CIB1 and CO interact to mediate CRY2-dependent regulation of flowering

Yawen Liu^{1,2} , Xu Li¹, Dingbang Ma^{1,2}, Ziru Chen^{1,2}, Jia-Wei Wang¹ & Hongtao Liu^{1,*}

Abstract

Cryptochromes are photolyase-like photoreceptors. *Arabidopsis* CRY2 (cryptochrome 2) primarily mediates the photoperiodic regulation of floral initiation. CRY2 has been shown to promote *FT* (FLOWERING LOCUS T) mRNA expression in response to blue light by suppressing the degradation of the CO (CONSTANS) protein and activating CIB1 (CRY2-interacting bHLH1). Although CIB1 and CO are both transcriptional activators of *FT*, their relationship is unknown. Here, we show that CIB1 physically interacts with CO and promotes *FT* transcription in a CO-dependent manner. CRY2, CIB1, and CO form a protein complex in response to blue light to activate *FT* transcription, and the complex is regulated by the photoperiod and peaks at dusk along with higher *FT* expression. We also determined that CRY2 was recruited to the *FT* chromatin by CIB1 and CO and that all three proteins are bound to the same region within the *FT* promoter. Therefore, there is crosstalk between the CRY2-CO and CRY2-CIBs pathways, and CIB1 and CO act together to regulate *FT* transcription and flowering.

Keywords CIB1; CO; cryptochrome; flowering time; FT

Subject Categories Development & Differentiation; Plant Biology; Transcription

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Introduction

Cryptochromes (CRYs) are photolyase-related blue light receptors that mediate light responses in plants and animals [1–3]. Photoexcited CRYs undergo several biophysical and biochemical changes, including circular electron transfer, phosphorylation, ubiquitination, and conformational changes, to alter gene and protein expression at both transcriptional and posttranslational levels [4,5]. The *Arabidopsis* genome encodes at least two cryptochromes, cryptochrome 1 (CRY1) and CRY2. The major function of CRY1 is to mediate blue light-dependent de-etiolation responses [6], whereas

the primary role of *Arabidopsis* CRY2 is the photoperiodic regulation of floral initiation [7].

CO (CONSTANS) and FT (FLOWERING LOCUS T) are among the most important regulators of floral initiation in response to photoperiod by integrating light and temporal signals [8,9]. CO is a transcriptional regulator that promotes flowering by activating *FT* mRNA expression [10,11]. FT is a RAF kinase inhibitor protein, which acts as a long-distance signal by migrating from the leaves through the vascular system to the apical meristem [12,13]. The amount of *FT* mRNA is very important for the flowering time. The transcriptional activation of *FT* in response to long-day (LD) conditions is the limiting step in the photoperiodic induction of flowering in *Arabidopsis*.

CRY2 has been shown to activate *FT* mRNA expression in response to blue light by suppressing the degradation of the CO protein [14–16] and by direct activation of the CIB1 (CRY2-interacting bHLH1) transcription factor [17–19]. The ability of CRY2 to stabilize the CO protein may be partially explained by the light-independent formation of the CRY2-COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1)-SPA1 (SUPPRESSOR OF PHYA-105) complex [20–22]. COP1 is an E3 ubiquitin ligase that targets CO and several other transcription factors for degradation [20–22], and photoexcited CRY2 interacts with SPA1 to suppress COP1-dependent degradation of CO. CIB1 is the first blue light-dependent CRY2-interacting protein to be described. It promotes flowering in a CRY-dependent manner by activating the transcription of *FT*. Several homologs of CIB1 (CIB2, CIB4, and CIB5) function redundantly with CIB1 to activate the *FT* transcription by forming different heterodimers. All of these homologs positively regulate CRY2-mediated photoperiodic flowering and *FT* transcription [17,18].

Although CIB1 and CO are both transcriptional activators of *FT*, their relationship is unknown. Here, we demonstrate that CIB1 and CRY2 promote *FT* transcription and flowering in a CO-dependent manner, while CO can act independently of CIBs. CIB1 and CO physically interact to induce *FT* transcription together. CRY2, CIB1, and CO form a protein complex in response to blue light. In addition, CRY2 is recruited to the *FT* promoter by CIB1 and CO, and they all bind to the same chromatin region; therefore, the CRY2-CO and CRY2-CIBs pathways interact to regulate *FT* transcription and flowering.

¹ National Key Laboratory of Plant Molecular Genetics, CAS Center for Excellence in Molecular Plant Sciences, Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China

² University of Chinese Academy of Sciences, Shanghai, China

*Corresponding author. Tel: +86 21 54924291; E-mail: htliu@sibs.ac.cn

Results and Discussion

CIB1 promotes FT transcription and flowering in a CO-dependent manner

CIB1 is the first blue light-dependent CRY2-interacting protein to be described, and it promotes FT transcription and flowering in a CRY2-dependent manner [17–19]. CO is another transcriptional activator of FT [10,11], but its relationship with CIB1 in the activation of FT is unknown. When we overexpressed CIB1 in *ft-1*, *co-1*, *co-2*, and *gi* mutants, our results indicated that the early flowering phenotype of *35S:Myc-CIB1* was abolished in the *ft-1* and *co* mutant backgrounds (Figs 1A–C and EV1A–F). These results indicated that the promotion of flowering by CIB1 was dependent on both CO and FT. In addition, CIB1 could not promote FT transcription in the *co* mutant backgrounds (Figs 1D and EV1G), even though the transcription and protein levels of CIB1 were similar or even higher in the *co* mutants than in the WT (wild type) background (Figs 1D and E, and EV1G and H). *ft-1* is a recessive mutant with a point mutation at amino acid 171 (G171E) which resulted in non-functional FT protein [23,24], CIB1 also promoted the transcription of the mutated FT in *ft-1*, though the mutated FT was un-functional (Fig EV1G and

H). CIB1 could not promote flowering in the *ft-1* and *co* mutant backgrounds not because *ft-1* and *co* mutants were late flowering, since CIB1 still could promote flowering and FT transcription in the *gi* (GIGANTEA) mutants which were also late flowering (Appendix Fig S1A–E). Blue light-dependent CRY2 phosphorylation, degradation, and also the interaction between CRY2 and CIB1 were not affected by GI protein, since CRY2 still gets phosphorylated and degraded under the blue light even in the *gi* mutant, and CRY2 still could co-precipitated with CIB1 in the *gi* mutant (Appendix Fig S2A and B). Interestingly, CIB1 could also promote FT transcription in the *cop1-6* mutant background, and *Myc-CIB1/cop1-6* showed similar early flowering phenotype as *cop1-6* (Appendix Fig S3). These data indicate that CIB1 promotes flowering and FT transcription in a CO-dependent manner.

CO can act independently of CIBs

There are about 170 bHLH proteins in *Arabidopsis thaliana*. CIB1 belongs to the 17-member bHLH subfamily 18; of this family, CIB4, CIB5, CIL1, and HBI1 are also involved in flowering [17,18,25], suggesting a functional redundancy among these genes. Indeed, although *cib1 cib5* double and *cib1 cib2 cib5* triple mutants showed

Figure 1. CIB1 promotes FT transcription and flowering in a CO-dependent manner and CO acts independently of CIBs.

A–J (A, F) 52-day-old (A), or 25-day-old plants (F) of the genotypes indicated grown in LDs. Scale bars: 5 cm. (B, C, G, H) The time to flowering and the number of rosette leaves at the time of flowering of the genotypes shown in (A) (for B and C), (F) (for G and H); the numbers indicate independent transgenic lines. The letters “a” to “c” indicate statistically significant differences between flowering time and leaf numbers of the indicated genotypes. (D, E, I, J) qPCR results showing mRNA expression of *CIB1*, *CO*, *FT* in the genotypes indicated. The numbers indicate independent transgenic lines. The transcript levels of *CIB1* or *CO* or *FT* in Col were set to 1.0. Samples were collected from 7-day-old LD-grown seedlings at ZT16. In (B, C, G, H), data are presented as mean $\pm$ SD, $n \geq 15$ per genotypes indicated. $P \leq 0.05$ (Tukey’s HSD test). For data in (D, E, I, J), error bars indicate standard error within three biological replicates and for each of these two technical replicates were performed.

statistically significant delays in flowering under the photoperiodic inductive condition, but not under the LD (long-day) condition [17,18], the transgenic lines expressing the dominant repressor version of CIB1 (CIB1-EAR) showed a very late flowering phenotype under LD (Appendix Fig S4) [18]. When CIB1-EAR was expressed in a *co* mutant background, it consistently showed a late flowering phenotype and decreased *FT* expression similar to the *co* single mutant (Appendix Fig S4). We generated a *cib4^c* mutant using the CRISPR/Cas9 system [26] and then made the *cib1 cib3 cib5 beel beel cib4^c* hexuple (*cibh*) mutant (Fig EV2A and B). The *cibh* mutants exhibited significantly late flowering under LD conditions though not as late as *cry2* mutant (Fig EV2C–E), whereas the expression of *FT* was lower in *cibh* mutants than in the WT plants (Fig EV2F). Interestingly, the *cibh* mutant partially suppressed the early flowering phenotype and elevated *FT* expression in *35S:Myc-CO* plants, even though the transcription and the protein levels of CO were similar in the *cibh* and WT plants (Figs 1F–J and EV2G). *gi* mutants could not suppress the early flowering phenotype and elevated *FT* expression in *35S:Myc-CO* plants though *gi* mutants exhibit more severe late flowering phenotype than *cibh* (Appendix Fig S1F–J), the expression pattern of CO was not affected by GI in our condition (Appendix Fig S2C), indicating that CO can act independently of CIBs, and CIBs may regulate CO activity. Taken together, these results demonstrate that CO can act independently of CIBs while *cibh* may attenuate CO activity in the activation of *FT* transcription.

CIB1 and CO are co-expressed

CIB1 promotes flowering and *FT* transcription in a CO-dependent manner, and CO can act independently of CIBs. To figure out the underlying mechanism, we first checked whether they affect each other's expression. Our quantitative PCR results indicated that the transcription of *CIB1* in WT and the *co-1* mutant were similar, and the transcription of *CO* in WT and CIB1-EAR transgenic lines were also similar (Fig EV3A and B). CIB1 and CO did not affect one another's transcription. Furthermore, using transgenic lines that overexpress CO and CIB1 in different reciprocal mutant background with similar expression level, we found CIB1 and CO did not affect one another's protein stability. The protein level of CO in WT, *cibh*, and Myc-CIB1 overexpression lines or CIB1 in WT and the *co-1* mutant were similar (Figs 2A–D and EV3C). Interestingly, CIB1 and CO were both found to be regulated by photoperiod and are co-expressed; their proteins both peaked in abundance early in the morning and again at dusk in LDs with *FT* expression (Fig 2A–D).

CIB1 and CO interact directly

Because CIB1 depends on CO in the induction of *FT* transcription and CIB1 and CO are co-expressed, we examined the interaction between CO and CIB1. CO and CIB1 were co-localized in the nuclei (Fig 3A); therefore, we examined their interaction in plant cells using the bimolecular fluorescence complementation (BiFC) assay. Strong fluorescence was detected in the nuclei of cells co-transformed with *cCFP-CO* and *nYFP-CIB1*, but no fluorescence was detected in cells transformed separately with either *cCFP-CO* or *nYFP-CIB1* (Fig 3B). Bimolecular luminescence complementation (BiLC) assays confirmed that CO interacted directly with CIB1

(Fig 3C). Next, we investigated the CO-CIB1 interaction using co-IP; our results indicated that CO co-immunoprecipitated with CIB1 (Fig 3D). Furthermore, the *in vitro* pull-down assay indicated that the C-terminus of CO interacted with the C-terminus of CIB1 (Figs 3E and F, and EV3D and E, and Appendix Fig S5), whereas CRY2 interacted with the N-terminus of CIB1 [17]. *co-2* is a recessive mutant with a point mutation at amino acid 59 (R59H) on B-BOX domain of CO protein. Consistent with that CCT domain interacted with the C-terminus of CIB1, the *in vitro* pull-down assays showed that CO-2 mutant protein could still interact with CIB1 (Fig EV3D). CO and CIB1 had been previously shown to bind to a similar region in the *FT* promoter [17,18,27]. To investigate whether the CO-CIB1 interaction could be detected in planta just because they both bind to the *FT* promoter and the DNA pull them together, we did a co-IP experiment with DNase treatment. Our results indicated that CO co-precipitated with CIB1 even after DNase treatment, and they interacted with each other without *FT* promoter (Fig EV3F). In addition to CIB1, CIB5 was shown to interact with CO by the BiFC assay (Fig EV3G), indicating that multiple CIBs interact with CO.

CIB1 and CO act together to induce *FT* transcription

To investigate the biological significance of the CIB1 and CO interaction, we checked the flowering phenotype of transgenic lines that co-overexpressed CO and CIB1, they flowered earlier and exhibited dramatically higher *FT* transcription than lines that only overexpressed CO (Figs 4A–E and EV4). Together with the finding that CIB1 promoted flowering and *FT* transcription in a CO-dependent manner, this result indicated that CO and CIB1 might act together to induce *FT* transcription. A transient dual-LUC assay was used to

Figure 2. CIB1 and CO are co-expressed.

A, B Immunoblots showing CIB1 (A) and CO (B) protein levels in WT and *co-1* backgrounds (A) or WT and *cibh* backgrounds (B) over the course of 20 h under LDs. Samples were separated on 10% SDS-PAGE, blotted, probed with the anti-Myc antibody, stripped and re-probed with the anti-actin antibody (ACT).
C, D The relative levels of CIB1 (A) and CO proteins (B) in WT and *co-1* backgrounds (A) or WT and *cibh* backgrounds (B) over the course of 20 h under LDs were calculated as described (see Materials and Methods). Error bars represent the standard deviation of three biological replicates of the Western blots.

Figure 3. CIB1 and CO interact directly.

A Fluorescent microscopy images showing that CIB1 co-localizes with CO in nuclear speckles. Scale bars: 2 μ m.

B BiFC assays to show *in vivo* protein interactions. Epidermal cells of *Nicotiana benthamiana* leaves were co-transformed with cCFP-CO and nYFP-CIB1, with cCFP-CO and nYFP, or with cCFP and nYFP-CIB1. BF, bright field. Merge, overlay of the YFP and bright field images. Scale bars: 50 μ m.

C Split-LUC assay showing that CIB1 interacts with CO. Leaf epidermal cells of *N. benthamiana* were co-transformed with CO-nLUC and cLUC-CIB1 or cLUC or nLUC with cLUC-CIB or cLUC.

D Co-IP assays of samples prepared from 10-day-old *35S:Myc-CIB1*, *35S:CO-Flag*, or *35S:Myc-CIB1/35S:CO-Flag* seedlings grown in LDs.

E Schematic representation of CO used in this work was showing. CO contains two B-Box domains and a CCT domain.

F *In vitro* pull-down assays showing the interaction between CIBC and CO, or CIBC and CO Δ N and the lack of interaction between CIB1C and CO Δ C. His-TF tagged CO, CO Δ N, CO Δ C, or His-TF tagged UVR8 was mixed with GST-CIB1C purified from *Escherichia coli*, and His antibody was used for the *in vitro* pull-down assay. The products were analyzed by immunoblots probed with the anti-CIB1C antibody or the anti-His antibody (UVR8, CO, CO Δ C, CO Δ N).

Data information: For each experiment, two or more biological replicates were performed.

further study whether CO and CIB1 act together to induce *FT* transcription. The co-expression of CIB1 and CO promoted the expression of the *FT promoter:LUC* to a much higher level compared with the expression of CIB1 or CO alone (Fig 4F and G). Consistent with the functional dependency of CIB1 on CO in the induction of *FT* transcription, the activities of CIB1, CO, and CIB1 plus CO were all decreased on the *FT* promoter lacking the CO binding site (*ProFTm2*; Fig 4F and G). The 1,034 bp *FT* promoter fragment contains 5 E-box

motifs, which are putative binding sites for CIB1 (Fig 4F and Appendix Fig S6A). The mutation of each E-box motif individually had no effect on CIB1-induced *FT* transcription, while the mutation of E-box 1 and E-box 2 (*ProFTE1E2m*) together or of all five E-box motifs (*ProFTm1*) significantly reduced CIB1-mediated activation of *FT* transcription. The mutation of all five E-box motifs (*ProFTm1*) also slightly reduced CO-mediated activation of *FT* transcription (Fig 4G and Appendix Fig S6B). CIB1 and CO could not promote the

transcription of the *FT* promoter lacking both the CO binding site and all E-box motifs (*proFTm3*; Fig 4G). These results indicate that CO and CIB1 bind to the DNA of the *FT* promoter together to activate its transcription, and CO and CIB1 act together to induce *FT* transcription.

CRY2 activates *FT* mRNA expression in response to blue light by suppressing the degradation of the CO protein [14–16] and by direct activation of the CIB1 [17–19]. CIB1 and CO acted together to regulate *FT* transcription, and CIB1 promoted flowering in a CO-dependent manner, then CRY2 might also promote flowering in a CO-dependent manner. Overexpression of the constitutively active CRY2 form CRY2^{W374A} leads to early flowering phenotype in both long-day and short-day conditions [28]. The early flowering phenotype of 35S:CRY2^{W374A} was abolished in *co-1* mutant background (Appendix Fig S7A–C), and the higher level of *FT* transcription detected in 35S:CRY2^{W374A} plants was not observed in the *co-1* mutant background (Appendix Fig S7D and E). Furthermore, *cry2 co* and *cry2 ft* double mutants exhibited late flowering that was similar to the late flowering phenotype observed in *co* and *ft* mutants (Appendix Fig S7F and G). These results indicate that CRY2 promotes flowering and the transcription of *FT* in a CO-dependent manner.

CIB1 and CO act together to mediate CRY2-dependent regulation of *FT* transcription

CIB1 physically interacts with both CRY2 and CO; the three proteins might be in the same protein complex. We observed that CRY2, CIB1, and CO co-localized in nuclear speckles of tobacco (Fig 5A), in agreement with the previous observation that CRY2-GFP formed distinct nuclear bodies in response to blue light [29]. More importantly, endogenous CRY2 could co-immunoprecipitate both CIB1 and CO in samples that were irradiated with white or blue light but not in samples irradiated with red light, indicating that CRY2, CIB1, and CO form a complex *in vivo* in a blue light-dependent manner (Figs 5B and EV5A). CIB1 and CO were both found to be regulated by photoperiod and they are co-expressed, whereas CRY2 was not (Figs 5C, and EV5B and C), and endogenous CRY2 could co-immunoprecipitate more CIB1 and CO when more CIB1 and CO proteins were present at dusk along with higher *FT* expression (Fig 5C). CO and CIB1 had been previously shown to bind to a similar region in the *FT* promoter [17,18,27]. CRY2 might also bind to the same sites as part of a complex with CIB1-CO. We therefore tested this possibility using ChIP. The ChIP-qPCR results indicated that CRY2, CIB1, and CO bound to the same chromatin region of *FT* (Fig 5D and E), suggesting that CIB1 and CO recruit CRY2 to the *FT* chromatin and that the three proteins form a complex to promote *FT* transcription. Our ChIP results also showed that CIB1 bound to the *FT* promoter in *co-1*, *gi-1*, and *cry2* mutant backgrounds, but the enrichment of CIB1 on the *FT* promoter was significantly lower in *cry2* mutant than in the WT, indicating that CRY2 might affect the binding of CIB1 to *FT* (Appendix Fig S8A–C). CO also bound to the *FT* promoter in *cry2* mutant background, indicating that the binding of CO to *FT* was not affected by CRY2 (Appendix Fig S8C). Interestingly, CRY2 bound to the *FT* promoter in *co-9* mutant and *cibh* mutant (Appendix Fig S8D), given that the late flowering of *cibh* was not as late as the flowering of *cry2*, suggesting that more CIB1 homologs are involved in flowering, other CIB1 homologs might mediated the binding of CRY2 to the *FT* promoter. Consistent with that CRY2, CIB1 and CO bound to the same chromatin region of *FT*, the co-expression of CRY2, CIB1, and CO promoted the expression of the *proFT:LUC* to a much higher level compared with the expression of CO alone or CO and CIB1 together (Fig 5F). These results indicate that CIB1 and CO

Figure 5. CRY2 forms a complex with CO and CIB1 in response to blue light to activate *FT* transcription.

A Fluorescent microscopy images showing that CRY2, CIB1, and CO co-localized to the nucleus. Scale bars: 2 μ m.

B Co-IP assays showing that CRY2, CIB1, and CO form a protein complex. Samples were prepared from 10-day-old WT or 35S:Myc-CIB1/35S:CO-Flag or 35S:Myc-CIB1/35S:CO-Flag/cry2-1 seedlings grown in LDs (16L/8D), and samples were collected at ZT16.

C Co-IP assays of samples prepared from 10-day-old 35S:Myc-CIB1/35S:CO-Flag 35S:Myc-CIB1/35S:CO-Flag/cry2-1 seedlings grown in LDs (16L/8D). Samples were collected at the times indicated over the course of 1 day.

D Diagram depicting the putative promoter and genomic region of *FT*. The diamond symbol indicates the position of CORE. The white circles indicate positions of E-boxes (CANNTG) located at proximal promoter of *FT*.

E Representative results of the ChIP-qPCR assays. Chromatin fragments (~500 bp) were prepared from 10-day-old 35S:Myc-CIB1/35S:CO-Flag seedlings, immunoprecipitated by the anti-CRY2, anti-Flag (CO-Flag), anti-Myc (Myc-CIB1) antibody, or anti-preimmune IgG, and the precipitated DNA was analyzed by qPCR using the primer pairs indicated. The IP/input ratio of anti-preimmune is set to 1.0.

F Box plots (the minimum, first quartile, median, third quartile, and maximum are shown, with data > 1.5 interquartile ranges denoted with circles) for relative reporter activity (LUC/REN) in plants expressing different reporters and effectors. The LUC/REN in plants expressing *ProFT* alone is set to 1.0. Immunoblots were done to show GFP-CRY2^{W374A}, Myc-CIB1, and Myc-CO protein levels in the transient Dual-LUC assay. Samples were separated on 10% SDS-PAGE, blotted, probed with the anti-Myc antibody and with the anti-GFP antibody.

G Model representing the action of CIB1 and CO on *FT* expression under long-day condition.

Data information: For data in (E), the relative IP/input ratios are shown with their standard deviation ($n = 3$). Two biological replicates were performed. For data in (F), the relative LUC activities normalized to the REN activity are shown (LUC/REN) as boxplots. The letters "a" to "f" indicate statistically significant differences determined by Tukey's HSD test ($P \leq 0.05$). Two biological replicates were performed.

act together to mediate CRY2-dependent regulation of *FT* transcription.

Flowering time is regulated by very complex regulatory networks to ensure reproductive success. The photoperiodic pathway mainly perceives external light signals from the environment to optimize seed production in specific environments. CO is the main component of the photoperiodic pathway and the main transcriptional activator of *FT* [8–11]. CIB1 is another transcriptional activator of *FT*, a key component of blue light signaling; however, the relationship between CIB1 and CO is unknown. Here, we showed that CIB1 promoted *FT* transcription and flowering in a CO-dependent manner, and they interacted physically and act together to induce *FT* transcription. Furthermore, CRY2 displayed a higher co-immunoprecipitation efficiency for CIB1 and CO in particular when their protein abundance peaked at dusk on one hand and overlapped with the peak in expression of *FT* on the other hand. Moreover, the observed physical interaction between CIB1 and CO strongly argues for a cooperation of the CIBs-CO complex with CRY2 to regulate *FT* transcription.

CO binds to the proximal region of the *FT* promoter by recognizing two CO responsive elements (CORE) that are required for *FT* activation [30]. One CORE is located at –221 to –210 bp, whereas the other one is located at –161 to –151 bp upstream of the *FT* transcription start site. CIB1 and its homologs (CIB2, CIB4, and CIB5) function redundantly to activate the transcription of *FT* by forming different heterodimers that bind to the E-boxes of the *FT* promoter. There are two E-box elements around the CORE, one located at –23 to –18 bp and the other one located at –325 to –320 bp. The DNA-binding sites of the CIBs and CO are close to one another. CIBs and CO all bind to the *FT* promoter directly, and they physically interact

with one another to promote each other's transcriptional activity. TARGET OF EAT1 (TOE1) can bind to the *FT* promoter near the CO binding site, and it interacts with CO to inhibit CO activity [31]. Nuclear factor Y (NF-Y) binds to a CCAAT element located 5.3 kb upstream of the transcriptional start site of *FT* [32], and certain NF-Y subunit paralogs are required for CO activation of *FT* [33]. NF-Y can also interact with CO directly to regulate the transcription of *SOC1* [34]. It has also been reported that ASYMMETRIC LEAVES 1 (AS1) physically interacts with CO to promote *FT* transcription and binds directly to the *FT* promoter [35]. Multiple transcription factors or DNA-binding proteins could interact with CO on the *FT* promoter to regulate its transcription.

Our ChIP data indicate that CRY2 associates with the *FT* promoter together with CIB1 and CO; therefore, the CIB1-CO transcription factors recruit CRY2 to the *FT* promoter, and CRY2 supports the transcriptional activity of CO-CIB1. Thus, we propose that CRY2, CIB1, and CO form a complex on the DNA so that changes in the external environment can rapidly lead to phenotypic changes. We also find that CRY2 affects the binding of CIB1 to *FT*, since the enrichment of CIB1 on the *FT* promoter was significantly lower in the *cry2* mutant than in the WT. It is therefore possible that CRY2 supports or stabilizes the binding of CIB1 to *FT* promoter. Along with Pedmale *et al*, we showed that the transcription factor PIF4 (PHYTOCHROME INTERACTING FACTOR 4) could recruit CRY1/CRY2 to the promoters of PIF4 targets [36,37]. CRYs are likely localized to DNA indirectly via interactions with other transcription

factors, similar to PhyB, which lacks a DNA-binding domain, is actively recruited to chromatin at PIF TF binding sites [38]. In animals, CRY interacts with CLOCK-BMAL1 at the promoter and inhibits CLOCK-BMAL1-dependent transcription [39,40]; therefore, in both plants and animals, CRYs interact with transcription factors on the DNA to regulate transcription. The molecular mechanisms underlying CRY2-mediated transcriptional inhibition and activation will need to be further investigated.

In summary, we found that CIB1 promoted *FT* transcription and flowering in a CO-dependent manner. CIB1 physically interacts with both CRY2 and CO, the three proteins could be found in the same protein complex, and the complex is regulated by both blue light and photoperiod. CIB1 and CO recruit CRY2 to the *FT* promoter, forming a protein complex to promote *FT* transcription (Fig 5G). Therefore, there is crosstalk between the CRY2-CO and CRY2-CIBs pathways, and CIB1 and CO act together to regulate *FT* transcription and flowering.

Materials and Methods

Plant materials

Plants were grown on soil or Murashige and Skoog (MS) media containing 1% sucrose in plant incubators (Percival Scientific) or phytotron at 22°C under full-spectrum white fluorescent light with a fluence rate of 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in long-day (16 h light/8 h dark) conditions. For the light treatment, red or blue light was provided by red LEDs or blue LEDs, respectively, with light intensities of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

For the transient assay, tobacco plants (*Nicotiana benthamiana*) were grown in phytotron at 22°C in long-day condition. The tobacco leaves were ready for infiltration 4–5 weeks after germination.

To analyze the flowering time, the number of rosette leaves were counted when inflorescences were seen. The days to flowering were calculated as how many days needed from the seed germination to the inflorescences be seen. All flowering experiments were repeated at least twice independently, and similar results were obtained. Western blot or qPCR or both were done to verify the overexpression of the transgenes.

The *cry2-1*, *co-1*, *co-2*, *co-9*, *ft-1*, *ft-10*, *gi-1*, *cib1 cib2 cib5*, *bee1*, *bee3*, and *CRY2*^{W374A} mutants and transgenic lines were described previously [7,8,18,23,28,41–48]. The T-DNA insertion mutants *cib3* (AT3G07340 SALK_091124) were obtained from ABRC. The *cry2 co-9*, *cry2 ft-10*, and *cib1 cib3 cib5 bee1 bee3 cib4^c* mutants were prepared by carrying out genetic crosses and their identities were verified by genotyping and RT-PCR. Transgenic *Arabidopsis* that overexpress 35S:Myc-CIB1 in the Col, Ler, *co-1*, *co-2*, *ft-1*, and *gi-1* backgrounds, 35S:MycCIB1EAR in the *co-2* background, 35S:CO-Flag, 35S:GFP-CO in Col background, 35S:Myc-CO expressed in the *cib1 cib2 cib5 bee1 bee3 cib4^c* backgrounds, 35S:HA-CO expressed in *gi-1* backgrounds, 35S:CRY2^{W374A} in the *co-1* mutant background were prepared by the floral dip method [49]. Transgenic *Arabidopsis* that overexpress 35S:Myc-CIB1 and 35S:CO-Flag together or 35S:Myc-CIB1 and 35S:GFP-CO together was prepared by carrying out genetic crosses between transgenic *Arabidopsis* that overexpress 35S:Myc-CIB1 and transgenic *Arabidopsis* that overexpress 35S:CO-Flag or 35S:GFP-CO. For every transformation, more than 10 independent

transgenic lines were generated. All the independent transgenic lines overexpress the same construct showed the same phenotype.

The binary plasmid encoding 35S:CO-Flag was pCambia 1306; the coding sequence of CO was inserted between BamHI and SalI restriction sites. To construct Myc-CO and Myc-CIB1, the coding sequences of CIB1 and CO were fused to the vector pEGAD by inserting the sequences between the EcoRI and XhoI restriction sites. 35S:HA-CO was prepared by gateway method and was cloned into pEarly-201. 35S:CO-YFP, 35S:CO-mCherry, 35S:CIB1-YFP, 35S:CRY2-CFP, and 35S:CIB1-mCherry were cloned into pCambia 1301 with modification by conventional restriction digestion and ligation method. The vectors pCold-TF (Takara) and pGEX4T-1 were used in the pull-down assays. BiFC and BiLC vectors were prepared using the GATEWAY method; the vectors backbone was described as before [50]. All of the prepared constructs were verified by DNA sequencing.

Immunoblots

Our attempts to prepare anti-CO and anti-CIB1 antibodies were unsuccessful (they were able to recognize CO and CIB1 expressed in *Escherichia coli* but not in plant extracts, and they were used in the *in vitro* pull-down assays). Therefore, all analyses of CO and CIB1 proteins were carried out using transgenic plants expressing the Myc-, Flag-, or GFP-tagged CO and CIB1. Immunoblots were carried out as described previously [51]. Anti-Myc antibody (Millipore, 05-724, diluted 1:4,000), anti-GFP antibody (Abiocode, M0802-3a, diluted 1:4,000), anti-Flag antibody (Sigma, F3165, diluted 1:3,000), anti-actin antibody (Abmart, M20009, diluted 1:3,000), anti-His antibody (MBL, D291-3, diluted 1:4,000), and anti-GST antibody (Abmart, M20007, diluted 1:5,000) were used.

CIB1 or CO signals were quantified by digitizing the band signal (ImageJ), subtracting the background signal, normalizing against the internal control (CRY1, ACT, or NS), and the relative level calculated against the value of CIB1, CO, or CRY2 in the WT background at the ZT0. The relative level of CIB1, CO, or CRY2 (Figs 2C and D, and EV5C) is calculated by the formula $[(\text{CIB1}_{\text{ZT}} - b_{\text{ZT}})/(\text{C}_{\text{ZT}} - b_{\text{ZT}})]/[(\text{CIB1}_{\text{ZTOW}} - b_{\text{ZTOW}})/(\text{C}_{\text{ZTOW}} - b_{\text{ZTOW}})]$, or $[(\text{CO}_{\text{ZT}} - b_{\text{ZT}})/(\text{C}_{\text{ZT}} - b_{\text{ZT}})]/[(\text{CO}_{\text{ZTOW}} - b_{\text{ZTOW}})/(\text{C}_{\text{ZTOW}} - b_{\text{ZTOW}})]$ in which CIB1_{ZT} or CO_{ZT} represents the signal intensity of CIB1 or CO from a sample collected at different ZT time (ZT0 to ZT20), b_{ZT} represents the background signal of the CIB1_{ZT} or CO_{ZT} lane in the immunoblot, C_{ZT} represents signal intensity of the loading control (CRY1 or ACT or NS), b_{ZT} represents the background signal of the respective C_{ZT} lane in the immunoblot, and $\text{CIB1}_{\text{ZTOW}}$, CO_{ZTOW} , C_{ZTOW} , b_{ZTOW} represent CIB1, CO, loading control, or background signals from sample in the WT background collected at ZT0.

Co-localization, BiLC, and BiFC assays

The co-localization assay was carried out as described previously with the following modifications [17,52]: CO was fused to YFP or mCherry, CIB1 was fused to GFP or mCherry, and CRY2 was fused to CFP. These constructs were then transformed into *Agrobacterium* strain GV3101 containing the pSoup-P19 plasmid that encodes the suppressor of gene silencing [53]. Overnight cultures of *Agrobacterium* were collected by centrifugation, re-suspended in MES buffer (10 mM MES pH 5.8, 10 mM MgCl₂, 200 μM acetosyringone) to an OD₆₀₀ of 0.8, mixed, and incubated at room temperature for 2 h

prior to infiltration. The *Agrobacteria* suspension was drawn into a 1-ml syringe (without the needle) and carefully press-infiltrated by hand into healthy leaves of 3-week-old *N. benthamiana* plants. The plants were left under continuous white light for 3 days after the infiltration. For the BiLC assay, 1 mM luciferin was infiltrated before LUC activity was monitored after 3 days. The LUC signal was photographed with a cool CCD camera (Andor DW936N-BV).

Co-immunoprecipitation (co-IP) experiments

The co-immunoprecipitation (co-IP) procedure was described previously [17]. Samples were ground in liquid nitrogen, homogenized in Binding Buffer [20 mM HEPES (pH 7.5), 40 mM KCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 1 mg ml⁻¹ Complete Protease Inhibitor Cocktail Tablets (Roche), 50 µg ml⁻¹ MG132], and centrifuged at 14,000 g for 15 min. One millilitre of supernatant was mixed with 25 µl of anti-CRY2-IgG-coupled protein A-Sepharose or anti-Myc affinity gel (Sigma E6654) incubated at 4°C for 20 min. Three microlitre of anti-CRY2 antiserum was incubated with 20 µl of protein A-Sepharose beads in 100 µl of Binding Buffer at 4°C for 2 h and used shortly after its preparation. The beads were washed (20 s each) three times with Washing Buffer [20 mM HEPES (pH 7.5), 40 mM KCl, 1 mM EDTA, 0.1% Triton X-100]. The bound proteins were then eluted from the affinity beads with 4X SDS-PAGE sample buffer (40% Glycerol, 0.4% Bromophenol Blue, 1% SDS, 400 mM Tris-HCl pH 6.8, 1% DTT) and analyzed by immunoblotting.

In vitro pull-down assays

The *in vitro* pull-down protein-protein interaction assay was described previously with modifications [18]. The coding sequences of CIB1 and CIB1C (residues 172–335) were fused to the C-terminus of GST in the vector pGEX4T (GE Healthcare) by inserting the sequences between the EcoRI and XhoI restriction sites. The coding sequences of CO, CO-2, COΔC (residues 1–253), COΔN (residues 106–374), CO-CCT (residues 208–374), and UVR8 (UV RESISTANCE LOCUS 8) were fused to the C-terminus of the His tag in the vector pCold-TF (Takara) by inserting the sequences between the EcoRI and SalI restriction sites. The fusion proteins were expressed in *E. coli* (strain BL21), purified and bound to Glutathione Sepharose 4B or Ni-NTA beads according to the manufacturer's instructions (Amersham).

Transient transcription dual-luciferase (dual-LUC) assays

Transient transcription dual-LUC assays in *N. benthamiana* plants were carried out as described previously [17,18]. To generate the constructs needed, 1,034 bp *FT* promoter was cloned into pBlueScript SK to generate *ProFT-SK* construct. The *ProFTm1-SK*, *ProFTm2-SK* and *ProFTm3-SK* constructs carrying mutated E-box element (CANNTG to AAAAAA) or deleted CORE element were generated by overlapping PCR reaction from the *ProFT-KS* construct. After verifying by DNA sequencing, the *ProFT*, *ProFTm1*, *ProFTm2*, and *ProFTm3* fragments were inserted to pGreen II 0800 to generate the final constructs for dual-LUC assay. All the primers used for dual-LUC assay were listed in Appendix Table S1. The LUC activity of the plant extracts was analyzed with a luminometer (GloMax 20/20, Promega) using LUC detection reagents according to the manufacturer's instructions (Promega).

mRNA expression analyses

Total RNA was isolated using the RNAiso Plus (Takara). cDNA was synthesized from 500 ng of total RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara). SYBR Premix Ex Taq (Takara) was used in the qPCR reactions, and the reactions were carried out using the Mx3000 System (Stratagene). Transcript levels for each sample were normalized to the level of *ACTIN7* mRNA expression (AT5G09810, Appendix Table S1). Biological replicates represent three independent experiments involving about 30 seedlings per experiment. Three technical replicates were done for each experiment.

Chromatin immunoprecipitation (ChIP) assays

ChIP was performed as described previously [17]. Two-week-old *Arabidopsis* seedlings grown on soil were harvested at ZT14 and were treated with 1% formaldehyde under vacuum for 16 min. The cross-linking was stopped by adding glycine to a final concentration of 0.125 M. The seedlings were rinsed with water, frozen in liquid nitrogen, and ground to a fine powder. Nuclei were extracted with Extraction Buffer 1 [10 mM Tris-HCl pH 8.0, 0.4 M sucrose, 10 mM MgCl₂, 0.1 mM PMSF, Complete Protease Inhibitor Cocktail Tablets (Roche)] and washed with Extraction Buffer 2 [10 mM Tris-HCl pH 8.0, 0.25 M sucrose, 10 mM MgCl₂, 1% Triton X-100, 0.1 mM PMSF, Complete Protease Inhibitor Cocktail Tablets (Roche)]. Nuclei were lysed in Nuclei Lysis Buffer [50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, 0.1 mM PMSF, Complete Protease Inhibitor Cocktail Tablets (Roche)] and sonicated to shear the DNA into fragments that were approximately 0.5–1 kb. The chromatin solution was diluted 10-fold with ChIP Dilution Buffer [16.7 mM Tris-HCl pH 8.0, 167 mM NaCl, 1.1% Triton X-100, 1.2 mM EDTA, 0.1 mM PMSF, Complete Protease Inhibitor Cocktail Tablets (Roche)]. Immobilized protein G (Pierce) was used for the immunoprecipitation. Anti-Myc antibody was prebound to the beads by incubating them together for 2 h in ChIP Dilution Buffer. Immunocomplexes were washed with Low Salt Buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 2 mM EDTA), High Salt Buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 2 mM EDTA), LiCl Wash Buffer (20 mM Tris-HCl pH 8.0, 0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA), and TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and then eluted with Elution Buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS). The cross-linking of the samples was reversed by incubating at 65°C overnight and then by treatment with Proteinase K to remove the proteins. DNA was purified by using the FastDNA Kit (MP Biomedicals) and eluted with 50 µl of TE buffer. One microlitre aliquots were used for qPCR. The primer pairs that were used to amplify the genomic sequence are listed in Appendix Table S1.

Expanded View for this article is available online.

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Author contributions

YL and HL conceived the project. YL performed most of the experiments, XL performed the pull-down assays, DM aided in the performance of the ChIP assays, ZC helped to prepare constructs, J-WW provided materials, YL and HL analyzed the data, and HL and YL wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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