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Authors
Yang, Liang
Mo, Weiliang
Yu, Xiaolan
et al.

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Cell Reports

Report

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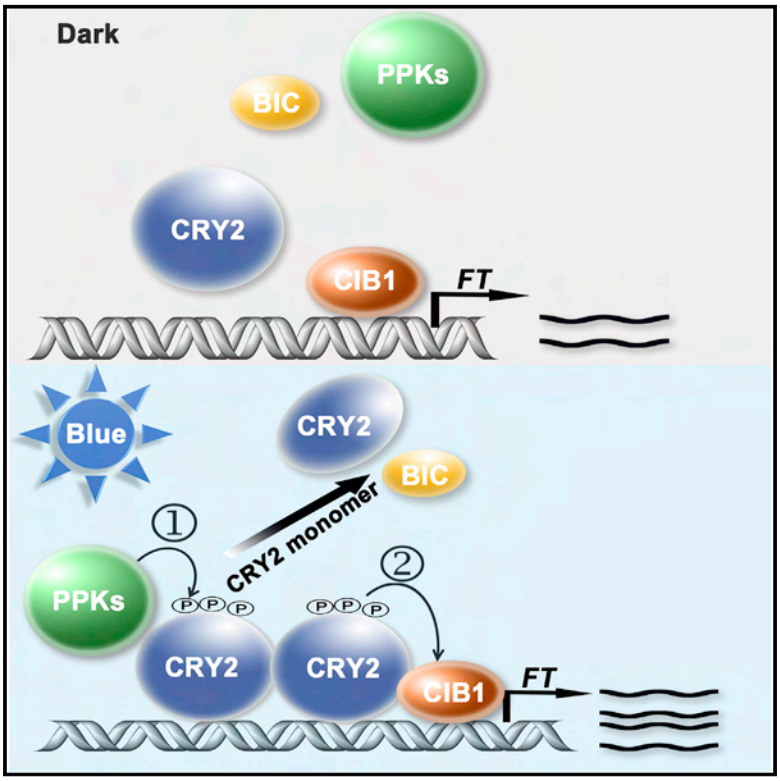
Graphical Abstract



Highlights

- *Arabidopsis* CRY2 directly binds DNA to activate transcription of *FT* under blue light
- Transcriptional activity of CRY2 is regulated by homodimerization
- CRY2 enhances the DNA affinity and transcriptional activity of CIB1
- CRY2 phosphorylation enhances the CRY2-CIB1 interaction to promote *FT* transcription

Authors

Liang Yang, Weiliang Mo, Xiaolan Yu, ..., Dehong Yang, Chentao Lin, Zecheng Zuo

Correspondence

zuozecheng@fafu.edu.cn

In Brief

Cryptochromes are the blue light receptors in plants. Yang et al. reconstitute the *Arabidopsis thaliana* CRY2 signaling pathway to investigate the mechanism of CRY2 transcriptional regulation in mammalian cells. They demonstrate that, besides being the light receptor, CRY2 binds DNA directly to activate transcription of *FT* in a blue-light-enhanced manner.

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Reconstituting *Arabidopsis* CRY2 Signaling Pathway in Mammalian Cells Reveals Regulation of Transcription by Direct Binding of CRY2 to DNA

Liang Yang,[1,2,4] Weiliang Mo,[2,4] Xiaolan Yu,[1,4] Nan Yao,[1,2] Zeng Zhou,[1] Xiaolu Fan,[1] Li Zhang,[2] Mingxin Piao,[1,2] Shiming Li,[1] Dehong Yang,[2] Chentao Lin,[3] and Zecheng Zuo[1,2,5,*]
[1]Basic Forestry and Proteomics Research Center, Fujian Agriculture and Forestry University, Fuzhou 350002, China
[2]Jilin Province Engineering Laboratory of Plant Genetic Improvement, College of Plant Science, Jilin University, 5333 Xi'an Road, Changchun 130062, China
[3]Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, Los Angeles, CA 90095, USA
[4]These authors contributed equally
[5]Lead Contact
*Correspondence: zuozecheng@fafu.edu.cn
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SUMMARY

In response to blue light, cryptochromes photoexcite and interact with signal partners to transduce signal almost synchronously in plants. The detailed mechanism of CRY-mediated light signaling remains unclear: the photobiochemical reactions of cryptochrome are transient and synchronous, thus making the monitoring and analysis of each step difficult in plant cells. In this study, we reconstituted the *Arabidopsis* CRY2 signaling pathway in mammalian cells and investigated the biological role of *Arabidopsis* CRY2 in this heterologous system, eliminating the interferences of other plant proteins. Our results demonstrated that, besides being the light receptor, *Arabidopsis* CRY2 binds to DNA directly and acts as a transcriptional activator in a blue-light-enhanced manner. Similar to classic transcription factors, we found that the transcriptional activity of CRY2 is regulated by its dimerization and phosphorylation. In addition, CRY2 cooperates with CIB1 to regulate transcription by enhancing the DNA affinity and transcriptional activity of CIB1 under blue light.

INTRODUCTION

Cryptochromes are photolyase-like proteins mediating the light regulation of gene expression and the circadian clock in plants and animals (Cashmore, 2003; Sancar, 2003). The cryptochromes originally identified in *Arabidopsis* include cryptochrome 1 (CRY1), which was found to mediate primarily plant photomorphogenesis under blue light, and its homolog (Cashmore, 1997) cryptochrome 2 (CRY2), which mainly regulates photoperiodic floral initiation (Guo et al., 1998). Differing from the animal cryptochromes, *Arabidopsis* cryptochromes mainly act as the blue light receptors that transduce the blue light signal by interacting with several signaling proteins (Liu et al., 2008; Pedmale et al., 2016; Zuo et al., 2012). However, the exact mechanism of *Arabidopsis* cryptochrome signal transduction remains to be elucidated (Liu et al., 2010, 2011). Because the idea of a non-plant-component reconstitution system was not previously entertained for the study of CRYs and CRY-related proteins, investigation into the biochemical characteristics of *Arabidopsis* cryptochromes has largely lagged. For example, photoexcited CRY2 interacts with several transcription factors (e.g., cryptochrome-interacting basic helix-loop-helix [CIB], phytochrome-interacting factor [PIF]) (Liu et al., 2013; Pedmale et al., 2016), and previous genetic studies implied that CRY2 plays a role in the transcriptional regulation of downstream genes (Liu et al., 2008; Pedmale et al., 2016); it has also been reported that CRY2 associates with chromosomes and is involved in the decondensation of chromatin (Cutler et al., 2000; van Zanten et al., 2012). However, because multiple components of the CRY2 signal network coexist in the plant nucleus, and the interference of CRY2-interacting protein is hard to eliminate in plant cells, it remains to be determined whether CRY2 regulates transcription and approaches chromatin directly by a cryptochrome-DNA interaction or indirectly via a protein-protein interaction (Lin and Shalitin, 2003; Liu et al., 2008; Pedmale et al., 2016). In addition, with the absence of a non-plant reconstitution system, dissecting the mechanism of CRY2-mediated signal transduction and analyzing individual biochemical reactions step by step are difficult tasks. For instance, the blue light-specific phosphorylation of CRY2 was identified >10 years ago (Shalitin et al., 2002) and is believed to play important roles in the function and regulation of CRY2 (Yu et al., 2007, 2009; Zuo et al., 2012). However, the exact functional role of CRY2 phosphorylation for the CRY2-mediated signaling pathway remains unknown (Liu et al., 2011, 2017). To resolve these technical impediments, we recently developed a plant protein expression system using HEK293T cells (Yang et al., 2016). This mammalian cell-based heterologous system offers numerous advantages over other expression systems we have used previously (Liu et al., 2008; Zuo et al., 2011) because it does not suffer from the major drawbacks of *E. coli*, yeast, and insect cells such as deficient chromophore, toxicity, low yield, and light-independent constitutive activities of *Arabidopsis* cryptochromes. In the present study, we reconstituted the minimal signal transduction

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pathway, from the perception of blue light to the expression regulation of the downstream light-responsive gene, in HEK293T cells. More important, the plant components and the biochemical state of CRY2 could be experimentally controllable in this reconstitution system. Each biochemical reaction involved in the CRY2-mediated signaling pathway was investigated individually. For example, we demonstrated that the phosphorylation of CRY2 is unnecessary for its protein-protein interaction; however, blue light-induced CRY2 phosphorylation could enhance the formation of CRY2-CIB1 heterodimerization. Furthermore, without the effect of other plant components, the biochemical profile of CRY2 was also recharacterized. This study shows that in addition to its previously well-documented role as the blue light receptor, *Arabidopsis* CRY2 functions as a transcriptional activator whose DNA affinity and transcriptional activity is induced by blue light. Under blue light, CRY2 directly binds to the G fragment of *FT* to promote transcription; at the same time, CRY2 enhances the transcriptional activity of CIB1 via the blue light-specific protein-protein interaction.

RESULTS

Reconstituting the CRY2-Mediated Blue Light Pathway in HEK293T Cells

We recently demonstrated a few biochemical characteristics of HEK293T-expressed CRY2, such as absorbing blue light via the chromophore flavin adenine dinucleotide (FAD), dimerization in response to blue light, and forming the blue light-specific photobodies (Liu et al., 2017; Wang et al., 2016; Yang et al., 2016). To reconstitute the plant blue light-signaling machinery in HEK293T cells, we further examined other reactions of CRY2 such as phosphorylation and degradation (Shalitin et al., 2002) in mammalian cells. As shown in Figure 1A (left), no phosphorylated CRY2 was detected in HEK293T cells after being irradiated with blue light, in contrast to previous observations of plant cells (Shalitin et al., 2003). However, after supplementing with the lysate of *cry1cry2* seedlings, HEK293T-expressed CRY2 was phosphorylated in a blue light-dependent manner (Figure 1A [right, shift band]), compared to the chromophore-deficient mutant of CRY2 ($CRY2^{D387A}$) (Figure S1A). Mass spectrometry analyses further confirmed that no phosphorylation sites in CRY2 were detected in HEK293T cells expressing only CRY2 (Figure 1B [blue bars]), unless photoregulatory protein kinases (PPKs) (Liu et al., 2017) were co-expressed with CRY2 (Figures 1B [gray bars] and S2). Similar to phosphorylation, HEK293T-expressed CRY2 was specifically degraded in blue light only after plant lysate was supplied (Figure 1C [bottom]). Taken together, these results suggest that the biochemical functions of CRY2 are normal in mammalian cells. More important, the biochemical state of recombinant CRY2 (e.g., phosphorylation, degradation, dimerization) could be experimentally controlled in HEK293T cells. This allowed the reconstitution of a controllable light-signaling pathway in mammalian cells and facilitated the investigation of the mechanism of the CRY2 signaling pathway (see below).

We next examined whether CRY2 communicates with its signaling partners properly in HEK293T cells. In addition to the CRY2-CIB1 interaction (Figure S1B) (Polstein and Gersbach, 2015; Taslimi et al., 2016), which is widely used as an optogenetics tool, recombinant CRY2 interacted with signaling partners in HEK293T cells in a blue-light-enhanced manner, e.g., SPA1 (suppressor of phyA), BICs (blue light inhibitor of cryptochrome), and PPKs, respectively (Figures S1C–S1G) (Liu et al., 2017; Wang et al., 2016; Zuo et al., 2011). Consistent with the co-immunoprecipitation findings, the results from fluorescence microscopy further support the blue light enhancement of co-localization of CRY2-GFP and its interaction partner (mCherry-CIB1, mCherry-SPA1) at photobodies in mammalian cells (Figures 1F and S1J). A uniform distribution of CIB1 and SPA1 was observed in the nucleus in the absence of CRY2-GFP (instead of GFP only) (Figures 1G and S1K) or co-expressed with $CRY2^{D387A}$-GFP (Figures S1L and S1M). It suggested that the blue light-specific formation of CIB1 and SPA1 photobodies occurs in a CRY2-dependent manner. Similar to plant cells (Liu et al., 2008; Zuo et al., 2011), COP1 constitutively interacts with CRY2 or CO in HEK293T cells (Figures S1H and S1I). In contrast, COP1 interacts with CRY2 in a blue-light-enhanced manner when SPA1 was additionally co-expressed in HEK293T cells (Figure 1D). Taken together, these results further suggest that the main steps and reactions of the CRY2 signaling pathway function properly in mammalian cells, thus mimicking the scenario found *in planta*. To investigate the biochemical mechanism through which CRY2 affects the activity of the SPA1/COP1 complex to transduce the blue light signal, we reconstituted the minimal set of components of the blue light-signaling pathway in HEK293T cells, from blue light perception to the downstream regulation of the functional protein (CO). In contrast to the CO-COP1 interaction occurring regardless of blue light irradiation in the absence of CRY2 and SPA1 (Figure S1I), we found that the interaction of CO and COP1 could be inhibited by the enhancement of CRY2-COP1 interaction under blue light (Figure 1E). Consistent with our previous hypotheses, this result demonstrated a photochemical mechanism, in which the photoexcited CRY2, SPA1, and COP1 form a new protein complex in response to blue light; this induces the COP1-SPA1-CO complex to disassemble (Figure 1E) and then protects CO from degradation and promotes floral initiation (Valverde et al., 2004). Because CRY2 could displace CO and interact with SPA1-COP1 under blue light, it corroborated the previous study in plant cells that demonstrated that CRY2 is degraded in a blue light-dependent manner via the formation of the CRY2-SPA1-COP1 complex (Weidler et al., 2012).

Arabidopsis CRY2 Is a Blue-Light-Regulated Transcription Regulator

To understand the exact biochemical mechanism of CRY2 in transcriptional regulation, we first examined the DNA-binding activity of CRY2 with chromatin immunoprecipitation (ChIP)-qPCR assay. Figure 2B shows that CRY2 could directly interact with several DNA fragments associated with *FT* genomic DNA (Figure 2A). The co-immunoprecipitation assay, using the biotin-labeled DNA fragments of *FT* genomic region as bait, further confirmed that CRY2 could bind DNA directly (Figure 2C) and exhibited the highest affinity with the G fragment (Figures 2B and 2C). We next examined whether the DNA affinity of CRY2 is responsive to blue light. As shown in Figure 2D, with

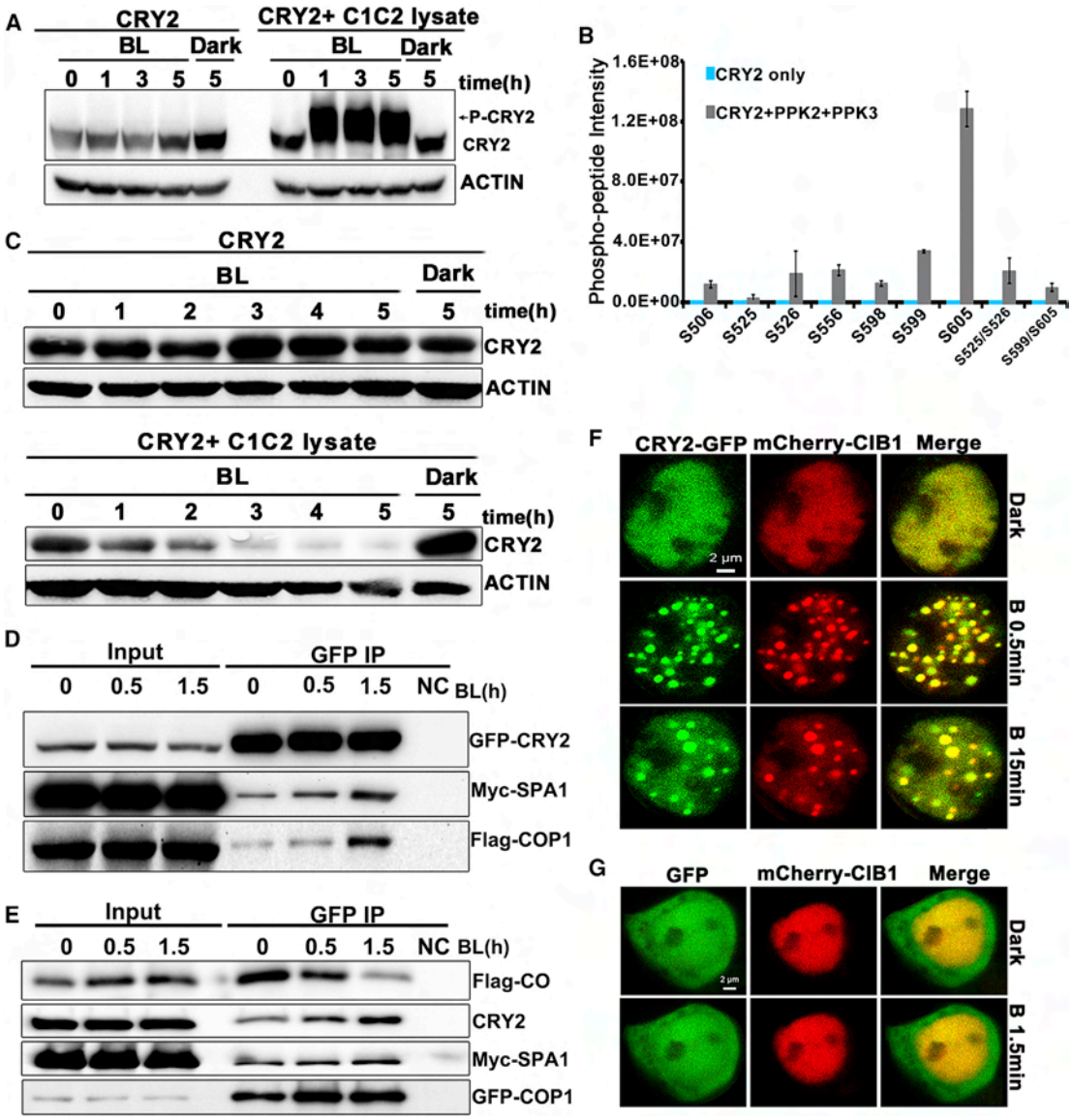

Figure 1. Reconstitution of the CRY2-Mediated Blue-Light-Signaling Pathway in Mammalian Cells

(A) HEK293T-expressing CRY2 was incubated with or without the *Arabidopsis cry1cry2* extraction mixture (C1C2 lysate) under blue light (35 μM m^{-2} s^{-1}) for 0, 1, 3, and 5 hr. Protease inhibitors were added. Samples were analyzed by western blot (WB) using the indicated antibodies.

(B) Bar graph showing that there is no phosphopeptide detected in HEK293T cells expressing CRY2 only (blue base on the x-axis, which indicates the intensity of phosphopeptide = 0) under blue light (35 μM m^{-2} s^{-1}), compared with HEK293T cells co-expressing CRY 2 and PPKs (gray bars).

(C) Similar to (A), without protease inhibitor.

(D) HEK293T cells co-expressing CRY2 and indicated proteins were kept in the dark or exposed to blue light (35 μM m^{-2} s^{-1}) for 0.5 and 1.5 hr. CRY2 was immunoprecipitated using GFP-trap beads, and CRY2-interacting proteins were detected by immunoblot with the indicated antibody.

(E) CRY2, MYC-SPA1, GFP-COP1, and FLAG-CO were co-transfected into HEK293T cells. GFP-COP1 was immunoprecipitated using GFP-trap beads, and the COP1-interacting proteins were detected by immunoblot.

(F and G) CRY2-AcGFP/mCherry-CIB1 (F) or AcGFP-only/mCherry-CIB1 (G) were co-expressed in HEK293T cells. After transfection, cells were treated with 488 nm laser light for the indicated time and imaged with a Zeiss LSM880 confocal scanning laser microscope.

an equal amount of CRY2 protein, CRY2 exhibited significantly higher affinity for the G fragment in the sample irradiated with blue light compared with the sample kept in the dark. We further verified the blue-light-enhanced DNA affinity of CRY2 with the co-immunoprecipitation (coIP) assay. As expected, CRY2 was co-immunoprecipitated by the biotin-labeled G fragment in the blue light-treated samples. In contrast, less CRY2 was co-immunoprecipitated by the G fragment under dark conditions (Figure 2E). If the DNA affinity of CRY2 is induced by blue light, then there is an expectation that the CRY2 apoprotein should

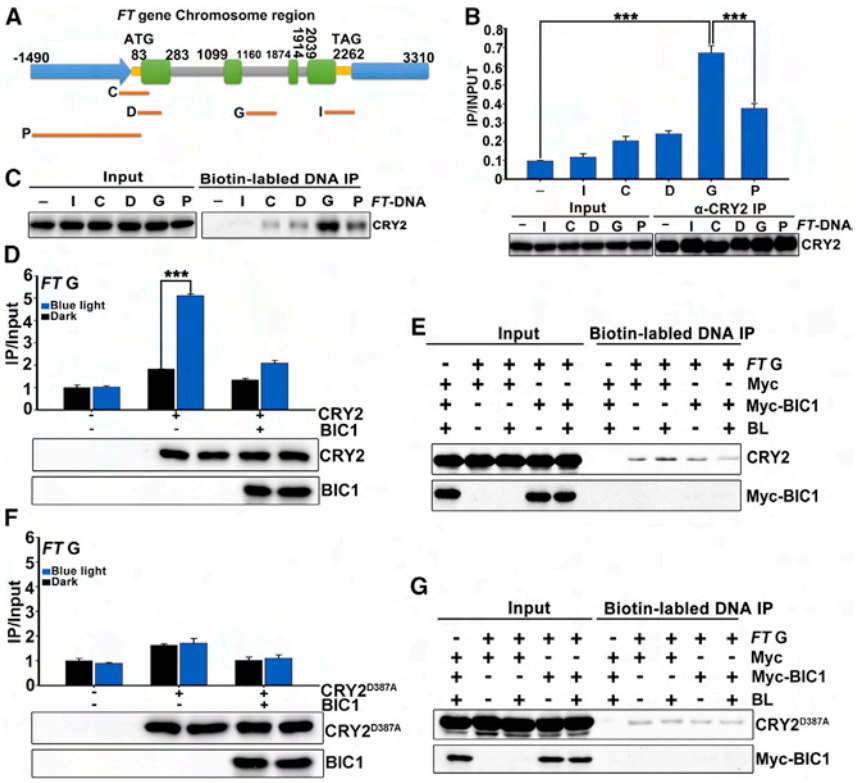

Figure 2. CRY2 Binds to Certain *FT* Fragments in a Blue-Light-Enhanced Manner

(A) Model of *FT* gene segments. Orange bars indicate the fragment of the *FT* chromatin region used to test DNA affinity and transcriptional activity of CRY2.

(B) ChIP-qPCR assay showed different CRY2 binding affinities to each *FT* fragment (I, C, D, G, and P) (top). CRY2 levels of each sample were detected by immunoblot (bottom).

(C) Biotin-labeled DNA immunoprecipitation assay indicated the different affinities of CRY2 for each *FT* fragment. Streptavidin agarose beads were used to immunoprecipitate the biotin-labeled *FT* fragments, and the immunoblot was performed to detect the co-immunoprecipitated CRY2 signal.

(D) Cells transfected indicated proteins were irradiated with blue light (35 μM m^{-2} s^{-1}) for 3 hr (blue bars) or kept in the dark (black bars). CRY2 was immunoprecipitated and the G fragment of the *FT* was analyzed using qPCR.

(E) Cells transfected indicated proteins were irradiated with blue light (35 μM m^{-2} s^{-1}) or kept in the dark. The biotin-labeled DNA coIP assays were performed as described in (C). BL, blue light.

(F and G) The blue light-deficient control of (D) and (E), respectively. $CRY2^{D387A}$ was expressed instead of CRY2 holoprotein.

Data are represented as means ± SDs (n = 3). $^{**}p < 0.01$; $^{***}p < 0.001$.

bind to DNA in a blue light-independent manner. The D387A mutant of CRY2 ($CRY2^{D387A}$), which is chromophore deficient and physiologically inactive, bound to the G fragment weakly and constitutively (Figures 2F and 2G), suggesting that the structure of the photoexcited holoprotein is important for the DNA affinity of CRY2.

To eliminate the potential effect of endogenous mammalian basic helix-loop-helix (bHLH) transcription factors, HEK293T-expressed His-CRY2 was purified and irradiated with blue light for ChIP-qPCR analysis (Figures S3C–S3F). None of the mammalian transcription factors was detected in the fractions containing high-level CRY2 (Figure S3F [fractions 10.5–11, 11–11.5, and 11.5–12]) using mass spectrometry (Table S1), and the amount of CRY2 protein and CRY2-immunoprecipitated G fragment exhibited a positive correlation (Figure S3F), suggesting that mammalian transcription factors are unlikely to affect our analyses of CRY2-DNA-binding activity. We next examined the DNA affinity of CRY2 in yeast. Compared to samples kept in the dark, a significantly stronger interaction of CRY2 with the G fragment was detected in yeast cells irradiated with blue light (Figure 3A). In addition, $CRY2^{D387A}$ exhibited a weak interaction with the G fragment and without blue light specificity in yeast cells (Figure S3A). These yeast-one-hybrid assays further confirmed that CRY2 directly binds to the DNA in a blue light-enhanced manner. It also implied that CRY2 may act as a transcriptional regulator to affect *FT* expression directly. To further determine the transcriptional activity of CRY2, we developed a transcription assay in HEK293T cells, based on the dual-luciferase assay. As shown in Figure 3B, the reporter LUC gene derived from the G fragment was activated in the HEK293T cells co-expressing CRY2 under blue light, compared with the cells co-expressing $CRY2^{D387A}$ (Figure S3B). Similar to the yeast-one-hybrid assay, the LUC report gene exhibited significantly higher activity in the cells irradiated with blue light compared to samples kept in the dark (Figure 3B). To exclude the potential interference of mammalian transcription factors in the transcriptional activity analyses, we analyzed the co-immunoprecipitated product of the G fragment and the co-immunoprecipitated CRY2 using mass spectrometry. None of the mammalian transcription factors could be co-immunoprecipitated by the G fragment and CRY2 simultaneously (Table S2). These results suggest that CRY2 could activate the G fragment of *FT* independently, and the transcriptional activity of CRY2 could also be enhanced in a blue-light-enhanced manner.

To prove the DNA-binding activity and the transcriptional activation function of CRY2 are of *in vivo* relevance in plants, we first investigated whether CRY2 associates with the G fragment in plant cells. As shown in Figure 3C, the ChIP-qPCR results suggested that CRY2 approaches the G fragment of the *FT* gene in a blue-light-enhanced manner in plant cells. Because there is no *Arabidopsis* CRY2-related feedback system existing in HEK293 cells (e.g., blue light-specific degradation of CRY2 and the inhibition of BICs), CRY2 exhibited a weaker DNA affinity in plant cells (Figure 3C) compared with HEK293 cells (Figure 2D). This suggests that the transcriptional activity of CRY2 is also under the regulation of feedback systems in plant cells. These results further demonstrated the advantages of our reconstitution system for plant mechanism studies because it facilitates the

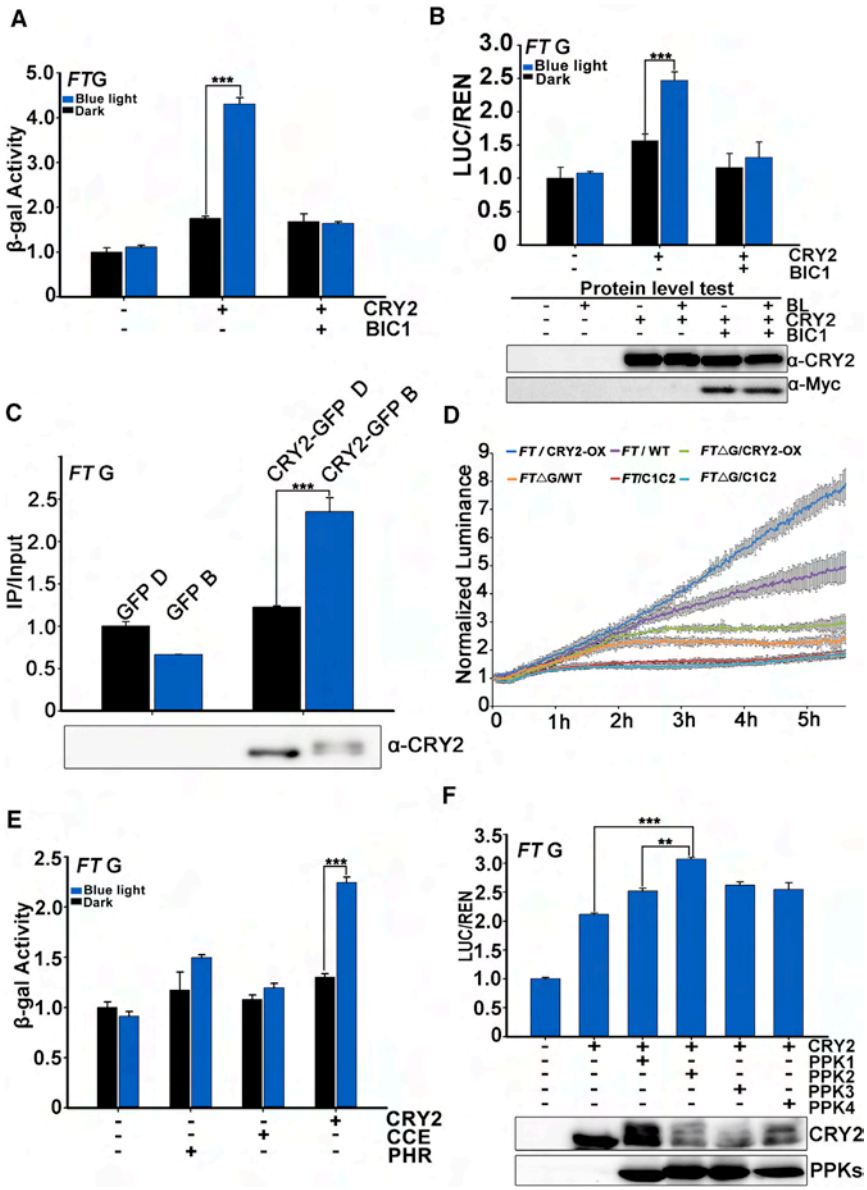

Figure 3. Blue Light Enhanced the Transcriptional Activity of CRY2

(A) Yeast cells transfected indicated proteins and G fragment were irradiated with blue light (35 μM m^{-2} s^{-1}) for 3 hr (blue bars) or kept in the dark (black bars). β-Galactosidase activities of each sample were assayed.

(B) The reporter (G fragment::Luc and SV40::Renilla), the effector (CRY2), and the inhibitor (BIC1) were transfected into HEK293T cells in the indicated order, − or +. Transfected cells were kept in the dark (black bars) or treated with blue light for 1 hr (blue bars). The relative firefly luciferase (LUC) activities were normalized to the Renilla luciferase (REN) activity. Protein expression was estimated by immunoblot.

(C) T3 seedlings, 14 days old, were transferred to the dark for 2 days and then were irradiated with blue light (35 μM m^{-2} s^{-1}) for 1 hr or kept in the dark. Immunoprecipitation was performed with GFP-trap beads, and the precipitated DNA was detected by qPCR. Immunoblots showed CRY2-GFP levels of input.

(D) pDT1(*FT*-LUC) or pDT1(*FT*ΔG-LUC) was transfected into different background seedlings (WT, *cry1cry2*, CRY2-OX) using the Agrobest method. *FT*-LUC and *FT*ΔG-LUC: see Figure S3G. Luciferase signals were collected at 1-min intervals every minute under blue light (15 μM m^{-2} s^{-1}) conditions; data were normalized using the value obtained from the first time point.

(E) PHR and CCE domains of CRY2 were transfected into yeast, and the β-galactosidase activities were detected as described in (A).

(F) HEK293T cells containing the reporter (G fragment::Luc and SV40::Renilla) were transfected with the indicated proteins. LUC/REN activities were detected as described in (B).

Data are represented as means ± SDs (n = 3–5). $^{**}p < 0.01$; $^{***}p < 0.001$.

discovery of the delicate mechanisms existing in plant signal transduction that can be difficult to identify by traditional genetic methods. Next, we examined whether the G fragment of *FT* plays a role in CRY2 regulating transcription. The *FT* or *FT*ΔG (deficient G fragment) gene with the native promoter of *FT* were fused to LUC (Figure S3G) and transformed into different genetic backgrounds (wild-type [WT], *cry1cry2*, and CRY2-OX [overexpression]). As shown in Figure 3D, the transcription level of *FT*-LUC increased in response to blue light in WT and CRY2-OX. In contrast, the increment of *FT*ΔG-LUC transcription is significantly suppressed because of the deficiency of the G fragment. It demonstrated that the G fragment is necessary for blue light to regulate the transcription of *FT*, thus suggesting that CRY2 regulates the transcription of *FT* under blue light in a G fragment-dependent manner. Furthermore, it demonstrated that the transcription of *FT* increased relative to CRY2 levels (Figure 3D), compared with *FT*ΔG. These biological phenomena occurring in plant cells are consistent with our finding in mammalian cells that CRY2 can directly bind to the G fragment of *FT* and regulate the transcription of *FT*.

The Transcriptional Activity of *Arabidopsis* CRY2 Is Regulated by Its Dimerization and Phosphorylation

We recently reported that like other photoreceptors, the photo-excited CRY2 could form homodimers and suppress by BICs (Wang et al., 2016). To further understand the biochemical mechanism of CRY2, we analyzed whether the homodimerization of CRY2 is necessary for its DNA affinity and transcriptional activity. Figures 2D and 2E show that with similar amounts of CRY2 (Figure 2D [bottom]) or biotin-labeled DNA, the DNA affinity of CRY2 is clearly suppressed under blue light in the HEK293T cells co-expressing BIC1. Likewise, in yeast cells, BIC1 also suppressed the interaction of CRY2 and the G fragment in blue light

(Figure 3A). As expected, the dual-luciferase assay indicated that BIC1 also inhibited the transcriptional activity of CRY2 in blue light (Figure 3B). These results suggested that the DNA affinity and transcriptional activity of CRY2 are dependent on its homodimerization. We hypothesized that the blue light-dependent photoexcitation and homodimerization of CRY2 may provide the proper conformation to enhance its DNA affinity and transcriptional activity. This hypothesis is consistent with the analyses of CRY2 domain activity. Neither the PHR domain (N-terminal photolyase homologous region) nor the CCE domain (C-terminal cryptochrome C-terminal extension) of CRY2 exhibited robust DNA-binding activity (Figures 3E and S4A) or transcriptional activity (Figure S4B) compared to the CRY2 holoprotein under blue light.

In the last decade, we have hypothesized that the blue light-specific phosphorylation of CRY2 may act as a signaling trigger for CRY2 signal transduction (Shalitin et al., 2002). However, without a reconstitution system of blue light signaling, this hypothesis has not been fully substantiated (Liu et al., 2017; Yu et al., 2010). Here, we show that the CRY2 is not phosphorylated in the absence of PPKs in HEK293T cells (Figures 1A and 1B). The protein-protein interaction data (Figures S1B–S1G) revealed that unphosphorylated CRY2 is sufficient to interact with CIB1, SPA1, COP1, and other proteins, thereby suggesting that the phosphorylation of CRY2 is unnecessary for CRY2 to interact with its signaling partners and to transduce the blue light signal. It therefore appears that the function of phosphorylated CRY2 must differ from our previous hypothesis. To further explore the biochemical characteristics of the CRY2 transcriptional machinery, we next checked whether the phosphorylation of CRY2 plays a role in its transcriptional activity. With similar or even lower amounts of CRY2 protein (Figure 3F [bottom]), phosphorylated CRY2 (co-expressing with PPK) exhibited higher transcriptional activities compared with the unphosphorylated form (Figure 3F [top]). In contrast, CIB1, which could not be phosphorylated by PPKs (Figure S4C [bottom]), exhibited similar transcriptional activity regardless of the presence of PPKs (Figure S4C [top]). Based on those observations, we concluded that the blue light-specific CRY2 phosphorylation could enhance the transcriptional activity of CRY2.

Photoexcited CRY2 Enhances the Transcriptional Activity of CIB1 under Blue Light

Given that CRY2 functions as a blue light-inducible transcription regulator (described above) and our previous results demonstrated that CRY2 blue light specifically interacts with the bHLH transcriptional factor CIB1 (Liu et al., 2008), we therefore investigated how CRY2 coordinates with CIB1 to regulate transcription. We first examined whether CIB1 interacts with CRY2 to regulate the DNA affinity of CRY2. As shown in Figure 4A, the DNA affinity of CRY2 exhibited no significant change in the blue light-irradiated HEK293T cell co-expressing CRY2 and CIB1 proteins, as compared with samples in which only CRY2 was expressed. We noticed that the DNA affinity of CRY2 increased in the dark in the presence of CIB1, a fact that could be explained by the weak interaction between CRY2 and CIB1 in the dark. It suggested the DNA affinity of CRY2 is robust enough in blue light, which could not be further enhanced by CIB1 in blue light but only in the dark. We next investigated whether the DNA affinity of CIB1 is induced by CRY2 or blue light. As expected, without CRY2 or other plant components, the DNA affinity of CIB1 was not regulated by blue light in HEK293T cells and was constant during the observed period (from dark to 30 min blue light treatment) (Figure 4B). In contrast, more CIB1 protein was co-immunoprecipitated by the G fragment of the *FT* in the blue light-treated HEK293T cells co-expressing CRY2 in comparison with HEK293T cells grown in the dark (Figure 4C). This suggested that the DNA affinity of CIB1 is enhanced by blue light in a CRY2-dependent manner. This result provided a molecular explanation for our previous observation that CIB1 promotes floral initiation by stimulating the *FT* mRNA expression in a CRY2-dependent manner. We further analyzed the transcriptional activity of the CRY2-CIB1 complex using the dual-luciferase assay. Similar to the result of Figure 4B, the transcriptional activity of CIB1 did not respond to blue light when CIB1 only was expressed in HEK293T cells. However, after being co-expressed with CRY2, the transcriptional activity of the CIB1-CRY2 complex exhibited blue light specificity (Figure 4D). Compared with those scenarios in which only CRY2 or CIB1 was expressed, the CRY2-CIB1 complex showed a significantly higher transcriptional activity in blue light conditions (Figure 4D), which suggested that CRY2 could enhance the transcriptional activity of CIB1 via the G fragment of the *FT* gene. Because a similar experiment is easy to perform and analyze in plants, which can act as a proof-of-concept for our reconstitution system mimicking the scenarios of plant cells, we examined whether CRY2 also enhances the DNA affinity of CIB1 *in planta*. As expected, CIB1 exhibited a higher and blue light-specific DNA affinity in wild-type seedlings. In contrast, no obvious activity increasing CIB1 or blue light specificity of CIB1 were detected in the *cry1cry2* mutant (Figure 4E).

We next investigated the role of CRY2 phosphorylation in the formation of the CRY2-CIB1 complex. The kinetics of the interactions between CRY2 and CIB1 were determined by the split LUC assay. As shown in Figure 4F, with similar expression levels of CRY2-nLUC and CIB1-cLUC, the interaction of CRY2-CIB1 was enhanced in HEK293T cells co-expressing PPKs, except PPK2, suggesting that CRY2 phosphorylated at most phosphorylation sites could increase the affinity for CIB1. The split LUC result was confirmed by the coIP assay, indicating that phosphorylated CRY2 exhibited a significantly higher affinity for CIB1 compared with unphosphorylated CRY2 (Figure 4F). The coIP assay results further confirmed that PPK1 enhances the CRY2-CIB1 interaction under blue light. It is interesting that with similar amounts of CIB1 and PPK expressed in HEK293T cells, the CRY2-CIB1 interaction is much stronger than the CRY2-PPK interaction in blue light (Figure 4G). This suggested that CRY2 preferentially interacts with downstream signaling partners to transduce the signal after phosphorylation, which is also consistent with our previous hypothesis that PPKs have a higher affinity for photoexcited and unphosphorylated CRY2 in blue light (Liu et al., 2017). Together with our previous results (Figures 1A, 1B, and S1B–S1G), these findings led us to conclude that although phosphorylation of CRY2 is not necessary for its protein-protein interaction, it may enhance the transcriptional activity of CRY2 and CRY2-CIB1 interaction to further promote the *FT* transcription and flower initiation.

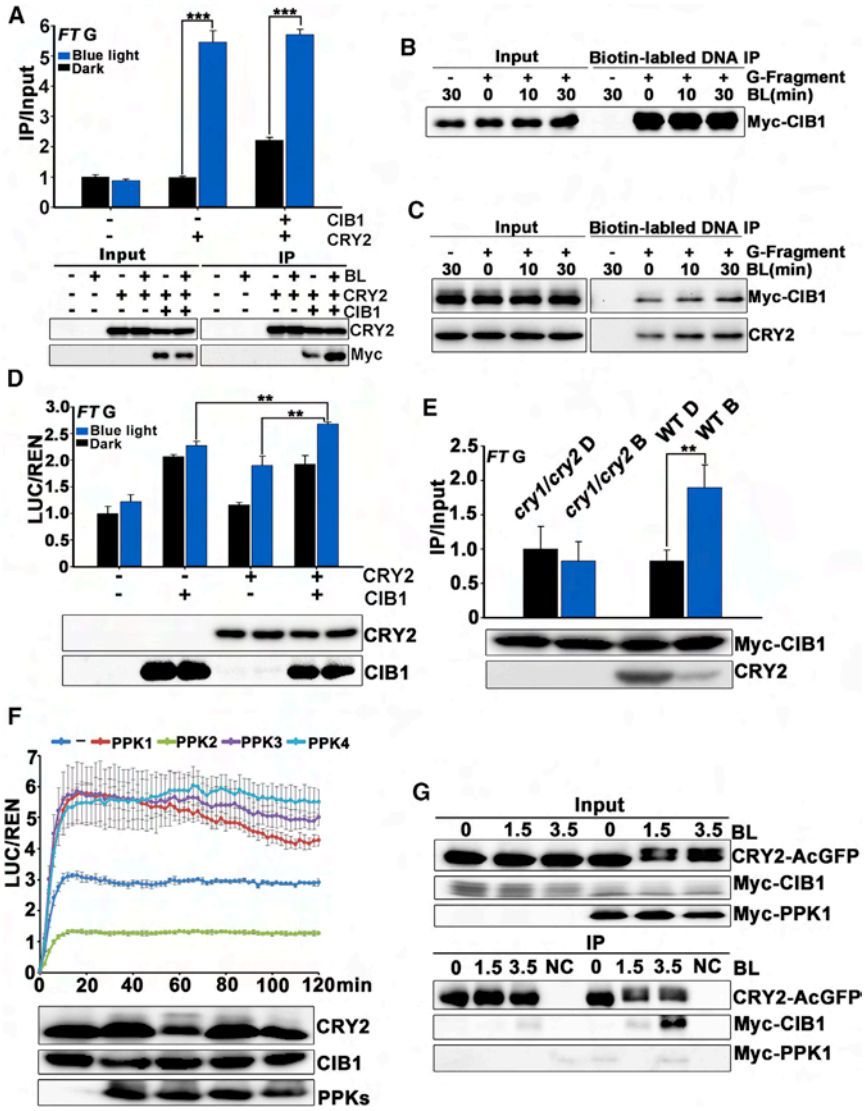

Figure 4. CRY2 Enhanced the DNA Affinity and Transcriptional Activity of CIB1 under Blue Light

(A) CRY2 antibody was used to perform the IP, and immunoprecipitated G fragments were detected using the qPCR assay. BL, blue light treatment.

(B and C) HEK293T cells expressing indicated protein were kept in the dark (0) or treated with blue light (35 μM m^{-2} s^{-1}) for 10 or 30 min. Co-immunoprecipitated CRY2 (B) and CIB1 (C) were detected by immunoblot with CRY2 and Myc antibody, respectively.

(D) HEK293T cells containing reporter, G fragment::Luc, and SV40::Renilla were transfected with indicated proteins. LUC/REN activities were measured as in Figure 3.

(E) Myc-CIB1 was transformed into *cry1cry2* double mutant and WT. Before the ChIP assay, T3 seedlings were irradiated with blue light (35 μM m^{-2} s^{-1}) for 1 hr or kept in the dark and used for the preparation of DNA fragment. The immunoprecipitation was performed with Myc-agarose beads and the precipitated DNA was detected by qPCR.

(F) HEK293T cells expressing cLUC-CRY2, nLUC-CIB1, and Renilla were co-transfected with FLAG-PPK or FLAG-only (control), respectively. LUC signal was collected every 2 min, from dark to 120 min blue light treatment.

(G) HEK293T cells expressing indicated proteins were irradiated with blue light (BL) (1.5 or 3.5 hr) or kept in the dark (0). CoIPs and immunoblots were performed as above with GFP-trap and Myc antibody, respectively.

Data are represented as means ± SDs (n = 3). $^{**}p < 0.01$; $^{***}p < 0.001$.

DISCUSSION

Cryptochromes evolved from common ancestral DNA photolyase binding to dimer-containing DNA. It is generally agreed that *Arabidopsis* cryptochromes transduce their signal by interacting with signaling proteins such as CIBs and SPA1. In the present study, we reconstituted the plant blue light-signaling pathways in mammalian cells. The biochemical characteristics of CRY2 were re-evaluated in HEK293T cells without the interference of other plant components involved in the CRY2 signal transduction pathway. The biochemical characteristic of CRY2 was re-checked, in that it binds to several DNA fragments of the *FT* gene directly. More interesting and differing from other DNA-protein interactions, the DNA affinity of *Arabidopsis* CRY2 can be enhanced in a blue light-dependent manner. In addition to HEK293T cells, the blue-light-enhanced DNA-binding activity of *Arabidopsis* CRY2 was further confirmed in yeast cells (Figure 3A) and in *in vitro* assays (Figures 2E and S3C–S3F). We also demonstrated that CRY2 has transcriptional activity via the G fragment of *FT*. Similar to its DNA-binding activity, the transcriptional activity of CRY2 could also be induced by blue light (Figure 3B). We noticed that CRY2, and even the transcription factor CIB1, did not exhibit a robust transcriptional activity in mammalian cells. However, the transcriptional activity of photoexcited CRY2 nearly reached that of CIB1 in blue light (Figure 4D [bars indicate only CRY2 or CIB1 expressed]), which implied that the transcriptional activity of CRY2 plays an important role in the blue light-signaling pathway. We further investigated the biological function of the G fragment and the biological relevance of CRY2 activity *in planta*. The plant cell-based results suggested that the transcription of *FT* increased relative to CRY2 levels and was regulated by CRY2 under blue light in a G fragment-dependent manner (Figures 3C and 3D), which is consistent with our discoveries in mammalian cells. It is interesting that both *Arabidopsis* CRY2 and CIB1 could recruit mammalian RNA polymerase to activate the transcription in mammalian cells, which may be explained by the fact that the transcriptional machinery is highly conserved in eukaryotes. It further suggested that a plant-specific general transcription factor or other component may exist in the plant transcriptional machinery, thus enhancing the recruitment of

RNA polymerase in plant cells. It is not fully understood whether CRY2 can directly regulate the transcriptional expression of other genes; in addition, the consensus DNA motif for CRY2 binding is not known. Further studies should focus on the systematic analysis of the CRY2-binding element or motif and other target genes to reveal more mechanisms of CRY2-based transcriptional regulation. For instance, the soybean CRY2 exhibited a different regulating activity compared with *Arabidopsis* CRY2 (Meng et al., 2013). The discovery of a different DNA motif or target gene associated with CRY2 in *Arabidopsis* and other species would provide clues to better understand the evolution and mechanisms of plant CRY2.

A mammalian cell-based reconstitution system is a valuable tool for the functional study of plant proteins involved in complex signaling networks, including multiple redundant components. More important, the post-translation modification can be experimentally controlled in a reconstitution system. For example, the function of CRY2 phosphorylation has been hypothesized for decades. However, the mechanism of blue light-induced phosphorylation of CRY2 is difficult to dissect and was always accompanied by and interfered with a series of photoreactions during CRY2 photoexcitation. Without direct evidence, the contribution of CRY2 phosphorylation in the blue light-signaling pathway remains unclear. In mammalian cells, CRY2 was phosphorylated only if PPKs were co-expressed, which means the state of CRY2 phosphorylation could be controlled experimentally and analyzed individually. Our results suggested that the phosphorylation of CRY2 was not necessary for CRY2-CIB1 interaction, but phosphorylated CRY2 enhanced the affinity for CIB1 and the transcriptional activity of the CRY2-CIB1 complex. Likewise, the mammalian cell reconstitution system facilitated the analyses of complex signaling networks. It has been suggested that CIB1 interacts with CRY2 and the function of CIB1 is dependent on CRY2. Because the transcription of *FT* is controlled by multiple components of the complex light-signaling network, the detailed mechanism of CRY2 regulating the transcription of *FT* via CIB1 remains unclear. However, without the interference of other plant components, we analyzed the mechanism through which CRY2 directly regulates the transcriptional activity of CIB1 in mammalian cells. To verify the results obtained with our reconstitution system, the regulation mechanism of CRY2-CIB1 was also verified in plant cells (Figure 4E). Our results indicated that photoexcited CRY2 enhanced the DNA affinity and the transcriptional activity of CIB1 under blue light independently, which may explain our previous observation that CIB1 activity is dependent on CRY2. These results are consistent with the principle of transcriptional synergy, which suggested that one transcription factor is much weaker than several bound TF molecules to attract the transcriptional machinery (Carey, 1998; Todeschini et al., 2014). It suggested that photoexcited CRY2 acts as a transcriptional regulator possessing the DNA affinity and transcriptional activity and cooperates with the transcription factor CIB1 to regulate *FT* transcription in blue light.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.06.069.

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AUTHOR CONTRIBUTIONS

Z. Zuo conceived the study, designed the experiments, and wrote the paper. L.Y. mainly focused on the dual-luciferase assay, the split-luciferase assay, and part of coIP. W.M. performed the yeast one-hybrid assay (Y1H), biotin DNA immunoprecipitation, and part of the biochemistry assay. X.Y. mainly conducted ChIP, part of the coIP, and the biochemistry assay. C.L. revised the manuscript. N.Y., Z. Zhou, X.F., L.Z., M.P., S.L., and D.Y. participated in the data process, vector construction, plant transfection, and liquid chromatography-mass spectrometry (LC-MS) analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-CRY2	N/A	N/A
Mouse monoclonal anti-Myc	MBL	M047-3
Mouse monoclonal anti-Flag	MBL	M185-3L
Rabbit polyclonal anti-GFP	MBL	598
Mouse monoclonal anti-ACTIN	MBL	M177-3
Chemicals, Peptides, and Recombinant Proteins		
MG132	Sigma	C2211-5MG
Cocktail	Roche	04693159001
streptavidin agarose	ThermoFisher	20349
Luciferin	Goldbio	Luck-1g
Forskolin	Sigma	F6886
Dex	Sigma	D1756
PMSF	PMSF	78830-5G
phosphatase phosphatase inhibitor	Roche	490683700
acrylamide	Sigma	A3553-500
bis-acrylamide	Sigma	146072
Ni-NTA Agarose	ThermoFisher	R90101
Lipofectamine 3000	ThermoFisher	L3000015
PEI-max	Polysciences	24765-1
FBS	Biological Industries	04-001-1ACS
DMEM	Biological Industries	06-1055-57-1ACS
TrypLE Express	ThermoFisher	12605-010
Critical Commercial Assays		
ChIP-DNA clean kit	Zymo research	D5205
Dual-Luciferase® Reporter Assay System	Promega	E1910
Experimental Models: Cell Lines		
HEK293T	ATCC	ATCC® CRL-11268TM
Experimental Models: Organisms/Strains		
Arabidopsis: 35S::*Myc-CIB1*/WT	Liu et al., 2008	N/A
Arabidopsis: 35S::*Myc-CIB1/cry1cry2*	Liu et al., 2008	N/A
Arabidopsis: 35S::*GFP*/WT	Liu et al., 2017	N/A
Arabidopsis: 35S::*GFP-CRY2*/WT	Liu et al., 2017	N/A
Arabidopsis: Col-4(WT)	Liu et al., 2008	N/A
Oligonucleotides		
Q-PCR for *FT* fragments	Liu et al., 2008	N/A
Other primers see Table S3	N/A	N/A
Recombinant DNA		
pNL 2.2	Promega	N1071
pCI(neo)	Promega	E1841
pDT1	Liu et al., 2017	N/A
pGAD424	Clonetech	K1605-B
pBridge	Clonetech	630404

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Dr. Zecheng Zuo (zuozecheng@fafu.edu.cn).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Arabidopsis

All *Arabidopsis* lines used in this work are of the Columbia (Col) accession. The 35S::*GFP-CRY2*/WT, 35S::*GFP*/WT, 35S::*Myc-CIB1*/WT and 35S::*Myc-CIB1/cry1cry2* were described as previously (Liu et al., 2008, 2017). Plants were grown in walk-in growth chambers at 22°C, 65% relative humidity under cool white fluorescent tubes. Long-day (LD) photoperiod is defined as 16 h light/8 h dark. Light-emitting diode was used to obtain monochromatic blue light (peak 450 nm; half-bandwidth of 20 nm).

Cell Culture

HEK293T cells were obtained from ATCC (CRL-11268TM) and routinely cultured in 75cm^2 flasks in Dulbecco's Modified Eagle Media medium (DMEM, 06-1055-57-1ACS, Biological Industries) with 10% fetal bovine serum (FBS, 04-001-1A, Biological Industries) under 37°C, 5% CO_2 condition.

METHOD DETAILS

HEK293T cell culture and transfection

HEK293T cells were routinely cultured as we previously described (Yang et al., 2016). And transfections were performed using Lipofectamine 3000 as manufacture manual or modified PEI-max (Akinc et al., 2005; Longo et al., 2013) method.

Biotin labeled DNA IP experiment

Biotin labeled DNA IP was performed as Kenneth K. Wu (Wu, 2006) method with some modifications. DNA was amplified by PCR, both forward and reverse primers were labeled with biotin. Purified DNA products were incubated with streptavidin agarose for 12h before pull down. The lysate of the transfected and light treated cells was incubated with beads and kept in the dark or transferred to blue light conditions for 3 hr, and then beads were washed with PBS for 5 times. Supernatants were detected by immunoblot using different antibodies.

Co-immunoprecipitation assays

Transfected cells were incubated in the dark prior to co-IP assay. For blue light treatment, cells were irradiated by blue light (35 μmol m^{-2} s^{-1}) for different lengths of time; then cells were collected. And co-IP experiments were performed similar to we described before (Yang et al., 2016).

Yeast one-hybrid assay

The prey vector pGAD-424 expressing CRY2 or CRY2^{D387A}; the expression vector pBridge-M (cutting off GAL4 DNA binding domain) expressing 4XMyc (as a control to BIC1) or BIC1 and the pLacZi vector including G fragment of *FT* or not (control) were transformed into the yeast strain YM4271. Prior to liquid assay, transformed yeast were cultured in YPDA medium for 16 h (in dark), and then irradiated with blue light (35 μmol m^{-2} s^{-1}) or kept in the dark for 3h. Yeast transfect, selected and β-galactosidase activity was performed according to the Clontech Yeast Protocols Handbook (YPH, PT3024-1).

Fluorescence microscopy

HEK293T cells were cultured in 35mm confocal Petri dish, and plasmids pCI-neo expressing indicated fluorescence fusion protein were transfected using the Lipofectamine 3000 method. 24 hr post transfection, cells were observed under a confocal laser scanning Zeiss LSM880 microscope. For photobody observations, cells were excited with 5% of 488nm laser light for 0.5min, 1.5min, 5min or 15min. Fluorescent signals were detected at 488nm for GFP and 561nm for the mCherry channel.

Dual Luciferase Assay

PNL 2.2 plasmid was modified by replacing the Hyg gene with Renilla; and the fragment of *FT* is also cloned into this vector to driver the report gene Luciferase. HEK293T cells were co-transfect the desired vectors using Lipofectamine 3000 method. Following 24h of incubation, cells were treated with blue light and LUC/REN assay were performed as manufacture manual.

Split Luciferase Assay

The plasmids expressing cLUC, nLUC fusion protein and Renilla were transfected using Lipofectamine 3000. After transfection and a 24-hour incubation, cells was washed with PBS (pH 7.2) and coated with 10ml DMEM without FBS medium, at 37°C, 5% CO_2 incubated 1h. Cells were resuspend with 1mL DMEM (without Phenol red) with 10% FBS and 1mM Luciferin, 1 μM Forskolin, 200nM Dex

medium (Ramanathan et al., 2012). After incubated in the dark for 20min, luciferase and renilla signals was detected by the luminometer (Berthold LB942).

ChIP-qPCR assay

Fragments of *FT* gene were amplified by PCR. Transfected HEK293T cells were then lysed using the co-IP lysis buffer, and 1 μg a particular *FT*'s PCR product was mixed with the lysed supernatant. 50 μl mixture was withdrawn and used as a loading control. Before immunoprecipitation, the CRY2 antibody was incubated with BSA and Salmon sperm DNA pretreated protein A agarose beads. IP was performed overnight, and beads were washed with different buffers. Elution buffer was used to elute DNA, and ChIP-DNA clean kit was used to purify DNA. Primers for individual *FT* fragments were used to carry out qPCR. Protein expression was detected by immunoblot.

Agrobest and transcriptional activity test in plant cell

A 3689bp DNA fragment of *FT* gene including the native promoter (−1509 to +2108) was cloned and fused with the LUC gene in the vector pDT1 for constructing pDT1(*FT*-LUC). For constructing pDT1(*FT* △G-LUC), two DNA fragment of *FT* gene (−1509 to +1314) and (+1582 to +2108)were amplified respectively,then amplified together by overlap PCR and cloned into pDT1,Plant transfect and Luciferase assay were performed as the Agrobest method described previously (Wu et al., 2014). In brief, seeds were kept dark and incubated at 4°C for 3 days,sterilized in 10% NaClO for 15min for 2 times,then rinsed 3 times with sterile water,resuspended with 1/2 MS at last time,20-30 seeds were transferred to 1 mL 1/2 MS liquid medium (1/2 MS salt supplemented with 0.5% sucrose (w/v), pH 5.5) in each well of a 6-well plate. Then took the 6-well plate in a growth incubator at 22°C under a 16-hr/8-hr light–dark cycle (75 μmol m^{-2} s^{-1}). Agrobacterium C58C1 should be streaked out from −80°C glycerol stock onto a 523 agar plate in the same day,after two days,several colonies from the plate were picked out into 5 mL 523 liquid medium containing 50ug/ml Kanamycin 50ug/ml Rifampicin and 10ug/ml Tetracycline antibiotics for shaking (220rpm) at 28°C for 24 hr. The cells were pelleted and re-suspended to OD600 0.2 by AB-MES (17.2 mM K2HPO4, 8.3 mM NaH2PO4, 18.7 mM NH4Cl, 2 mM KCl, 1.25 mM MgSO4,100 μM CaCl2, 10 μM FeSO4, 50 mM MES, 2% glucose(w/v), pH 5.5) with 200 μM AS without any antibiotics. then shaken (220 rpm) at 28°C for 16 hr, the Agrobacterium were pelleted and re-suspended to OD600 0.02 by ABM-MS (1/2 AB-MES, 1/4 MS, 0.25% sucrose (w/v), pH 5.5) with 200 μM AS,1ml re-suspended cells were added into each well of 6-well plate, the co-cultured in a growth incubator at 22°C under a 16-hr/8-hr light–dark cycle (100 μM m^{-2} s^{-1}) for 3 days. Seedlings were washing 3 times with 1/2 MS containing 100 μM Timentin and transferred into a 96-well plate which contained 1/2 solid MS with 3mM luciferin and 100 μM Timentin, after 12h incubation under continuous dark,Luciferase signals was detected by the luminometer (Berthold LB960) every one minute under blue light(15 μM $m^{-2}s^{-1}$) condition.

Gel filtration

Cells were cultured in 150 cm^2 as above and CMV-His-CRY2 (pCI-neo His-CRY2) was transfected. Transfected cells were lysed by co-IP lysis buffer (with inhibitor). Ni-NTA Agarose was used for immunoprecipitation, after 2.5h incubation, agarose beads were washed 5 times by co-IP lysis buffer (with reduced detergent). His-CRY2 was eluted by 250mM imidazole. Phenomenex BioSep-SEC-S3000 column and Thermo UltiMate 3000 HPLC system were used for gel filtration. PBS (pH 7.2) was used for mobile phase with 1ml/min flow rate. From 6 min-14 min, fraction was collected every 30 s.And Immunoblot detected CRY2 fraction. Fraction from 10min to 12.5min were concentrated by 30KD ultrafiltration tube. His-CRY2 was diluted with 400 μL ChIP dilution buffer (1% Triton X-100,2mM EDTA pH8.0,20mM Tris-HCL pH8.0,150mM NaCl with inhibitor), and mixed with 1 μg *FT* G DNA, and CRY2 antibody for ChIP. Western blot and Q-PCR was performed as above.

LC-MS/MS

Phosphorylation of CRY2

HEK293T expressing CRY2 (pCI-neo-CRY2) were co-transfected with CMV::Flag-PPK2(pCMV-Flag-PPK2s) /CMV:: Flag-PPK3 (pCMV-Flag-PPK3s) or empty control vector, and then kept in the dark for 24h, prior to being irradiated with 35 μM m^{-2} s^{-1} blue light. Transfected cells were lysed using the phosphorylation lysis buffer (Tris HCl 50mM, pH 7.4, EDTA 1mM, NaCl 150mM, NP40 1%, Na deoxycholate 0,25%, Protease inhibitors and Phos-stop). CRY2 antibody was used for immunoprecipitation, and Coomassie brilliant blue stained SDS-PAGE gels to identify phosphorylation modification. Proteins digestion and mass spectrometry were performed as previously described (Liu et al., 2017). Briefly, Orbitrap-Fusion-Tribrid mass spectrometer (Thermo Scientific) was used for MS analyses, and the data dependent acquisition (DDA) and parallel reaction monitoring (PRM) method was used to collect data. For DDA, MS1 mass resolution was set as 60 K with m/z 350-1550 and MS/MS resolution was set as 30 K under HCD mode. The dynamic exclusion was set as n = 1, and the dynamic exclusion time was 30 s, AGC target is 5e4, max injection time is 80 ms. DDA raw data were analyzed using Maxquant (1.5.6.0) (Tyanova et al., 2016). For PRM, AGC target was 2e5 and the maximum injection time was set to 100 ms, and the resolution of MS1 full scan was set to 60,000; the resolution of multiple PRM scans (MS2) was 60,000. Precursors of each phosphorylated peptide were selected by the quadrupole mass analyzer (1.2 Da isolation window). Raw PRM data were analyzed using Skyline daily (version 3.5) (MacLean et al., 2010).

Identification of Immunoprecipitation product

Immunoprecipitation product were digested as previously described (Liu et al., 2017). For Table S2, the Immunoprecipitation product of G fragment and CRY2 were digested and mass-spec analyzed, respectively. The mass spectrometer was run under data dependent acquisition mode. MS1 mass resolution was set as 60 K with m/z 350-1550 and MS/MS resolution was set as 30 K under HCD mode. The dynamic exclusion was set as n = 1, and the dynamic exclusion time was 30 s, AGC target is 5e4, max injection time is 80 ms (Zhang et al., 2013). MS/MS raw data of three biological replicates were analyzed using Protein Discoverer 2.1 (Thermo Fisher Scientific, San Jose, CA, USA; version 2.1) (Zhang and Elias, 2017). Sequest was set up to search Human+ *Arabidopsis* CRY2 fasta library. Sequest was searched with a fragment ion mass tolerance of 0.020 Da, a parent ion tolerance of 10.0 ppm and max miss cleavage of 2. Carbamidomethyl of cysteine was specified in Sequest as fixed modifications. Oxidation of methionine and acetyl of the n-terminus were specified in Sequest as variable modifications. Protein identifications was controlled by FDR < 0.01 and unique peptides ≥ 2. Transcription factor (or presumed) was determined with HGNC (Yates et al., 2017) and Human transcription factors database (Lambert et al., 2018).

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical data were collected in an Excel, ANOVA with two-tailed Student's-t test (Schmittgen and Livak, 2008) were used to evaluate statistical significance in excel,while $^{**}p < 0.01$, $^{***}p < 0.001$. All data were reported as mean ± SD.