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

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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 bHLH3). 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

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.
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
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 cibf mutants using the CRISPR/Cas9 system [26] and then made the cib1 cib3 cib5 bee1 bee3 cibf cibf 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...
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

Figure 3. CIB1 and CO interact directly.
A Fluorescent microscopy images showing that CIB1 co-localizes with CO in nuclear speckles. Scale bars: 2 μm.
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 μm.
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 nLUC with cLUC-CIB or nLUC.
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 CIB1C and CO, or CIB1C and COΔΝ and the lack of interaction between CIB1C and COΔΝ. His-tagged CO, COΔΝ, COΔΝ, or His-TF tagged UVR 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, COΔΝ, COΔΝ).

Data information: For each experiment, two or more biological replicates were performed.
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<sup>W374A</sup> leads to early flowering phenotype in both long-day and short-day conditions [28]. The early flowering phenotype of 35S:CRY2<sup>W374A</sup> was abolished in co-1 mutant background (Appendix Fig S7A–C), and the higher level of FT transcription detected in 35S:CRY2<sup>W374A</sup> 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 mediate 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

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.
CIB1 and CO cooperatively activate FT

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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 μm.
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-pre-immune IgG, and the precipitated DNA was analyzed by qPCR using the primer pairs indicated. The IP/input ratio of anti-pre-immune 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, MYC-CIB1, and CO-Flag 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-Flag 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 LUC activities normalized to the REN activity 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 ≤ 0.05). Two biological replicates were performed.
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 phototrons at 22°C under full-spectrum white fluorescent light with a fluence rate of 90 μmol m⁻² 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 μmol m⁻² s⁻¹.

For the transient assay, tobacco plants (Nicotiana benthamiana) were grown in phototrons 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 CRY2W374A 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 cry2−1, cry2 ft−10, and cib1 cib3 cib5 bee1 bee3 cib4 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 backgrounds, 35S:HA-CO expressed in gi−1 backgrounds, 35S:CRY2W374A 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 Sall 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 \[\frac{\text{signal of the CIB1ZT lane in the immunoblot, CIB1ZT represents the signal intensity of CIB1 or CO from a sample collected at different ZT time} (\text{ZT0 to ZT20}), \text{bZT represents the background signal of the CIB1ZT or COZT lane in the immunoblot}, \text{CZT represents signal intensity of the loading control (CRY1 or ACT or NS), bZT represents the background signal of the respective CZT lane in the immunoblot, and bZT represents background signal from sample in the WT background collected at ZT0.} \]

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 Agrobacteria 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 Cocktails (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 millilitre 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 pH6.8, 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 CIB1 and CIB1C (residues 172–335) were fused to the C-terminus of 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 pBlue-Script SK to generate ProFT-SK construct. The ProFTm1-SK ProFTm2-SK and ProFTm3-SK constructs carrying mutated E-box element (CANNNG to AAAAAA) or deleted CORE element were generated by overlapping PCR reaction from the ProFT-SK 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 RNeasy 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 pH8.0, 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 pH8.0, 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 microtitre aliquots were used for qPCR. The primer pairs that were used to amplify the genomic sequence are listed in Appendix Table S1.

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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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