Arabidopsis cryptochrome 2 (CRY2) functions by the photoactivation mechanism distinct from the tryptophan (trp) triad-dependent photoreduction

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Cryptochromes are blue-light receptors mediating various light responses in plants and animals. The photochemical mechanism of cryptochromes is not well understood. It has been proposed that photoactivation of cryptochromes involves the blue-light–dependent photoreduction of flavin adenine dinucleotide via the electron transport chain composed of three evolutionarily conserved tryptophan residues known as the "trp triad." We investigated this hypothesis by analyzing the photochemical and physiological activities of *Arabidopsis* cryptochrome 2 (CRY2) mutations altered in each of the three trp-triad residues. We found that all trp-triad mutations of CRY2 tested lost photoreduction activity in vitro but retained the physiological and biochemical activities in vivo. Some of the trp-triad mutations of CRY2 remained responsive to blue light; others, such as CRY2^{W374A}, became constitutively active. In contrast to wild-type CRY2, which undergoes blue-light–dependent interaction with the CRY2-signaling proteins SUPPRESSOR OF PHYA 1 (SPA1) and cryptochrome-interaction basic helix–loop–helix 1 (CIB1), the constitutively active CRY2^{W374A} interacts with SPA1 and CIB1 constitutively. These results support the hypothesis that cryptochromes mediate blue-light responses via a photochemistry distinct from trp-triad–dependent photoreduction and that the trp-triad residues are evolutionarily conserved in the photolyase/cryptochrome superfamily for reasons of structural integrity rather than for photochemistry per se.

photomorphogenesis | photoreceptor | redox

Cryptochromes are photolyase-like proteins that regulate photomorphogenic development in plants and the circadian clock in plants and animals (1–4). *Arabidopsis* cryptochrome 1 (CRY1) and cryptochrome 2 (CRY2) mediate light stimulation of de-etiolation and photoperiodic control of floral initiation (5–7). Members of the photolyase/cryptochrome superfamily contain three uniformly conserved tryptophan residues near the flavin adenine dinucleotide (FAD)-binding pocket of the photolyase-homologous region (PHR) domain, which are referred to as the "trp triad" (8–10). It has been proposed that the photoactivation of cryptochromes involves a trp-triad–dependent photoreduction (8, 11). According to this hypothesis, cryptochromes at the resting state contain oxidized FAD; upon photoactivation, FAD is reduced to semireduced flavin adenine dinucleotide (protonated) ($FADH^{\bullet}$) via an electron transport chain composed of the trp-triad residues; $FADH^{\bullet}$ is the signaling-active state that triggers subsequent conformational changes and signal transduction of the photoreceptor; $FADH^{\bullet}$ can be reduced further to fully reduced $FADH^{-}$ by green light or can be oxidized to FAD via dark conversion to complete the photocycle (9, 11–16). Most evidence supporting the photoreduction hypothesis came from biochemical and biophysical studies in vitro. However, it is known that photoreduction is not required for the enzymatic activity of *Escherichia coli* photolyase in vivo, although it is required for the photolyase activity in vitro (17, 18). It was reported that site-specific mutations of *Arabidopsis* CRY1 at the two trp-triad residues abolished the physiological activities of CRY1 in transgenic plants expressing the mutant proteins (12), representing the only genetic evidence thus far supporting the trp-triad photoreduction hypothesis. In contrast, a number of genetic studies have demonstrated that trp-triad–dependent photoreduction is not required for the physiological activities of animal type 1 cryptochromes that act as photoreceptors (10, 19–21). These discrepancies raise an intriguing question: Is there a fundamental difference in photochemistry between plant and animal cryptochromes?

To investigate this question, we examined the biochemical and physiological properties of *Arabidopsis* CRY2 mutations altered site specifically in each of the evolutionarily conserved trp-triad residues. Our results demonstrate that *Arabidopsis* CRY2 mediates blue-light responses via a photoactivation mechanism distinct from trp-triad–dependent photoreduction and that the trp-triad residues are evolutionarily conserved in CRY2 for reasons of structural integrity, such as the formation of the FAD-binding pocket, but not for photochemical requirements of those light-dependent enzymes and photoreceptors.

Results

Trp-Triad Residues Are Required for in Vitro Photoreduction of CRY2. A sequence alignment of *Arabidopsis* CRY2 with photolyases and cryptochromes from different organisms and a structural comparison of the computer-modeled structure of the PHR domain of CRY2 and the crystal structure of the PHR domain of CRY1 indicate that W321, W374, and W397 are the trp-triad residues of CRY2 (Fig. 1*A* and Fig. S1*A*). We replaced each of the three trp-triad residues in CRY2 by either alanine (W-to-A mutants) or phenylalanine (W-to-F mutants), using the site-directed mutagenesis method. Both W-to-A and W-to-F mutations of the trp-triad residues have been shown previously to abolish photoreduction of photolyases and cryptochromes (10–12, 15, 18, 19). We expressed the histidine-tagged CRY2 and CRY2-mutant proteins in insect Sf9 cells. The W-to-F CRY2-mutant proteins were expressed poorly, at levels less than 1/10 of those in wild-type CRY2 (Fig. S1*B*), so we were unable to purify sufficient amounts of the W-to-F mutant proteins for photospectrometry analysis (Fig. S1*B*). In addition, all three W-to-F CRY2-mutant proteins migrated significantly more slowly than the wild-type CRY2 in SDS/PAGE (Fig. S1*B*), suggesting that replacement of tryptophan with phenylalanine in any of the three trp-triad residues may alter the conformation of CRY2 in a similar way. On the other hand, all three

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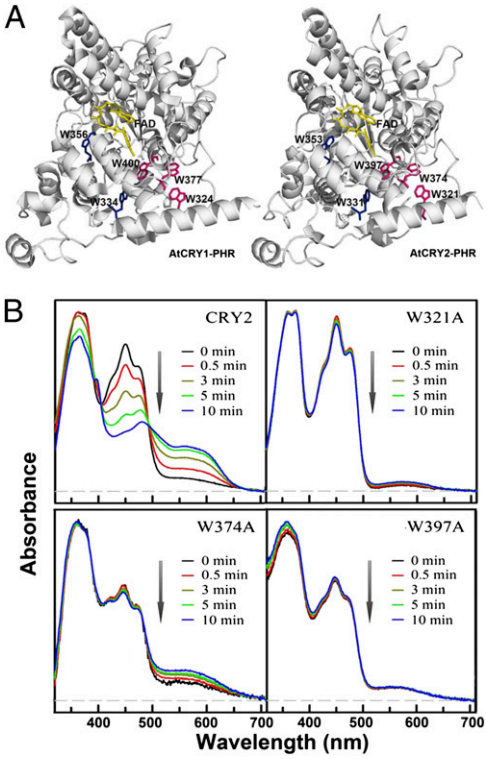

Fig. 1. Trp-triad residues and photoreduction of *Arabidopsis* CRY2. (*A*) A comparison of the crystal structure of the PHR domain of *Arabidopsis* CRY1 [Protein Data Bank (PDB) ID code 1U3C] (*Left*) and a structural model of the PHR domain of *Arabidopsis* CRY2 generated by Pymol (*Right*). FAD (yellow), the trp-triad tryptophan residues (pink), and two other conserved tryptophan residues (blue) of the two proteins are indicated. (*B*) Photoreduction of the wild-type CRY2 protein and the lack of photoreduction of the CRY2 trp-triad–mutant proteins. The absorption spectra of CRY2 and the CRY2 trp-triad mutants were recorded at indicated times after blue-light illumination (450 ± 15 nm, 2 mW·cm^{-2}) under aerobic conditions at 20 °C, in the presence of 10 mM β-mercaptoethanol as the external electron donor.

W-to-A CRY2-mutant proteins were expressed well in insect cells; they were purified with yields similar to those in wild-type CRY2, and they migrated normally in SDS/PAGE (Fig. S1*B*). We therefore determined the photoreduction activity of the W-to-A CRY2-mutant proteins. Fig. 1*B* shows that the wild-type CRY2 protein was photoreduced rapidly in vitro under aerobic conditions, as reported previously (Fig. 1*B*) (14). A mutation at any of the trp-triad residues of CRY2 effectively reduced or abolished the photoreduction activity (Fig. 1*B*). No photoreduction was detected in CRY2^{W321A} and CRY2^{W397A}, but residual photoreduction was detected in CRY2^{W374A} (Fig. 1*B*). We concluded that the trp triad is required for the in vitro photoreduction of *Arabidopsis* CRY2.

Trp-Triad–Dependent Photoreduction Is Not Required for Physiological Functions of CRY2 in *Arabidopsis*. *Arabidopsis* CRY2 mediates blue-light inhibition of hypocotyl elongation, blue-light stimulation of gene expression, and long-day (LD) promotion of floral initiation (6, 7). Because the GFP-CRY2 fusion protein behaved similarly to endogenous CRY2 in all the physiological and biochemical responses tested, and because its subcellular localization can be monitored easily (22), we tested all six trp-triad mutations of CRY2 as GFP-fusion proteins. We prepared transgenic *Arabidopsis* lines expressing each of the W-to-A and W-to-F trp-triad mutants of CRY2 under the control of the constitutive 35S promoter in the *cry1cry2* mutant background. For simplicity and clarity, the GFP-fusion CRY2-mutant proteins (GFP-CRY2^{W321A}, GFP-CRY2^{W321F}, GFP-CRY2^{W374A}, GFP-CRY2^{W374F}, GFP-CRY2^{W397A}, and GFP-CRY2^{W397F}) are referred to as CRY2^{W321A}, CRY2^{W321F}, CRY2^{W374A}, CRY2^{W374F}, CRY2^{W397A}, and CRY2^{W397F}, respectively, in the text, and they are labeled W321A, W321F, W374A, W374F, W397A, and W397F, respectively, in the figures of this article. All GFP-fusion proteins of the CRY2 mutants accumulated in the nucleus, as did the wild-type GFP-CRY2 control (Fig. S1*C*). The physiological activity of each CRY2 trp-triad–mutant protein was investigated in detail using one representative transgenic line (Figs. 2–4), but the phenotype was confirmed in multiple independent transgenic lines of the respective transgene (Fig. S2). A semiquantitative analysis of the transgenic lines analyzed in detail in this report (Fig. 2 and Figs. S2–S5) indicates that the expression levels of CRY2^{W321A}, CRY2^{W321F}, and CRY2^{W397F} were comparable to or slightly lower than that of the GFP-CRY2 control, whereas the expression levels of CRY2^{W397A}, CRY2^{W374A}, and CRY2^{W374F} were only about one-fourth that of the GFP-CRY2

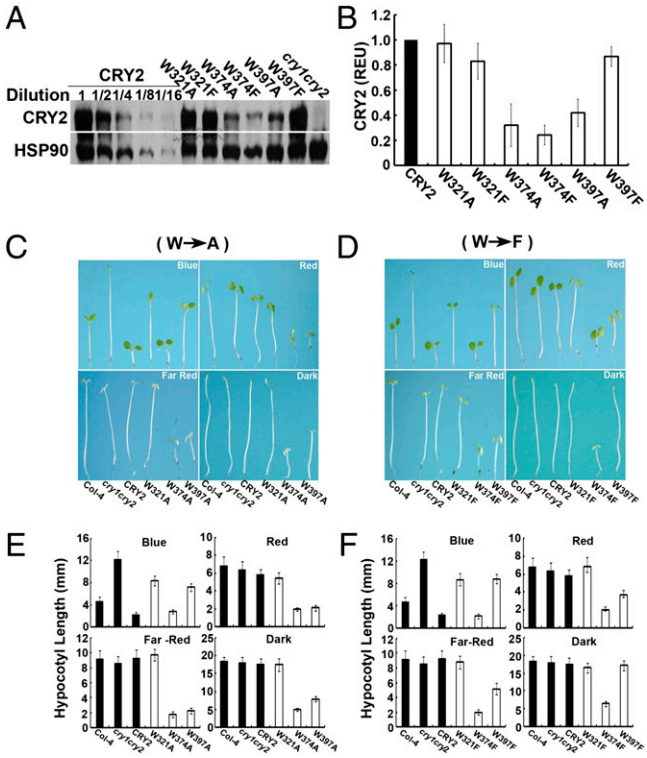

Fig. 2. CRY2 trp-triad mutants actively promote de-etiolation. (*A*) Immunoblot showing the level of protein expression of the GFP-fusion proteins of CRY2 and the trp-triad mutants of CRY2 from the respective transgenic lines. Seven-day-old seedlings of indicated genotypes were grown in Murashige and Skoog medium under continuous white light. Total protein extracts were fractioned by a 10% (wt/vol) SDS/PAGE gel and were blotted, and probed with anti-CRY2. Then the blot was stripped and reprobed with anti-heat shock protein 90 (HSP90; Santa Cruz Biotechnology) as the loading control. The serial dilutions of the GFP-CRY2 control were included to help compare the relative levels of protein expression. (*B*) The relative level of CRY2 expression represented by the relative expression unit (REU) is calculated by the formula [CRY2^{mt}/HSP90^{mt}]/ [CRY2^{wt}/HSP90^{wt}], in which "CRY2" and "HSP90" designate the digitized band intensity of CRY2 or HSP90 in the respective CRY2 mutants or in wild-type GFP-CRY2 as control. (*C* and *D*) Images of representative 7-d-old seedlings of indicated genotypes grown in compound soil under continuous blue light (20 μmol·m^{-2}·s^{-1}), red light (10 μmol·m^{-2}·s^{-1}), far-red light (3 μmol·m^{-2}·s^{-1}), or in darkness. All transgenic lines are in the *cry1cry2* background. (*E* and *F*) Hypocotyl lengths of indicated genotypes grown under conditions indicated in *C* and *D* were measured and are shown with SDs ($n \geq 20$).

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control (Fig. 2*B*); ranking by expression lever are [GFP-CRY2] ~ [CRY2^{W321A}] ~ [CRY2^{W321F}] ~ [CRY2^{W397F}] > [CRY2^{W397A}] ≥ [CRY2^{W374A}] ~ [CRY2^{W374F}].

The parental *cry1cry2* mutant is impaired in the de-etiolation responses, in that it showed a long hypocotyl and small/unopened cotyledons when grown in continuous blue light (Fig. 2 *C–F*). As shown in Fig. 2, all six CRY2-mutant proteins tested rescued the long hypocotyl phenotype of the parental line (Fig. 2 *C–F*), although none of the mutants tested showed significant photoreduction activity in vitro (Fig. 1*B*). In comparison with the parental *cry1cry2* seedlings, the seedlings expressing CRY2^{W321A} or CRY2^{W321F} exhibited shorter hypocotyls and larger expanded cotyledons when grown in blue light, although the activity of CRY2^{W321A} and CRY2^{W321F} was relatively weaker than the GFP-CRY2 control (Fig. 2 *C–F*). Importantly, the seedlings expressing these two CRY2-mutant proteins exhibited hypocotyl lengths similar to those of the wild type, the parental *cry1cry2*, or the control seedlings expressing GFP-CRY2 when grown in darkness, in red light, or in far-red (FR) light (Fig. 2 *C–F*), demonstrating that the activity of CRY2^{W321A} and CRY2^{W321F} is blue light-specific. On the other hand, seedlings expressing the other four trp-triad mutants of CRY2 (CRY2^{W374A}, CRY2^{W374F}, CRY2^{W397A}, and CRY2^{W397F}) exhibited shorter hypocotyls not only when grown in blue light but also when grown in darkness, in red light, and in FR light (Fig. 2 *C–F*), suggesting that those four CRY2-mutant proteins are constitutively active. Seedlings expressing CRY2^{W397A} and CRY2^{W397F} showed blue-light–independent activity, although CRY2^{W397A} appeared to exhibit relatively weaker activity in blue light, for unknown reasons (Fig. 2 and Fig. S2). Seedlings expressing CRY2^{W374A} and CRY2^{W374F} showed the strongest inhibition of hypocotyl growth in all light conditions tested (Fig. 2 *C–F*), although the relative levels of expression of CRY2^{W374A}- and CRY2^{W374F}-mutant proteins were lower than other mutants tested (Fig. 2*B*). This result indicates that CRY2^{W374A} and CRY2^{W374F} have the highest specific activity among the seven CRY2 proteins tested, including the GFP-CRY2 control. To examine further the activity of these CRY2-mutant proteins, we analyzed the hypocotyl growth response under different fluence rates of blue light (Fig. S3). The result of this experiment confirmed that CRY2^{W321A} and CRY2^{W321F} possess blue-light–specific and fluence rate-dependent activity suppressing hypocotyls elongation (Fig. S3). Interestingly, although the four CRY2 mutations affecting residues W374 and W397 clearly showed constitutive activity (Fig. 2 *C–F*), they still are responsive to the change in fluence rates of blue light (Fig. S3). The hypocotyl inhibition activity of the CRY2^{W374A}, CRY2^{W374F}, and CRY2^{W397A} mutants are responsive to light at both lower and high fluence rates, whereas the activity of CRY2^{W397F} appears more responsive to blue light at higher fluence rates (Fig. S3).

In addition to mediating blue-light inhibition of hypocotyl elongation, cryptochromes are well known for mediating blue-light induction of chalcone synthase (*CHS*) and chlorophyll A/B-binding protein (*CAB*) mRNA expression (23). As expected, the mRNA expression of the *CAB* and *CHS* genes showed at least 15- to 20-fold induction in etiolated wild-type seedlings exposed to blue light but not in the *cry1cry2* mutant (Fig. S4). Similar to observations for hypocotyl growth response, all the CRY2 trp-triad mutants tested were active in mediating blue-light stimulation of mRNA expression of *CAB* and *CHS*, although their blue-light responsiveness varied (Fig. S4). Moreover, the blue-light responsiveness of the trp-triad mutants in *CHS* and *CAB* gene expression appeared similar to that shown in the hypocotyl response: Transgenic expression of CRY2^{W321A} and CRY2^{W321F} resulted in blue-light–dependent induction of the mRNA expression of both *CAB* and *CHS* genes, whereas transgenic expression of the other four trp-triad mutations affecting the residues W374 and W397 resulted in activation of *CAB* and *CHS* mRNA expression in a blue-light–independent manner (Fig. S4).

The *cry1cry2*-mutant parent flowered markedly later in the LD photoperiod but not in the short-day (SD) photoperiod (Fig. 3) (7). Transgenic expression of GFP-CRY2 fully rescued the late-flowering phenotype of the *cry1cry2* mutant grown in LD photoperiods but had little effect on the flowering time of plants grown in SD photoperiods (Fig. 3) (24). Transgenic expression of all CRY2 trp-triad mutants rescued the late-flowering phenotype of the parental *cry1cry2* mutant grown in LD photoperiods (Fig. 3) and expression of the flowering locus T (*FT*) gene (Fig. S5), demonstrating their activity in promoting floral initiation. Analogous to their activity in de-etiolation, the trp-triad mutants showed either photoperiod-dependent or constitutive activity in the promotion of floral initiation. The two CRY2 mutations affecting the residue W321 showed photoperiod-dependent regulation of flowering time (Fig. 3): The CRY2^{W321A}/*cry1cry2* and CRY2^{W321F}/*cry1cry2* plants flowered markedly earlier than the *cry1cry2* parent in the LD photoperiods but not in the SD photoperiods (Fig. 3). The other four trp-triad mutations affecting residues W374 and W397 demonstrated "photoperiod-independent" activity promoting floral initiation: The CRY2^{W374A}/*cry1cry2*, CRY2^{W374F}/*cry1cry2*, CRY2^{W397A}/*cry1cry2*, and CRY2^{W397F}/*cry1cry2* lines exhibited accelerated flowering in both LD and SD photoperiods in comparison with the *cry1cry2* parent (Fig. 3). Consistent with their accelerated flowering time in SD photoperiods, the transgenic plants expressing the CRY2^{W374A}, CRY2^{W374F}, CRY2^{W397A}, or CRY2^{W397F} mutants also showed elevated mRNA expression of the *FT* gene (Fig. S5).

In summary, all six trp-triad CRY2 mutations tested are capable of promoting de-etiolation responses, activating gene-expression changes, and stimulating floral initiation. The CRY2-mutant proteins containing mutations at residue W321 showed little photoreduction and retained blue-light–dependent physio-

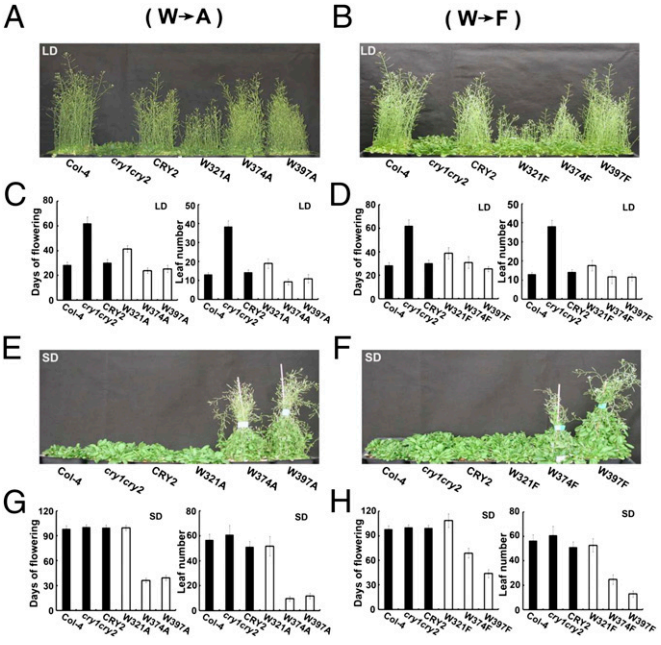

Fig. 3. CRY2 trp-triad mutants actively stimulate floral initiation. An analysis of flowering time of the respective genotypes grown in LD (*A–D*) or SD (*E–H*) conditions. Images of 50-d-old (*A–D*) or 80-d-old (*E–H*) plants of the indicated genotypes grown in LD (16 h light/8 h darkness) or SD (9 h light/15 h darkness) are shown in *A*, *B*, *E*, and *F*. The flowering time, measured as days to flowering, and leaf number at the time of flowering (± SD) are shown in *C*, *D*, *G*, and *H* (≥20).

logical activities, albeit at reduced levels. Mutations at CRY2 residues W374 and W397 reduced or eliminated photoreduction, but the mutant proteins exhibited robust but light-independent physiological activities in all three physiological responses tested. The differences in light responsiveness of the CRY2 mutations affecting the trp-triad residues W321, W374, and W397 are not caused by the level of transgene expression, because there is no correlation between the relative levels of protein expression and the relative strengths of physiological activities tested in the respective CRY2trp-triad mutants (Figs. 2 and 3). We concluded that the trp-triad–dependent photoreduction of CRY2 is not required for its physiological activities in plants.

Trp-Triad–Dependent Photoreduction Is Not Required for Blue-light–Dependent Proteolysis of CRY2. *Arabidopsis* CRY2 undergoes blue-light–dependent ubiquitination and degradation in the nucleus (22). It has been reported previously that green light suppresses blue-light–dependent CRY2 degradation, a finding that was interpreted as an evidence that photoreduction is critical to the photochemistry of CRY2 (11). However, we found that green light and red light had similar effects on the blue-light–induced CRY2 degradation (Fig. S6), raising doubt about the previous interpretation. To investigate this question further, we examined the blue-light–induced degradation of the CRY2 trp-triad–mutant proteins (Fig. 4). We found that all the CRY2 trp- triad–mutant proteins were degraded in response to blue light, although most of the CRY2-mutant proteins showed a reduced velocity of degradation in comparison with the GFP-CRY2 control (Fig. 4). The velocities of degradation of different CRY2 mutants showed no correlation between the W321A or W397A mutants that exhibited little photoreduction in vitro and the W374A mutant that retained residual photoreduction in vitro (Fig. 1) or between the light-responsive W321 mutants and the constitutively active W374 and W397 mutants (Figs. 2–4). These results suggest that it is more likely that the decreased velocity of degradation of the CRY2 mutants is caused by structural alternation than by the lack of photoreduction per se. Furthermore, the lack of correlation between the physiological activity and the rate of degradation in response to blue light suggests that different structural elements of CRY2 are responsible for the blue-light–dependent physiological activity and the blue-light–dependent ubiquitination/proteolysis of CRY2. We concluded that, similar to the animal type 1 cryptochromes reported previously (19), trp-triad–dependent photoreduction is not required for the blue-light–dependent degradation of *Arabidopsis* CRY2.

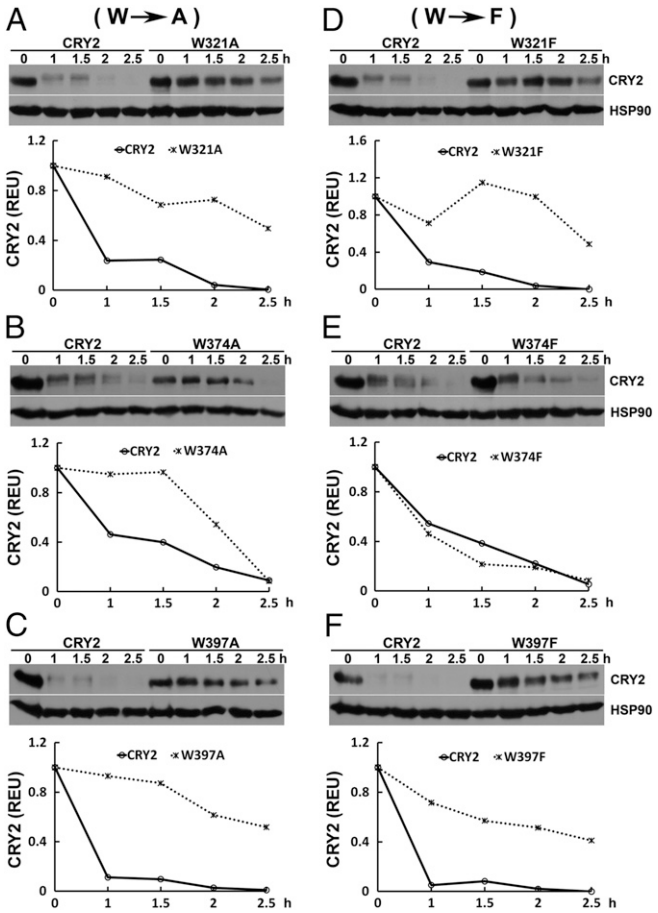

Fig. 4. Blue-light–induced proteolysis of the CRY2 trp-triad mutants. Immunoblots showing the level of CRY2 and trp-triad CRY2 mutants in the indicated transgenic lines are quantified and the results are shown underneath the respective immunoblot. Seedlings were grown in continuous red light for 7 d and were transferred to blue light (76 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for the indicated time (h). The total protein extracts were analyzed in 10% (wt/vol) SDS/PAGE for immunoblot analyses, which were probed with anti-CRY2 (CRY2), stripped, and reprobed with anti-HSP90 (HSP90). The relative levels of CRY2 expression (REU) is calculated by the formula $[CRY2^t/HSP90^t]/[CRY2^0/HSP90^0]$, in which "CRY2" and "HSP90" denote digitized band intensities of CRY2 or HSP90 in the respective samples collected at time 0 or at the indicated time (t) after blue-light exposure).

Why Is a Trp-Triad CRY2 Mutant Constitutively Active? Contrary to what would be expected from the trp-triad–dependent photoreduction hypothesis, we found that the four CRY2 trp-triad mutants associated with the residues W374 and W397 are constitutively active, to various extents. The transgenic plants expressing these CRY2 mutants showed constitutive photomorphogenic phenotypes in de-etiolation, gene expression, and floral initiation (Figs. 2 and 3 and Fig. S4), resembling phenotypes of constitutive photomorphogenic (*cop*) mutants (25). Among the four constitutively active CRY2 mutants, $CRY2^{W374A}$ is particularly interesting because it showed the highest specific activities in all the responses tested (Figs. 2 and 3). It had been reported previously that transgenic expression of various fusion proteins or truncated cryptochromes or cryptochrome mutants, including β-glucuronidase (GUS)-CCT1, GUS-CCT2, GUS-NC80, and CRY2-GFP (22, 26, 27) and a point mutation, $CRY1^{G380R}$ (28), caused constitutive photomorphogenic phenotypes in plants. It was proposed that these cryptochrome fusion proteins or mutants possess conformation similar to that of a photoactivated photoreceptor to become constitutively active regardless of the presence of blue light (26–28). It is conceivable that the mutations at the W374 and W397 trp-triad residues of CRY2 also may cause conformational changes so that CRY2 becomes constitutively active.

To test this hypothesis, we investigated whether a mutation in the trp-triad residue may change its interaction with the CRY2-signaling proteins. We and others have shown recently that photoexcited CRY2 undergoes blue-light–dependent interaction with the basic/helix-loop-helix (bHLH) transcription factor cryptochrome-interaction bHLH 1 (*CIB1*) and the WD-repeat protein SUPPRESSOR OF PHYA 1 (SPA1) to trigger light-dependent changes of gene expression and plant development (29–32). Because the blue-light dependency of the CRY2–CIB1 or CRY2–SPA1 interaction most likely results from the conformational changes of the photoactivated CRY2, one might expect that a constitutively active CRY2 mutant, such as $CRY2^{W374A}$, could interact constitutively with one or both CRY2-signaling proteins. We first compared the physical interactions of CRY2 and $CRY2^{W374A}$ with CIB1 and SPA1 by the yeast two-hybrid assay (Fig. 5 *A* and *B*). As reported previously, CRY2–SPA1 interaction was detected only in yeast cells illuminated with blue light but not in cells illuminated with red light or grown in darkness (Fig. 5*A*). In contrast, $CRY2^{W374A}$ interacted constitutively

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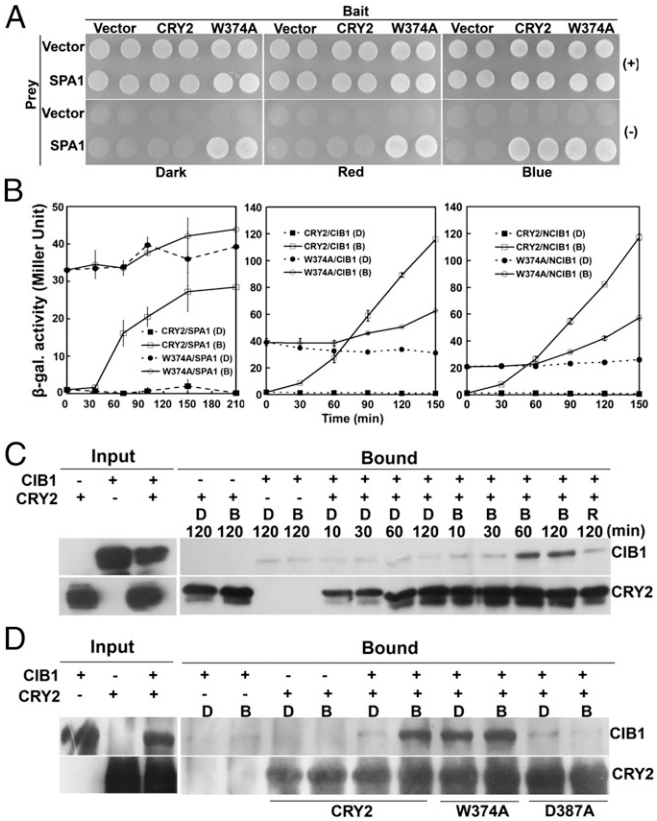

Fig. 5. Constitutively active CRY2^{W374A} mutant interacts constitutively with the CRY2-signaling proteins CIB1 and SPA1. (*A*) The histidine auxotrophy assay showing blue-light–dependent interaction between CRY2 and SPA1 and the blue-light–independent interaction between CRY2^{W374A} (W374A) and SPA1. Yeast cells containing plasmids encoding the indicated proteins were grown on medium in the presence (+) or absence (–) of histidine and adenine under blue light (Blue; 25 μmol·m^{-2}·s^{-1}), red light (Red; 25 μmol·m^{-2}·s^{-1}), or in darkness (Dark) for 3 d. (*B*) The β-galactosidase assay showing the blue-light–dependent interactions of CRY2–SPA1, CRY2–CIB1, and CRY2–NCIB1 in contrast to the blue-light–independent interactions of CRY2^{W374A} (W374A)–SPA1, CRY2^{W374A} (W374A)–CIB1, and CRY2^{W374A} (W374A)–NCIB1, Yeast cells were illuminated with blue light (B; 25 μmol·m^{-2}·s^{-1}) or kept in darkness (D) for the indicated time. (*C*) The in vitro pull-down assay showing blue-light–dependent formation of the CRY2–CIB1 complex. The anti-CRY2 IgG coupled to protein A agarose beads was mixed with the lysate of insect cells expressing CRY2 and with the lysate of *E. coli* cells expressing CIB1; the mixture was incubated for the indicated time under blue light (B), red light (R), or in darkness (D). The bound proteins were eluted after washing and were analyzed by immunoblot probed with anti-CRY2 (CRY2) or anti-CIB1 (CIB1). (*D*) The In vitro pull-down assay showing the blue-light–dependent interaction between CRY2 and CIB1, the constitutive interaction between CRY2^{W374A} (W374A) and CIB1, and the lack of interaction between the FAD-deficient mutant CRY2^{D387A} (D387A) and CIB1. The assays were carried out as described in *C*, except that the lysates were precleaned by mixing with 20 μL protein A/G agarose suspension before the addition of the anti-CRY2/IgG–coupled protein A/G agarose beads. The reactions were exposed to blue light for 60 min (*B*) or were left in darkness (*D*).

with SPA1 in yeast cells grown in darkness, in red light, or in blue light (Fig. 5 *A* and *B*). Similarly, in yeast cells CRY2 interacted with CIB1 or with the N-terminal domain of CIB1 (NCIB1) only in response to blue light, whereas CRY2^{W374A} interacted with CIB1 and NCIB1 constitutively (Fig. 5*B*).

We then examined the effect of blue light on the CRY2–CIB1 interaction using a newly established in vitro blue-light–dependent pull-down assay (Fig. 5*C* and Fig. S7). The CRY2–CIB1 complex was detected only in blue light, not in darkness or in red light (Fig. 5*C* and Fig. S7*A*), demonstrating a blue-light–specific interaction between CRY2 and CIB1 in vitro. The blue-light-dependent CRY2–CIB1 interaction has been previously shown in vivo (29). The results presented here (Fig. 5 and Fig. S7) demonstrate that the wavelength-specific CRY2–CIB1 interaction can also be observed in vitro. The formation of the CRY2–CIB1 complex was detectable 30 min after blue-light illumination, and it reached a saturated level of interaction within 60 min of light exposure (Fig. 5*C*). The rate of CRY2–CIB1 complex formation is slightly slower than that observed in plant cells, probably because of the low temperature (~4 °C) used in the in vitro experiment. Interestingly, green light did not suppress the blue-light–dependent CRY2–CIB1 interaction (Fig. S7*B*), supporting the view that photoreduction is not required for the CRY2 photoactivation. We next performed a similar test for the trp-triad mutant CRY2^{W374A} (Fig. 5*D*). In contrast to CRY2, which interacted with CIB1 specifically in the presence of blue light, CRY2^{W374A} interacted with CIB1 constitutively regardless of blue light, whereas the FAD-deficient mutant CRY2 (D378A) (29) showed no interaction with CIB1 regardless of blue light treatment (Fig. 5*D*). We concluded that the CRY2^{W374A} mutant interacts constitutively with the CRY2-signaling proteins SPA1 and CIB1. These results argue strongly that mutations at CRY2^{W374A}, and probably other trp-triad mutants of CRY2, cause conformational changes resembling that of the photoactivated CRY2, so that they act constitutively in plant cells.

Discussion

In the present study, we showed that trp-triad–dependent photoreduction is not required for the function of *Arabidopsis* CRY2 *in planta*. The three trp-triad residues are required for the CRY2 photoreduction in vitro (Fig. 1), but none of the physiological and biochemical activities tested, including de-etiolation responses in seedlings, floral initiation in adult plants, blue-light–specific CRY2 degradation, and protein–protein interactions, was eliminated in any of the trp-triad mutations as predicted by the photoreduction hypothesis (Figs. 2–5). To the contrary, several CRY2 trp-triad mutants are constitutively active (or more active) in all physiological responses examined. A comparison of two CRY2 trp-triad mutants, CRY2^{W321A}, and CRY2^{W374A}, revealed a particularly striking contrast to what would be predicted by the photoreduction hypothesis. The CRY2^{W321A} mutant showed no detectable photoreduction in vitro (Fig. 1*B*), but it remained physiologically active in a blue-light–dependent manner (Figs. 2 and 3). The CRY2^{W374A} mutant retained a residual photoreduction activity in vitro (Fig. 1*B*) but was constitutively active (Figs. 2 and 3). In other words, elimination of trp-triad–dependent photoreduction of the CRY2^{W321A} mutant failed to abolish the photoreceptor activity in response to blue light, but reduced photoreduction of the CRY2^{W374A} mutant appears to make it constitutively active. Similar results were observed, although to a lesser extent, for three other CRY2 trp-triad mutants tested (CRY2^{W374F}, CRY2^{W397A}, and CRY2^{W397F}) (Figs. 2 and 3). These results demonstrated unequivocally that trp-triad–dependent photoreduction is not required for the photoactivation of a plant cryptochrome. Taken together, our results argue strongly that, similar to bacterial photolyases and animal cryptochromes, plant cryptochromes mediate light responses via a photoactivation mechanism distinct from trp-triad–dependent photoreduction.

If the trp-triad residues are not needed for photoactivation of photolyases and cryptochromes, why are they uniformly conserved in those photoactive proteins throughout evolution? Based on the following observations, we propose that these tryptophan residues are evolutionarily conserved for structural reasons. First, although trp-triad residues are structurally conserved in all members of the photolyase/cryptochrome superfamily examined, they are not photobiologically required for most proteins studied so far, including the animal type 1 cryptochromes, animal type 2 cryptochromes, and photolyases (18–20, 33). Sec-

ond, trp-triad residues are not the only tryptophan residues conserved in the photolyase/cryptochrome superfamily. Approximately one-fourth of the amino acid residues of the region encompassing the trp triad, including two additional tryptophan residues, are conserved uniformly in photolyases and cryptochromes (Fig. S1). This conservation indicates that the three trp-triad residues are not unique from a sequence-conservation point of view. Moreover, the trp-triad residues are located close to the FAD-binding pocket, which is one of the most conserved regions of the photolyase/cryptochrome superfamily (Fig. 1*A*) (34). Transgenic expression of four CRY2 trp-triad mutants, such as $CRY2^{W374A}$, caused constitutive photomorphogenic phenotypes similar to that caused by GUS-CCT and CRY2-GFP fusion proteins that contain no trp-triad mutations (22, 26). The simplest explanation for the similar activity found in the cryptochrome-fusion proteins and the CRY2 trp-triad mutants would be that they possess similar structure alterations necessary to exert the similar physiological activities in plants. Our observation that the $CRY2^{W374A}$-mutant protein interacts constitutively with the CRY2-signaling proteins SPA1 and CIB1 (Fig. 5) provides a strong argument for this proposition and a biochemical explanation of why a CRY2 trp-triad mutant, such as W374A, is physiologically active in the absence of light. Taken together, our results support a hypothesis that the trp-triad residues are conserved evolutionarily because they are important for maintaining the native structure of cryptochromes.

Materials and Methods

All *Arabidopsis* lines used in this report were derived from the Col accession. Transgenic plants expressing the GFP-fusion protein of the CRY2 trp-triad mutants were prepared in the *cry1cry2*-mutant background, as was GFP-CRY2, described previously (24). Briefly, single-amino acid substitutions of tryptophan to alanine or phenylalanine were introduced into the CRY2 coding region at positions 321, 374, and 397, using the QuikChange Site-Directed Mutagenesis system according to the manufacturer's instructions (Stratagene). After sequence verification, mutated CRY2 cDNAs were fused to the C terminus of GFP as described previously (22, 35). The transgenes are under the control of the cauliflower mosaic virus 35S promoter. Preparation and phenotypic analyses of transgenic plants, including genetics analysis, hypocotyl inhibition response, quantitative PCR, and immunoblots were performed as previously described (22, 24). To measure flowering time, plants were grown in a temperature-controlled growth chamber (Conviron E7/2) in LD (16 h light/8 h darkness) or SD (9 h light/15 h darkness) at 21 °C until floral buds became visible. Other methods and materials used in this report can be found in *SI Materials and Methods*.

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