NcoI–BamHI fragment of pCHF30-phyA was then replaced by the PHYA promoter NcoI–BamHI fragment of pSV-SPORT I-phyA to obtain pphyA30-phyA.

The FHL RNAsi construct was obtained as follows. The sense fragment of FHL was PCR amplified from genomic DNA (ecotype Columbia) using a 5'-primer including KpnI and BamHI sites (5'-CCG GGT ACC GGA GCT TCC TGG TCA ATG AAG AAT CA-3') and a 3'-primer that contained a PstI site (5'-AAA ACT GCA GAT GTG TGG TTT CTT GAG AG-3'). The PCR fragment was digested with KpnI and PstI, and ligated into the KpnI–PstI site of pBS II KS. The FHL antisense fragment was PCR amplified from genomic DNA using 5' and 3' primers including PstI and XbaI sites (5'-AAA ACT GCA GTG ACA CTC CAC CTT GCA GA-3' and 5'-GCT CTA GAC TGC ATG CTC AAG AAT CA-3'). The PCR fragment was digested with PstI and XbaI, and ligated into the PstI–XbaI site of pBS II KS containing the FHL sense fragment. The FHL sense-antisense fragment was then cut with BamHI and XbaI, and ligated into the BamHI–XbaI site of pCHF5.

PCR-amplified fragments have been verified by DNA sequencing.
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References


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