Blue-light-dependent interaction of cryptochrome 1 with SPA1 defines a dynamic signaling mechanism

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Article published online ahead of print. Article and publication date are February 28, 2011.

Received December 22, 2010; revised version accepted February 28, 2011.

Light is the primary environmental cue that is essential for plant growth and development. In plants, the light signal is perceived by multiple photoreceptors, including the blue/ultraviolet-A [320- to 500-nm]-light-absorbing cryptochromes (CRY1 and CRY2) (Cashmore et al. 1999; Lin and Shalitin 2003; Li and Yang 2007) and phototropins (PHOT1 and PHOT2) (Briggs and Christie 2002), and the red [600- to 700-nm]- and far-red [700- to 750-nm]-light-absorbing phytochromes [phyA to phyE] (Quail 2002).

Cryptochromes (CRYs) are blue-light photoreceptors that mediate various light responses in plants and animals. The signaling mechanism by which CRYs regulate light responses involves their physical interactions with COP1. Here, we report that CRY1 interacts physically with SPA1 in a blue-light-dependent manner. SPA acts genetically downstream from CRYs to regulate light-controlled development. Blue-light activation of CRY1 attenuates the association of COP1 with SPA1 in both yeast and plant cells. These results indicate that the blue-light-triggered CRY1–SPA1 interaction may negatively regulate COP1, at least in part, by promoting the dissociation of COP1 from SPA1. This interaction and consequent dissociation define a dynamic photosensory signaling mechanism.

Results and Discussion

The C-terminal domain of CRY1 interacts with SPA1 in yeast cells

It has been shown that the C-terminal WD40 domain of COP1 mediates the interaction with CRY1 or CRY2 (Wang et al. 2001; Yang et al. 2001), and that SPA1 and COP1 share a highly structurally related coiled-coil region and a C-terminal WD40 domain (Hoecker et al. 1999; Hoecker and Quail 2001). To examine whether CRY1 might also interact with SPA1, a yeast two-hybrid assay was performed. The results indicate that the CRY1 C-terminal domain (CCT1) strongly interacts with SPA1, a yeast two-hybrid assay.
interacts with the C-terminal WD40-containing domain of SPA1 (SCT1, referring to as CT509) (Yang and Wang 2006; Liu et al. 2011) but hardly with the N-terminal domain (SNT1) or coiled-coil domain (SCC1) of SPA1 (Supplemental Fig. S2A–C; Seo et al. 2003). These data indicate that, like COP1 (Yang et al. 2001), SPA1 interacts with CRY1 through the C-terminal WD40 domain. We further truncated SPA2, SPA3, and SPA4 corresponding to SCT1 (Supplemental Fig. S3) and determined that CTT1 interacts with the C-terminal WD40-containing domain of SPA2, SPA3, and SPA4 proteins (SCT2, SCT3, and SCT4, respectively) (Supplemental Fig. S2A,B,D).

The full-length CRY1 and CRY2 interact with SPA1 in a blue-light-dependent manner in yeast cells

To determine whether the full-length CRY1 interacts with SPA1, we prepared bait constructs expressing a variety of SPA protein domains (Fig. 1A) and a prey construct expressing the full-length CRY1 (Fig. 1B). We then performed yeast two-hybrid assays in the dark and in blue light, respectively. Strikingly, we found that full-length CRY1 interacts with SPA1 under blue light, but not in the dark (Fig. 1C; Supplemental Fig. S4). CRY1 also interacts with SCT1 in a blue-light-dependent manner, but fails to interact with SNT1 (Fig. 1C). We further observed that blue-light irradiation clearly promoted the interaction of SCT2, SCT3, and SPA4 with CRY1, although SPA4 is able to interact with CRY1 in darkness (Fig. 1C). Likewise, we found that full-length CRY2 interacts with SPA1 in a blue-light-dependent manner (Fig. 1C; Supplemental Fig. S4). It has been shown that, when fused to β-glucuronidase (GUS), CCT1 is constitutively active in both darkness and light and mediates a strong constitutive light response in Arabidopsis (Yang et al. 2000; Yang et al. 2005). We then tested whether GUS-CCT1 interacts with SCT1, SCT2, SCT3, and SPA4 under blue light and in the dark in yeast cells. The results demonstrated a clear constitutive interaction for each of these pairs independent of blue light (Fig. 1C).

Next, we investigated the blue-light fluence rate response of the CRY–SPA1 interaction. As shown in Figure 1D, CRY1–SPA1 and CRY2–SPA1 interactions increased in yeast cells exposed to increasing fluence rates of blue light. CRY1–SPA1 and CRY2–SPA1 interactions were barely detectable in yeast cells treated with the lowest fluence of blue light tested (0.1 μmol/m²/sec for 120 min). When the fluence rate was increased to the highest [100 μmol/m²/sec for 120 min], the levels of CRY1–SPA1 and CRY2–SPA1 interactions were increased by 15-fold and sixfold, respectively (Fig. 1D). We then demonstrated that CRY1–SPA1 and CRY2–SPA1 interactions increased in yeast cells with increasing irradiation time. As shown in Figure 1E, the levels of CRY1–SPA1 and CRY2–SPA1 interactions in yeast cells exposed to 20 μmol/m²/sec blue light for 240 min were approximately seven times and three times greater, respectively, than those in yeast cells exposed to 20 μmol/m²/sec blue light for 15 min.

Figure 1. CRY interacts with SPA in a blue-light-dependent manner in yeast cells. (A) Yeast two-hybrid bait constructs. All proteins are fused with the LexA DNA-binding domain (LexA), [SNT1] N-terminal domain of SPA1, [SCT1] C-terminal domain of SPA. (B) Yeast two-hybrid prey constructs. All proteins are fused with the B42 activation domain (AD). (C) Yeast two-hybrid analyses of the CRY–SPA interactions and the interactions of GUS-CCT1 with SPA under blue light (BL) and in darkness (DK). [D, E] Quantitative yeast two-hybrid analyses of the CRY–SPA1 interactions under the indicated fluence rates of blue light ([D] and 20 μmol/m²/sec blue light for the indicated exposure time ([E]). Data are mean ± SD (n = 12).
SPA acts genetically downstream from CRY to regulate light-controlled development

To examine whether CRY1 and SPA might genetically interact, we generated a spa1 spa2 spa3 spa4 quadruple mutant and a cry1 spa1 spa2 spa3 spa4 quintuple mutant and analyzed their photomorphogenic phenotypes under blue light. The results showed that the cry1 spa1 spa2 spa3 spa4 quintuple mutant exhibited an enhanced photomorphogenic phenotype similar to that of the spa1 spa2 spa3 spa4 quadruple mutant [Fig. 4A,C]. Consistent with these phenotypes, Western blotting analysis using an antibody prepared against HY5 indicated that the cry1 spa1 spa2 spa3 spa4 quintuple mutant and spa1 spa2 spa3 spa4 quadruple mutant seedlings accumulated similarly high levels of HY5 protein under blue light (Supplemental Fig. S6). It has been shown that CRY1 mediates blue-light-triggered stomatal development, and that SPA acts to repress stomatal development (Kang et al. 2009). We then analyzed stomatal development in these mutants and found that the stomatal phenotype of the cry1 spa1 spa2 spa3 spa4 quintuple mutant under blue light resembled that observed for the spa1 spa2 spa3 spa4 quadruple mutant, as shown by the stomata developing in clusters and the stomatal index (the ratio of stomata to stomata plus other epidermal cells) [Fig. 4E]. We also generated cry2 spa1 spa2 spa3 and cry2 spa1 spa3 spa4 quadruple mutants and investigated their photomorphogenic phenotypes. The results showed that the phenotypes of the cry2 spa1 spa2 spa3 and cry2 spa1 spa3 spa4 quadruple mutants under blue light were similar to those of the spa1 spa2 spa3 and spa1 spa3 spa4 triple mutants, respectively [Fig. 4B,D]. We then determined the flowering time phenotype under a long day photoperiod (16 h light/8 h dark) and found that the cry2 spa1 spa2 spa3 and cry2 spa1 spa3 spa4 quadruple mutants flowered essentially as early as the spa1 spa2 spa3 and spa1 spa3 spa4 triple mutants, respectively [Fig. 4F].

These results suggest that SPA acts genetically downstream from CRY1 and CRY2 to regulate photomorphogenic development, stomatal development, and flowering time.

The COP1–SPA1 interaction is repressed by blue-light-activated CRY1 in both yeast and plant cells

The COP1–SPA1 association shows dynamic characteristics. In the dark, COP1 strongly interacts with SPA1, while light promotes their dissociation (Saijo et al. 2003). The demonstration that CRY1–SPA1 exhibits blue-light-induced interaction prompted us to examine whether CRY1 is directly involved in the regulation of the dynamic interaction of COP1 and SPA1 under blue light through yeast three-hybrid assays. We generated a construct expressing COP1 bait and the full-length CRY1 bridge protein or a mock CRY1 bridge [mCRY1] that does not express CRY1 and a construct expressing SPA1 prey (Fig. 5A). As shown in Figure 5B, the COP1–SPA1 interaction decreased in yeast cells expressing CRY1 but not mCRY1, even when exposed to a higher fluence rate of blue light. The level of COP1–SPA1 interaction in yeast cells exposed to the highest fluence of blue light tested (50 μmol/m²/sec) decreased by ~80% compared with that in yeast cells exposed to the lowest fluence of blue light tested (0.5 μmol/m²/sec).

It is possible that inhibition of COP1–SPA1 interaction by CRY1 is mediated through the CRY1–SPA1 and/or CRY1–COP1 interaction. To investigate these possibilities further, we first prepared a construct expressing the coiled-coil-containing domain of COP1 bait [CC1], which does not interact with CRY1 [Fig. 5A, Supplemental Fig. S7], and performed yeast three-hybrid assays under different fluence rates of blue light.

Figure 2. Colocalization of CRY1 and CRY2 with SPA1 in onion epidermal cells. Onion peels were cobombarded with the DNA constructs indicated. CRY1 and CRY2 localize together with SPA1 to the NBs in onion epidermal cells. (Dic) Differential interference contrast in light microscope mode. Bars, 10 μm.

Figure 3. CRY1 interacts with SPA1 in a blue-light-dependent manner in vivo. (A–C) Co-IP using anti-CCT1 antiserum in the extracts from Myc-SPA1/spa1 seedlings grown under continuous white light for 5 d and then transferred to darkness for another 3 d [A,B], or seedlings grown in continuous darkness for 6 d [C] before being exposed to the indicated light conditions for 10 h [A], the indicated fluence rates of blue light [B], or 10 μmol/m²/sec blue light for the indicated exposure time [C]. [D] Darkness; [B] blue light (30 μmol/m²/sec); [R] red light (30 μmol/m²/sec); [FR] far-red light (5 μmol/m²/sec). The immunoprecipitates were probed with anti-CCT1 and anti-Myc antibodies, respectively.
fluorescence rates of blue light. As shown in Figure 5C, the CRY1–SPA1 interaction was inhibited in yeast cells expressing CRY1 but not mCRY1 as the fluence rate was increased by ~26% and 42% on 2 and 4 h of blue-light irradiation compared with that on 0.5 h of irradiation, respectively (Fig. 5E). In contrast, in the cry1 mutant background, blue-light-induced COP1–SPA1 dissociation was inhibited (Fig. 5F). These data indicate that blue-light-mediated dissociation of COP1 from SPA1 is achieved, at least in part, through the blue-light-induced interaction of CRY1 with SPA1.

Genetic and biochemical studies demonstrate that COP1 activity is regulated by multiple components, such as SPA, the CDD complex, and CSN (Chamovitz et al. 1996; Suzuki et al. 2002; Saijo et al. 2003; Seo et al. 2003; Yamaizawa et al. 2004). We proposed previously that the outcome of the light activation of CRY1 through its physical interaction with COP1 is the disruption of the negative regulation of COP1 exerted on its substrates (Yang et al. 2001; Liu et al. 2008). Based on a previous study showing that SPA1 can enhance the COP1–mediated ubiquitination of LAF1 in vitro (Seo et al. 2003) and our results, we tentatively propose that the blue-light-triggered CRY1–SPA1 interaction might result in a change in SPA1 properties, as a result of which the COP1–SPA1 interaction is inhibited, COP1 activity is depressed, and the HY5 protein is relieved from the COP1–SPA1-mediated proteolysis and performs its role in regulating photomorphogenesis (Fig. 5G). Moreover, it is possible that, upon blue light irradiation, the CRY1–COP1 interaction might, on the hand, suppress COP1 activity, and, on the other hand, also inhibit COP1 from associating with SPA1, further attenuating COP1 activity. In the dark, COP1 localizes to the nucleus, whereas, in the light, it localizes to the cytoplasm (von Arnim and Deng 1994). Thus, future studies should investigate whether regulation of COP1 by CRY1–COP1 and CRY1–SPA1 interactions involves regulation of the E3 ubiquitin ligase activity and the cellular localization properties of COP1.

We provide here new insights into the signaling mechanism of CRY1. The blue-light-induced CRY1–SPA1 interaction promotes the dissociation of COP1 from SPA1 to regulate COP1 activity negatively, a regulation that defines the dynamic nature of photosensory signaling induced by photoactivated CRY1 (Fig. 5G; Supplemental Fig. S1B). It has been established that COP1–SPA tetramer complexes exhibit different forms in response to various light conditions (Zhu et al. 2008). Our results indicate that the transformation among different forms of COP1–SPA complexes may result from the dynamic interactions and/or different association capacities of photoreceptors and SPA proteins. Given that CRY2 also interacts with COP1 (Wang et al. 2001) and SPA1 in a blue-light-dependent manner in yeast cells (Fig. 1C–E) and colocalizes in the same NBs with SPA1 in plant cells (Fig. 2; Supplemental Fig. S5), and that SPA acts genetically downstream from CRY2 to regulate photomorphogenic development and flowering time (Figs. 5E,F, Sang et al. 2005). The results demonstrated that, in the presence of CRY1, the COP1–SPA1 interaction was reduced progressively with prolonged exposure to blue light, with the amount of COP1 coimmunoprecipitated with Myc-SPA1 being reduced by 26% and 42% on 2 and 4 h of blue-light irradiation compared with that on 0.5 h of irradiation, respectively (Fig. 5E).
mutants were obtained from the Arabidopsis Biological Resource Center, spa3 (SALK_120736) from the Versailles Genetics and Plant Breeding Laboratory Arabidopsis thaliana Resource Center, and Myc-SPA1/spa1-3, cry1-104, and cry1-1 mutants were generated previously [Bruggemann et al. 1996; Guo et al. 1996; Yang and Wang 2006]. The spa3 spa2 spa3, spa1 spa3 spa4, spa1 spa2 spa3 spa4, cry1 spa1 spa2 spa3 spa4, cry2 spa1 spa2 spa3, cry2 cry1 spa2 spa3, and Myc-SPA1/cry1 mutants were obtained by genetic crossing. The resulting mutants harboring a mutation in SPA3 were backcrossed into the Col background four times to reduce the Ws background. All of the genotypes were confirmed by PCR and phenotypic analysis.

**COP1 antibody preparation**

Rabbits were vaccinated with recombinant Histagged peptide corresponding to amino acids 1-212 of COP1 purified in Escherichia coli to generate anti-COP1 antisera. The anti-COP1 antibody was further purified from the antiserum using protein A beads.

**Co-IP study**

Co-IP was performed by methods described previously [Shalitin et al. 2002; Zhu et al. 2008], with minor modifications. Myc-SPA1/spa1 seeds were harvested in dim green safe light and homogenized in lysis buffer (Zhu et al. 2008). After centrifugation, total protein concentration was determined by Bradford assay [Bio-Rad]. Equal amounts of total protein [1-3 mg] in 1 mL of lysis buffer were incubated with 5 μL of anti-CCT1 antisera (Sang et al. 2008) for 1 h at 4°C, then 20 μL of protein A-Sepharose beads [bed volume, GE healthcare] was added to the mixture and incubated for another hour at 4°C. The immunoprecipitates were washed four times with wash buffer (Shalitin et al. 2002). For anti-Myc co-IP, equal amounts of total protein [1 mg, unless otherwise stated] in 1 mL of lysis buffer from Myc-SPA1/spa1 and Myc-SPA1/cry1 seedlings were incubated with 40 μL of anti-Myc agarose beads [bed volume, Sigma] for 2 h at 4°C. The immunoprecipitates were washed three times with wash buffer. Eluting and concentrating of the immunoprecipitates were performed as described previously (Zhu et al. 2008). The concentrates were resuspended with 2× SDS sample buffer and boiled for 5 min, then subjected to Western blotting analysis. The immunoblots were quantified with ImageJ [http://rsb.info.nih.gov/ij].

**Yeast three-hybrid assay**

A yeast three-hybrid assay was performed according to the manufacturer’s instructions except that the yeast strain SPY526 was used. Transformed colonies were selected on synthetic complete medium lacking Leu and Trp. Three independent clones with four respective replicates were used in each performance. Cell cultures were placed under indicated light conditions and incubated at 30°C until the OD600 was between 0.5 and 0.8 with the conditional expression of the bridge proteins. Calculations of relative β-galactosidase activities were performed as described previously [Yang et al. 2001]. At least three independent experiments were performed, and the result of one representative is shown.

**Acknowledgments**

We thank W.R. Briggs for critically reading our manuscript, C. Lin for discussions and communication of data, H.Y. Wang for Myc-SPA1 seeds, Y.J. Wu and A.R. Cashmore for discussions and communication of data, H.Y. Wang for Myc-SPA1 seeds, and X.W. Deng for helpful comments. This work was supported by the National Natural Science

**Materials and methods**

The plant materials and growth conditions, yeast two-hybrid assay, and subcellular colocalization study were as described previously [Yang et al. 2001; Sang et al. 2005; Liu et al. 2008]. Arabidopsis thaliana Columbia accession [Col] was used as the wild type.

**Construction of mutants**

The Arabidopsis mutants used for this report were in the Col background, except for the spa2 mutant, which was in the Wassilewskija [Ws] background. Spa1 (SALK_023840), spa2 (SALK_083331), and spa4 (SALK_082740)
Foundation of China [30830012 to H.-Q.Y.], a National Special Grant for Transgenic Crops [2009ZX0009-0018 to H.-Q.Y.], the Science and Technology Commission of Shanghai Municipality [10XD140300 to H.-Q.Y.], and the Shanghai Leading Academic Discipline Project [B209].

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