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RESEARCH ARTICLE

The Signaling Mechanism of Arabidopsis CRY1 Involves Direct Interaction with COP1

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Dark-grown transgenic Arabidopsis seedlings expressing the C-terminal domains (CCT) of the cryptochrome (CRY) blue light photoreceptors exhibit features that are normally associated only with light-grown seedlings, indicating that the signaling mechanism of Arabidopsis CRY is mediated through CCT. The phenotypic properties mediated by CCT are remarkably similar to those of the *constitutive photomorphogenic1* (*cop1*) mutants. Here we show that Arabidopsis cryptochrome 1 (CRY1) and its C-terminal domain (CCT1) interacted strongly with the COP1 protein. Coimmunoprecipitation studies showed that CRY1 was bound to COP1 in extracts from both dark- and light-grown Arabidopsis. An interaction also was observed between the C-terminal domain of Arabidopsis phytochrome B and COP1, suggesting that phytochrome signaling also proceeds, at least in part, through direct interaction with COP1. These findings give new insight into the initial step in light signaling in Arabidopsis, providing a molecular link between the blue light receptor, CRY1, and COP1, a negative regulator of photomorphogenesis.

INTRODUCTION

The Arabidopsis cryptochromes (CRY1 and CRY2) mediate a variety of blue light–induced responses, including hypocotyl shortening, cotyledon expansion, and anthocyanin production (Ahmad and Cashmore, 1993; Ahmad et al., 1998a; Lin et al., 1998). CRY1 and CRY2 share sequence similarity to photolyases, a family of proteins that catalyze the repair of UV light–damaged DNA (Sancar, 1994). However, the Arabidopsis cryptochromes have a distinguishing C-terminal domain that is absent in photolyases (Ahmad and Cashmore, 1993; Cashmore et al., 1999) and lack photolyase activity (Ahmad and Cashmore, 1993; Lin et al., 1995).

Several lines of evidence indicate an interaction between cryptochromes and phytochromes, the red/far-red photoreceptors in plants (Mohr, 1994). In Arabidopsis, a synergism has been described for the activity of the two classes of photoreceptors (Casal and Mazzella, 1998). Furthermore, Arabidopsis CRY1 has been shown to be phosphorylated by the protein kinase activity of oat phyA, and select *cry1* alleles are early flowering (Ahmad et al., 1998b), a property like that of mutants in the *PHYB* gene (Bagnall et al., 1995). Similarly, the late flowering phenotype of Arabidopsis *cry2* alleles is postulated to reflect the wild-type CRY2 protein negatively affecting phyB signaling (Mockler et al., 1999). CRY2 and phyB were found to interact in extracts of transgenic Arabidopsis plants overexpressing CRY2, and by fluorescence resonance energy transfer, these same photoreceptors were shown to be associated in nuclear speckles that form in a light-dependent manner (Mas et al., 2000).

Insight into the signaling pathway of Arabidopsis cryptochrome was obtained through the demonstration that transgenic plants expressing the C-terminal domain of either CRY1 (CCT1) or CRY2 (CCT2) fused to β-glucuronidase (GUS) display a constitutive photomorphogenic (COP) phenotype (Yang et al., 2000). These data suggest that CRY1 and CRY2 signaling in response to light activation is mediated through their C-terminal domains. This phenotype was not observed for transgenic plants expressing mutant CCT1 proteins corresponding to loss-of-function *cry1* alleles, indicating that the COP phenotype observed for CCT is physiologically meaningful. In view of the similarity of the CCT-mediated phenotype to that of mutants of both COP1 and the COP9 signalosome complex, it was proposed that CRY signaling may involve a direct interaction with one of these COP proteins.

The *COP*/*DET*/*FUS* loci are pleiotropic negative regulators of plant development (Chory et al., 1989; Deng et al., 1991; Misera et al., 1994; Kwok et al., 1996). The *COP1* gene is one of the best characterized of these, with dark-grown

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mutant *cop1* seedlings displaying a COP phenotype that includes shortened hypocotyls, anthocyanin production, and chloroplast development, which are characteristic of light-grown seedlings (Deng et al., 1991). Some *cop1* mutants are adult lethal, whereas others develop plants with severely reduced rosette size and low fertility (Deng and Quail, 1992; McNellis et al., 1994). The *COP1* gene encodes a protein with three distinguishing structurally recognized domains: an N-terminal ring finger domain, a coiled-coil region, and C-terminal WD40 repeats (Deng et al., 1992; McNellis et al., 1994).

In the dark, COP1 localizes to the nucleus, whereas in the light, it localizes to the cytoplasm (von Arnim and Deng, 1994). Other COP loci include those that encode members of the COP9 signalosome. In contrast to COP1, this COP9 signalosome complex is localized constitutively to the nucleus (Chamovitz et al., 1996), where it is required for the accumulation of COP1 (Kwok et al., 1998; Osterlund et al., 1999). In dark-grown seedlings, the COP1 protein negatively regulates HY5, a bZIP transcription factor (Oyama et al., 1997; Ang et al., 1998; Torii et al., 1998). COP1 binds to HY5, and this interaction targets HY5 for proteasome-mediated degradation in the nucleus (Osterlund et al., 2000).

Mutations of *COP1*, and those of genes encoding members of the COP9 signalosome complex, are epistatic to mutations in *CRY* and *PHY* genes in both dark- and light-grown seedlings (Ang and Deng, 1994; Wei et al., 1994). These genetic data indicate that photoreceptor function is not required for the photomorphogenic phenotype characteristic of the *cop* mutants. These results have been interpreted to suggest that the COP proteins function as negative regulators downstream, and on the direct signaling pathway, of the photoreceptor light-signaling pathway (Ang and Deng, 1994; Osterlund et al., 1999).

Here it is demonstrated that CCT1 and the full-length CRY1 protein interact physically with the COP1 protein both in the yeast two-hybrid system and in binding studies in vitro. Furthermore, we show by coimmunoprecipitation studies that CRY1 was bound to COP1 in extracts from both dark- and light-grown Arabidopsis. We interpret these findings to indicate that the signaling mechanism of the Arabidopsis CRY1 protein involves direct interaction with the COP1 protein. Such an interaction is presumed to counteract, in a blue light–dependent manner, the negative regulatory properties of COP1, thus initiating the photomorphogenic processes mediated by the CRY1 photoreceptor.

RESULTS

Arabidopsis CRY1 C-Terminal Domain Interacts with COP1 in Yeast Cells

The finding that the phenotype of transgenic Arabidopsis plants expressing CCT1 remarkably resembles that of loss-of-function mutations of *COP1* prompted us to determine whether CRY1 signaling involved a direct interaction with COP1. To do this, we used the yeast two-hybrid system. We prepared bait constructs expressing the LexA DNA binding domain fused to a variety of CRY protein domains (Figure 1A). We also prepared prey constructs expressing the B42 transcriptional activation domain (B42 AD) fused to COP1 and related proteins (Figure 1B). The bait construct expressing the LexA DNA binding domain covalently joined to Arabidopsis CCT1 showed significant background, as seen by β-galactosidase activity in yeast cells coexpressing the control prey B42 AD polypeptide (Figure 1D, sample 5). However, this activity increased dramatically when the CCT1 bait was coexpressed with the prey construct comprising B42 AD fused to Arabidopsis COP1 (Figure 1D, sample 8). Indeed, the level of activity observed in these cells was remarkably strong, comparable to that observed in cells expressing a control fusion protein in which the GAL4 activation domain is linked covalently to the LexA DNA binding domain (Figure 1D, samples 1 and 2).

To test the specificity of this interaction, a prey construct was made expressing MSL1, an Arabidopsis protein not involved in light signaling but containing a WD40 repeat domain such as COP1 (Ach et al., 1997). Analysis of β-galactosidase activity in yeast cells coexpressing the CCT1 bait fusion protein and the MSL1 prey fusion protein demonstrated no interaction between these proteins (Figure 1D, sample 6). A more striking test of specificity was obtained by evaluating the interaction of CCT1 with the WD40 domain of the human ortholog of COP1; this region of the human protein shows 47% amino acid sequence identity with the corresponding region of Arabidopsis COP1 (Wang et al., 1999). No interaction was observed between CCT1 and the human COP1 sequence (Figure 1D, sample 7).

Our previous studies on the expression of CCT1 in transgenic plants involved a GUS–CCT1 fusion protein. For this reason, we examined the activity of this fusion protein in yeast and demonstrated that coexpression of bait and prey fusion proteins, containing GUS–CCT1 and COP1, respectively, produced the same level of interaction as that observed between CCT1 and COP1 (Figure 1D, samples 8 and 11). No interaction was observed between GUS and COP1 (Figure 1D, sample 3).

Transgenic plants expressing the N-terminal photolyase-like domain of CRY1 (CNT1), in contrast to those expressing CCT1, do not display a COP phenotype (Yang et al., 2000). Nevertheless, we tested whether CNT1 could interact with COP1 and failed to detect an interaction (Figure 1D, sample 4). This finding indicates that the domain of CRY1 that mediates the constitutive light response in Arabidopsis (i.e., CCT1) is the domain that interacts with COP1 in yeast.

Transgenic Plants Expressing CCT1 Show Severe Dwarfism

Given the strong interaction observed between CCT1 and COP1, we repeated our earlier screen of GUS–CCT1

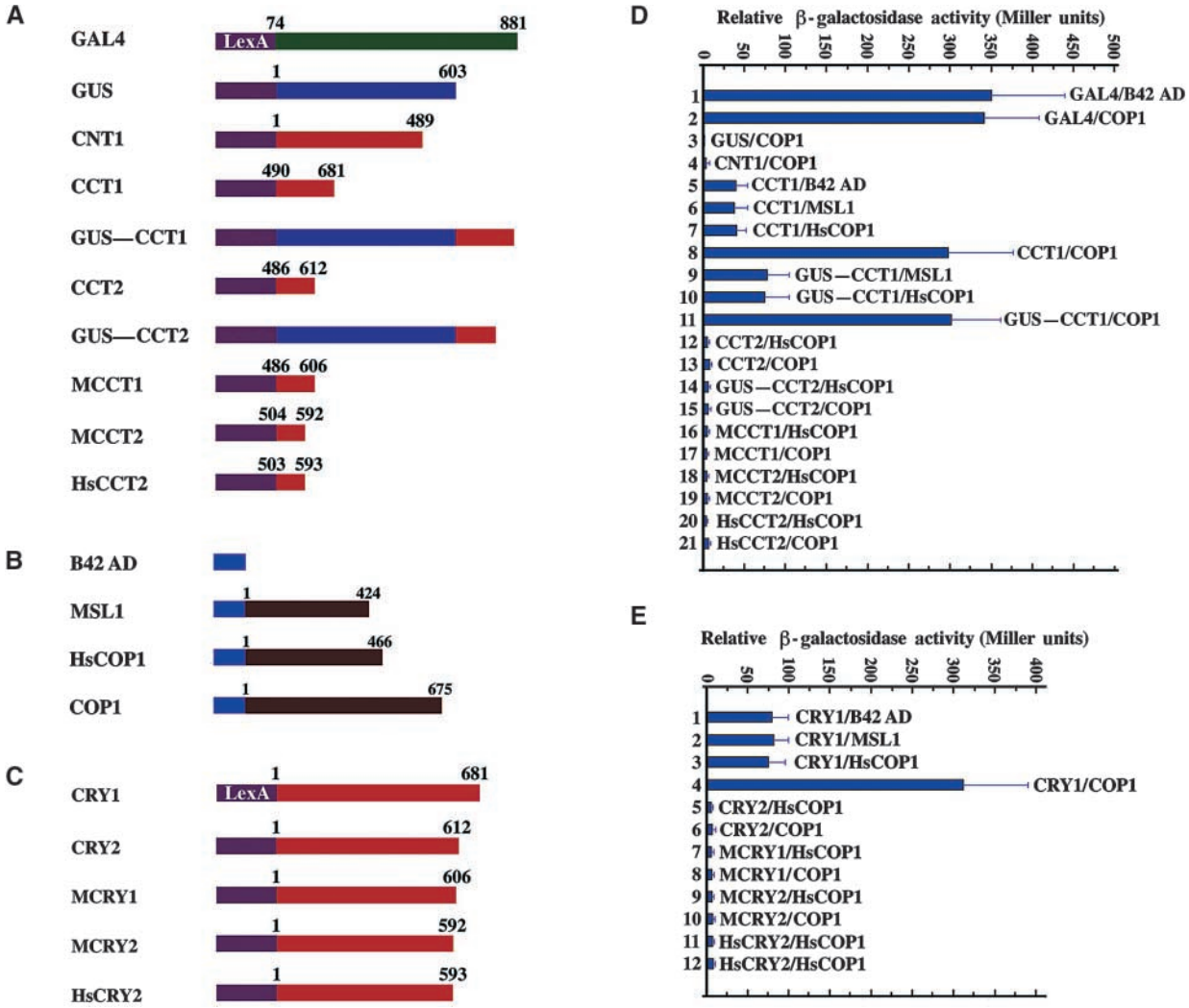

Figure 1. Arabidopsis CCT1 Interacts with COP1 in Yeast.

(A) CCT and CNT bait proteins. All proteins are fusions with the LexA DNA binding domain (LexA) and contain, in addition, the following proteins: GAL4, Gal4 protein containing the activation domain; GUS, β-glucuronidase; CNT1, Arabidopsis CRY1 N-terminal domain; CCT1, Arabidopsis CRY1 C-terminal domain; GUS–CCT1; CCT2, Arabidopsis CRY2 C-terminal domain; GUS–CCT2; MCCT1, mouse CRY1 C-terminal domain; MCCT2, mouse CRY2 C-terminal domain; and HsCCT2, human CRY2 C-terminal domain. CCT1-10, CCT1-19, and CCT1-20 are Arabidopsis CCT1 mutants.

(B) Prey proteins. All proteins are fusions to the B42 activation domain (B42 AD), which in turn is fused to a hemagglutinin epitope tag; MSL1 is an Arabidopsis protein that, like COP1, contains WD40 repeats; HsCOP1, C-terminal 466–amino acid polypeptide of human COP1; COP1, Arabidopsis COP1.

(C) CRY bait proteins. All proteins are fusions with the LexA DNA binding domain (LexA) fused to various CRY proteins. CRY1, Arabidopsis CRY1; CRY2, Arabidopsis CRY2; MCRY1, mouse CRY1; MCRY2, mouse CRY2; HsCRY2, human CRY2.

(D) Arabidopsis CCT1 interacts strongly with COP1. The interaction strength was determined by quantitative yeast two-hybrid interaction assay. In this and other figures, all vector combinations are given as bait/prey. The CCT1/COP1 interaction is almost as strong as those of the positive controls, GAL4/B42 AD and GAL4/COP1, in which the Gal4 protein (including its activation domain) is joined covalently to the LexA DNA binding domain (LexA). In these experiments, the prey vectors B42 AD and COP1 were included, although presumably they do not contribute to the reaction. Ten independent original transformants were analyzed for each vector combination. Standard deviations are indicated by error bars.

(E) Full-length CRY1 interacts strongly with COP1. The yeast two-hybrid assay showed a strong interaction between Arabidopsis CRY1 and COP1 but no interaction (above background) between CRY1 and either MSL1 (an Arabidopsis protein that, like COP1, contains a WD40 repeat) or human COP1 (HsCOP1). Similarly, no interaction was observed between Arabidopsis CRY2 or the mouse or human CRY proteins and either the Arabidopsis or human COP1 proteins.

Figure 2. Transgenic Arabidopsis Plants Expressing CCT1 Exhibit a Dwarf Phenotype.

CCT1 plants display severe dwarfism. The plant at left is Columbia wild type. Approximately 10% of CCT1 transformants exhibited a dwarf phenotype, and the two plants at right are representative of this phenotype. Both wild-type and CCT1 plants are 20 days old. Bar = 1 cm.

transgenic plants to determine whether these plants displayed the additional phenotypes characteristic of strong *cop1* mutants, namely dwarfism and reduced fertility (Deng and Quail, 1992; McNellis et al., 1994). Approximately 240,000 T1 seed, grown in the dark on Murashige and Skoog (1962) plates without kanamycin, were screened for seedlings showing a COP phenotype. We obtained 247 such seedlings, of which 101 plants survived after transfer to soil. Of these, 22 displayed severe dwarfism, as illustrated in Figure 2. Protein gel blot analysis was conducted on extracts from leaves of the dwarf CCT1 and wild-type plants using antibody against CCT1. The GUS–CCT1 fusion protein levels in severe dwarf plants were significantly higher than those in the plant showing no dwarfism (data not shown).

These CCT1 plants were much smaller than wild-type plants and had a much lower seed set, producing only a few siliques; these were shorter than wild-type siliques with few or no seed. Some of the plants showed more severe phenotypes, including lethality. In parallel, by following the same procedure, we grew seedlings expressing the CCT1-19 transgene, which corresponds to a loss-of-function *cry1* allele (Yang et al., 2000). Of 80 seedlings that were transferred to soil, all survived and none of the resulting plants showed either dwarfism or reduced fertility (data not shown).

C-Terminal Domain of Arabidopsis CRY2 Does Not Interact with COP1 in Yeast

Although the Arabidopsis CRY2 C-terminal domain (CCT2) shares little sequence similarity with CCT1, on fusion with GUS it also confers a COP phenotype (Yang et al., 2000). We investigated whether CCT2, like CCT1, interacted with COP1 in the yeast two-hybrid system. Surprisingly, neither CCT2 nor GUS–CCT2 showed an interaction with COP1 (Figure 1D, samples 13 and 15). This result did not reflect the instability of the polypeptide, because protein blots of yeast extracts demonstrated that the CCT2 proteins were expressed at levels comparable to those found for CCT1 (data not shown). Although these results could reveal differences in the signaling mechanisms of CRY1 and CRY2, we think this unlikely (see Discussion).

C-Terminal Domains of Mammalian Cryptochromes Do Not Interact with Human COP1

Cryptochromes are found in animals as well as plants (Cashmore et al., 1999), and in both cases they play a role in the functioning of the circadian clock (Somers et al., 1998;

Stanewsky et al., 1998; Thresher et al., 1998; van der Horst et al., 1999). Although Arabidopsis CCT1 did not interact with human COP1, this did not eliminate the possibility that mammalian cryptochromes interact with mammalian COP proteins. To address this question, we coexpressed in yeast cells bait fusion proteins containing the C-terminal domain of either mouse CRY1 or CRY2 or human CRY2 with a prey fusion protein containing human COP1; the latter protein differs from mouse COP1 in only three amino acids in its entire sequence (Wang et al., 1999). As shown in Figure 1D (samples 16 to 21), none of the mammal cryptochrome C termini interacted with human COP1.

Full-Length Arabidopsis CRY1 Protein Interacts with COP1 in Yeast Cells

To determine if the full-length Arabidopsis CRY1 also was able to interact with COP1, we made a bait construct expressing the LexA–CRY1 fusion protein (Figure 1C). Although this fusion protein conferred even greater background activity than that observed with CCT1 (Figure 1E, sample 1), full-length CRY1 clearly interacted strongly with COP1 (Figure 1E, sample 4). As in the results obtained for the C-terminal domain, CRY1 did not interact with either the Arabidopsis protein MSL1 or human COP1 (Figure 1E, samples 2 and 3). We also investigated the activity of the full-length Arabidopsis CRY2 and the mouse and human CRY proteins. As observed for their C-terminal domains, none of these proteins interacted with either Arabidopsis COP1 or the human COP1 sequences (Figure 1E, samples 5 to 12).

Arabidopsis phyB C-Terminal Domain Interacts with COP1

In earlier studies, it was shown that neither the C-terminal domain of phyA nor that of phyB mediated a COP phenotype (Yang et al., 2000). However, it has been demonstrated that the nuclear abundance of GUS–COP1 is affected by phytochromes (Osterlund and Deng, 1998), and genetic epistasis analysis indicates that COP1 functions in the phytochrome signaling pathway (Ang and Deng, 1994). Therefore, we were interested in determining whether the C-terminal domain of either phyA or phyB could interact with Arabidopsis COP1 (Figure 3). A very clear interaction was obtained between the C-terminal domain of phyB and COP1 (Figure 3B, sample 4). Although this interaction was weaker by at least 5-fold in terms of β-galactosidase activity, relative to that observed for CCT1, it still was markedly stronger than that observed for background samples (~25-fold greater), including that demonstrating no interaction between the C-terminal domain of phyB and human COP1 (Figure 3B, sample 2). No interaction was observed between the C-terminal domain of phyA and Arabidopsis COP1 (Figure 3B, sample 3).

In our earlier studies, we had entertained the possibility that the COP phenotype mediated by CCT might reflect a direct interaction between cryptochrome and HY5, a bZIP

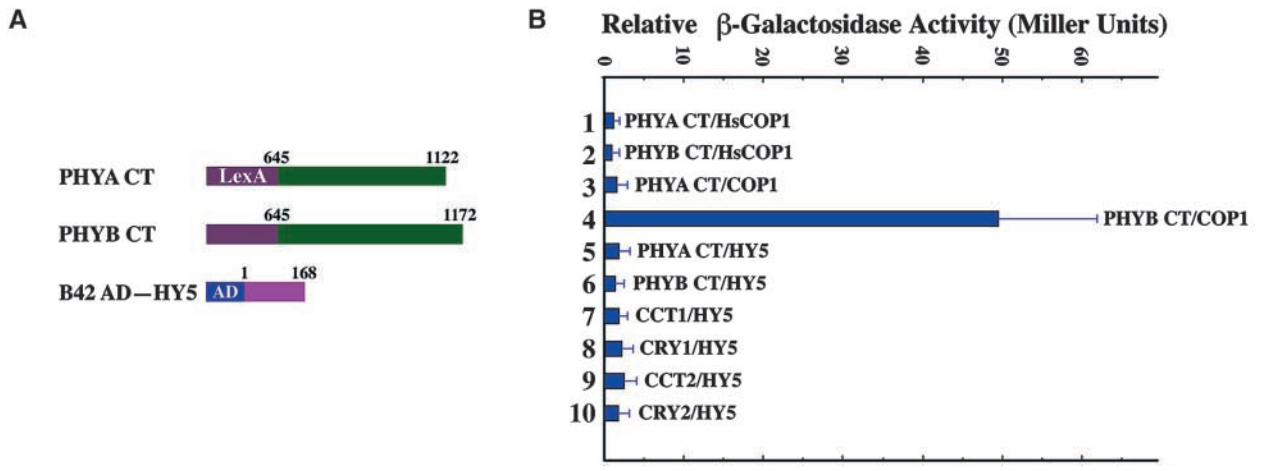

Figure 3. The phyB C-Terminal Domain Interacts with COP1.

(A) Bait and prey constructs. The bait constructs comprise the phyA and phyB C-terminal (CT) domains fused to the LexA DNA binding domain (LexA). The prey construct comprises HY5 fused to the B42 AD.

(B) Yeast two-hybrid assays. The phyB C-terminal domain (PHYB CT), but not the phyA C-terminal domain (PHYA CT), interacted with Arabidopsis COP1. Neither the phyA nor the phyB fragments interacted with human COP1 (HsCOP1). Neither full-length Arabidopsis CRY1 or CRY2, nor their C-terminal domains (CCT1 and CCT2), interacted with HY5. Similarly, no reaction was observed between the phyA or phyB C-terminal fragments and HY5.

Error bars indicate ±SE.

DNA binding protein that interacts physically with, and whose activity is suppressed by, COP1 (Ang et al., 1998; Torii et al., 1998). However, we found no evidence for any interaction between HY5 and either the C-terminal fragments (CCT1 or CCT2) or the full-length proteins (CRY1 or CRY2). Nor did we detect any interaction between HY5 and the C-terminal domains of either phyA or phyB (Figure 3B).

C-Terminal Domain of COP1, Containing the WD40 Repeat, Is Essential and Sufficient to Mediate the Interaction with CCT1

Arabidopsis COP1 has three distinguishing domains: the zinc binding ring finger, the coiled-coil region, and the WD40 repeat domain. Deletion of either of the latter two domains of COP1 significantly reduces its ability to interact with HY5 (Ang et al., 1998). To define the domains within COP1 that are required for its interaction with CCT1, we prepared constructs corresponding to a series of COP1 deletion fragments (Figure 4A). A protein gel blot experiment, conducted on extracts from yeast cells coexpressing CCT1 and any one of these deletion fragments, demonstrated that all of the COP1 fragments were expressed at a similar protein level (data not shown). Deletion of either the zinc binding ring finger or the coiled-coil domain, or deletion of both of these domains, had little effect on the capacity of COP1 to interact with CCT1 (Figure 4B, samples 2 to 4). Furthermore, an N-terminal fragment containing both of these domains failed to show an interaction (Figure 4B, sample 5). In contrast, the COP1 C-terminal fragment (C210-675), which contains the WD40 repeats, retained essentially all of the capacity observed for full-length COP1 to interact with CCT1 (Figure 4B, sample 6). Subfragments of this C-terminal COP1 domain (C210-386 and C387-675) showed dramatically reduced activity (Figure 4B, samples 7 and 8). Therefore, the COP1 region from amino acids 210 to 675 is essential and sufficient to mediate the interaction with CCT1.

Effects of Point Mutations within CCT1 on Interaction with the COP1 WD40 Domain

Many *cry1*/*hy4* mutant alleles have been characterized, and several of the mutations reside within the C-terminal domain (Ahmad et al., 1995). In describing the COP phenotype of transgenic plants expressing CCT1, it was noted that three mutations in the CCT1 sequence, corresponding to loss-of-function *cry1* alleles, failed to confer a COP phenotype (Yang et al., 2000). We argued that these observations provided good evidence in favor of the physiological significance of the observed COP phenotype. We prepared bait constructs expressing mutant CCT1s (CCT1-9, -10, -19, -20, -22, -23, and -24; Figure 4C) corresponding to several of the previously described *cry1* alleles (Ahmad et al., 1995), and yeast two-hybrid assays were performed to determine the ability of these mutant proteins to bind to the COP1 WD40 domain (Figure 4D). The mutation in CCT1-22 eliminated its interaction with the COP1 WD40 domain (Figure 4D, sample 6). Similarly, the mutation in CCT1-19 also reduced its activity dramatically (Figure 4D, sample 4). The other mutations had less effect on the interaction with the WD40 domain (Figure 4D, samples 2, 3, 5, 7, and 8). The mutant CCT1 polypeptides were expressed normally in yeast cells, as indicated by a protein gel blot using antibody against the LexA DNA binding domain (data not shown). These observations suggest that although binding of CRY1 (and CCT1) to COP1 may be necessary for signaling, it is not sufficient. There must be an additional property, distinct from binding to COP1, that is deficient in these CCT1 mutants.

Effects of Point Mutations within the COP1 WD40 Domain on Interaction with the CRY1 C-Terminal Domain

Several mutations within the WD40 domain of COP1, some of which correspond to loss-of-function *cop1* alleles, have been shown to eliminate the binding of COP1 to HY5 in yeast (Holm et al., 2001). Other mutations within this domain have been shown to enhance the binding of COP1 to HY5 dramatically (Holm et al., 2001). To further characterize the features of the COP1 WD40 region important for its interaction with CCT1, we made some of these point mutations within the WD40 domain and tested their effect on the binding to CCT1 in yeast (Figures 4E and 4F).

Neither WD40–467 nor WD40–524, the latter of which corresponds to the lethal *cop1-9* allele, showed significant interaction with CCT1 (Figure 4F, samples 4 and 5). These two sites also were found to be crucial for COP1-HY5 interaction (Holm et al., 2001). Other mutations had less effect on the interaction with CCT1 (Figure 4F, samples 2, 3, 6, and 7); in contrast, two of these mutations (WD40–422 and WD40–592) were found to increase the interaction with HY5 (Holm et al., 2001). The mutant WD40 polypeptides were expressed normally in yeast cells, as shown by a protein gel blot using antibody against the hemagglutinin epitope tag (data not shown). These results indicate that there are both similarities and differences in the structural requirements of the COP1 WD40 repeat for the binding of HY5 and CRY1.

Interaction between Arabidopsis CRY1 and COP1 in Yeast Cells Is Light Independent

The experiments described so far were performed under standard laboratory lighting conditions, with no attempt to determine the effect of light. To determine whether light could influence the binding, we examined the interaction of Arabidopsis CRY1 and CCT1 with COP1 in yeast cells, either illuminated with blue light or in darkness. The results demonstrated an absence of any light effect, with strong

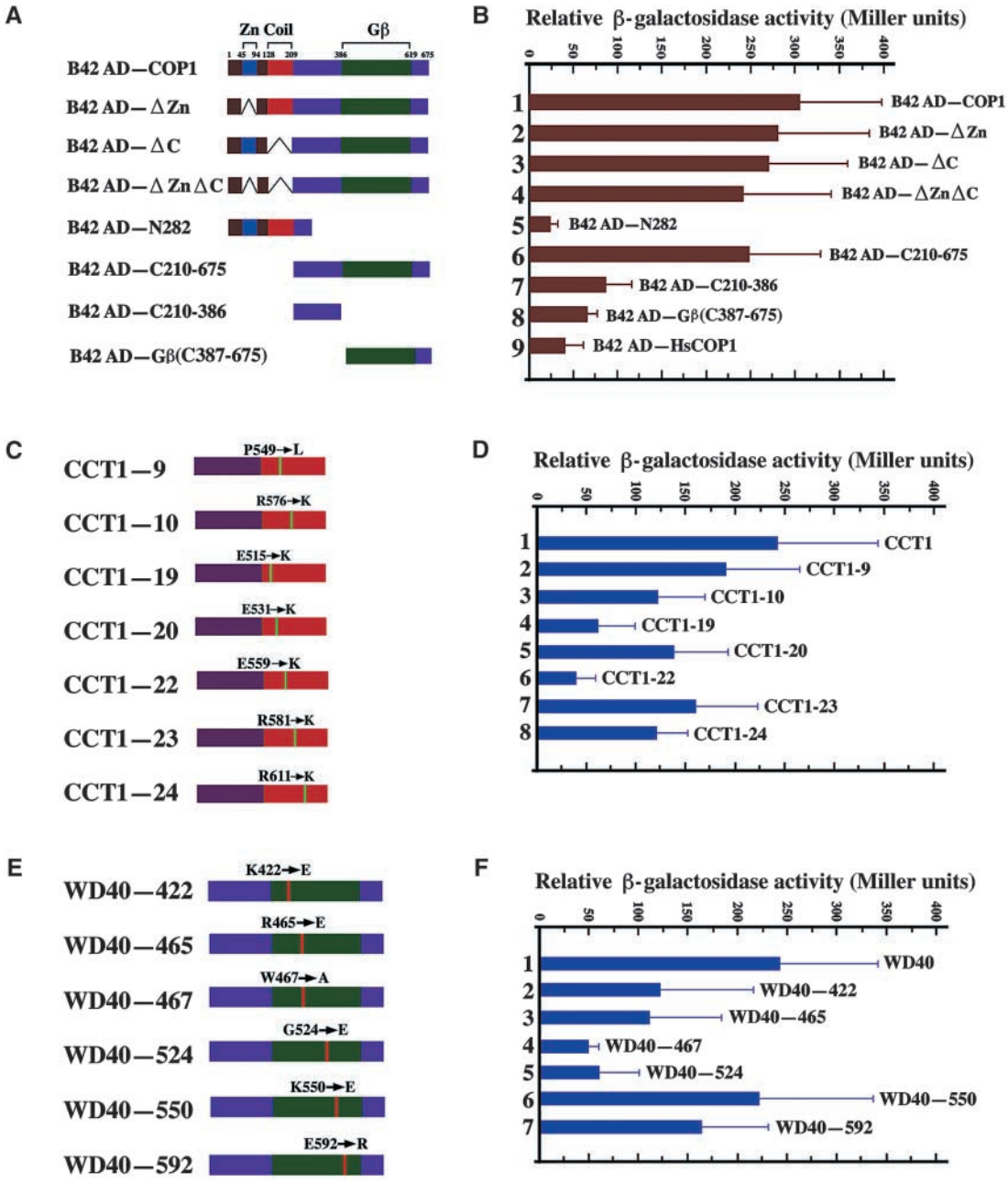

Figure 4. Identification of Domains in COP1 and Amino Acid Sites within CCT1 and WD40 of COP1 That Affect the COP1–CRY1 Interaction.

(A) Prey constructs comprising COP1 fragments fused to the B42 AD. Zn, zinc binding ring finger domain; Coil, coiled-coil region; Gβ, WD40 repeats.
(B) A C-terminal fragment of Arabidopsis COP1, containing the WD40 repeats, is essential and sufficient to mediate the interaction with CCT1. Yeast two-hybrid assays were performed to determine the interaction between the various COP1 prey fragments and a bait fusion protein containing Arabidopsis CCT1. Strong interaction was observed between CCT1 and all COP1 polypeptides that included residues C terminal to amino acid 210 and contained the WD40 repeats (C210-675).
(C) Bait constructs containing point mutations within CCT1 corresponding to previously identified *cry1* mutant alleles (Ahmad et al., 1995).
(D) The effects of point mutations within CCT1 on its interaction with the COP1 WD40 domain. Mutations in CCT1-19 and CCT1-22 almost completely abolished the ability to interact with the COP1 WD40 domain, whereas other mutations did not affect their activity significantly. CCT1-10, CCT1-19, and CCT1-20 mutant proteins are completely ineffective in their ability to confer a COP phenotype in transgenic plants (Yang et al., 2000).
(E) Prey constructs comprising point mutations within the WD40 domain of COP1 (C210-675 in **[A]**). WD40–524 corresponds to the lethal *cop1-9* allele. Mutation in this site (WD40–524) and in WD40–467 almost completely abolished the capacity to interact with CCT1. These two sites also were found to be essential for the COP1–HY5 interaction (Holm et al., 2001). Other mutations did not affect the ability to interact with CCT1 in yeast cells significantly.
(F) The effects of point mutations within the COP1 WD40 domain on its interaction with CCT1. Mutations in WD40–467 and WD40–524 almost completely compromised the inability to interact with CCT1, whereas other mutations affected activity to varying degrees.
Error bars indicate ±SE.

interaction being observed between full-length CRY1 and COP1 in both darkness and blue light (Figure 5A, samples 4 and 8). As expected, the interaction of CCT1 and COP1 also was unaffected by light (Figure 5A, samples 3 and 7), because CCT1 lacks the flavin binding domain. We examined protein levels after growth under these different conditions. No differences in CRY1 or CCT1 protein levels were detected for the yeast cells grown in darkness compared with those grown in blue light (Figure 5B).

Arabidopsis CRY1 Interacts with COP1 in Vitro

To further extend our findings, we performed protein interaction studies in vitro. Vectors were prepared expressing the GAL4 activation domain (GAD) fused to either the full-length or the C-terminal domain of Arabidopsis CRY1 (Figure 6A). These proteins, as well as the Arabidopsis and human COP1 proteins (Figure 6A), were synthesized as radioactively labeled polypeptides by in vitro transcription/translation (Figure 6B, lanes 1 to 4). The GAD–CRY fusion proteins were immunoprecipitated using monoclonal antibody prepared against GAD, and the ability of these proteins to bind the corresponding COP proteins was determined. Both Arabidopsis CCT1 and CRY1 were observed to bind to Arabidopsis COP1 but not to human COP1 (Figure 6B, lanes 5 to 8).

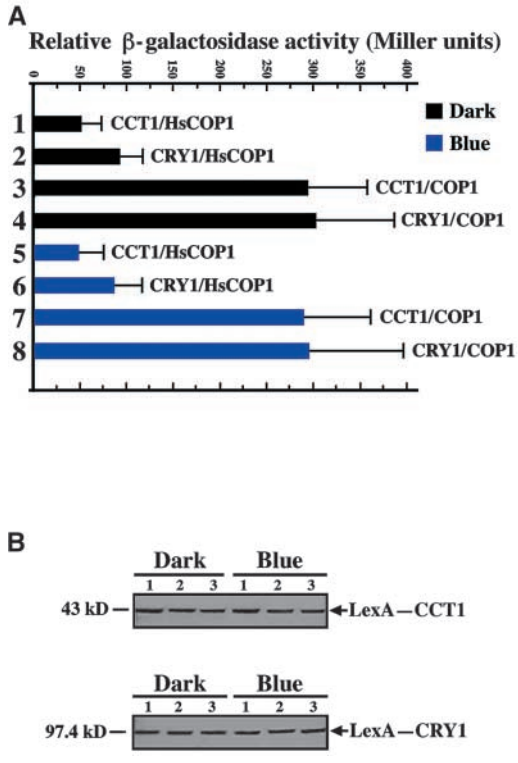

Figure 5. Arabidopsis CRY1 Binds to COP1 in Yeast in Both Blue Light and Dark.

(A) Yeast two-hybrid assays. Arabidopsis CRY1 and CCT1 interacted with COP1 in yeast cells when grown under blue light or in darkness. The activity observed for the human COP1 samples (HsCOP1) represents background levels.

(B) CRY1 and CCT1 protein levels in yeast are unaffected by blue light irradiation. Protein was detected with gel blots using LexA antibody.

Error bars indicate ±SE.

Arabidopsis CRY1 Is Bound Constitutively to COP1 in Arabidopsis Seedlings

To determine if CRY1 binds to COP1 in Arabidopsis, we conducted coimmunoprecipitation studies. Plant extracts were made from 6-day-old dark-grown and blue light–grown seedlings overexpressing the green fluorescent protein (GFP)–COP1 fusion protein. In parallel, control extracts were prepared from seedlings overexpressing GFP as well as from wild-type and *cry1* mutant seedlings. Both GFP and the GFP–COP1 fusion proteins were isolated using monoclonal GFP antibody, and these proteins then were detected on immunoblots (Figure 6C, lanes 1, 2, and 4). The ability of the COP1 protein to bind to CRY1 was determined by probing the same blot with antibody against CCT1. As shown in Figure 6D, CRY1 was bound to GFP–COP1 in both the dark- and light-grown Arabidopsis samples (lanes 2 and 4). No CRY1 protein was detected in the immunoprecipitate from the control plants (Figure 6D, lane 1), even though these plants expressed substantially higher levels of GFP (Figure 6C, lane 1) than plants expressing the corresponding GFP–COP1 protein (Figure 6C, lanes 2 and 4). The authenticity of the CRY1 band in these experiments (Figure 6D, lanes 2 and 4) was demonstrated by its presence in wild-type Arabidopsis extract (Figure 6D, lane 6) and its absence from a *cry1* mutant extract (Figure 6D, lane 5).

DISCUSSION

We demonstrated previously that the signaling mechanism of Arabidopsis cryptochromes is mediated through the C-terminal domain (Yang et al., 2000). On fusion with GUS, the CCT of both CRY1 and CRY2 mediates a constitutive light response. In this report, we further our understanding of the Arabidopsis cryptochrome signaling mechanism by demonstrating, in both yeast and Arabidopsis, a direct physical interaction between Arabidopsis CRY1 and COP1. The physiological significance of our findings is attested to by the demonstration that certain mutations, corresponding to loss-of-function alleles of *cry1* or *cop1*, affect this interaction negatively. We demonstrate in Arabidopsis and in yeast that CRY1 binds to COP1 in both dark- and light-grown samples. We propose from these observations that blue

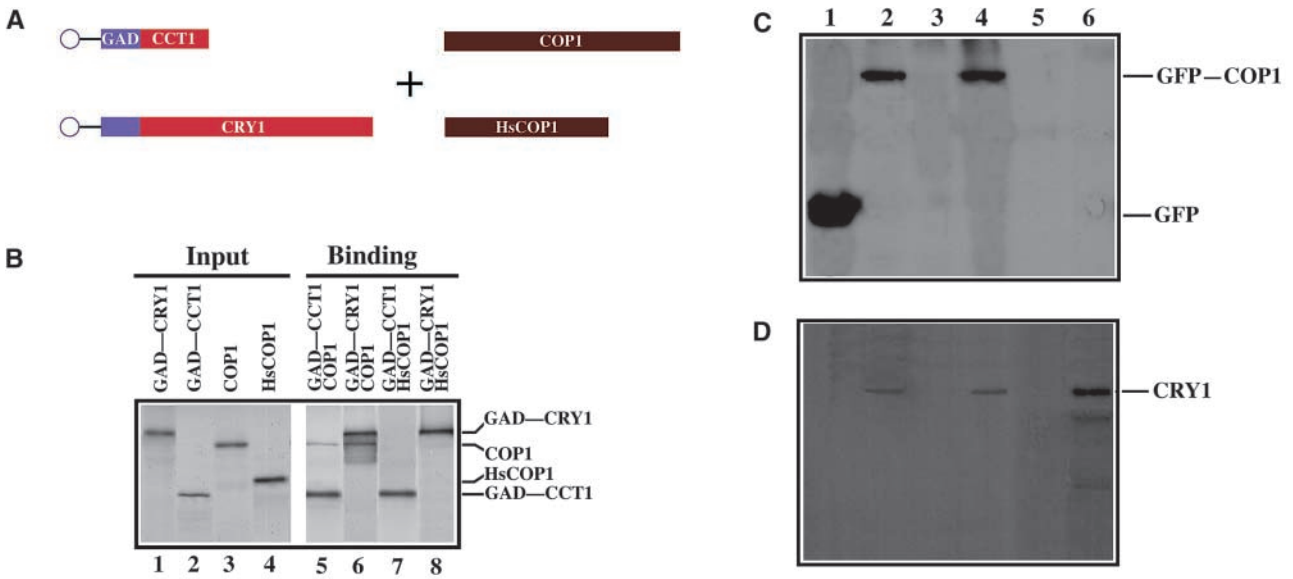

Figure 6. In Vitro Binding of Arabidopsis CRY1 to COP1 and Coimmunoprecipitation Study of CRY1 and COP1 in Arabidopsis.

(A) Bait and prey constructs. Full-length Arabidopsis CRY1 and CCT1 were prepared as fusion proteins with the GAL4 activation domain (GAD). The Arabidopsis COP1 and human COP1 (HsCOP1) proteins were prepared without the GAD fusion.

(B) Antibody pulldown and SDS-PAGE show binding of CRY1 and CCT1 to COP1. Proteins were synthesized by in vitro transcription/translation in the presence of ^{35}S-methionine. The GAD fusion proteins were immobilized by incubating with GAD monoclonal antibody and protein A–agarose beads (circles in **[A]**), and then the COP1 proteins were added and incubated. The beads were isolated, and bound proteins were identified by SDS-PAGE and autoradiography. Both Arabidopsis CRY1 and CCT1 bound to Arabidopsis COP1 but not to HsCOP1.

(C) Immunoblot showing GFP and GFP–COP1 fusion protein expression in transgenic Arabidopsis seedlings. Total protein extracts were prepared from 6-day-old dark- and blue light–grown Arabidopsis seedlings expressing GFP and GFP–COP1 fusion proteins. The extracts were incubated with GFP monoclonal antibody and protein A–agarose beads. The beads were isolated, and bound proteins were detected by immunoblot analysis using GFP antibody and the enhanced chemiluminescence kit (Amersham). Lane 1, sample from blue light–grown seedlings expressing GFP protein; lanes 2 and 4, samples from dark- and blue light–grown seedlings, respectively, expressing GFP–COP1 fusion protein; lane 3, sample from blue light–grown wild-type seedling; lanes 5 and 6, nonimmunoprecipitated samples from blue light–grown *cry1* mutant (*hy4-104*) and wild-type seedlings, respectively.

(D) CRY1 is bound constitutively to COP1 in Arabidopsis. The same blot shown in **(C)** was reprobed with CCT1 antibody. CRY1 protein was detected in samples prepared from both dark- and blue light–grown seedlings expressing GFP–COP1 fusion protein immobilized with GFP antibody (lanes 2 and 4). The CRY1 protein signal in the blot was confirmed by comparing nonimmunoprecipitated samples prepared from wild-type (lane 6) and *cry1* mutant (lane 5) seedlings.

light–dependent CRY1 signaling involves a modification of this CRY1–COP1 interaction that counteracts the repression of photomorphogenesis mediated by COP1 in the dark. These conclusions are consistent with earlier genetic studies demonstrating that the constitutive photomorphogenic phenotype of *cop1* mutants is epistatic to *cry1*/*hy4* mutants in both light and dark (Ang and Deng, 1994).

Arabidopsis CRY1 Signaling Mechanism Involves Physical Interaction between CRY1 and COP1

The properties of transgenic Arabidopsis plants expressing CCT1 (Yang et al., 2000), including the dwarf and fertility phenotypes described here, are remarkably similar to those of *cop1* mutants. Specifically, the following phenotypes described for CCT1 overexpression plants also are characteristic of *cop1* loss-of-function mutants. (1) Dark-grown CCT1 and *cop1* seedlings have shortened hypocotyls, fully opened and expanded cotyledons, and visible accumulation of anthocyanin (Deng et al., 1991; Deng and Quail, 1992; Yang et al., 2000). (2) Light-regulated gene expression and chloroplast development is enhanced in dark-grown CCT1 and *cop1* mutant seedlings (Deng et al., 1991, 1992; Deng and Quail, 1992; Yang et al., 2000). (3) The phenotypes of CCT1 plants and *cop1* mutants are epistatic to those of *phyA*, *phyB*, *hy1*, and *cry1* mutants (Ang and Deng, 1994; Yang et al., 2000). (4) The *cop1* mutants flower early in short-day light conditions (McNellis et al., 1994), as do CCT1 plants (Yang et al., 2000). (5) Plants expressing high levels of

CCT1 displayed a dwarf phenotype (this report) similar to that of select alleles of *cop1* (Deng and Quail, 1992; McNellis et al., 1994). (6) Both CCT1 and COP1 localize to the nucleus in the dark, whereas they both localize to the cytoplasm in the light (von Arnim and Deng, 1994; Yang et al., 2000).

The results presented here demonstrate that both Arabidopsis CCT1 and the full-length CRY1 protein interact strongly with COP1 in yeast cells and in vitro. Furthermore, we show that CRY1 is bound to COP1 in extracts from both dark- and light-grown Arabidopsis. Combining the earlier genetic epistasis analysis (Ang and Deng, 1994) and the transgenic data (Yang et al., 2000; this report) with these biochemical interaction data indicates that the Arabidopsis cryptochrome signaling mechanism involves physical interaction between CRY1 and COP1.

Mode of Action of CRY2

A surprising finding in this study is that neither the C-terminal domain (CCT2) nor full-length CRY2 interacted with COP1 in yeast. Although CCT1 and CCT2 share little sequence similarity, upon fusion with GUS both mediate a COP phenotype (Yang et al., 2000). Whereas the COP phenotype conferred by CCT2 is less severe than that conferred by CCT1, it seemed likely that both phenotypes resulted from the same molecular mechanism. How does this interpretation fare in light of our present findings concerning the lack of affinity in yeast cells of CCT2, in contrast to CCT1, for COP1?

The most conservative interpretation of these results is that CCT2 (and CRY2) does in fact function in a manner that is mechanistically similar to that of CCT1 (and CRY1). This interpretation is favored by evolutionary arguments and is in keeping with our earlier finding that CRY1–CRY2 fusion proteins are functional (Ahmad et al., 1998a). According to this line of thinking, CRY2 probably does function through interaction with COP1, and our inability to demonstrate such an interaction in yeast reflects the weaker nature of this interaction and/or the absence of additional factors that facilitate the reaction. Here it is interesting to recall our finding that mutant CCT1-22 is unable to bind to the COP1 WD40 domain (Figures 4C and 4E); this mutation (E559K), which corresponds to a loss-of-function *cry1* allele (Ahmad et al., 1995), resides within a small cluster of acidic amino acids that is conserved between the largely divergent C-terminal domains of CRY1 and CRY2 (Lin et al., 1998). One interpretation of these findings is that this acidic domain plays an important role in the binding of both CRY1 and CRY2 to COP1; for the reasons discussed, we were unable to detect the latter interaction. In keeping with these arguments, we have on occasions observed weak binding of CRY2 to COP1 in the in vitro binding assay (data not shown). Furthermore, in a recent publication, it was demonstrated that CRY2 does interact with COP1 in yeast (in contrast to our findings), and by coimmunoprecipitation these two proteins were shown to interact in Arabidopsis extracts (Wang et al., 2001). In the same study, CRY1 and COP1 were shown to colocalize in onion cells as nuclear speckles and cytoplasmic inclusion bodies.

Mammalian Cryptochromes Do Not Bind to COP1

One indication of the specificity of the reaction demonstrated here between Arabidopsis CRY1 and CCT1 with COP1 was the absence of any reaction between these cryptochrome sequences and human COP1. Of more interest, we failed to detect an interaction between the human COP1 sequence and either full-length mouse CRY1 or CRY2 or human CRY2 or the C-terminal domains of these proteins. These observations suggest that mammalian cryptochromes function in a manner distinct from their plant counterparts. Mammalian cryptochromes play an essential role in the circadian clock (Thresher et al., 1998; van der Horst et al., 1999), and this activity apparently involves binding with PER and negative regulation of CLOCK:BMAL1-mediated transcription (Kume et al., 1999). We have postulated, on the basis of sequence analysis, that plant and animal cryptochromes have markedly distinct evolutionary histories (Cashmore et al., 1999); the apparent disparate modes of action of these cryptochromes, in reference to the role of COP1, is in keeping with this proposal.

Phytochrome Signaling May Involve Direct Interaction between phyB and COP1

The short hypocotyl phenotype of both dark- and light-grown mutant *cop1* seedlings is epistatic to the long hypocotyl phenotype of *hy1*, *hy2*, and *phyB* mutants, indicating an absence of any requirement for phytochrome activity for these *cop1* phenotypes (Ang and Deng, 1994). Mutations in both *PHYA* and *PHYB* also affect the nuclear abundance of COP1 in far-red-light– and red light–grown seedlings, respectively, suggesting a positive regulatory role for phytochrome in the nuclear to cytoplasmic translocation of COP1 (Osterlund and Deng, 1998). Whereas these findings commonly have been argued to favor models in which COP1 lies directly on phytochrome signaling pathways (Osterlund et al., 1999), other models have not been excluded (Millar et al., 1994). The finding that the C-terminal domain of phyB interacts with COP1 suggests strongly that, like cryptochrome, phytochrome signaling proceeds in part through direct interaction with COP1. Although the interaction between the c-terminal domain of phyB and COP1 was not as strong as that observed for CRY1 or CCT1, the interaction was at least 20-fold greater than background, and the specificity of the reaction was demonstrated by the absence of an interaction with the human COP1 protein. Furthermore, like CCT1, the WD40 domain of COP1 is essential and suffi-

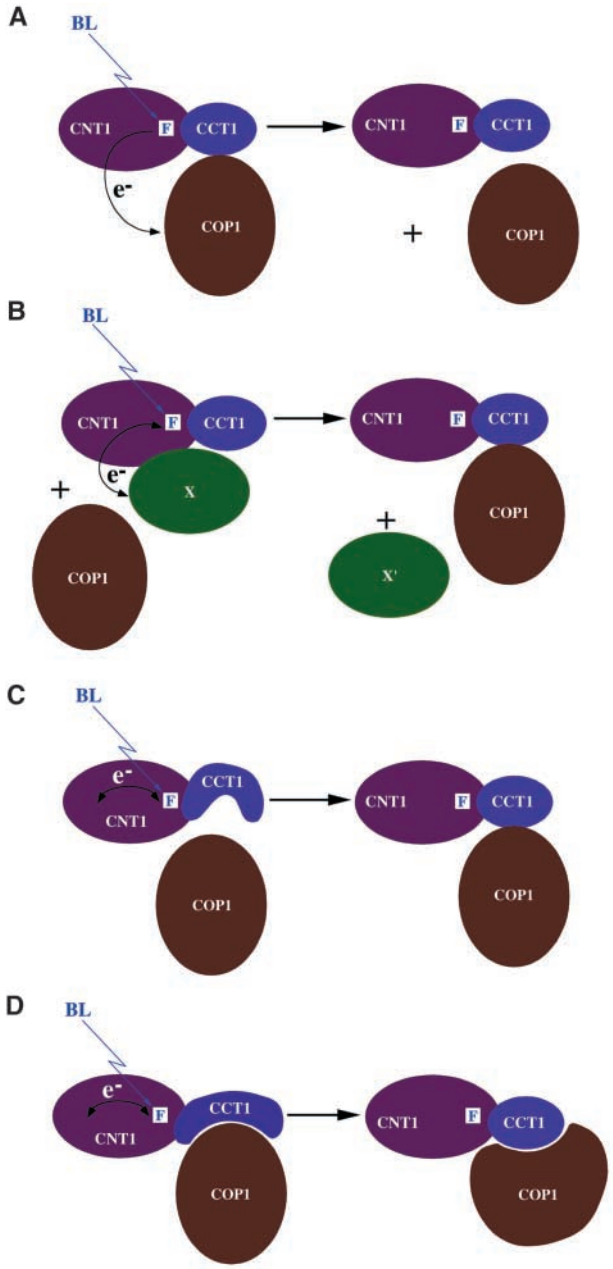

Figure 7. Alternative Models Describing the Interaction between CRY1 and COP1.

All models assume that the primary signaling event is a light-activated redox reaction.

(A) This model, based on the mode of action of photolyase, was discounted in earlier studies (see text and Yang et al. [2000]).

(B) This intermolecular redox model explains the light-independent response of CRY1 in yeast cells if it is postulated that yeast lacks the presumptive regulatory molecule X. However, this model does not explain the light-independent binding of CRY1 to COP1 observed in Arabidopsis.

(C) This model, involving an intramolecular redox reaction, is not favored, because this reaction would be expected to occur in yeast cells and confer a light response. Similarly, this model does not explain the light-independent binding of CRY1 to COP1 observed in Arabidopsis.

(D) In this model (the favored model), COP1 is bound to CRY1 constitutively. The light signal is transduced through an intramolecular redox reaction, resulting in a change in CCT1, which in turn alters the properties of bound COP1.

BL, blue light; F, flavin; e^-, electron.

cient for the interaction with the phyB C terminus in yeast (data not shown). It will be of interest to examine the binding of full-length phytochromes to COP1 and to study the dependence of any such interaction on light.

Of interest in regard to these findings is the demonstration that SPA1, a phyA-specific signaling intermediate, has been demonstrated to interact with COP1 (Hoecker and Quail, 2001). It is clear that COP1 plays an important intermediary role in many distinct signaling pathways; the pleiotropic phenotype of *cop1* mutants is in keeping with this conclusion (Deng and Quail, 1999).

A Model Describing the Interaction of CRY1 and COP1

In our earlier study, we discussed three models for the mechanism by which light may activate CRY1 (Yang et al., 2000). In one model (Figure 7A), the presumptive signaling partner (now identified as COP1) is bound to the C-terminal domain of CRY1 (CCT1), where it is activated through a light-dependent intermolecular redox reaction. This model, based on the mode of action of photolyase, was discounted because it did not explain the constitutive signaling properties of CCT1. In a second model (the intermolecular redox model), a regulatory molecule bound to CRY1 is displaced through a light-dependent intermolecular redox reaction, enabling CRY1 to interact with its signaling partner (Figure 7B). Although this model could be accommodated by our yeast studies, by postulating the absence of the presumptive regulatory component, it is not consistent with our studies demonstrating the constitutive binding of CRY1 to COP1 in Arabidopsis.

In an alternative model, we proposed a light-dependent intramolecular redox reaction resulting in a change in the structure of CRY1 that facilitates the binding of CRY1 to its signaling partner. This model (Figure 7C) does not explain the constitutive binding of CRY1 to COP1 we observed in Arabidopsis. Furthermore, if this intramolecular model were correct, we might have expected that light-dependent binding of CRY1 to COP1 would occur in yeast in a manner similar to the light-dependent binding of Drosophila CRY1 to

TIMELESS in yeast (Ceriani et al., 1999). However, we saw no effect of light on the binding of CRY1 to COP1 in yeast.

Our studies in Arabidopsis and in yeast demonstrate that CRY1 is bound to COP1 in the dark as well as in the light. Because genetic studies implicate both of these molecules in blue light signaling (Koornneef et al., 1980; Ahmad and Cashmore, 1993; Ang and Deng, 1994; Ahmad et al., 1995; Osterlund and Deng, 1998; Osterlund et al., 2000), we argue that light must induce a change in this CRY1–COP1 complex without dramatically affecting the affinity of these molecules for one another. We propose that CRY1 undergoes an intramolecular, light-dependent redox reaction that is transduced through a change in CCT1 (Figure 7D). This change in CCT1, which is mimicked in the CCT1 plants (Yang et al., 2000), somehow negatively affects the properties of COP1. This model could readily accommodate our findings that certain mutant CCT1 proteins, although deficient in mediating a COP phenotype in transgenic plants (Yang et al., 2000), bind COP1 in a manner that is not dramatically different from that of the wild-type CCT1 protein (Figure 4D, samples 2, 3, 5, 7, and 8). These latter findings indicate that although a capacity of CCT1 to bind COP1 may be a requirement for generating a COP phenotype, it is not sufficient.

In view of the findings and arguments just presented, we tentatively conclude that CRY1 is activated by light through a redox reaction, resulting in turn in a change in the properties of COP1. We propose 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 proteins such as HY5 (Ang et al., 1998). In this manner, HY5 is relieved from COP1 and COP9 signalosome-dependent proteolysis and is able to perform its role in photomorphogenesis (Osterlund et al., 2000).

METHODS

Construction of Vectors for the LexA Yeast Two-Hybrid System

Bait Constructs

The polymerase chain reaction (PCR)-amplified fragments of β-glucuronidase (GUS), *Arabidopsis thaliana* cryptochrome1 (CRY1) N-terminal domain (CNT1), C-terminal domain (CCT1), and full-length CRY1, GUS–CCT1, Arabidopsis CRY2 C-terminal domain (CCT2), GUS–CCT2, mouse CRY1 C-terminal domain (MCCT1) and CRY1 full-length (MCRY1), mouse CRY2 C-terminal domain (MCCT2), and human CRY2 C-terminal domain (HsCCT2) were cloned into EcoRI and XhoI sites of pLexA. The Arabidopsis phyA C-terminal domain and phyB C-terminal domain sequences were amplified by PCR and inserted into the BamHI and SalI sites of pLexA. Arabidopsis CRY2 full-length (CRY2), mouse CRY2 full-length (MCRY2), and human CRY2 full-length (HsCRY2) were amplified by PCR and integrated into the XhoI site of pLexA. The correct orientation was defined through appropriate enzyme digestion and DNA sequencing. The bait constructs containing the mutant Arabidopsis CCT1 fragments (Figure 4C) were made with the Stratagene In Vitro Mutagenesis Kit using pLexA–CCT1 (Figure 1A) as a template. All of the clones used were confirmed by DNA sequencing.

Prey Constructs

The PCR-amplified Arabidopsis MSL1 and HY5 cDNA sequences were cloned into EcoRI and XhoI sites of pJG4-5, respectively. The human constitute photomorphogenic1 (COP1) sequence (HsCOP1) encoding the C-terminal 466 amino acids was ligated into the XhoI site of pJG4-5. The COP1 cDNA fragments encoding the N-terminal 282 amino acids, the C-terminal 289 amino acids (from 387 to 675) containing the WD40 repeat domain, the C-terminal 466 amino acids (from 210 to 675) containing the WD40 repeat domain, and the internal 177 amino acids (from 210 to 386) were amplified separately and cloned into EcoRI and XhoI sites of pJG4-5. The fragment lacking the zinc finger ring domain was made by two rounds of PCR. First, the fragments encoding the N-terminal 45 amino acids with a SpeI site at the 3′ end and the C-terminal 582 amino acids with a SpeI site at the 5′ end were amplified separately by PCR, cleaved with SpeI, and then ligated. Second, the desired fragment was obtained by PCR using the ligation product as a template. Similar procedures were followed to generate the fragment lacking the coiled-coil region. The fragment deficient in both the zinc ring finger domain and the coiled-coil region was made through three rounds of PCR. First, the fragments encoding the N-terminal 45 amino acids with a SpeI site at the 3′ end and the 35 polypeptides (from 95 to 128) with a SpeI site at the 5′ end and a NcoI site at the 3′ end were amplified by PCR, cut with SpeI, and then ligated. Second, the fragment lacking the zinc finger ring domain was obtained using the ligation product as a template, digested with NcoI, and ligated to a NcoI-cut PCR product encoding the C-terminal 466–amino acid fragment with a NcoI site at the 5′ end. Finally, this ligation product was used as a template to obtain the desired fragment by PCR. All deletion fragments were cut with EcoRI and SalI and cloned into EcoRI and XhoI sites of pJG4-5. The prey vectors bearing WD40 mutants of Arabidopsis COP1 (Figure 4E) were made using pJG4-5–C210-675 (Figure 4A) as a template.

Yeast Two-Hybrid Assay

All combinations of prey and bait constructs and the reporter vector pSH18-34 were cotransformed into the yeast strain EGY48 according to the procedures described previously (Chen et al., 1992). The selection for transformants and the analysis of the relative β-galactosidase were essentially as described (McNellis et al., 1996). The expression of LexA and B42 transcriptional activation domain fusion proteins was detected by gel blot analysis using antibodies against LexA and hemagglutinin (Santa Cruz Biotechnology, Santa Cruz, CA). The calculation of relative β-galactosidase activities was as described by Ausubel et al. (1994). Ten clones for each combination of prey and bait constructs were taken randomly and analyzed to generate the data in Figures 1D, 1E, 3B, 4B, 4D, 4F, and 5A. The data for yeast cells coexpressing CCT1 and COP1 generated as shown in Figures 1D, 4B, and 5A were obtained from three different sets of 10 randomly taken independent clones. For all of the two-hybrid experiments described in Figures 1, 3, and 4, the yeast cells were grown under standard laboratory lighting conditions. In contrast, for the experiment described in Figure 5, the yeast cells were either grown in darkness or illuminated with blue light of 35 $\mu mol \cdot m^{-2} \cdot sec^{-1}$. Extracts for these latter samples were prepared under either red or blue light, respectively.

Transformation, Growth of Plants, and Protein Expression Studies

Wild-type plants in the Columbia background were transformed with the GUS-CCT1 transgene according to the procedure described previously (Yang et al., 2000). More than 240,000 sterile T1 seed, ~1000 wild-type seed, and ~1000 CCT1-19 seed carrying a mutant CCT1 transgene were spread on Murashige and Skoog (1962) plates without kanamycin, put to 4°C for 3 to 4 days, induced for germination in white light for 24 hr, and finally transferred to the dark for 6 days. All of the T1 seedlings showing a COP phenotype obtained from the screen, 80 wild-type seedlings and 80 CCT1-19 seedlings, were transferred to soil and grown at 22°C in constant light at a fluence rate of 300 $\mu mol \cdot m^{-2} \cdot sec^{-1}$. Total protein extracted from the leaves of CCT1 and wild-type plants was subjected to protein gel blot analysis according to the procedures described previously using the antibody against the Arabidopsis CRY1 C-terminal domain (Lin et al., 1996).

In Vitro Transcription/Translation and in Vitro Binding Assay

The chimeric GAL4 activation domain (GAD)–CCT1 and GAD–CRY1 fragments were amplified by PCR from the Gal4 yeast two-hybrid prey vector pACT2 carrying CCT1 and CRY1 sequences and ligated into XhoI and XbaI sites and SpeI and SacI sites, respectively, of pBluescript SK− (Stratagene, La Jolla, CA) under the control of the T7 and T3 promoters. The Arabidopsis COP1 sequence was cloned into KpnI and EcoRI sites of pBluescript SK− under the control of the T3 promoter, and the HsCOP1 sequence was integrated into the XhoI site of pBluescript SK− under the control of the T7 promoter. The GAD–CCT1 and GAD–CRY1 fusion proteins and COP1 and HsCOP1 proteins were synthesized and labeled with ^{35}S-methionine in the T'n'T In Vitro Transcription/Translation System (Promega). The preparation of bait agarose beads and the in vitro interaction assay were performed essentially as described (Ni et al., 1999; Jarillo et al., 2001).

Coimmunoprecipitation Studies

Total protein extracts were prepared from 6-day-old dark- and blue light–grown Arabidopsis seedlings expressing green fluorescent protein (GFP)–COP1 fusion protein. Monoclonal antibody against GFP (Clontech, Palo Alto, CA) was bound to agarose beads for the coimmunoprecipitation assays using standard procedures. Proteins were detected by immunoblot analysis, first using the GFP antibody and then by reprobing the blots with anti-CCT1 antibody.

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