Repression of light signaling by Arabidopsis SPA1 involves post-translational regulation of HFR1 protein accumulation

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Received 2 March 2005; revised 9 April 2005; accepted 20 April 2005.
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Summary
Arabidopsis uses two major classes of photoreceptors to mediate seedling de-etiolation. The cryptochromes (cry1 and cry2) absorb blue/ultraviolet-A light, whereas the phytochromes (phyA–phyE) predominantly regulate responses to red/far-red light. Arabidopsis COP1 represses light signaling by acting as an E3 ubiquitin ligase in the nucleus, and is responsible for targeted degradation of a number of photomorphogenesis-promoting factors, including HY5, LAF1, phyA, and HFR1. Distinct light signaling pathways initiated by multiple photoreceptors (including both phytochromes and cryptochromes) eventually converge on COP1, causing its inactivation and nuclear depletion. Arabidopsis SPA1, which encodes a protein structurally related to COP1, also represses light signaling under various light conditions. In this study, we present genetic evidence supporting that HFR1, which encodes a photomorphogenesis-promoting bHLH transcription factor, acts downstream of SPA1 and is required for different subsets of branch pathways of light signaling controlled by SPA1 under different light conditions. We show that SPA1 physically interacts with HFR1 in a yeast two-hybrid assay and an in vitro co-immunoprecipitation assay. We demonstrate that higher levels of HFR1 protein accumulate in the spa1 mutant background under various light conditions, including far-red, red, blue, and white light, whereas a marginal increase in HFR1 transcript level is only seen in dark- and far-red light-grown spa1-100 mutants. Together, our data suggest that repression of light signaling by Arabidopsis SPA1 likely involves post-translational regulation of HFR1 protein accumulation.

Keywords: Arabidopsis, photomorphogenesis, HFR1, SPA1, COP1, proteolysis.

Introduction
Light is one of the most influential environmental factors that regulates diverse aspects of plant growth and development throughout their entire life cycle, including seed germination, seedling de-etiolation, gravitropism, phototropism, chloroplast relocation, shade avoidance, circadian rhythms, and flowering time (Deng and Quail, 1999; Wang and Deng, 2003). Among these photoreponses, light control of seedling development is best understood. Arabidopsis seedlings grown in the dark follow a skotomorphogenesis (etiolation) developmental program and exhibit long hypocotyls, closed cotyledons with enclosed apical hooks, and development of the proplastids into etioplasts. In contrast, light-grown seedlings undergo photomorphogenesis (de-etiolation) and display short hypocotyls, open and expanded cotyledons, and development of the proplastids into green mature chloroplasts (McNellis and Deng, 1995).

Arabidopsis uses two major classes of photoreceptors to mediate seedling de-etiolation: the blue/UV-A (320–500 nm) absorbing cryptochromes (cry1 and cry2), and the red/far-red light (600–750 nm) sensing phytochromes (phyA–phyE) (Briggs and Olney, 2001; Kendrick and Kronenberg, 1994; Lin, 2002). cry1 and cry2 act redundantly to mediate seedling de-etiolation under blue light with differential fluence requirements. cry1 plays a major role in response to high intensities of blue light, whereas cry2 is the primary photoreceptor for low-intensity blue light (Lin et al., 1998). Among the phytochromes, phyB–phyE predominantly regulate light responses under continuous red and white light, with phyB playing a dominant role. phyA is the primary, if not sole,
photoreceptor responsible for mediating various far-red light responses, including inhibition of hypocotyl elongation, opening of the apical hook, expansion of cotyledons, accumulation of anthocyanin, and far-red light pre-conditioned blocking of greening (Nagatani et al., 1993; Neff et al., 2000; Whitelam et al., 1993).

Intensive molecular genetic studies have identified numerous signaling intermediates for phytochromes and cryptochromes in mediating Arabidopsis seedling de- etiolation (for reviews, see Quail, 2002; Wang and Deng, 2004). Some signaling components are specific for individual photoreceptors, while others are shared by multiple types of photoreceptors. For example, FHY1, FHY3, FAR1, PAT1, LAF1, LAF3, LAF6, FIN219, SPA1, AFR, and EID1 are specific for phyA signaling (Ballesteros et al., 2001; Bolle et al., 2000; Desnos et al., 2001; Dieterle et al., 2001; Hare et al., 2003; Harmon and Kay, 2003; Hoecker et al., 1998, 1999; Hsieh et al., 2000; Hudson et al., 1999; Møller et al., 2001; Wang and Deng, 2002); GI, ELF3, ELF4, ARR4, PIF4, and SRR1 are specific for phyB signaling (Huq and Quail, 2002; Huq et al., 2000; Khanna et al., 2003; Liu et al., 2001; Staiger et al., 2003; Sweere et al., 2001); whereas PIF3, NDPK2, PKS1, COG1, PFT1, and PRR7 are shared by both phyA and phyB (Cerdan and Chory, 2003; Choi et al., 1999; Fankhauser et al., 1999; Kaczorowski and Quail, 2003; Ni et al., 1998; Park et al., 2003). SUB1 and PP7 repress and promote cryptochrome signaling, respectively (Guo et al., 2001; Møller et al., 2003).

In addition, HFR1 is required for both phyA-mediated far-red and cry1-mediated blue light signaling (Duek and Fankhauser, 2003; Fairchild et al., 2000), whereas HRB1 and OB35 regulate both phyB-mediated red and cryptochrome-mediated blue light signaling (Kang et al., 2005; Ward et al., 2005). Furthermore, a group of COP/DET/FUS proteins act downstream of both phytochromes and cryptochromes to repress photomorphogenesis (Serino and Deng, 2003; Wei and Deng, 1996), whereas a bZIP transcription factor, HY5, acts to promote photomorphogenesis under all light conditions (Oyama et al., 1997).

Among the COP/DET/FUS proteins, the RING finger protein COP1 appears to define a rate-limiting regulatory component of light signaling in Arabidopsis (Deng et al., 1992; McNellis et al., 1994). Recently, it was demonstrated that COP1 functions as an E3 ubiquitin ligase and is responsible for the ubiquitination and degradation of a number of photomorphogenesis-promoting factors, including HY5, LAF1, phyA, and HFR1, thus desensitizing light signaling (Duek et al., 2004; Jang et al., 2005; Osterlund et al., 2000; Saijo et al., 2003; Seo et al., 2003, 2004; Yang et al., 2005). Distinct light signaling pathways initiated by multiple photoreceptors (including both phytochromes and cryptochromes) eventually converge on COP1, and lead to its inactivation; however, little is known about the molecular mechanisms linking photoreceptor activation and COP1 activity repression. Although COP1 displays a light-mediated nucleo-cytoplasmic repartitioning, being enriched in the nucleus in the dark and depleted from the nucleus in the light (Osterlund and Deng, 1998; von Arnim and Deng, 1994), the slow kinetics of COP1 nuclear depletion (could take up to 24 h) and rapid degradation of HFR1, HY5, and phyA (Duek et al., 2004; Osterlund et al., 2000; Seo et al., 2004; Yang et al., 2005) suggest that at least one additional regulatory event is required for the rapid inactivation of COP1 by light.

Recent studies suggested that repression of COP1 activity by blue light signaling might involve direct physical interaction between the cryptochrome photoreceptors and COP1 (Wang et al., 2001; Yang et al., 2001). Although the red light photoreceptor phyB has also been reported to interact with COP1 in a yeast two-hybrid assay (Yang et al., 2001), the physiological relevance of phyB-COP1 interaction remains to be established. In addition, there is a gap in our understanding linking phyA activation and COP1 repression.

Arabidopsis SPA1 (suppressor of phyochromosome A-105), a negative regulator of phyA signaling, encodes a nuclear-localized novel protein that is structurally related to COP1 in their C-terminal WD-repeat domains (Hoecker et al., 1999) and it can physically interact with COP1 (Hoecker and Quail, 2001). The direct interaction between SPA1 and COP1 might provide a molecular basis for the convergence of phyA-mediated signaling and COP1. It has been suggested that SPA1 may function in concert with COP1 to target proteins, such as HY5, for degradation, thereby repressing photomorphogenesis in the dark and preventing excess photomorphogenesis in the light (Hoecker and Quail, 2001; Saijo et al., 2003; Seo et al., 2003).

In this study, we show that HFR1, a photomorphogenesis-promoting bHLH protein (Duek and Fankhauser, 2003; Fairchild et al., 2000; Yang et al., 2005), is required for a number of exaggerated photoresponses of the spa1 mutants, including hypocotyl elongation and light-responsive gene expression under specific light conditions. We demonstrate that SPA1 physically interacts with HFR1 and is required for proper degradation of HFR1 under various light conditions, including far-red, red, blue, and white light, but not under darkness. On the contrary, slightly elevated expression of HFR1 was only seen in dark- and far-red light-grown spa1 mutants. Our results support the proposition that Arabidopsis SPA1 represses light signaling by modulating COP1-dependent degradation of HFR1 at a post-translational level.

**Results and discussion**

hfr1-201 suppresses the short-hypocotyl phenotype of spa1-100 in far-red and blue light

Previous studies showed that the spa1 mutants exhibit exaggerated photoresponses (including inhibition of
hypocotyl elongation and anthocyanin accumulation) to far-red and red light, but not under darkness (Hoecker et al., 1998), whereas the hfr1/rep1/rsf1 loss-of-function mutants (hereafter referred as hfr1) display reduced photomorphogenesis under both far-red and blue light conditions, but not under red light and darkness (Duek and Fankhauser, 2003; Fairchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000). To investigate the epistasis relationship between SPA1 and HFR1, we generated a spa1-100/hfr1-201 double homozygous mutant. The spa1-100 mutant allele was isolated from The Syngenta Arabidopsis Insertion Library (SAIL) T-DNA insertion mutant population (Sessions et al., 2002). spa1-100 carries a T-DNA insertion in the second exon of the SPA1 gene (Figure 1a). We chose this allele to construct a double mutant with hfr1-201 (Soh et al., 2000, a putative null allele) because of their same ecotype background (Columbia), whereas the previously published spa1 mutant alleles are all derived from the RLD ecotype background (Hoecker et al., 1998). Northern blot analysis revealed that no SPA1 mRNA accumulation was detected in the spa1-100 mutant (Figure 1b). Thus, it likely represents a null allele. As reported previously, the spa1-100 mutant had much reduced hypocotyl length under both far-red and red light conditions, and slightly reduced hypocotyl length under blue light, although it etiolated normally under darkness. Thus, it appears that SPA1 acts to repress light signaling under all light conditions. The hfr1-201 mutant etiolated normally and had a hypocotyl length similar to wild-type plants under red light, but had more elongated hypocotyls under far-red and blue light. Strikingly, the hfr1-201 mutation largely suppressed the short-hypocotyl phenotype of spa1-100 under far-red and blue light conditions, but not under red light (Figure 1c,d), suggesting that hfr1-201 is epistatic to spa1-100 under far-red and blue light conditions, thus HFR1 likely acts downstream of SPA1. The failure of hfr1-201 to suppress the short-hypocotyl phenotype of spa1-100 under red light is consistent with the lack/limited role of HFR1 under red light and suggests that SPA1 suppression of photomorphogenesis under red light, which is a phyA-dependent very-low-fluence response (Baumgardt et al., 2002; Zhou et al., 2002), likely involves protein(s) other than HFR1.

We also compared the accumulation of anthocyanin of spa1-100, hfr1-201 and their double mutants under various light conditions. As shown in Figure 2, the wild type, hfr1-201, spa1-100, and the hfr1-201/spa1-100 double mutants all had very low levels of anthocyanin accumulation under darkness. Under continuous far-red, red, and blue light conditions, the spa1-100 mutants and the hfr1-201/spa1-100 double mutants clearly accumulated higher levels of anthocyanin, compared with wild type and hfr1-201 mutant seedlings. This result confirms the notion that HFR1 does

![Figure 1](image1.png)

**Figure 1.** hfr1-201 suppresses the short-hypocotyl phenotype of spa1-100. (a) Diagram of the genomic structure of the SPA1 gene and the T-DNA insertion (represented by the triangle). Black rectangles represent the exons and lines are introns. (b) Northern blot analysis showing that SPA1 mRNA accumulation is abolished in the spa1-100 mutant. An 18S rRNA blot is shown below as a loading control. (c) Phenotypes of Columbia wild type (Col), spa1-100, hfr1-201/spa1-100, and hfr1-201 seedlings under various light conditions. Bar: 1 mm. (d) Quantification of hypocotyl lengths (average of 20 seedlings) under various light conditions. Bars stand for standard deviations. Lane 1, wild type (Col); lane 2, spa1-100; lane 3, hfr1-201/spa1-100; lane 4, hfr1-201.

![Figure 2](image2.png)

**Figure 2.** Measurement of anthocyanin accumulation. Relative anthocyanin content of wild type (Col), spa1-100, hfr1-201/spa1-100, and hfr1-201 seedlings under various light conditions. Bars represent standard deviations from triplicate experiments. Lane 1, wild type (Col); lane 2, spa1-100; lane 3, hfr1-201/spa1-100; lane 4, hfr1-201.
not play a significant role in regulating anthocyanin accumulation under different light conditions (Fairchild et al., 2000; Soh et al., 2000).

hfr1-201 suppresses the elevated expression of CAB3 and RBCS genes conferred by spa1-100 specifically under far-red light

To provide molecular evidence for the observed genetic interaction between the hfr1-201 and spa1-100 mutations, we examined expression of CAB3 and RBCS in the hfr1-201, spa1-100 mutants, and their double mutants under various light conditions. As shown in Figure 3(a), for seedlings grown under continuous darkness for 5 days, the expression of CAB3 and RBCS was significantly elevated in spa-100 mutants and was comparable in the hfr1-201 mutants and wild-type plants. The expression level of RBCS in the hfr1-201/spa1-100 double mutant was similar to that in the spa1-100 single mutant, but CAB3 expression was slightly higher in the double mutants. For seedlings grown under continuous darkness for 5 days and then transferred to far-red light for 24 h, the expression of CAB3 and RBCS was significantly higher in the spa1-100 mutants, but lower in the hfr1-201 mutants, compared with wild-type plants. The elevated expression of CAB3 and RBCS conferred by the spa1-100 mutation was partially suppressed by the hfr1-201 mutation (Figure 3b). For seedlings grown under darkness for 5 days and then transferred to red light or blue light for 24 h, the spa1-100 mutant had much higher RBCS expression, but the expression of CAB3 was comparable with wild-type plants. Under these light conditions, CAB3 and RBCS expression in the hfr1-201/spa1-100 double mutant was essentially similar to the spa1-100 single mutants (Figure 3c,d). This result indicates that HFR1 is required for the elevated expression of CAB3 and RBCS in the spa1-100 mutants specifically under far-red light. This finding, together with the observed suppression of the short-hypocotyl phenotype of spa1-100 by the hfr1-201 mutation under both far-red and blue light conditions (Figure 1c,d), indicates that HFR1 acts down-stream of SPA1, and is involved in different subsets of branch pathways of light signaling regulated by SPA1 under different light conditions.

HFR1 physically interacts with SPA1

Previous studies have shown that SPA1 can physically interact with COP1 and HY5, and is involved in the regulated degradation of HY5 under far-red light (Hoecker and Quail, 2001; Saijo et al., 2003). Recently, a few groups, including ours, have demonstrated that COP1 also serves as an E3 ubiquitin ligase targeting HFR1 for degradation, thus desensitizing light signaling (Duek et al., 2004; Jang et al., 2005; Yang et al., 2005). To test whether SPA1 might physically interact with HFR1, we first used a yeast two-hybrid assay. We found that full-length HFR1 as well as a number of deletion mutant forms of HFR1, including HFR1-NT189, HFR1-NT131, HFR1-CT161, and HFR1-ΔHLH, were all capable of interacting with full length SPA1, whereas HFR1-CT106 and HFR1-ΔHLH were not able to interact with SPA1 (Figure 4a). These observations suggest that the overall three-dimensional structure of HFR1 might be important for HFR1–SPA1 interaction. Full-length HFR1 also interacted with full-length SPA1 and a number of mutant derivatives of SPA1, including SPA1-NT696, SPA1-CC, and SPA1-CT509. SPA1 deletion mutants lacking the coil-coil domain (such as SPA1-NT545 and SPA1-ΔCC) did not interact with HFR1 (Figure 4b). Thus, the coiled-coil region of SPA1 appears to be required, and sufficient, for mediating the interaction between SPA1 and HFR1, although other domains of SPA1 may also contribute to the interaction.

To verify these protein–protein interactions, we conducted an in vitro interaction assay using recombinant proteins (Hoecker and Quail, 2001). SPA1 and GAL4 activation domain (GAD)-tagged HFR1 were synthesized by coupled in vitro transcription and translation (TnT). Co-immunoprecipitation was carried out with antibodies against GAD. Full-length SPA1 as well as SPA1-NT696 were able to interact with GAD-tagged HFR1 and be pulled down by anti-GAD

![Image](https://example.com/image.png)

Figure 3. hfr1-201 suppresses the elevated expression of CAB3 and RBCS under far-red light. Lane 1, wild type (Col); lane 2, spa1-100; lane 3, hfr1-201/spa1-100; lane 4, hfr1-201. For each blot, a UV fluorescence image of rRNA of the duplicating gels was shown below as a loading control.
antibody conjugated with agarose beads, whereas SPA1-NT545 lacking the coiled-coil domain was not able to interact with GAD-HFR1 (Figure 5a). The coiled-coil domain itself (SPA1-CC) also interacted with HFR1 (Figure 5b). Quantification of the immunoprecipitates indicated that approximately 4% of the SPA1 full-length protein, or about 2.3% of SPA1-NT696, or about 1.2% SPA1-CC added to the assay were co-precipitated by GAD-HFR1, whereas <0.2% of the added SPA1 or its deletion mutants were precipitated by the control bait GAD (Figure 5c). These results are consistent with the results of our yeast two-hybrid interaction studies.

Degradation of HFR1 is defective in the spa1-100 mutants

In a previous study, we generated transgenic plants over-expressing a GFP-tagged full-length HFR1 gene (GFP-HFR1, driven by the 35S promoter). These transgenic plants exhibit much more enhanced photomorphogenesis under various light conditions, although they etiolate normally in darkness (Yang et al., 2005). The GFP-HFR1 protein is degraded in darkness through a 26S proteasome-dependent pathway, and this process is mediated by COP1. Light, irrespective of its quality, enhances HFR1 protein accumulation in the nucleus by promoting its stabilization (Yang et al., 2005). The observed physical interaction between SPA1 and HFR1 suggests that SPA1 might also be involved in the regulation of HFR1 protein accumulation. To test this possibility, we introduced the GFP-HFR1 transgene into the spa1-100 mutant background. As shown in Figure 6, GFP-HFR1 transgenic plants (in the hfr1-201 mutant background, line B4) are hyper-photomorphogenic (with drastically shorted hypocotyls) in response to far-red, red, and blue light, with normal etiolation. As expected, this GFP-HFR1 transgene further enhanced the exaggerated photomorphogenesis phenotype of the spa1-100 mutants under all light conditions (including far-red, red, and blue), whereas etiolation of spa1-100 mutants under darkness was not apparently affected by the GFP-HFR1 transgene (Figure 6).

To determine the effect of the spa1-100 mutation on HFR1 protein accumulation, levels of the GFP-HFR1 fusion protein in the spa1-100 mutant background were compared with the parental GFP-HFR1 transgenic plants under various light conditions, using both fluorescence microscopy examination and immunoblot analysis. No apparent accumulation of GFP-HFR1 was detected in either the hypocotyl or root cells of dark-grown spa1-100 mutants harboring the GFP-HFR1 transgene or the parental GFP-HFR1 plants, suggesting that
SPA1 is not involved, or has a limited role in COP1-mediated degradation of HFR1 under darkness. In contrast, higher levels of GFP-HFR1 were observed in both the hypocotyl and root cells in the spa1-100 mutant background for seedlings grown under continuous darkness for 4 days and then transferred to far-red light for 24 h (Figure 7). Therefore, the observed increase in GFP-HFR1 protein accumulation in the spa1-100 mutant background under all light conditions (far-red, red, blue, and white light) is likely due to reduced proteolysis, rather than stabilization of the HFR1 transcript.

Our genetic, molecular, and biochemical evidence appears to support the notion that repression of light signaling by Arabidopsis SPA1 primarily involves post-translational regulation of HFR1 protein accumulation. This is in line with a previous study which showed that more HY5 accumulates in the spa1-3 mutant seedlings than wild-type seedlings under continuous far-red light, and that HY5 begins to accumulate more rapidly in the spa1-3 mutant than in the wild type, upon transfer of seedlings from darkness to continuous far-red light (Saijo et al., 2003). These studies, together, provided compelling evidence that SPA1 may function in concert with COP1 to target proteins (such as HY5 and HFR1) for degradation, thereby repressing photomorphogenesis in the dark and preventing excess photomorphogenesis in the light (Hoecker and Quail, 2001; Saijo et al., 2003). It is conceivable that upon illumination, the efficiency of COP1 in ubiquitinating substrate proteins in the nucleus will become weakened due to the reduced abundance of nuclear COP1 (von Arnim and Deng, 1994). SPA1, through direct physical interaction with COP1 and direct interaction with substrate proteins (such as HY5 and HFR1), may help in capturing the substrate proteins and bring them to the proximity of COP1 for degradation. SPA1 might also synergize with COP1 in the dark to deplete transcription factors like HY5, LAF1, and HFR1 more rapidly, to prepare plants for anticipated light signals. This model is also in good agreement with the result of a physiological study which showed the spa1 mutants to be more persistent in both the very-low-fluence response and continuous far-red light-induced high-irradiance responses (Baumgardt et al., 2002), possibly due to the delayed photomorphogenesis observed in the spa1-100/GFP-HFR1 plants under various light conditions, and suggests that SPA1 is required for normal degradation of HFR1 under all light conditions.

To ascertain the increased GFP-HFR1 accumulation in the spa1-100 mutant background under various light conditions is due to impaired protein degradation, rather than increased transcript accumulation, we examined the effects of the spa1-100 mutation on HFR1 transcript accumulation under different light conditions. Compared with wild-type plants, slightly more HFR1 transcript accumulated in the spa1-100 mutant seedlings that were grown under continuous darkness for 5 days or under continuous darkness for 4 days and then transferred to far-red light for 24 h (Figure 8a,b). HFR1 transcript levels were similar, or slightly reduced in the spa1-100 mutant seedlings that were grown under continuous darkness for 4 days and then transferred to red or blue light for 24 h, compared with wild-type plants (Figure 8c,d). Therefore, the observed increase in GFP-HFR1 protein accumulation in the spa1-100 mutant background under all light conditions (far-red, red, blue, and white light) is likely due to reduced proteolysis, rather than stabilization of the HFR1 transcript.

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Figure 6. The GFP-HFR1 transgene enhances the hyper-photomorphogenesis phenotype conferred by the spa1-100 mutation under various light conditions. (a) Seedling morphology under different light conditions. Photographs of seedlings from each light condition were taken at the same magnification. Bars: 1 mm. (b) Quantification of hypocotyl lengths of various mutants under different light conditions. Bars stand for standard deviations.
turnover of these photomorphogenesis-promoting transcription factors.

SPA1 might also regulate COP1-mediated protein degradation through modification of the E3 ubiquitin ligase activity of COP1. In the absence of COP1, SPA1 alone does not show any detectable E3 activity, but significantly reduces the E3 activity of COP1 on HY5 and greatly enhances the auto-ubiquitination activity of COP1 (Saijo et al., 2003). However, Seo et al. (2003) reported that the coiled-coil domain of SPA1 has no effect on COP1 auto-ubiquitination, but facilitates LAF1 (an MYB transcription factor promoting photomorphogenesis specifically under far-red light, Balles-teros et al., 2001) ubiquitination at low COP1 concentrations. The discrepancies in the effect of SPA1 on COP1 E3 activity could be due to different SPA1 proteins used in the assays (full-length versus coiled-coil domain only), different substrates (HY5 versus LAF1), or different assay conditions (different E1 and E2 were used). Obviously, these discrepancies need to be resolved in future studies. In addition, as the exaggerated photoresponses of the spa1 mutant are fully dependent on the presence of a functional phyA (Hoecker et al., 1998), it will be of great interest to determine
how phyA and light regulate SPA1 activity. One possibility is that phyA could directly bind to SPA1 and may cause phosphorylation of SPA1, as phyA possesses kinase activity (Yeh and Lagarias, 1998) and light treatment induces its import into the nucleus (Kircher et al., 1999; Yamaguchi et al., 1999), where SPA1 localizes (Hoecker et al., 1999). In addition, in the nucleus, phyA co-localizes with COP1 in nuclear bodies (speckles), where COP1 acts as an E3 ligase promoting phyA degradation and subsequent termination of phyA signaling (Seo et al., 2004). Conversely, SPA1, which contains a kinase-like domain at the N-terminus (Hoecker et al., 1999), could regulate phyA stability or activity by acting as a kinase for phyA. This scenario would be consistent with the finding that phyA activity is reduced by phosphorylation (Jordan et al., 1997; Ryu et al., 2005; Stockhaus et al., 1992). Alternatively, interaction of SPA1 with phyA could reduce the efficiency of phyA signaling by competing with a positive regulator of phyA for binding to phyA. Determining the functional relationship between phyA and SPA1 could represent a major leap in our understanding of the molecular mechanisms governing light signaling in Arabidopsis.

Experimental procedures

Plant materials and growth conditions

The wild type, various mutants and transgenic plants were of the Columbia (Col) ecotype background. The hfr1-201 mutant and the GFP-HFR1 transgenic line (B4) were described previously (Soh et al., 2000; Yang et al., 2005). The spa1-100 mutant was isolated from SAIL T-DNA insertion population (Sessions et al., 2002) and has been backcrossed once with Columbia wild type to remove/reduce potential background mutations. The T-DNA insertion sites were confirmed by PCR and sequencing. The primers used were the left border-specific primer (LB3) 5'-TAG CAT CTG AAT TTC ATA ACC A-3' and the SPA1-specific primer 5'-GAT GCC TGT TAT GGA AAG AG-3'. Homozygous lines were selected by their phenotype and further verified by PCR genotyping. Growth conditions for the seedlings were described previously (Yang et al., 2005). Far-red, red, and blue lights were supplied by LED light sources, with irradiance fluence rates of approximately 0.5, 30, and 5 μmol m⁻² sec⁻¹ respectively, unless otherwise indicated (measured with International Light Inc. model IL1400A with sensor model SEL-033/F/W; Newburyport, MA, USA). White light was supplied by cool-white fluorescent lamps.

Construction of double mutants

The spa1-100/hfr1-201 and spa1-100/GFP-HFR1 double mutant combinations were derived from genetic crosses of their respective two single parental mutants (or transgenic lines). Putative double mutants were selected in the F₂ generation and confirmed in the F₃ generation based on the mutant phenotype and/or antibiotic selection markers.

Anthocyanin measurement

Measurement of anthocyanin content was conducted according to Fankhauser and Casal (2004). Briefly, 20 seedlings from each of the light treatments/genotype were incubated overnight (with gentle shaking in the dark) in 150 μl of methanol acidified with 1% HCl. Then 100 μl of distilled water and 250 μl of chloroform were added, vortexed and a quick spin was performed to separate anthocyanin from chlorophyll. Total anthocyanin was determined by measuring the A₅₃₀ and A₆₅₇ of the aqueous phase using a spectrophotometer. The relative amount of anthocyanin per seedling was calculated by subtracting A₆₅₇ from A₅₃₀.

Northern blotting and immunoblot analysis

For Northern blotting, Arabidopsis seedlings were grown under different light conditions. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The procedure for probe labeling and hybridization was essentially as described (Lin and Wang, 2004). Protein extraction method and immunoblot procedure were described previously (Yang et al., 2005). The GFP-HFR1 fusion protein was detected with rabbit anti-GFP polyclonal antibodies (Molecular Probes, Eugene, OR, USA) and visualized by incubating with goat-anti-rabbit secondary antibodies conjugated with alkaline phosphatase in the presence of 5-bromo-4-
chloro-3-indolyl-phosphate and nitro blue tetrazolium as substrates.

Fluorescence microscopy

To visualize the GFP-HFR1 fusion proteins, spa1-100 mutant seedlings harboring the GFP-HFR1 transgene or the parental GFP-HFR1 transgenic line (in the hfr1-201 mutant background, Yang et al., 2005) were mounted on slides and examined with an Axioskop fluorescence microscope (Zeiss, Oberkochem, Germany) with GFP filter sets. Representative images were documented by photography with a digital Axiocam camera system (Zeiss). All images were taken from the same regions of hypocotyls or roots with identical exposure.

In vitro interaction assay

The procedure and constructs for the in vitro expression of GAD, SPA1, SPA1-NT696, SPA1-NT545, and SPA1-CC have been described in Hoecker and Quail (2001). The GAD-HFR1 construct has been described in Yang et al. (2005).

Yeast two-hybrid assay

The assay system and all the procedures have been described by Serino et al. (1999). Various LexA and GAD constructs used in this study were described previously (Saijo et al., 2003; Yang et al., 2005).

Acknowledgements

We thank Georg Jander and Elizabeth Estabrook for their reading and comments on the manuscript. We also thank Syngenta for providing the spa1-100 mutant allele (Sessions et al., 2002). This research was supported by set-up funds from the Boyce Thompson Institute, the Triad Foundation, and the National Science Foundation (MCR-0420932) to H.W., and by the Deutsche Forschungsgemeinschaft (SFB590) to U.H.

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