Trp triad-dependent rapid photoreduction is not required for the function of Arabidopsis CRY1

Jie Gao\textsuperscript{a,b,1}, Xu Wang\textsuperscript{b,c,1}, Meng Zhang\textsuperscript{d,e,1}, Mingdi Bian\textsuperscript{a,1}, Weixian Deng\textsuperscript{a}, Zecheng Zuo\textsuperscript{a}, Zhenming Yang\textsuperscript{b,2}, Dongping Zhong\textsuperscript{d,e,2}, and Chentao Lin\textsuperscript{b,2}

\textsuperscript{a}Laboratory of Soil and Plant Molecular Genetics, College of Plant Science, Jilin University, Changchun 130062, China; \textsuperscript{b}Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, CA 90095; \textsuperscript{c}Basic Forestry and Proteomics Research Center, Fujian Agriculture and Forestry University, Fuzhou 350002, China; \textsuperscript{d}Department of Physics, Ohio State University, Columbus, OH 43210; \textsuperscript{e}Department of Chemistry and Biochemistry, Ohio State University, Columbus, OH 43210

Edited by Aziz Sancar, University of North Carolina at Chapel Hill, Chapel Hill, NC, and approved May 27, 2015 (received for review March 3, 2015)

Cryptochromes in different evolutionary lineages act as either photoreceptors or light-independent transcription repressors. The flavin cofactor of both types of cryptochromes can be photoreduced in vitro by electron transportation via three evolutionarily conserved tryptophan residues known as the “Trp triad.” It was hypothesized that Trp triad-dependent photoreduction leads directly to photoexcitation of cryptochrome photoreceptors. We tested this hypothesis by analyzing mutations of Arabidopsis cryptochrome 1 (CRY1) altered in each of the three Trp-triad tryptophan residues (W324, W377, and W400). Surprisingly, in contrast to a previous report all photoreduction-deficient Trp-triad mutations of CRY1 remained physiologically and biochemically active in Arabidopsis plants. ATP did not enhance rapid photoreduction of the wild-type CRY1, nor did it rescue the defective photoreduction of the CRY1\textsuperscript{W324A} and CRY1\textsuperscript{W400F} mutants that are photophysiological activities of plant cryptochromes argue that the Trp triad-dependent photoreduction is not required for the function of cryptochromes and that further efforts are needed to elucidate the photoexcitation mechanism of cryptochrome photoreceptors.

Significance

The Trp triad-dependent photoreduction of the flavin chromophore has been widely accepted as the photoexcitation mechanism of cryptochrome photoreceptors. However, the experimental evidence supporting this hypothesis derived primarily from the biophysical studies in vitro, except for one genetics study of Arabidopsis cryptochrome 1 (CRY1). In contrast to the previous report, we found that all Trp-triad mutations of Arabidopsis CRY1 remained physiologically active in plants, and this result cannot be readily explained by the ATP-dependent enhancement of Trp triad-dependent photoreduction. Our results challenge the widely accepted Trp triad hypothesis and call for further investigation of alternative electron transport mechanisms to explain cryptochrome photoexcitation.

Author contributions: D.Z. and C.L. designed research; J.G., X.W., M.Z., and M.B. contributed equally to this work; W.D. and Z.Z. performed research; Z.Y. contributed new reagents/analytic tools; D.Z. analyzed data; and C.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

See Commentary on page 8811.

\textsuperscript{1}J.G., X.W., M.Z., and M.B. contributed equally to this work.

\textsuperscript{2}To whom correspondence may be addressed. Email: zmyang@jlu.edu.cn, zhong.28@osu.edu, or clin@mcdb.ucla.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1504404112/-/DCSupplemental.

How chemical or physical properties of proteins observed in vitro are associated with their functions in vivo is often a challenging question in biology. For example, free flavins and flavin cofactors of flavoproteins commonly undergo photoreduction in vitro, whereby oxidized flavins are converted to a reduced form upon exposure to light, although few of those “photoreducible” flavoproteins, such as D-amino acid oxidase and glucose oxidase, possess light-dependent functions in vivo (1, 2). Cryptochromes are photolyase-like flavoproteins that act as photoreceptors regulating light responses in plants and Drosophila or as light-independent transcription repressors of the circadian clock in mammals (3–6). Like other flavoproteins, cryptochromes can be photoreduced in vitro regardless of their photobiological activity in vivo (7–10). There are five evolutionarily conserved tryptophan residues within a region associated with the flavin adenine dinucleotide (FAD)-binding pocket of the photolyase-homologous region (PHR) domain of cryptochromes (Fig. 1A). Three of those five tryptophan residues, referred to as the “Trp triad,” are required for the in vivo photoreduction of cryptochromes (8, 11–13). Photoreduction of cryptochromes has been studied extensively and is widely accepted as the photoexcitation mechanism of cryptochrome photoreceptors (6).

Most evidence supporting the photoreduction hypothesis came from in vitro biophysical studies (6, 14). The only genetics study reported so far in support of the hypothesis that Trp triad-dependent photoreduction is required for the physiological activities of cryptochrome photoreceptors came from a study of Arabidopsis cryptochrome 1 (CRY1) (15). It was reported that mutations in two of the three Trp-triad residues of Arabidopsis CRY1—W324 and W400—abolished or dramatically reduced the photophysiological activities of CRY1 in transgenic plants (15). In contrast, all other genetics studies reported to date showed that mutations at the Trp-triad residues of the photolyase/cryptochrome proteins do not significantly affect the in vivo activity of the respective mutants. For example, Trp triad-dependent photoreduction is not required for the in vivo DNA-repairing activity of Escherichia coli photolyase (16–18), the photobiological and magnetosensing activities of Drosophila cryptochrome (10, 13, 19–21), or the photobiological and photophysiological activities of Arabidopsis CRY2 (22). It was proposed recently that ATP or other metabolites may enhance the rapid photoreduction of CRY2 to “rescue” its defects in photoreduction and physiological activities in vivo (23). However, because Trp-triad mutations were reported to inactive CRY1 (15) but not CRY2 in vivo (22), this interpretation raises the intriguing possibility that Arabidopsis CRY1 might possess a photoexcitation mechanism distinct from both the closely related Arabidopsis CRY2 and the remotely related E. coli photolyase and Drosophila cryptochrome. To investigate this puzzle and the photoactivation mechanism of cryptochromes in general, we analyzed the Trp-triad mutants of CRY1, including the previously reported mutants W324 and W400 (15), to test whether photoreduction or ATP enhancement of photoreduction is required for the function of CRY1.
Lack of Correlation Between ATP-Enhanced Photoreduction and Trp Triad-Dependent Photoreduction of CRY1.

Results

Cryo1 protein (Fig. 1 and SI Appendix, Fig. S1). As expected, the wild-type CRY1 protein showed a rapid (within 10 min) photoreduction in vitro under anaerobic conditions, as reported previously (Fig. 1C) (7). Mutations of any Trp-triad tryptophan residues (W400, W377, or W324) by either alanine or phenylalanine abolished or dramatically reduced the rapid photoreduction activity of the respective mutant CRY1 protein (Fig. 1C–N). In contrast, mutations of the other two evolutionarily conserved tryptophan residues, W334 and W356, showed rapid photoreduction activity similar to that of the wild-type CRY1 protein (SI Appendix, Fig. S1). These results confirmed that only the Trp-triad residues are required for the rapid in vitro photoreduction of Arabidopsis CRY1.

It is well known that flavins and flavoproteins are photoreducible in vitro and that many small molecules, such as EDTA, urea, flavin, NADH, and amino acids, can enhance in vitro flavin photoreduction (1, 2, 25–27). It has been reported recently that metabolites such as ATP or NADH enhance photoreduction of Arabidopsis CRY2 (23), suggesting a compelling explanation of why the Trp-triad mutations of Arabidopsis CRY2 that do not undergo photoreduction in vitro are photophysioactively active in vivo. ATP-enhanced photoreduction also has been reported for the PHR domain of CRY1 (28, 29), supporting the hypothesis that metabolites such as ATP may act as regulators of cryptochromes in cells. We tested this hypothesis by investigating whether ATP may enhance photoreduction of Arabidopsis CRY1 and whether ATP may rescue the defective photoreduction of the Trp-triad mutants of CRY1. In contrast to the full-length CRY2 or truncated CRY1, ATP failed to enhance the rapid photoreduction of the full-length Arabidopsis CRY1 under conditions similar to those reported previously for CRY2 (Fig. 1C and F and SI Appendix, Fig. S2). Importantly, ATP also failed to rescue the defective photoreduction of the Trp-triad mutant W400F of CRY1 (Fig. 1D and G and SI Appendix, Fig. S2) and even slightly inhibited the residual photoreduction of the Trp-triad mutant W324A (Fig. 1E and H and SI Appendix, Fig. S3). ATP did enhance photoreduction of other Trp-triad mutants of CRY1 (W324F, W377A, and W377F) that are defective in photoreduction. ATP also accelerated or enhanced photoreduction of the non–Trp-triad CRY1 mutation W334F and W356F that showed no apparent defect in photoreduction (Fig. 1 and SI Appendix, Figs. S1–S5). If the in vivo activity of Trp-triad mutants was caused by ATP-enhanced photoreduction, ATP would be expected to rescue light-dependent flavin reduction of the Trp-triad mutants that possess light-dependent physiological activities in vivo and to exert light-independent enhancement of flavin reduction of the Trp-triad mutants that exhibit light-independent or constitutive activities in vivo. In contrast to both expectations, it had been reported previously that ATP enhanced light-dependent flavin reduction or photoreduction of the W374A mutant of CRY2 that is constitutively active in vivo (22, 23) but failed to enhance photoreduction of the W397F mutant of CRY2 that showed light-dependent activities in plants (22, 23). Similarly, ATP failed to enhance photoreduction of the W324A and W400F mutants of CRY1 (Fig. 1), both of which exhibit light-dependent physiological activities in plants (see below). Taken together, these results demonstrate a lack of correlation between the ATP-enhanced photoreduction and light-dependent functions of CRY1 and CRY2.

*Fig. 1.* The Trp triad-dependent photoreduction of Arabidopsis CRY1. (A) The structure of CRY1 (Protein Data Bank ID code 1U3G) showing the Trp-triad residues (blue), and the two evolutionarily conserved non–Trp-triad tryptophan residues (green) are indicated. (B) Sequence alignment of the Trp-triad region of *Synechococcus elongates* photolyase (SePhr), *E. coli* photolyase (EcPhr), *Drosophila* cryptochrome (DmPhr), human CRY1 and CRY2 (hCRY1, hCRY2), and Arabidopsis CRY1 and CRY2 (AtCRY1, AtCRY2). The Trp-triad residues (blue) and the other two conserved non–Trp-triad tryptophan residues (green) are indicated. The positions of the five conserved tryptophan residues in AtCRY1 are labeled at the bottom. The alignment was generated by ClustalX and modified by GeneDoc. (C–N) Photoreduction of the wild-type CRY1 and the indicated CRY1 mutant proteins expressed and purified from Sf9 insect cells is shown. The scanning absorbance spectra (C–E and J–L) were recorded after blue light (450 ± 15 nm, 1.8 mW/cm², or 70 μmol·m⁻²·s⁻¹) treatment for 10 min in the absence (B10) or presence of 1 mM ATP (B10 (+ ATP)) under anaerobic conditions at 20 °C, in the presence of 10 mM β-mercaptoethanol as external electron donor. The difference spectra of indicated proteins are shown in F–H and L–N, a.u., arbitrary units.
Trp Triad-Dependent Photoreduction Is Not Required for the Photophysiological Activities of CRY1. CRY1 mediates blue light inhibition of hypocotyl growth, blue light stimulation of anthocyanin accumulation, and blue light induction of mRNA accumulation (30–33). To test the photophysiological activities of the Trp-triad mutants of CRY1, we prepared transgenic Arabidopsis lines expressing wild-type or mutant CRY1 as the GFP-CRY1 fusion proteins under the control of the constitutive 35S promoter in the cry1 mutant background. For simplicity, the different GFP-CRY1 fusion proteins (GFP-CRY1, GFP-CRY1W324A, GFP-CRY1W324F, GFP-CRY1W377A, GFP-CRY1W377F, GFP-CRY1W400A, and GFP-CRY1W400F, GFP-CRY1W334A, GFP-CRY1W334F, GFP-CRY1W356A, GFP-CRY1W356F) are referred to hereafter as “CRY1,” “W324A,” “W324F,” “W377A,” “W377F,” “W400A,” “W400F,” “W334A,” “W334F,” “W356A,” and “W356F,” respectively. All CRY1 mutant proteins accumulated in the nucleus and cytoplasm as the wild-type CRY1 control (SI Appendix, Figs. S6 and S7) (34). To ensure that the observed physiological activities of the Trp-triad mutants of CRY1 are not caused by higher levels of protein expression, transgenic lines expressing the mutant CRY1 proteins at the levels similar to or slightly lower than that of the wild-type GFP-CRY1 control (Fig. 2 and SI Appendix, Figs. S8 A and B and Table S1) were selected for phenotypic studies. The genotypes of the transgenic lines used for detailed phenotypic analyses (SI Appendix, Fig. S6A) were verified by genomic sequencing, and the hypocotyl inhibition activity of all transgenics was confirmed in three independent lines (SI Appendix, Figs. S9 and S10). The overall results are summarized in SI Appendix, Table S1.

Surprisingly, in contrast to a previous report (15), none of the Trp-triad mutations of CRY1 abolished physiological activity inhibiting hypocotyl elongation (Fig. 2 C and D and SI Appendix, Figs. S8–S10). It was reported previously that mutations of the W324 or W400 residue of CRY1 were inactive in mediating blue light inhibition of hypocotyl elongation (15). However, in our experiment, mutations in all Trp-triad residues of CRY1, including W324 and W400, remained active in mediating blue light inhibition of hypocotyl elongation (Fig. 2). For example, the W324F and W324A mutants of CRY1 fully rescued the long hypocotyl phenotype of the cry1 parent (Fig. 2 C and D and SI Appendix, Figs. S8 C and D). The specific activity of the W324F and W324A mutants is comparable to or higher than that of the wild-type GFP-CRY1 control, because the protein level of the W324A or W324F mutants was about 60–70% that of the GFP-CRY1 control (SI Appendix, Table S1). Among the six Trp-triad mutants of CRY1 examined, four (W324F, W324A, and W324F) showed blue light-specific activity in growth inhibition (Fig. 2 D and SI Appendix, Fig. S8D), whereas two mutants (W377A and W400A) exhibited activity stronger than that of the wild-type GFP-CRY1 control in growth inhibition without the blue light specificity (SI Appendix, Fig. S8 C and D). Seedlings expressing W377A showed light-dependent hypocotyl inhibition in both blue light and red light, whereas seedlings expressing W400A exhibited constitutive growth inhibition in both dark and light (SI Appendix, Fig. S8 ), a finding that is reminiscent of the W377A and W374F mutants of Arabidopsis CRY2 (22).

To measure the photophysiological activity of the Trp-triad mutants of CRY1 more accurately, we performed a fluence rate response analysis (SI Appendix, Fig. S11). In this experiment, the hypocotyl lengths of seedlings expressing each of the Trp-triad mutants of CRY1 were compared with the hypocotyl length of the parent cry1 mutant (cry1), the wild type, and the transgenic line expressing the wild-type GFP-CRY1 control (CRY1) (SI Appendix, Fig. S11). SI Appendix, Fig. S11 shows that the apparent growth inhibition activity of the six Trp-triad mutants of CRY1 can be ranked in the following order in comparison with the wild-type GFP-CRY1 control: W400A > W377A > W324A ~ W324F ~ CRY1 > W377F ~ W400F. Taking into consideration the level of relative protein expression, we found that four (W400A, W377A, W324A, and W324F) of the six Trp-triad mutants tested exhibited apparent specific activities similar to or higher than that of the wild-type GFP-CRY1 control. For example, hypocotyl length in seedlings expressing W324A was indistinguishable from that of seedlings expressing the wild-type GFP-CRY1 control, although the level of relative protein expression in W324A is about 70% that of the GFP-CRY1 control (SI Appendix, Fig. S11 and Table S1). This result demonstrates that the photophysiological activity of W324A is at least similar to that of the wild-type CRY1. Seedlings expressing the W400A mutant (and to a lesser extent the W377A mutant) showed a constitutive photomorphogenic phenotype (SI Appendix, Fig. S11) that is reminiscent of the W374A mutant of CRY2 (22).
It is noteworthy that the constitutively active W400A mutation of CRY1 affects the Trp-triad residue (W400) that is the most proximate to FAD, whereas the constitutively active W374A mutation of CRY2 affects the Trp-triad residue (W374) located in the middle of the Trp triad. It also is interesting that both the W374F and W374A mutants of CRY2 are constitutively active (22), whereas only the W400A mutant but not the W400F mutant of CRY1 showed constitutive activities (Fig. 2 and SI Appendix, Figs. S8 and S11). Because (i) all Trp-triad mutants tested were able to rescue the cry1 mutant (Fig. 2 and SI Appendix, Fig. S8), (ii) more than half of the six Trp-triad mutants tested exhibited specific activity similar to or higher than that of the GFP-CRY1 control (SI Appendix, Fig. S11 and Table S1), and (iii) ATP failed to enhance photoreduction of the wild-type CRY1 or to rescue the defective photoreduction of at least two Trp-triad mutants of CRY1 that are photophysically active in plants (Fig. 1 C–E), we propose that Trp-triad-dependent photoreduction is not required for the photophysiological activity of CRY1.

To test this proposition further, we examined whether the Trp-triad mutants can mediate the blue light-induced anthocyanin accumulation and changes of gene expression in plants. Again, in contrast to the previous report (15), all Trp-triad mutants of CRY1 were able to mediate blue light stimulation of anthocyanin accumulation (Fig. 3). As shown in Fig. 3, transgenic plants expressing all six Trp-triad mutant proteins of CRY1 accumulated significantly higher ($P < 0.01$) levels of anthocyanin than the cry1 mutant parent or the wild-type plants when grown in continuous blue light. Interestingly, the W-to-A Trp-triad mutants appear to exhibit higher activity than the W-to-F mutants of the same Trp-triad residues in both anthocyanin accumulation and hypocotyl inhibition responses (Fig. 3 and SI Appendix, Fig. S11). Because the phenylalanine replacement of tryptophan is expected to result in less structural perturbation (and thus in higher activity) than the alanine replacement, this observation seems counterintuitive.

The W-to-A mutants, W400A (and, to a lesser extent, W377A) caused constitutive accumulation of higher levels of anthocyanin than the controls (Fig. 3A and B), indicating again that W400A is constitutively active. All Trp-triad mutants of CRY1 were active in mediating blue light induction of mRNA expression of the three genes (CHS, SIG5, and Psbs) known to be induced by blue light (SI Appendix, Fig. S12). The constitutively active W400A mutant of CRY1 (and, to a lesser extent, W377A) exhibited constitutive activity stimulating mRNA expression of the CHS and SIG5 genes but a light-dependent activity stimulating mRNA expression of the Psbs gene (SI Appendix, Fig. S12), suggesting that W400A may retain residual photo-responsiveness. In summary, all photoreduction-deficient Trp-triad mutants of CRY1 remained physiologically active in the three different blue light responses examined: inhibition of hypocotyl growth, promotion of anthocyanin accumulation, and change of gene expression. This result argues that the Trp-triad-dependent rapid photoreduction is not required for the photophysiological activity of CRY1.

Trp Triad-Dependent Photoreduction Is Not Required for the Photobiological Activities of CRY1. A simple interpretation of the observation that all photoreduction-deficient CRY1 mutants retained physiological activities would be that the Trp-triad mutation did not disrupt the essential photochemical properties of CRY1 that are important to its functions. To test this proposition, we first examined the blue light-dependent phosphorylation of CRY1. Arabidopsis cryptochromes undergo blue light-dependent protein phosphorylation that is required for their photophysiological activities (35–39). Because phosphorylated CRY1 exhibits a blue light-dependent electrophoretic mobility upshift typical of phosphorylated proteins (36), we used the mobility upshift assay to test the blue light-dependent phosphorylation of the W-to-A Trp-triad mutants. As expected, the endogenous CRY1 and the wild-type GFP-CRY1 exhibited the blue light-dependent electrophoretic mobility upshift (Fig. 4 A and B). Similarly, the W324A and W377A Trp-triad mutants of CRY1, as well as mutations of the two non–Trp-triad tryptophan residues (W344A and W356A), also showed light-dependent mobility upshift (Fig. 4 B–D). The observation that both Trp-triad and non–Trp-triad mutants retained the photobiological activity is consistent with the results showing that both types of mutants retained the photophysiological activities (Fig. 3 and SI Appendix, Figs. S8 and S11). Importantly, the W400A mutant exhibited a constitutive mobility upshift that can be eliminated by the protein phosphatase treatment, demonstrating that W400A is constitutively phosphorylated independent of light (Fig. 4C). This result also is consistent with the fact that W400A is constitutively active in vivo (Fig. 3 and SI Appendix, Figs. S8 and S11).

Another known photobiological activity of CRY1 is its blue light-dependent physical interaction with SPA1 (SUPPRESSOR OF PHYTOCHROME A 1) (40, 41). Therefore, we tested whether the constitutively active Trp-triad mutation W400A may interact constitutively with SPA1. We first compared the activity of CRY1 and W400A, using the HEK 293 coexpression system and a commounprecipitation assay. Fig. 4 shows that both the wild-type CRY1 protein and the W400A mutant protein commounprecipitated SPA1 (Fig. 4 E and F). However, in
triad mutations of CRY1 can interact with SPA1 in plant cells, (40, 41), is relatively easier to express, we tested whether the Trp-triad tryptophan residues of CRY1 and SPA1 were fused to the N-terminal fragment of desmin previously (42). In this experiment, the Trp-triad mutants using the bimolecular fluorescence complementation (BiFC) assay were exposed to blue light (30 μmol·m⁻²·s⁻¹) for 10, 30, or 60 min. Samples were extracted and fractionated in a 6% SDS/PAGE gel, blotted, probed with anti-CRY1 antibody, stripped, and reprobed with anti-HSP90 antibody. An aliquot of the W400A mutant from seedlings exposed to blue light for 30 min was treated with Lambda Protein Phosphatase (30 min (+Pase)). White dotted lines are drawn to help distinguish the upshifted (i.e., phosphorylated) bands. Levels of proteins from different immunoblots are not directly comparable.

Fig. 4. The Trp-triad mutants of CRY1 are biochemically active. (A–D) Mobility upshift assay showing blue light-dependent phosphorylation of the endogenous CRY1 (Col-4), the GFP-CRY1 control (CRY1), and the indicated mutants of CRY1 and light-independent phosphorylation of the W400A mutant of CRY1. Seven-day-old seedlings grown on MS medium in darkness (0) were exposed to blue light (30 μmol·m⁻²·s⁻¹) for 10, 30, or 60 min. Samples were extracted and fractionated in a 6% SDS/PAGE gel, blotted, probed with anti-CRY1 antibody, stripped, and reprobed with anti-HSP90 antibody. An aliquot of the W400A mutant from seedlings exposed to blue light for 30 min was treated with Lambda Protein Phosphatase (30 min (+Pase)). White dotted lines are drawn to help distinguish the upshifted (i.e., phosphorylated) bands. Levels of proteins from different immunoblots are not directly comparable.

Fig. 5. The Trp-triad mutants of CRY1 are constitutively active and reconstituted YFP activity. A, Col-4 and cry1; B, CRY1 and W324A; C, W377A and W400A; D, W334A and W356A; E, FlagSPA1 and MycCRY1; F, FlagSPA1 and MycCRY1; G, BiFC signal (%). CRY1 interacts with SPA1 and W400A and SPA1 were coexpressed in HEK293 cells. The transfected cells were cultured in darkness before exposure to blue light (40 μmol·m⁻²·s⁻¹) for 2 h or were kept in darkness. The total protein extracts (Input) and immunoprecipitation product (IP-Flag) were fractionated by a SDS/PAGE gel, blotted, probed with the anti-Flag antibody, stripped, and reprobed with the anti-CRY1 antibody. G) Results of BiFC assays showing the interaction of the CRY1 or mutant proteins with SPA1 in n. benthamiana plants. Three-week-old tobacco plants grown in long-day conditions (16 h light/8 h dark) were cotransformed with Agrobacteria harboring the plasmids encoding nYFP-CRY1W-to-A mutants and cCFP-SPA1 in n. benthamiana plants. The transformed plants were incubated for 12 h in the dark and then were transferred to white light for 48–72 h. The infected leaf spots were excised and examined under a fluorescence microscope. Images were taken at 10X magnification and are shown in SI Appendix, Fig. S14. The percentage of cells that exhibit BiFC fluorescence signals were calculated by the formula [(number of YFP fluorescent nuclei/number of DAPI-stained nuclei)]% Data are shown as means and SD (n = 3).

Discussion

We show in the present study that the Trp triad-dependent rapid photoreduction is not required for the photochemical and photophysiological activities of Arabidopsis CRY1 in plants. This result is consistent with similar findings in E. coli photolyase (16, 17), Drosophila cryptochrome (10, 13, 19–21), and Arabidopsis CRY2 (22). It has been proposed recently that ATP-enhanced photoreduction may rescue the defects of the Trp-triad mutants of Arabidopsis CRY2 in both photoreduction and physiological activities in plants (23). This hypothesis is attractive, because cryptochrome is known to bind ATP, and ATP may act as the phosphate donor for the light-dependent phosphorylation of cryptochromes (35–37). However, several observations are in contrast with what would be expected based on the ATP-enhanced photoreduction model. First, ATP enhances rapid photoreduction of wild-type CRY2 but not wild-type CRY1 (Fig. 1 C and F) (23). Second, ATP enhances light-dependent flavin reduction of the Trp-triad mutant W374A of CRY2 that is constitutively active in plants (22, 23), suggesting that the ATP-enhanced photoreduction is irrelevant to the light-independent physiological activities of the W374A mutant of CRY2. Third, ATP enhances photoreduction of two non–Trp-triad mutants of CRY1 (W334F and W356F) that show no defect in photoreduction or physiological activity. Finally, ATP does not enhance rapid photoreduction of the W397F mutant of CRY2 (23) or the W400F and W324A mutants of CRY1 (Fig. 1 D, E, G, and H), all of which possess light-dependent activities in plants (Figs. 2 and 3 and SI Appendix, Figs. S8–S12) (22). These observations demonstrate a lack of correlation between the ATP-enhanced photoreduction in vitro and the photophysiological activities of both CRY1 and CRY2 in vivo.

If the Trp-triad residues are not required for the photoexcitation of cryptochromes, why are they uniformly conserved in the photolyase/cryptochrome superfamily? To address this question, we analyzed the other two conserved tryptophan residues of CRY1 in this conserved region of the photolyase/cryptochrome proteins (Fig. 1b). We found that mutations of the evolutionarily conserved non–Trp-triad tryptophan residues of CRY1, W334 and W356, showed no apparent defects in photoreduction (Fig. 1 and SI Appendix, Fig. S1) or in the photophysiological or photochemical activities examined (Figs. 1–4 and SI Appendix, Figs. S8–S12). The observations that (i) the five tryptophan residues of this region are evolutionarily conserved regardless of a role in photoreduction and (ii) individual mutations of these conserved tryptophan residues failed to abolish CRY1 activities regardless of their respective photoreduction activities are consistent with the hypothesis that both the Trp-triad residues and the non–Trp-triad residues are evolutionarily conserved for reasons not directly associated with photoreduction per se. Taking together these results and those of previous studies, we argue that there is presently no genetic evidence to support the hypothesis that Trp triad-dependent photoreduction is the photoexcitation mechanism of cryptochromes. Renewed efforts are needed to elucidate the photoexcitation mechanism of the cryptochrome photoreceptors. For example, only rapid photoreduction (within 10 min) has been analyzed in this and previous studies, because the in vivo photochemical and photophysiological activities of plant cryptochromes...
Materials and Methods

Single amino acid substitutions of tryptophan to alanine or phenylalanine were introduced into the CRY1 coding region at the Trp-triplet positions of 324, 377, and 400 and at the non-Trp-triplet positions of 334 and 356, using the QuickChange Site-Directed Mutagenesis system according to the manufacturer’s instruction (Stratagene). Transgenic lines expressing the recombiant GFP-CRY1 and GFP-CRY1 (mutant) proteins were prepared in the cry1 mutant background (cry1-204, Col accession) (44). Additional methods of protein expression, purification, and analyses and the preparation and phenotypic analyses of transgenic plants are described in SI Appendix, SI Materials and Methods.

ACKNOWLEDGMENTS. This work was supported in part by National Institutes of Health Grants GM56265 (to C.L.) and GM074813 (to D.Z.) and by research funds from Jinlin University (research support to Laboratory of Soil and Plant Molecular Genetics) and Fujian Agriculture and Forestry University (research support to Basic Forestry and Proteomics Research Center).

SUPPORTING INFORMATION (SI)

Trp triad-dependent rapid photoreduction is not required for the function of Arabidopsis CRY1

Jie Gao\textsuperscript{a,b,1}, Xu Wang\textsuperscript{b,c,1}, Meng Zhang\textsuperscript{d,e,1}, Mingdi Bian\textsuperscript{h,1}, Weixian Deng\textsuperscript{a}, Zecheng Zuo\textsuperscript{a}, Zhenming Yang\textsuperscript{a,2}, Dongping Zhong\textsuperscript{d,e,2}, and Chentao Lin\textsuperscript{b,2}

Supplemental Materials and Methods

Site-specific mutagenesis

Individual tryptophan residues of CRY1 at positions 324, 334, 356, 377, or 400 were substituted to alanine or phenylalanine using QuikChange Site-Directed Mutagenesis system according to the instruction manual (Stratagene). The sequences of primers used in site-directed mutagenesis are shown in the following:

W324A-F, CCATCTAAAGTTCTTCCCT\textsuperscript{GCC}GCTGTGGATGAGAACACTAT  
W324A-R, ATAGTTCTCATCACACACGCCAAGGAAAGACTTTAGG  
W377A-F, GTAAAGTGCTTCAATTACCA\textsuperscript{GCC}GATGGGGGATGAAGTATTTC  
W377A-R, GAAATACTTCTCATCCCCCAT\textsuperscript{GCC}TGTTGAATTGAGACTTTAAAC  
W400A-F, GAAACCGAGTCATTGCTTGGC\textsuperscript{GCC}CATAACATTACCAGG  
W400A-R, GTACCGGTAATGGTATTTG\textsuperscript{GCC}ACCAAGAGCATCGCTTTC  
W334A-F, TGGAAGACTTCTTACAGGACGCCAGGCAAGGCGACTGG  
W334A-R, CCACGGCGCCCTTTGACCT\textsuperscript{GCC}TGCTTTGAAATAGTTCTA  
W356A-F, GAGGTTGATGGGCTACTGTTG\textsuperscript{GCC}GATGGGGGATGAAGTATTTC  
W356A-R, CTCTTGAGATGCGCAACGACCAGTACCGCTTTCGCTCTCTC  
W324F-F, CCATCTAAAAGTTCTTCCCT\textsuperscript{TCC}GCTGTGGATGAGAAC  
W324F-R, GTTTCTCATCCACAG\textsuperscript{AAA}GGAAGGCAACTTTAGG  
W377F-F, GTGCTTCAATTACCA\textsuperscript{TCC}AGGCAAGGCCGGAC  
W377F-R, CTCTTCTTACAGGACGCCAGGCAAGGCGACTGG  
W400F-F, CCGCGCCCTTTGACCT\textsuperscript{TCC}GCTGTGGATGAGAAC  
W400F-R, GTACCGGTAATGGTATTTG\textsuperscript{TCC}ACCAAGAGCATCGCTTTC  
W334F-F, GAGGTTGATGGGCTACTGTTG\textsuperscript{TCC}GCTGTGGATGAGAAC  
W334F-R, CTCTTCTTACAGGACGCCAGGCAAGGCGACTGG  
W356F-F, GAGGTTGATGGGCTACTGTTG\textsuperscript{TCC}GCTGTGGATGAGAAC  
W356F-R, CTCTTCTTACAGGACGCCAGGCAAGGCGACTGG

Expression, purification, and photoreduction assay

The mutated CRY1 cDNAs were cloned to the vector pFastBacHTA, by fusing CRY1 in frame to the C-terminus of His tag at the EcoRI and XhoI restriction sites of pFastBacHTA. The recombinant pFastBacHTA plasmids were transformed to DH10Bac\textsuperscript{TM} E. coli to generate the recombinant Bacmids, which were used to transfect SF9 insect cells using the Bac-to-Bac Baculovirus expression system according to the manufacturer's instruction (Invitrogen). The wild-type His-CRY1 protein and trp-triad mutant His-CRY1 proteins were purified using the Ni-NTA Purification System (Invitrogen) with minor modification. Briefly, the virus-infected
cells were centrifuged at 5,000 rpm for 5 min, suspended with Native Binding Buffer [500 mM NaCl, 50 mM NaH₂PO₄ (pH 8.0), 0.5% Triton X-100, 1:3,000 β-mercaptoethanol, with 1 mM PMSF added freshly, pH 8.0]. The cells were lysed by sonication (model VC505; Sonics & Materials, Inc.) for 5 × 10 s at 30% amplitude until the solution became watery, and centrifuged at 14,000 rpm for 30 min at 4 °C. The supernatant was filtered with a 0.22-μm filter. Proteins were further purified according to the user manual of Ni-NTA Purification System (Invitrogen).

For the photoreduction assays, the proteins were illuminated by blue light (450±15 nm, 1.8 mW·cm⁻² or approximately 70 μmol·m⁻²·s⁻¹) and absorption spectra were recorded at indicated times under anaerobic conditions at 20 °C, in the presence of 10 mM β-mercaptoethanol as external electron donor.

**Plant materials**

The mutated CRY1 cDNAs were cloned into the pEGAD vector, by fusing in frame to the C-terminus of GFP as described previously (1, 2). Transgenic lines expressing 35S::GFP-CRY1, 35S::GFP-CRY1⁴⁻³⁺⁴⁻⁰, and 35S::GFP-CRY1⁴⁻⁰⁻⁰(mutant) were prepared in the cry1-304 mutant background of the Col-0 accession (3). The cry1 mutations of indicated transgenic lines were confirmed by genomic PCR, using primer pairs: CRY1⁴⁻³⁺⁴⁻⁰ (5' TTGCCA-GAGAGGCACCTCAGAG and 3' GGTTGGAAGAGGGACTCAAGGG) spanning the sequence encoding the N-terminal fragment of CRY1.

Our repeated attempts to obtain the previously published transgenic lines expressing the W324F and W400F triad mutants (4) were unsuccessful. Therefore, a direct comparison of the transgenic lines reported here and those reported previously (4) could not be performed.

**Hypocotyl inhibition assay**

Seedlings were prepared as described previously (2). Briefly, seeds were sterilized and sown on MS medium, stratified at 4 °C in darkness for 4 d, treated with white light for 24 h to induce germination, transferred to the different light conditions for 5 d. Hypocotyl lengths were measured as described (5).

**Anthocyanin extraction and quantification**

Anthocyanin contents of transgenic lines expressing the CRY1⁴⁻³⁺⁴⁻⁰(mutant) and CRY1⁴⁻⁰⁻⁰(mutant) were measured as previously reported (6, 7). Seedlings were grown in continuous blue light (20 μmol·m⁻²·s⁻¹) or darkness for 5 d. 30 seedlings per sample were frozen in liquid nitrogen, ground in liquid nitrogen in a 1.5 ml tube and total plant pigments were extracted overnight at room temperature in 0.8 ml methanol with 1% HCl. Samples were centrifuged in a microcentrifuge for 10 min, ca. 0.6 ml supernatant were moved to a new tube, and mixed with 0.4 ml H₂O and 0.6 ml chloroform. Samples were shaken for 1 h, centrifuged to separate chlorophyll from anthocyanin, levels of anthocyanin in the methanol phase (upper) was determined spectrophotometrically and calculated by the formula (A530-A657)/ number of seedling ×1000 as described previously (8).

**Phosphorylation and dephosphorylation assays**

Seven-day-old etiolated transgenic seedlings grown on MS in darkness for were exposed to blue light (30 μmol·m⁻²·s⁻¹) for the time indicated. For dephosphorylation assays, total protein was firstly extracted with protein extraction buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100], treated with Lambda Protein Phosphatase (NEB P0753S) in a reaction conditions [50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35, 1 mM MnCl₂] according to the user manual (NEB), incubated at 30°C for 30 min. The dephosphorylation
Protein samples were fractionated by a 6% (wt/vol) SDS/PAGE gel, blotted, probed with anti-CRY1 antibody, stripped and reprobed with HSP90 antibody as a loading control.

**Protein expression in HEK293 (Human Embryonic Kidney) cells and co-immunoprecipitation assay**

HEK293 (Human Embryonic Kidney) cell was cultured according to the Cell Culture Basics Handbook (invitrogen). Briefly, cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Gibco), cultured in 37°C incubator with a humidified atmosphere of 5% CO2 in air. HEK293 cells were transfected by the calcium phosphate transfection method. Briefly, cells were plated the night before and grown to 60-70% confluence by the day of transfection. To transfect one well of cells in a 6-well plate, add < 2 μg DNA (for 6-well plates) to a 1.5 ml sterile tube, add ddH2O to make the total volume of 180 μl, add 20 μl 2.5 M CaCl2. When the DNA mixture is ready, add 200 μl of 2xHeBS [50 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4, 12 mM Dextrose(Mw: 180.16) and 50 mM Hepes(PH 7.5)] dropwise while vortex. Aspirate media from 6-well plates, slowly add the mixture to one side of the dish, and rotate to coat the whole well. Add 2 ml fresh media with 25 μM Chloriquine into the well, and incubate in 37°C incubator for 6 h to overnight. Aspirate the media with 25 μM Chloriquine, then change into 2 ml fresh media without Chloriquine. 30-48 h after transfection, the transfected cell cultured in darkness were exposed to blue light (40 μmol·m−2·s−1) or kept in darkness for 2 h. Cells were lysed in lysis buffer [1% Brij 35, 50 mM Tris pH=8.0, 150 mM NaCl, 1mM EDTA], and centrifuged to remove insoluble debris. The supernatants were pre-cleaned for 30 min with 20 ul protein G beads (PIERCE, Cat# 20398), mixed with EZview™ Red ANTI-FLAG® M2 Affinity Gel (SIGMA, Cat#F2426) and incubated for 1h. Beads were washed 5 times, spun briefly to remove supernatant. SDS/PAGE sample buffer was added into the immunoprecipitation products and boiled for 5 min. Total protein extracts (Input) and immunoprecipitation product (IP-Flag) were fractionated by a SDS/PAGE gel, transferred to nitrocellulose membranes, probed with the anti-Flag antibody, stripped, and reprobed with the anti-CRY1 antibody. In the co-IP assays, the plasmids encoding Myc-CRY1/Myc-W400A, Flag-SPA1 and Myc-COP1 were co-expressed in HEK293 cells.

**BiFC assay**

The plasmids encoding nYFP-CRY1/CRY1W-to-A mutants and cCFP-SPA1CT509 were constructed, transformed into Agrobacterium strain: Agl-0. The agrobacterium was cultured overnight in the LB medium [10 mMMES, 20 mM AS (Acetosyringone)] overnight, collected by centrifugation, washed and re-suspended in the infiltration buffer [10 mM MES, 150 mM AS, 10 mM MgCl2, 0.5% glucose]. Different Agrobacterial cultures were diluted to OD600 = 0.5 and equal amounts of mixed, incubated at room temperature for 3 h, and infiltrated into three-week-old *Nicotiana benthamiana* leaves using a syringe as described before (9). The BiFC fluorescence signals were detected 2~3 d after infiltration by a Zeiss AxioImager Z1 microscope, images were taken by a Hamamatsu Orca-ER camera at 10×magnification or 100×magnification and processed by Zeiss Axiovision software.

**mRNA expression assay**

Total RNAs isolation, cDNA preparation and quantitative RT-PCR (qPCR) reactions were proceeded as described previously (2, 10). The qPCR signals of the CHS (At5g13930, chalcone synthase), SIG5 (At5g24120, sigma factor 5), and Psbs (At1g44575, Chlorophyll a/b binding protein of photosystem II) genes were normalized to that of ACT2 (At3g18780, actin 2) control. The primers used in the qPCR reaction of CHS and ACT2 genes were as described (2). Primers used in the qPCR reaction of other genes are:
AtSIG5F (5'-TGATATAGTGAGCTTGGACTGG),
AtSIG5R (5'-CTTGAGCTCTACCTATTTTG);
PsbsF (5'-CTCTTCAAACCCAAACCAAGCT),
PsbsR (5'-GCCCTTGTGAAACCAATCCCA).

References:


**Supplemental Table**

Table S1

<table>
<thead>
<tr>
<th>CRY1 Proteins</th>
<th>Mutation</th>
<th>Trp-triad</th>
<th>Photoreduction</th>
<th>ATP-enhanced Photoreduction</th>
<th>Relative Expression</th>
<th>Photophysiological Activities</th>
<th>Photobiochemical Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hypocotyl Anthocyanin inhibition accumulation</td>
<td>Gene expression</td>
</tr>
<tr>
<td>WT</td>
<td>no</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
<td>0.3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cry1</td>
<td>null</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CRY1</td>
<td>no</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CRY1&lt;sup&gt;W32A&lt;/sup&gt;</td>
<td>W32A</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0.7</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>CRY1&lt;sup&gt;W34F&lt;/sup&gt;</td>
<td>W34F</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>0.6</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CRY1&lt;sup&gt;W37A&lt;/sup&gt;</td>
<td>W37A</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>0.4</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>CRY1&lt;sup&gt;W37F&lt;/sup&gt;</td>
<td>W37F</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>0.5</td>
<td>++</td>
<td>n/a</td>
</tr>
<tr>
<td>CRY1&lt;sup&gt;W40A&lt;/sup&gt;</td>
<td>W40A</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
<td>0.7</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>CRY1&lt;sup&gt;W40F&lt;/sup&gt;</td>
<td>W40F</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0.9</td>
<td>++</td>
<td>n/a</td>
</tr>
<tr>
<td>CRY1&lt;sup&gt;W34A&lt;/sup&gt;</td>
<td>W34A</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
<td>0.8</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CRY1&lt;sup&gt;W34F&lt;/sup&gt;</td>
<td>W34F</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>0.9</td>
<td>++</td>
<td>n/a</td>
</tr>
<tr>
<td>CRY1&lt;sup&gt;W35A&lt;/sup&gt;</td>
<td>W35A</td>
<td>-</td>
<td>+</td>
<td>n/a</td>
<td>1.1</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CRY1&lt;sup&gt;W35F&lt;/sup&gt;</td>
<td>W35F</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>0.7</td>
<td>++</td>
<td>n/a</td>
</tr>
</tbody>
</table>

**Table S1.** The level of protein expression and activities of the trp-triad mutants of CRY1 in Arabidopsis plants.

The level of protein expression and light-responsive biochemical or physiological activities of the endogenous CRY1 (WT), the wild-type GFP-CRY1 control (CRY1) and the indicated mutants of CRY1 expressed in the cry1 mutant parent are shown. The symbols “+” indicates light-dependent activity comparable to that of the endogenous CRY1 of the wild-type plants (WT), “++” indicates light-dependent activity comparable to the GFP-CRY1 control (CRY1), “+++” indicates constitutive activity, “na” indicates not analyzed.
Supplemental Figures

Fig. S1

Fig. S1 Expression and purification of the CRY1 and indicated mutant proteins and photoreduction of the non-trp-triad W334F and W356F mutant proteins.

(A) Wild-type CRY1 and the CRY1 mutant proteins were expressed and purified from insect cells (Sf9), fractionated in 10% SDS/PAGE gel, stained with Coomassie Brilliant Blue (upper) or proceeded with an immunoblot (lower), probed with anti-CRY1 antibody. Expression Levels of proteins from different gels or immunoblots are not directly comparable.

(B-C) The scanning absorption spectra of the non-trp-triad mutants W334F and W356F were recorded after blue light (450±15 nm, 1.8 mW·cm⁻² or 70 μmol m⁻²·s⁻¹) treatment for 10 min in the absence (B10) or presence of 1mM ATP [B10 (+ATP)] under anaerobic conditions at 20 °C, in the presence of 10 mM β-mercaptoethanol as external electron donor. The absorption spectra of W334F in darkness are included, because the W334F protein tends to aggregate during experiment in darkness in the presence of ATP, reducing absorption and causing distorted differential spectrum shown in D.

(D-E) The difference spectra of W334F (D) and W356F (E) in the absence (B10) or presence of ATP [B10(+ATP)]. The ATP enhancement of photoreduction of W334F was slightly underestimated due to protein aggregation of W334F in darkness in the presence of ATP (see B).
Fig. S2 Photoreduction of the wild-type CRY1 protein and the trp-triad W400F mutant protein.

(A-B) The scanning absorption spectra of the wild-type CRY1 protein purified from insect cells were recorded after blue light (450±15 nm, 1.8 mW·cm⁻² or 70 μmol m⁻²s⁻¹) treatment for the time indicated in the absence (A) or presence of 1mM ATP (B) under anaerobic conditions at 20 °C, in the presence of 10 mM β-mercaptoethanol as external electron donor.

(C-D) The scanning absorption spectra of the mutant W400F protein purified from insect cells were recorded after blue light (450±15 nm, 1.8 mW·cm⁻² or 70 μmol m⁻²s⁻¹) treatment for the time indicated in the absence (C) or presence of 1mM ATP (D) under anaerobic conditions at 20 °C, in the presence of 10 mM β-mercaptoethanol as external electron donor.

(E-F) The difference spectra of the wild-type CRY1 proteins in the absence (E) or presence (F) of ATP.

(G-H) The difference spectra of W400F in the absence (G) or presence (H) of ATP.
Fig. S3 Photoreduction of the trp-triad W324F and W324A mutant proteins.  
(A-D) The scanning absorption spectra of the W324F (A-B) and W324A (C-D) mutant proteins purified from insect cells were recorded after blue light (450±15 nm, 1.8 mW·cm⁻² or 70 μmol m⁻²s⁻¹) treatment for the time indicated in the absence (A, C) or presence of 1mM ATP (B, D) under anaerobic conditions at 20 °C, in the presence of 10 mM β-mercaptoethanol as external electron donor.  
(E-H) The difference spectra of the W324F (E-F) and W324A (G-H) mutant proteins in the absence (E, G) or presence (F, H) of ATP.
**Fig. S4 Photoreduction of the trp-triad W377F and W377A mutant proteins.**

**(A-D)** The scanning absorption spectra of the W377F (A-B) and W377A (C-D) mutant proteins purified from insect cells were recorded after blue light (450±15 nm, 1.8 mW·cm⁻² or 70 μmol m⁻²s⁻¹) treatment for the time indicated in the absence (A, C) or presence of 1 mM ATP (B, D) under anaerobic conditions at 20 °C, in the presence of 10 mM β-mercaptoethanol as external electron donor.

**(E-H)** The difference spectra of the W377F (E-F) and W377A (G-H) mutant proteins in the absence (E, G) or presence (F, H) of ATP.
Fig. S5 Photoreduction of the non-trp-triad W334F and W356F mutant proteins.

(A-D) The scanning absorption spectra of the W334F (A-B) and W356F (C-D) mutant proteins purified from insect cells were recorded after blue light (450±15 nm, 1.8 mW·cm⁻² or 70 μmol m⁻²s⁻¹) treatment for the time indicated in the absence (A, C) or presence of 1mM ATP (B, D) under anaerobic conditions at 20 °C, in the presence of 10 mM β-mercaptoethanol as external electron donor.

(E-H) The difference spectra of the W334F (E-F) and W356F (G-H) mutant proteins in the absence (E, G) or presence (F, H) of ATP.
Fig. S6 Characterization of the GFP-CRY1 control and the trp-triad GFP-CRY1 mutant proteins. 

(A) Verification of the cry1 mutant background of the indicated transgenic lines. A diagram showing the CRY1 locus and the positions of primer pairs used in the PCR reactions (Top), and DNA gels showing the genomic PCR product amplified from the genomic DNA isolated from the indicated genotypes (Bottom) are shown. The genomic DNAs were extracted using CTAB method from the 3-week-old respective transgenic lines grown in 16h light/8h darkness condition.

(B-C) Subcellular localization of the GFP-CRY1 and GFP-CRY1 mutants. 5~7-day-old seedlings were grown on MS medium under continuous white light, epidermis of hypocotyl was peeled for fluorescence microscopy analyses. Images were taken using a Zeiss AxioImager Z1 microscope, by a Hamamatsu Orca-ER camera at 10×Magnification, and processed by the Zeiss Axiovision software. Enlarged partial images (red square) are shown in Fig.S7.
Fig. S7. The GFP-fusion proteins of the trp-triad mutants of CRY1 and the wild-type GFP-CRY1 controls are located in both the nucleus and cytosol. Seedlings grown condition and images capture were processed as described in Fig. S6. The amplified images of GFP-CRY1^{W-to-A} and GFP-CRY1^{W-to-F} trp-triad mutants in Fig. S6 are shown in (A) and (B), respectively.
Fig. S8. The trp-triad mutants (W-to-A) of CRY1 are active in mediating blue light inhibition of hypocotyl elongation.

(A) Immunoblot showing the expression levels of GFP-fusion proteins of the trp-triad (labeled in blue) and non-trp-triad mutants of CRY1 in transgenic plants. All transgenic lines are in the cry1 background. Samples were extracted from seedlings grown on MS medium under continuous white light, fractionated in SDS/PAGE (10%), blotted, probed with anti-CRY1 antibody (CRY1), stripped, and reprobed with anti-HSP90 antibody (HSP90; Santa Cruz Biotechnology) as the loading control. A series dilution of the sample prepared from plants expressing the GFP-CRY1 control (CRY1), and the parental cry1 mutant (cry1) are included to facilitate semi-quantification.

(B) The relative expression unit (REU) showing the semi-quantification of the indicated proteins was calculated by the formula \[ \frac{\text{CRY1}^{\text{mt}}/\text{HSP90}^{\text{mt}}}{\text{CRY1}^{\text{wt}}/\text{HSP90}^{\text{wt}}} \]. The “CRY1” and “HSP90” signals of immunoblots were digitized and quantified by ImageJ.

(C) Images showing the representative seedlings of indicated genotypes. Seedlings were grown on MS medium under continuous blue light (20 µmol·m\(^{-2}\)·s\(^{-1}\)), red light (18 µmol·m\(^{-2}\)·s\(^{-1}\)), far-red light (1.5 µmol·m\(^{-2}\)·s\(^{-1}\)), or in darkness for 5 d.

(D) Hypocotyl lengths of indicated genotypes grown under conditions indicated in C were measured (n≥20). The wild-type plants (Col-4), the cry1 mutant parent (cry1), and transgenic plants expressing the wild-type GFP-CRY1 (CRY1) controls are included. Error bars indicate SD.
Fig. S9. Hypocotyl inhibition phenotype of independent transgenic lines expressing wild-type GFP-CRY1 or the W-to-A mutants. Seedlings were grown under different light conditions as indicated, and hypocotyl lengths of indicated genotypes in independent transgenic lines were measured and shown with SD (n≥20). The trp-triad transgenic lines are highlighted in blue.
Fig. S10. Hypocotyl inhibition phenotype of independent transgenic lines expressing wild-type GFP-CRY1 or the W-to-F mutants. Seedlings were grown under different light conditions as indicated, and hypocotyl lengths of indicated genotypes in independent transgenic lines were measured and shown with SD (n≥20). The trp-triad transgenic lines are highlighted in blue.
5-day-old seedlings were grown on MS medium under continuous blue light of different fluence rates or in darkness. Hypocotyl lengths of indicated genotypes were measured and shown with SD (n≥20). The fluence rates are shown in the logarithmic scale, and the darkness is indicated by ∞.
Fig. S12.  Blue light-induced gene expression of transgenic lines expressing the indicated CRY1 mutants.

qPCR analysis of the mRNA expression of chalcone synthase (A, CHS), Sigma Factor 5 (B, SIG5) and photosystem II subunits (C, Psbs) genes in plants expressing the GFP-CRY1 or GFP-CRY1<sup>W324A</sup> trp-triad mutants. Total RNA was extracted from 7-d-old seedlings grown in darkness or continuous blue light (20 μmol·m<sup>-2</sup>·s<sup>-1</sup>). The qPCR signals of the respective genes are normalized to that of ACT2 and shown with SD (n=3).
Fig. S13. BiFC assay showing interaction between CRY1<sup>W-to-A</sup> mutants and the CRY1-interacting protein SPA1<sup>CT509</sup>. Three-week-old *Nicotiana benthamiana* plants grown in long-day (LD, 16h Light/8h Dark) were co-transformed with Agrobacterial strains harboring the plasmids encoding nYFP-CRY1 or nYFP-CRY1<sup>W400A</sup> mutants and cCFP-SPA1<sup>CT509</sup>. The plants were incubated for 12 h in the darkness after infection, transferred to white light for 48-72 h, and analyzed by the fluorescence microscopy. Images were taken by a camera at 100× magnification, the overlay of Cy3 autofluorescence (red) and DAPI (4′,6-diamidino-2-phenylindole) nuclear signal (blue), and the overlay of Cy3 (Red) and YFP BiFC signal (green) of guard cells are shown.
Fig. S14. BiFC assay showing interaction between CRY1 W-to-A mutants and the CRY1-interacting protein SPA1 CT509.

BiFC images of CRY1-SPA1 CT509 (A), W400A-SPA1 CT509 (B), W324A-SPA1 CT509 (C), W377A-SPA1 CT509 (D), W334A-SPA1 CT509 (E) and W356A-SPA1 CT509 (F) in Nicotiana benthamiana. The Co-transformation was performed as described in Fig.4. Images were taken by a camera at 10× magnification, The DAPI (4',6-diamidino-2-phenylindole) nuclear signal (blue), the YFP BiFC signal (green) and merge images are shown.