PCP
PLANT & CELL PHYSIOLOGY

Regular Paper

# Three Transcription Factors, HFR1, LAF1 and HY5, Regulate Largely Independent Signaling Pathways Downstream of Phytochrome A

In-Cheol Jang[1,2], Rossana Henriques[1] and Nam-Hai Chua[1,*]

[1]Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA
[2]Present address: Temasek Life Sciences Laboratory, National University of Singapore, Singapore 117604.
*Corresponding author: E-mail, chua@mail.rockefeller.edu; Fax, +1 212 327 8327.
(Received January 7, 2013; Accepted March 9, 2013)

**Among signaling components downstream of phytochrome A (phyA), HY5, HFR1 and LAF1 are transcription factors that regulate expression of phyA-responsive genes. Previous work has shown that FHY1/FHL distribute phyA signals directly to HFR1 and LAF1, both of which regulate largely independent pathways, but the relationship of HY5 to these two factors was unclear. Here, we investigated the genetic relationship among the genes encoding these three transcription factors, *HY5*, *HFR1* and *LAF1*. Analyses of double and triple mutants showed that HY5, a basic leucine zipper (bZIP) factor, HFR1, a basic helix–loop–helix (bHLH) factor, and LAF1, a Myb factor, independently transmit phyA signals downstream. We showed that HY5 but not its homolog, HYH, could interact with HFR1 and LAF1; on the other hand, FHY1 and its homolog, FHL did not interact with HY5 or HYH. Together, our results suggest that HY5 transmits phyA signals through an FHY1/FHL-independent pathway but it may also modulate FHY1/FHL signal through its interaction with HFR1 and LAF1.**

**Keywords:** Double and triple mutants • Light signaling • Phytochrome A • Protein interaction • Signaling cascade • Transcription factors.

**Abbreviations:** bHLH, basic helix–loop–helix, bZIP; basic leucine zipper, Col; Columbia, FAR1; FAR-RED-IMPAIRED RESPONSE, FHL; FHY1-LIKE, FHY1; FAR-RED ELONGATED HYPOCOTYL 1, FHY3; FAR-RED ELONGATED HYPOCOTYLS 3, FR; far-red, FRc; continuous far-red, GST; glutathione *S*-transferase, HFR1; LONG HYPOCOTYL IN FAR-RED 1, HY5; LONG HYPOCOTYL 5, LAF1; LONG AFTER FAR-RED LIGHT 1, L*er*; Lansberg *erecta*, MBP; maltose-binding protein, NLS; nuclear localization signal, PhyA; phytochrome A, RNAi; RNA interference, RT–PCR; reverse transcription–PCR, WT; wild type.

## Introduction

As sessile and photo-autotrophic organisms, plants use light not only as an energy source for photosynthesis but also as an environmental cue to provide them with positional information to adjust and adapt their physiological responses throughout their life cycle. To perceive changes in light quality, fluences, direction and duration, Arabidopsis possesses four classes of photoreceptors: phytochromes (phyA–phyE), cryptochromes (cry1 and cry2), phototropins (phot1 and phot2) and Zeitlupe family members (ZTL, FKF1 and LKP2). Cryptochromes, phototropins and the Zeitlupe family specifically detect ultraviolet-A/blue light, whereas phytochromes absorb primarily red and far-red (FR) light (Kami et al. 2010).

Light regulates many developmental events during the early stages of plant development, e.g. seed germination and inhibition of hypocotyl elongation and greening of the emerged seedling (Quail 2002). Among the five members of Arabidopsis phytochromes, phyA plays a major role in such early developmental processes. Genetic analysis has uncovered >10 mutants affected in either positive or negative regulatory components of phyA signaling; the responsible genes have been identified and their products characterized. However, many aspects of their site of action, their inter-relationship and their hierarchical location in the phyA signaling pathway remain unresolved. Among the positive regulatory components of phyA signaling, the basic leucine zipper (bZIP) factor HY5 (LONG HYPOCOTYL 5) (Oyama et al. 1997), the basic helix–loop–helix (bHLH) factor HFR1 (LONG HYPOCOTYL IN FAR-RED 1) (Fairchild et al. 2000, Fankhauser and Chory 2000, Soh et al. 2000) and the Myb factor LAF1 (LONG AFTER FAR-RED LIGHT 1) (Ballesteros et al. 2001) are known to be transcription factors, and all three have been shown to be substrates of the COP1 E3 ligase (Seo et al. 2003, Saijo et al. 2003, Jang et al. 2005, Yang et al. 2005).

Light-induced phytochrome nuclear import is a crucial regulatory step to trigger a light signaling cascade that underpins the ensuing biological responses. This event has been investigated in some detail for phyB. The C-terminal PAS-related domain of phyB contains a putative nuclear localization signal (NLS) which in the dark is masked by the N-terminal bilin lyase domain (BLD) and the PHY domain through direct interaction (Chen et al. 2005). It has been suggested that light

*Plant Cell Physiol.* 54(6): 907–916 (2013) doi:10.1093/pcp/pct042, available online at www.pcp.oxfordjournals.org
© The Author 2013. Published by Oxford University Press on behalf of Japanese Society of Plant Physiologists.
All rights reserved. For permissions, please email: journals.permissions@oup.com

triggers a conformational change which unmasks the NLS to facilitate phyB nuclear import (Chen et al. 2005). As phyA does not contain any NLS, it is logical to assume that other signaling components assist nuclear import of the photoreceptor. Among the identified components, two plant-specific proteins, FHY1 (FAR-RED ELONGATED HYPOCOTYL 1) and its homolog FHL (FHY1-LIKE) (Zeidler et al. 2001, Zhou et al. 2005), contain an NLS at their N-terminus and interact preferentially with light-activated Pfr phyA (Zeidler et al. 2004, Hiltbrunner et al. 2005, Hiltbrunner et al. 2006). Consistent with their role in phyA nuclear import, *fhy1fhl* double mutant plants are similar although not identical to *phyA* mutant plants with respect to early light seedling responses such as germination, hypocotyl elongation and cotyledon greening (Rösler et al. 2007). The transcription of *FHY1* and *FHL* depends on two transposase-derived transcription factors, FHY3 (FAR-RED ELONGATED HYPOCOTYLS 3) and its homolog FAR1 (FAR-RED-IMPAIRED RESPONSE), which together indirectly control phyA nuclear import (Lin et al. 2007). Recently, it has been reported that FHY3/FAR1-activated *FHY1/FHL* expression is repressed by HY5 in a negative feedback loop of phyA signaling (Li et al. 2010).

In addition to their role in facilitating nuclear import of phyA, FHY1/FHL also directly interact with HFR1 and LAF1 to transmit phyA signals (Yang et al. 2009). The general picture that emerges is that FHY1/FHL may nucleate a signaling complex with HFR1 and LAF1 to execute their functions, but whether FHY1/FHL also interact other factors is not clear. In addition, since FHY1/FHL deficiency does not completely abolish phyA signaling, other FHY1/FHL-independent signaling branches must exist.

PhyA signaling mutants deficient in *HY5*, *HFR1* and *LAF1* are hyposensitive to continuous far-red (FRc) light, showing longer hypocotyls than those of the wild type (WT). The relationships between these three factors have been investigated to some extent by comparative analyses of single and double mutants. For example, the *hfr1laf1* or *hy5hfr1* double mutant shows an additive hypocotyl phenotype of the two single mutants, indicating that the transcription factors function largely independently. Nevertheless, under FRc light, these double mutants are still shorter than *fhy1-3* or *fhy1fhl1* (Kim et al. 2002, Jang et al. 2007, Yang et al. 2009). This observation suggests that other factor(s) may operate downstream of the phyA signaling cascade through either FHY1/FHL or FHY3/FAR1, or via some as yet unidentified component.

Here, we addressed the relationship between HY5 and FHY1, and between HY5 and the other two transcription factors, HFR1 and LAF1. We found that the *hy5hfr1laf1* triple mutant has an additive hypocotyl phenotype compared with each of the double mutants and demonstrated that HY5 interacted with HFR1 and LAF1, but not with FHY1 or FHL in vitro and in vivo. These results led us to conclude that HY5 probably transmits phyA signals through an FHY1/FHL-independent pathway.

## Results

### The *hy5laf1* double mutant has an additive phenotype compared with either single mutant

Previous studies (Kim et al. 2002, Jang et al. 2007) have shown that the *hfr1hy5* and *hfr1laf1* double mutants have an additive hypocotyl phenotype compared with the single mutants. These results suggest that HFR1, LAF1 and HY5 control largely independent pathways downstream of phyA. If this is true, then the *hy5laf1* double mutant should also display an additive phenotype with respect to hypocotyl length compared with the single mutants, *hy5* and *laf1*.

To generate the *hy5laf1* double mutant for comparative analysis with the single mutants, we used RNA interference (RNAi) to suppress *LAF1* expression (Jang et al. 2007) in the *hy5-1* background. More than 10 RNAi lines (*LAF1RNAi/hy5*; hereafter referred to as the *hy5laf1* double mutant) were obtained and three lines were selected for further analysis. **Fig. 1A** and **Supplementary Fig. S1A** show that *LAF1* expression levels were highly reduced in the three selected *hy5laf1* double mutant lines as monitored by reverse transcription–PCR (RT–PCR) (**Supplementary Fig. S1A**) as well as by quantitative real-time PCR (**Fig. 1A**). Phenotypic analysis showed that the *hy5laf1* double mutants displayed an additive phenotype, with longer hypocotyls than those of the *hy5-1* and *laf1* single mutants, but shorter than those of *phyA* mutant (**Fig. 1B**; **Supplementary Fig. S1B**). Similar results were obtained over a range of FRc fluences (**Fig. 1B**; **Supplementary Fig. S1B**). Note that our *hy5laf1* double mutant along with the previously reported *hfr1hy5* (Kim et al. 2002) and *hfr1laf1* mutants (Jang et al. 2007) provide all possible double mutants with deficiency in two of the three transcription factors, HFR1, LAF1 and HY5.

### HFR1, LAF1 and HY5 regulate largely independent pathways in phyA signaling

To examine further the genetic relationship among *HFR1*, *LAF1* and *HY5*, we generated a *LAF1RNAi/hy5hfr1* triple mutant (referred to hereafter as the *hy5hfr1laf1* triple mutant). Because *hy5-1hfr1-201* (referred to hereafter as the *hy5hfr1* double mutant) and *laf1* are in different genetic backgrounds (Ballesteros et al. 2001, Kim et al. 2002), we used the same *LAF1* RNAi construct to suppress *LAF1* expression in the *hy5hfr1* double mutant background. **Fig. 2A** and **Supplementary Fig. S2** show that *LAF1* transcript levels were highly reduced in the three representative triple mutant lines. This *LAF1* transcript reduction (**Fig. 2A**) resulted in an additive hypocotyl phenotype in the *hy5hfr1laf1* triple mutant compared with the *hy5hfr1* double mutant (**Fig. 2B**).

We have previously shown that HFR1 and LAF1 interact with FHY1 and function downstream of the latter factor (Jang et al. 2007). **Fig. 2C** shows that the *hy5hfr1laf1* triple mutant was longer than each of the double mutants deficient in two of the three transcription factors, HFR1, LAF1 and HY5; moreover, it was similar in length to or slightly longer than

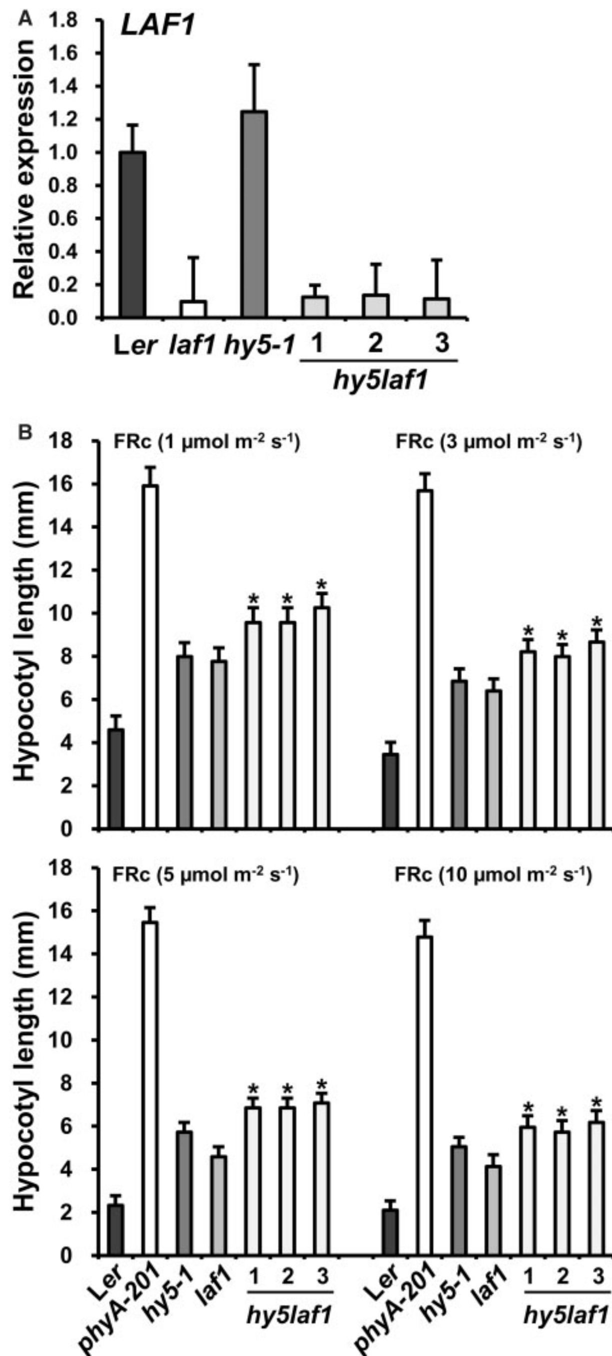


**Fig. 1** Phenotypes of the *hy5laf1* double mutant under continuous far-red (FRc) light. (A) Quantitative real-time PCR analysis showing reduction of *LAF1* transcript in *hy5laf1* lines. (B) Hypocotyl length of seedlings of WT (*Ler*), *phyA-201*, *hy5-1*, *laf1* and *hy5laf1* lines (lines #1–3) after irradiation under different fluence rates (1, 3, 5 and 10 μmol $m^{-2}$ $s^{-1}$) of FRc light. Data are presented as average hypocotyl length ± SD ($n = 40$). An asterisk denotes significant differences from single mutants (*hy5-1* and *laf1*) based on Student's *t*-test ($P < 0.01$).

*fhy1-3*. The latter result suggests that signal transmission through HY5 is independent of and/or in part dependent on FHY1. On the other hand, the hypocotyl length of the *hy5hfr1laf1* triple mutant was clearly shorter than those of *fhy1fhl* and *phyA* at the range of fluence rates tested (Fig. 2C).

## HY5 mutation in *fhy1-3* causes an additive effect under FRc light

To see if the HY5 function in FR-mediated signaling depends on FHY1, we generated *HY5RNAi/fhy1* lines by introducing *HY5RNAi* into the *fhy1-3* mutant background. First, we tested the efficacy of the *HY5* RNAi construct in Arabidopsis WT [Lansberg *erecta* (*Ler*)] plants. Fig. 3A and Supplementary Fig. S3A show that *HY5* transcript levels were greatly reduced in *HY5RNAi* (*HY5Ri*) lines. In addition, these lines mimic the *hy5-1* mutant phenotype under FRc light, indicating a high silencing efficiency of our *HY5* RNAi construct (Fig. 3B; Supplementary Fig. S3B). Next, we introduced the same *HY5* RNAi construct into the *fhy1-3* mutant background to generate *HY5RNAi/fhy1* lines (hereafter referred to as the *fhy1hy5* double mutant) for further study. Fig. 3C and Supplementary Fig. S3C show that *HY5* transcript levels were highly reduced in *fhy1hy5* double mutants. Comparative analysis of seedlings under FRc light showed that the hypocotyls of three independent *fhy1hy5* double mutants were clearly longer than those of *fhy1-3*, indicating independent function of HY5 and FHY1 (Fig. 3D; Supplementary Fig. S3D). Similar additive effects of the *fhy1* mutation and *HY5RNAi* were obtained at different FR fluence rates. These results are in contrast to those of *fhy1hfr1* and *fhy1laf1* double mutants in which the *fhy1* mutation was shown to be epistatic (Yang et al. 2009).

## HY5 interacts with HFR1 and LAF1 in vitro and in vivo

We have previously shown that phyA signals are transmitted to HFR1 and LAF1 through a complex containing FHY1/FHL (Yang et al. 2009). In the case of HY5, our genetic results showed that this transcription factor acts independently of FHY1. To investigate this issue further, we examined whether FHY1 would interact with HY5 in vitro. Fig. 4A shows that FHY1 interacted with itself as well as with HFR1, confirming previous results (Yang et al. 2009), but FHY1 did not bind to HY5 or to its homolog, HYH. Since FHL is an FHY1 homolog, we also tested its ability to associate with HY5 or HYH. The same results were obtained when FHL was used as a bait protein for in vitro pull-down assays (Fig. 4A). This lack of physical interaction between FHY1 or FHL and HY5 confirmed their independent mode of action.

Previous work showed that HFR1 can associate with LAF1 and function independently and interdependently in phyA signaling (Jang et al. 2007). Therefore, we examined possible direct interactions between HFR1 and HY5 and/or LAF1 and HY5 by in vitro pull-down assays. Fig. 4B shows that HY5 interacted

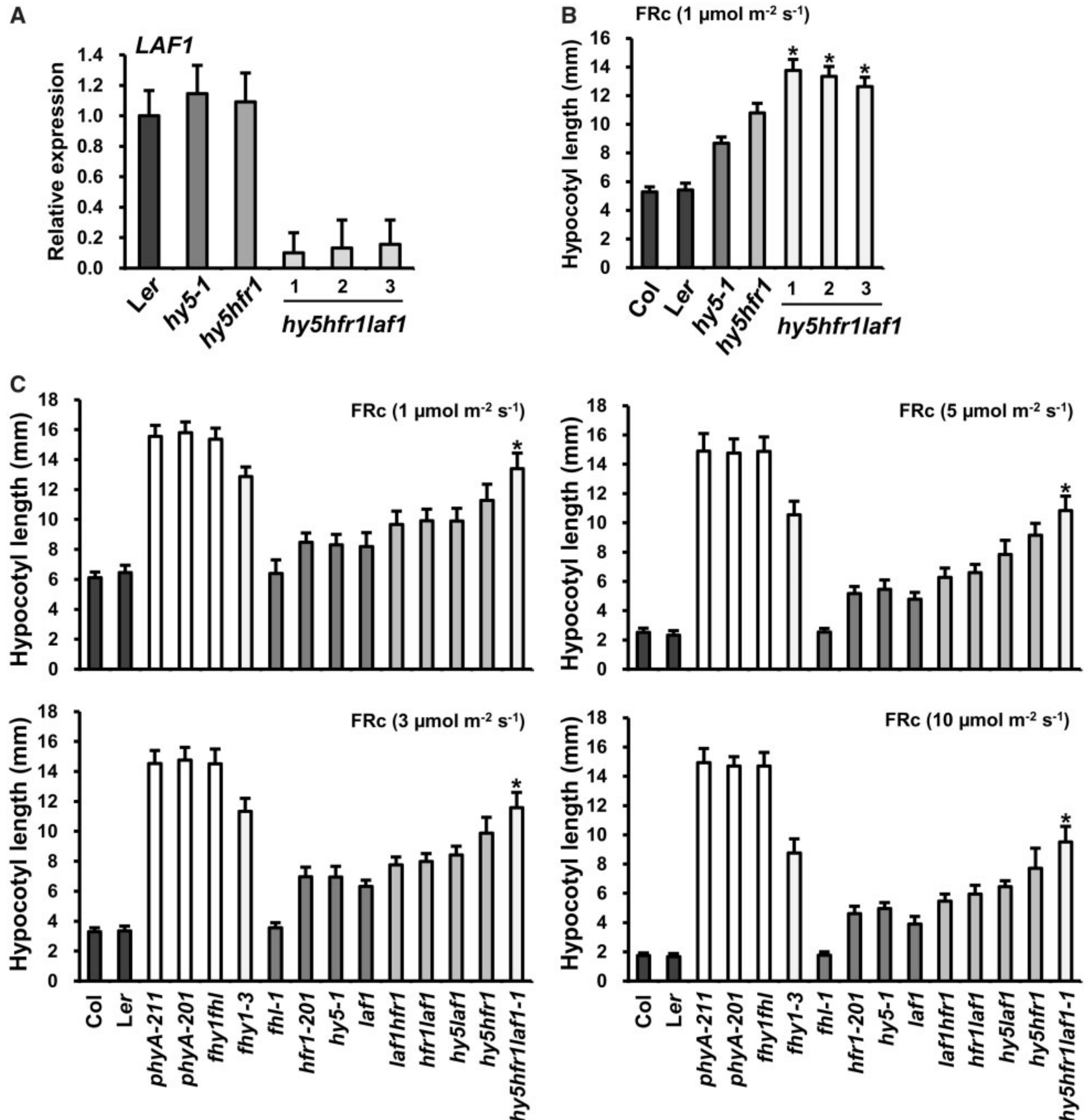


**Fig. 2** Phenotypes of the *hy5hfr1laf1* triple mutant under continuous far-red (FRc) light. (A) Quantitative real-time PCR analysis showing reduction of the *LAF1* transcript in *hy5hfr1laf1* lines. (B) Hypocotyl length of WT, *hy5-1*, *hy5hfr1* and *hy5hfr1laf1* seedlings after irradiation with FR light (1 $\mu$mol m$^{-2}$ s$^{-1}$). Data are presented as average hypocotyl length ± SD ($n = 40$). An asterisk denotes significant differences from *hy5hfr1* based on Student's *t*-test ($P < 0.01$). (C) Responses of the *hy5hfr1laf1* triple mutant under different fluence rates (1, 3, 5 and 10 $\mu$mol m$^{-2}$ s$^{-1}$) of FRc light. WT, *phyA* mutants (*phyA-211* and *phyA-201*), *fhy1fhl*, *fhy1-3*, *fhl-1*, *hfr1-201*, *hy5-1*, *laf1*, *laf1hfr1*, *hfr1laf1*, *hy5laf1*, *hy5hfr1* and *hy5hfr1laf1* were used and hypocotyl lengths were measured. Data are presented as average hypocotyl length ± SD ($n = 40$). An asterisk denotes significant differences from double mutants (*laf1hfr1*, *hfr1laf1*, *hy5laf1* and *hy5hfr1*) based on Student's *t*-test ($P < 0.01$).

with HFR1 and also with LAF1 whereas the HY5 homolog HYH interacted with neither. To confirm the interactions between HFR1 and HY5 and/or LAF1 and HY5 in vivo, we generated double transgenic plants co-expressing HY5-3HA/HFR1-6Myc or HY5-3HA/LAF1-6Myc. An estradiol-inducible system was used to express HFR1-6Myc or LAF1-6Myc (Zuo et al. 2000). Fig. 4C and D show that immunoprecipitates of HFR1-6Myc or LAF1-6Myc, which was expressed only upon inducer treatment, contained HY5-3HA, verifying HY5–HFR1 and HY5–LAF1 associations in vivo.

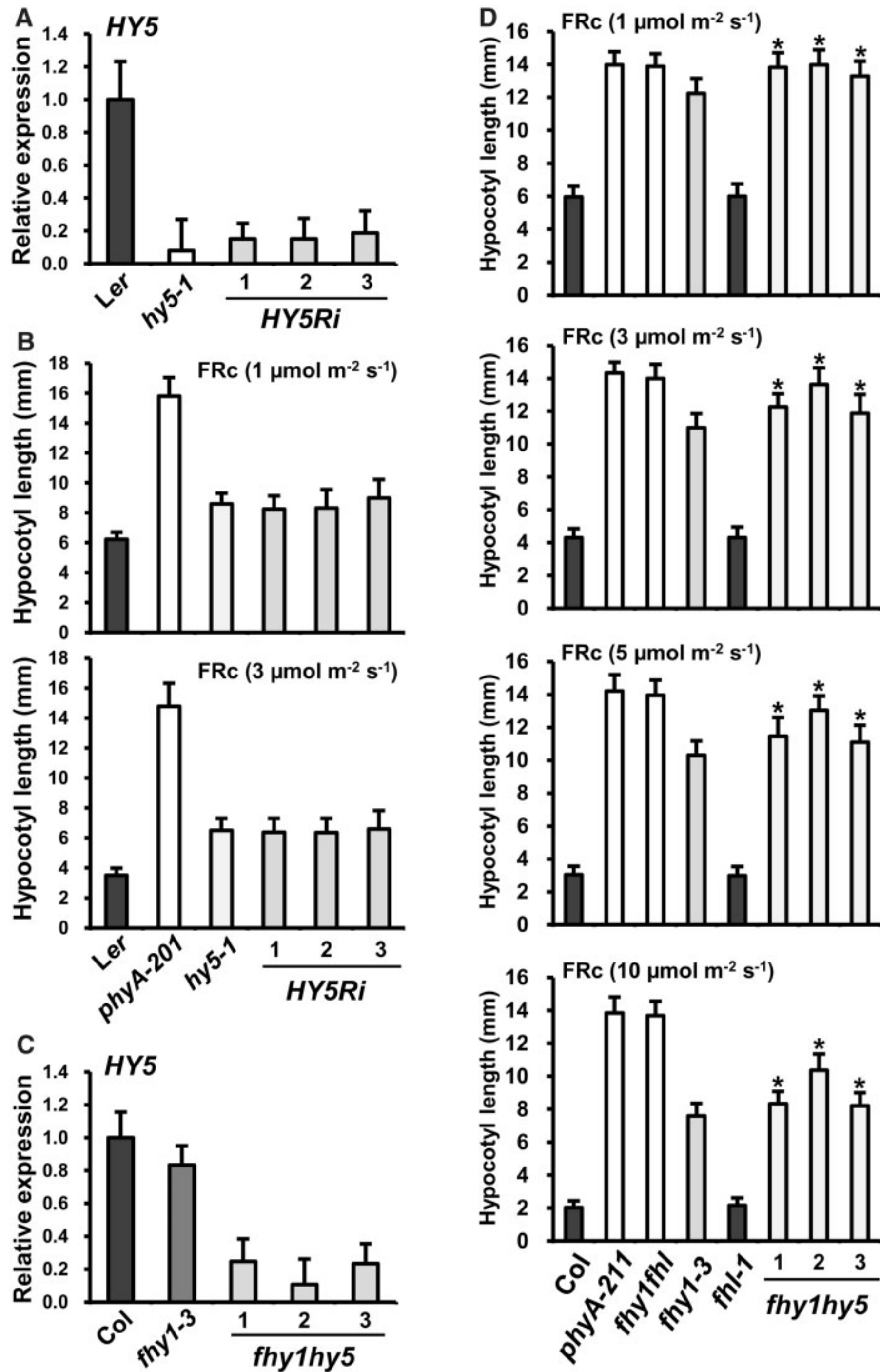


**Fig. 3** Phenotypes of the *fhy1hy5* double mutant under continuous far-red (FRc) light. (A) Quantitative real-time PCR analyses showing reduction of the *HY5* transcript in *HY5Ri* lines. (B) Hypocotyl length of seedlings of WT (L*er*), *phyA-201*, *hy5-1* and *HY5Ri* lines (lines #1–3) after irradiation under different fluence rates (1 and 3 μmol $m^{-2}$ $s^{-1}$) of FRc light. Data are presented as average hypocotyl length ± SD ($n$ = 40). (C) Quantitative real-time PCR analyses showing reduction of the *HY5* transcript in *fhy1hy5* lines. (D) Hypocotyl length of seedlings of WT (Col), *phyA-211*, *fhy1fhl*, *fhy1-3*, *fhl-1* and *fhy1hy5* lines (lines #1–3) after irradiation under different fluence rates (1, 3, 5 and 10 μmol $m^{-2}$ $s^{-1}$) of FRc light. Data are presented as average hypocotyl length ± SD ($n$ = 40). An asterisk denotes significant differences from *fhy1-3* based on Student's *t*-test ($P < 0.01$).

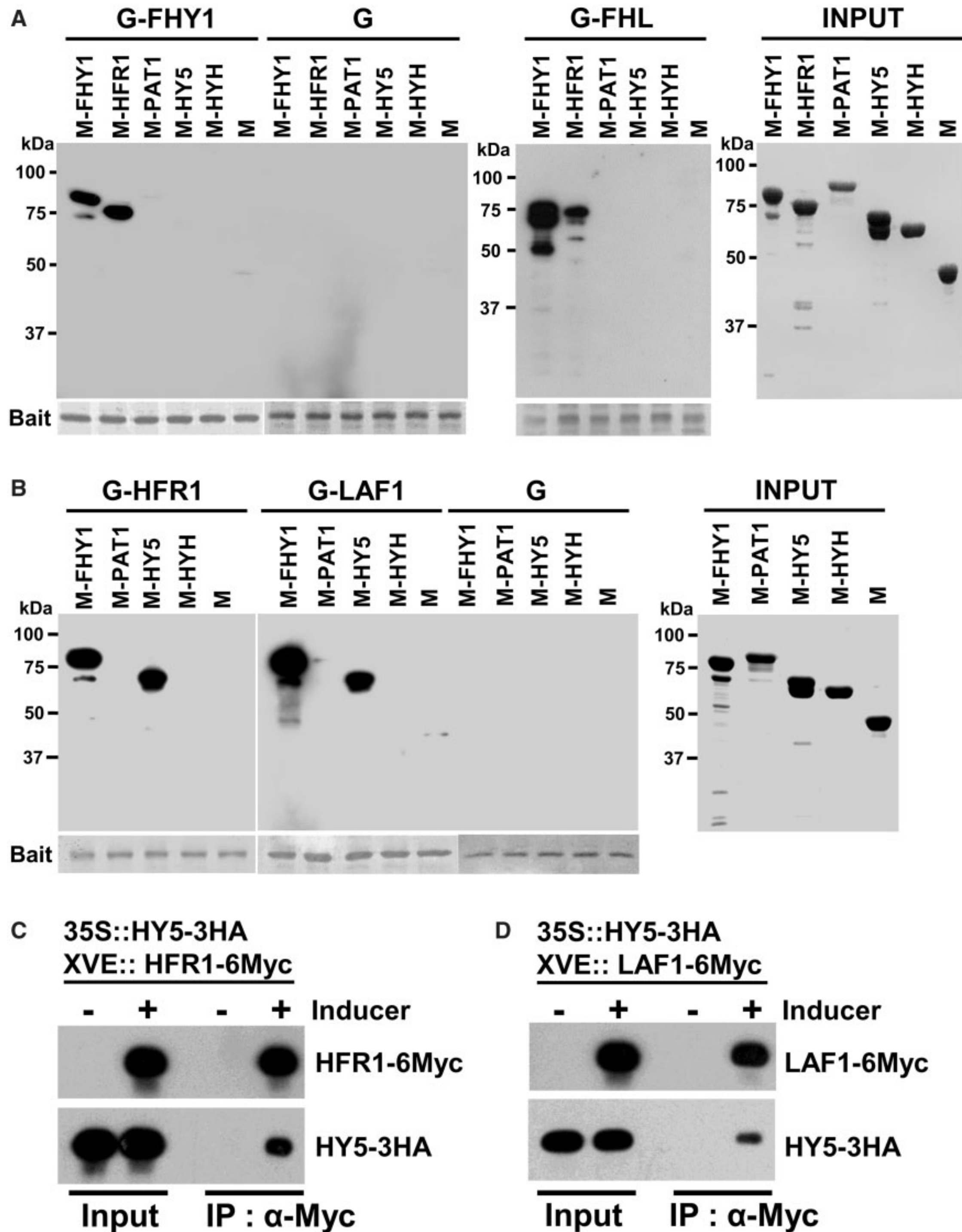


**Fig. 4** HY5 interacts with HFR1 and LAF1, but not with FHY1. (A) In vitro pull-down assay of full-length GST-tagged FHY1 (G-FHY1), FHL (G-FHL) or GST alone (G) with other proteins. A 500 ng aliquot of target proteins was pulled down with G-FHY1, G-FHL or GST protein (1 μμg each) and detected by anti-MBP antibody. (B) In vitro pull-down assay of full-length GST-tagged HFR1 (G-HFR1), GST-tagged LAF1 (G-LAF1) or GST alone (G) with other proteins as described above. Purified target proteins used for pull-down assay in (A) and (B) were loaded on SDS–PAGE and labeled as input proteins. (C) and (D) In vivo co-immunoprecipitation showing interaction between HY5 and HFR1 (C) or HY5 and LAF1 (D).

## Discussion

Being the major photoreceptor in imbibed seed, phyA is mainly responsible for early seedling de-etiolation and its transition from heterotrophic to phototrophic growth (Quail 2002). In the past two decades, genetic and molecular analyses of Arabidopsis mutants with hypocotyl phenotypes in FRc light have led to the identification of >10 signaling intermediates. Of these, only three signaling intermediates, HFR1, a bHLH factor, LAF1, a Myb factor, and HY5, a bZIP factor, are known to be transcription factors. Presumably, these three transcription factors are located at the endpoints of phyA signaling pathways, executing their functions through direct binding to promoter regions of phyA-responsive genes to modulate their transcription.

Because HFR1, LAF1 and HY5 belong to three different families of transcription factors, it is reasonable to assume they recognize different *cis*-elements on responsive promoters. This notwithstanding, our earlier work showed that HFR1 and LAF1 can interact in vitro as well as in vivo (Jang et al. 2007). One consequence of this association is to allow binding of the heterodimers to two adjacent sites on certain responsive promoters. Examples of this can be found in the well-characterized bHLH–Myb heterodimers in activating anthocyanin biosynthetic genes (Goff et al. 1992, Quattrocchio et al. 2006). Another consequence of this association is to delay the post-translational degradation of the HFR1–LAF1 interacting partners in FRc light, thereby increasing their transcriptional capacity (Jang et al. 2007). However, not all functions of HFR1 and LAF1 are executed through heterodimerization since the *hfr1laf1* double mutant has hypocotyls longer than those of the single mutants (Jang et al. 2007). Together, these results provide evidence that HFR1 and LAF1 have independent but also overlapping functions.

Here, we found that HY5, a bZIP factor, is able to bind not only to HFR1 but also to LAF1, suggesting that these factors may execute their shared functions through heterodimerization. This is perhaps not surprising since interactions of bZIP and bHLH factors as well as bZIP and Myb factors have been previously documented (Ness 1999, Amoutzias et al. 2008). Similar to HFR1 and LAF1, HY5 is also a target of the COP1 E3 ligase (Saijo et al. 2003). Although not specifically addressed here, it is reasonable to assume that HY5–HFR1 and HY5–LAF1 interactions have a similar effect of prolonging the half-life of the interacting partners as has been documented for HFR1 and LAF1 (Jang et al. 2007). Similar to the case of the *hfr1laf1* double mutant, we found that the *hy5hfr1laf1* triple mutant displays longer hypocotyls compared with the three possible combinations of double mutants, *hfr1laf1*, *laf1hy5* and *hfr1hy5*. The simplest interpretation of these results is that, in addition to their overlapping functions, these three transcription factors also have independent roles in phyA signaling.

HFR1 and LAF1 have been shown to transmit phyA signals via direct interaction with FHY1 (Yang et al. 2009). In contrast to HFR1 and LAF1, we found that HY5 does not interact with

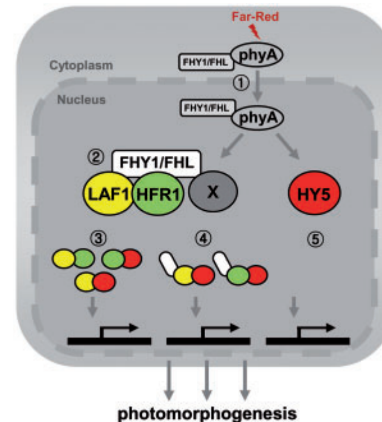


**Fig. 5** Schematic diagram of proposed phyA signaling. (1) Upon FRc exposure, phyA localizes from the cytosol into the nucleus through direct interaction with FHY1/FHL (Genoud et al. 2008). (2) In the nucleus, the phyA signal is transmitted to two major transcription factors (HFR1 and LAF1) through FHY1/FHL to promote photomorphogenesis (Yang et al. 2009). However, FHY1/FHL does not directly transmit phyA signal to one of the major transcription factors, HY5. The unidentified factor X may be involved in the FHY1/FHL-dependent pathway. (3) Direct interaction between HY5 and HFR1 or LAF1 may modulate transcription factor abundance and hence signaling strength. Taken together, we proposed here that HY5 may share the FHY1/FHL signal through interactions with HFR1 and/or LAF1 (4). Alternatively, HY5 may transmit phyA signals through a FHY1/FHL-independent pathway (5). Gray arrows represent the direction of the phyA signaling pathway.

FHY1 in vitro, suggesting that HY5 transduces phyA signals via an FHY1-independent pathway. This notion is supported by analysis of the *fhy1hy5* double mutant which displays hypocotyl lengths longer than that of *fhy1* and *hy5* single mutants. However, the *hy5hfr1laf1* triple mutant was not clearly longer in hypocotyl length than *fhy1-3*. There is the possibility that phyA signaling through HY5 is in part dependent on FHY1, presumably through direct interactions with HFR1 and/or LAF1.

Our results, together with those reported earlier, can be explained by a working model depicted in Fig. 5. In this model, three transcription factors act downstream of FHY1/FHL. Direct interactions of FHY1/FHL with HFR1 and LAF1 have been demonstrated (Jang et al. 2007, Yang et al. 2009). HY5 does not associate with FHY1 or its homolog, FHL, but it has the capacity to bind to HFR1 and LAF1. It should be emphasized that the functions of each of the three factors cannot be executed solely through heterodimers; otherwise, no additive hypocotyl phenotype would be observed. Dimerization of transcription factors may be one way to modulate their stability and hence coordinate signaling strengths of different signaling branches.

Although HY5 transmits phyA signals independently of FHY1/FHL, its upstream activator has not yet been identified. One possible candidate is FHY3/FAR1, and indeed HY5 has

been shown to bind to FHY3/FAR1. However, complexes with different subunit compositions appear to execute different functions in de-etiolating seedlings and in adult plants (Li et al. 2010, Li et al. 2011). In seedlings, direct interaction of HY5 with FHY3/FAR1 negatively regulates *FHY1/FHL* transcription in phyA signaling (Li et al. 2010), whereas in adult plants HY5/FHY3 activate ELF4 expression by directly binding to its promoter during the day in the circadian clock, thus providing the molecular mechanism connecting light/dark perception and circadian clock function (Li et al. 2011). So far, only two transcription factors, HFR1 and LAF1, have been identified as transmitting signals downstream of FHY1/FHL, but the hypocotyl length of the *hfr1laf1* double mutant is still shorter than that of the *fhy1fhl* mutant. This observation implies that some other as yet unidentified factor, but not HY5, must be involved in the FHY1/FHL-dependent pathway. Future work should be directed toward the identification and characterization of this factor.

## Materials and Methods

### Plant materials

We used WT [Columbia (Col)-0 and L*er*], *phyA-211* (Reed et al. 1994), *phyA-201* (Reed et al. 1994), *fhy1fhl* (Rösler et al. 2007), *fhy1-3* (Zeidler et al. 2001, Zeidler et al. 2004), *fhl-1* (Zhou et al. 2005), *hfr1-201* (Kim et al. 2002), *hy5-1* (Oyama et al. 1997), *laf1* (Ballesteros et al. 2001), *HFR1RNAi/laf1* (designated as *laf1hfr1* in **Fig. 2C**) (Jang et al. 2007), *LAF1RNAi/hfr1-201* (designated as *hfr1laf1* in **Fig. 2C**) (Jang et al. 2007) and *hy5hfr1* (Kim et al. 2002) as plant materials.

### Light treatments

Surface-sterilized WT (Col-0) and mutant seeds were kept for 4 d at 4°C in darkness and then transferred to FRc light for 4 d at 21°C after white light exposure for 1 h. FR fluence rates of 1, 3, 5 and 10 $\mu mol\ m^{-2}\ s^{-1}$ were used. As an FR light source, we used 600 light-emitting diodes (LEDs; maximum spectral output, 740 nm) consisting of four arrays with each array containing 150 (15 × 10) LEDs. The fluence rates were measured using a detector with an IL1400A photometer (SED033, International light Inc.).

### Construction of LAF1Ri/hy5, LAF1Ri/hy5hfr1, HY5Ri/Ler and HY5Ri/fhy1

We used hairpin RNA technology to silence *LAF1* or *HY5* in a *hy5-1* (Oyama et al. 1997) and *hy5hfr1* (Kim et al. 2002) or L*er* and *fhy1-3* (Zeidler et al. 2001, Zeidler et al. 2004) background. Vector construction for LAF1-RNAi was previously described (Jang et al. 2007). The HY5-RNAi contained a DNA fragment of about 260 bp which was amplified by PCR using the following oligos: 5′-gaacaagcgactagctctttagct-3′ and 5′-ttctctttctccg ccggtgtc-3′. The fragment was cloned into pENTR/D (Invitrogen) and followed by LR reaction (Invitrogen) with pBA-DC-RNAi (Jang et al. 2007) to generate pBA-RNAi-HY5 which conferred Basta resistance. The LAF1-RNAi or HY5-RNAi construct was transformed into WT (L*er*), *hy5-1*, *hy5hfr1* or *fhy1-3* by *Agrobacterium* strain EHA105 using the floral dip method. Homozygous $T_3$ Basta-resistant mutants were selected and used for further analysis.

### RNA extraction and quantitative RT–PCR

Total RNA was extracted from 2-week-old Arabidopsis seedlings grown under long-day conditions (16 h light/8 h dark) at 22°C with white light (80 $\mu mol\ m^{-2}\ s^{-1}$) using Qiagen RNeasy Plant Mini Kits. Reverse transcription was performed using a SuperScript II RT kit (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using using a SYBR premix Ex Taq (TAKARA) with gene-specific primers in a Bio-Rad CFX96 real-time system and each sample was analyzed in triplicate in a PCR. *ACTIN2* was used as an internal normalization in each quantitative real-time PCR. The oligonucleotide sequences for quantitative real-time PCR were as following; 5′-ccacaccgattattcctctg-3′ and 5′-acgtcgttgtt-gatggagaa-3′ for *LAF1* amplification; 5′-gtttggaggagaagctgtcg-3′ and 5′-tcttgcttgctgagctgaaa-3′ for *HY5* amplification; and 5′-acatcgttctcagtggtggttc-3′ and 5′-acctgactcatcgtactcactc-3′ for *Actin 2* amplification.

### Plasmids and preparation of recombinant proteins

Plasmids for expression of the recombinant proteins glutathion *S*-transferase (GST)–FHY1 (G-FHY1), GST–FHL (G-FHL) GST–HFR1 (G-HFR1), GST–LAF1 (G-LAF1), maltose-binding protein (MBP)–FHY1 (M-FHY1), MBP–HFR1 (M-HFR1) and MBP–PAT1 (M-PAT1) were described previously (Jang et al. 2005, Jang et al. 2007, Yang et al. 2009). cDNAs encoding full-length HY5 and HYH were amplified by PCR, cloned into pENTR/D vector and then transferred into pMBP-DC (Jang et al. 2007) by recombination using the LR clonase enzyme (Invitrogen) to generate MBP–HY5 and MBP–HYH, respectively. All constructs used in this study were verified by sequencing. Constructs were transformed into *Escherichia coli* BL21 cells, and recombinant proteins were purified from bacterial extracts after isopropyl-β-D-thiogalactoside induction as described (Jang et al. 2005).

### In vitro pull-down and in vivo co-immunoprecipitation

Experimental procedures for in vitro pull-down and in vivo immunoprecipitation were essentially identical to those described before (Jang et al. 2005, Jang et al. 2007).

For in vivo co-immunoprecipitation, 2-week-old transgenic Arabidopsis seedlings (35S::HY5-3HA/XVE::HFR1-6Myc or 35 S::HY5-3HA/XVE::LAF1-6Myc) grown under long-day conditions (16 h light/8 h dark) at 22°C with white light (80 $\mu mol\ m^{-2}\ s^{-1}$) were treated with MG132 (25 μM) or MG132 (25 μM) plus β-estradiol (10 μM) for 12 h under FRc light (10 $\mu mol\ m^{-2}\ s^{-1}$). Approximately 1 mg of total protein

and 5 µg of anti-Myc polyclonal antibody (Santa Cruz Biotechnology) were used for co-immunoprecipitation reactions. Pulled down proteins from protein A agarose beads (Roche) were analyzed by Western blotting using anti-HA monoclonal (Santa Cruz Biotechnology) and anti-Myc monoclonal (Monoclonal Antibody Core Facility, MSKCC) antibodies.

## Supplementary data

Supplementary data are available at PCP online.

## Funding

The National Institutes of Health [grant No. GM44640 to N.-H.C.].

## Acknowldgements

We thank Dr. Moon-Soo Soh for *hy5-1hfr1-201*.

## References

Amoutzias, G.D., Robertson, D.L., Van de Peer, Y. and Oliver, S.G. (2008) Choose your partners: dimerization in eukaryotic transcription factors. *Trends Biochem. Sci.* 33: 220–229.

Ballesteros, M.L., Bolle, C., Lois, L.M., Moore, J.M., Vielle-Calzada, J.P., Grossniklaus, U. et al. (2001) LAF1, a MYB transcription activator for phytochrome A signaling. *Genes Dev.* 15: 2613–2625.

Chen, M., Tao, Y., Lim, J., Shaw, A. and Chory, J. (2005) Regulation of phytochrome B nuclear localization through light-dependent unmasking of nuclear-localization signals. *Curr. Biol.* 15: 637–642.

Fairchild, C.D., Schumaker, M.A. and Quail, P.H. (2000) HFR1 encodes an atypical bHLH protein that acts in phytochrome A signal transduction. *Genes Dev.* 14: 2377–2391.

Fankhauser, C. and Chory, J. (2000) RSF1, an Arabidopsis locus implicated in phytochrome A signaling. *Plant Physiol.* 124: 39–45.

Genoud, T., Schweizer, F., Tscheuschler, A., Debrieux, D., Casal, J.J., Schäfer, E. et al. (2008) FHY1 mediates nuclear import of the light-activated phytochrome A photoreceptor. *PLoS Genet.* 4: e1000143.

Goff, S.A., Cone, K.C. and Chandler, V.L. (1992) Functional analysis of the transcriptional activator encoded by the maize B gene: evidence for a direct functional interaction between two classes of regulatory proteins. *Genes Dev.* 6: 864–875.

Hiltbrunner, A., Tscheuschler, A., Viczián, A., Kunkel, T., Kircher, S. and Schäfer, E. (2006) FHY1 and FHL act together to mediate nuclear accumulation of the phytochrome A photoreceptor. *Plant Cell Physiol.* 47: 1023–1034.

Hiltbrunner, A., Viczián, A., Bury, E., Tscheuschler, A., Kircher, S., Tóth, R. et al. (2005) Nuclear accumulation of the phytochrome A photoreceptor requires FHY1. *Curr. Biol.* 15: 2125–2130.

Jang, I.C., Yang, J.Y., Seo, H.S. and Chua, N.H. (2005) HFR1 is targeted by COP1 E3 ligase for post-translational proteolysis during phytochrome A signaling. *Genes Dev.* 19: 593–602.

Jang, I.C., Yang, S.W., Yang, J.Y. and Chua, N.H. (2007) Independent and interdependent functions of LAF1 and HFR1 in phytochrome A signaling. *Genes Dev.* 21: 2100–2111.

Kami, C., Lorrain, S., Hornitschek, P. and Fankhauser, C. (2010) Light-regulated plant growth and development. *Curr. Top. Dev. Biol* 91: 29–66.

Kim, Y.M., Woo, J.C., Song, P.S. and Soh, M.S. (2002) HFR1, a phytochrome A-signalling component, acts in a separate pathway from HY5, downstream of COP1 in Arabidopsis thaliana. *Plant J.* 30: 711–719.

Ness, S.A (1999) Myb binding proteins: regulators and cohorts in transformation. *Oncogene* 18: 3039–3046.

Li, G., Siddiqui, H., Teng, Y., Lin, R., Wan, X.Y., Li, J. et al. (2011) Coordinated transcriptional regulation underlying the circadian clock in Arabidopsis. *Nat. Cell Biol.* 13: 616–622.

Li, J., Li, G., Gao, S., Martinez, C., He, G, Zhou, Z. et al. (2010) Arabidopsis transcription factor ELONGATED HYPOCOTYL5 plays a role in the feedback regulation of phytochrome A signaling. *Plant Cell* 22: 3634–3649.

Lin, R., Ding, L., Casola, C., Ripoll, D.R., Feschotte, C. and Wang, H. (2007) Transposase-derived transcription factors regulate light signaling in Arabidopsis. *Science* 318: 1302–1305.

Oyama, T., Shimura, Y. and Okada, K. (1997) The Arabidopsis HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. *Genes Dev.* 11: 2983–2995.

Quail, P.H. (2002) Phytochrome photosensory signaling networks. *Nat. Rev. Mol. Cell Biol* 3: 85–93.

Quattrocchio, F., Verweij, W., Kroon, A., Spelt, C., Mol, J. and Koes, R. (2006) PH4 of petunia is an R2R3 MYB protein that activates vacuolar acidification through interactions with basic-helix–loop–helix transcription factors of the anthocyanin pathway. *Plant Cell* 18: 1274–1291.

Reed, J.W., Nagatani, A., Elich, T.D., Fagan, M. and Chory, J. (1994) Phytochrome A and phytochrome B have overlapping but distinct functions in Arabidopsis development. *Plant Physiol.* 104: 1139–1149.

Rösler, J., Klein, I. and Zeidler, M. (2007) Arabidopsis fhl/fhy1 double mutant reveals a distinct cytoplasmic action of phytochrome A. *Proc. Natl Acad. Sci. USA* 25: 10737–10742.

Saijo, Y., Sullivan, J.A., Wang, H., Yang, J., Shen, Y., Rubio, V. et al. (2003) The COP1–SPA1 interaction defines a critical step in phytochrome A-mediated regulation of HY5 activity. *Genes Dev.* 17: 2642–2647.

Seo, H.S., Yang, J.Y., Ishikawa, M., Bolle, C., Ballesteros, M.L. and Chua, N.H. (2003) LAF1 ubiquitination by COP1 controls photomorphogenesis and is stimulated by SPA1. *Nature* 26: 995–999.

Soh, M.S., Kim, Y.M., Han, S.J. and Song, P.S. (2000) REP1, a basic helix–loop–helix protein, is required for a branch pathway of phytochrome A signaling in Arabidopsis. *Plant Cell* 12: 2061–2074.

Yang, J., Lin, R., Sullivan, J., Hoecker, U., Liu, B., Xu, L. et al. (2005) Light regulates COP1-mediated degradation of HFR1, a transcription factor essential for light signaling in Arabidopsis. *Plant Cell* 17: 804–821.

Yang, S.W., Jang, I.C., Henriques, R. and Chua, N.H. (2009) FAR-RED ELONGATED HYPOCOTYL1 and FHY1-LIKE associate with the Arabidopsis transcription factors LAF1 and HFR1 to transmit phytochrome A signals for inhibition of hypocotyl elongation. *Plant Cell* 21: 1341–1359.

Zeidler, M., Bolle, C. and Chua, N.H. (2001) The phytochrome A specific signaling component PAT3 is a positive regulator of Arabidopsis photomorphogenesis. *Plant Cell Physiol.* 42: 1193–1200.

Zeidler, M., Zhou, Q., Sarda, X., Yau, C.P. and Chua, N.H. (2004) The nuclear localization signal and the C-terminal region of FHY1 are required for transmission of phytochrome A signals. *Plant J.* 40: 355–365.

Zhou, Q., Hare, P.D., Yang, S.W., Zeidler, M., Huang, L.F. and Chua, N.H. (2005) FHL is required for full phytochrome A signaling and shares overlapping functions with FHY1. *Plant J.* 43: 356–370.

Zuo, J., Niu, Q.W. and Chua, N.H. (2000) An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant J.* 24: 265–273.