Cryptochrome 1 Inhibits Shoot Branching by Repressing the Self-Activated Transcription Loop of PIF4 in Arabidopsis

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ABSTRACT

Cryptochrome 1 (CRY1) is an important light receptor essential for de-etiolation of Arabidopsis seedlings. However, its function in regulating plant architecture remains unclear. Here, we show that mutation in CRY1 resulted in increased branching of Arabidopsis plants. To investigate the underlying mechanism, we analyzed the expression profiles of branching-related genes and found that the mRNA levels of Phytochrome Interaction Factor 4 (PIF4) and PIF5 are significantly increased in the cry1 mutant. Genetic analysis showed that the pif4 or pif4pif5 mutant is epistatic to the cry1 mutant, and overexpression of PIF4 conferred increased branching. Moreover, we demonstrated that PIF4 proteins physically associate with the G-box motif within the PIF4 promoter to form a self-activated transcriptional feedback loop, while CRY1 represses this process in response to blue light. Taken together, this study suggests that the CRY1–PIF4 module regulates gene expression via forming a regulatory loop and shoot branching in response to ambient light conditions.

Key words: cryptochrome, PIF, branching, basic helix-loop-helix, photoreceptor, Arabidopsis

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INTRODUCTION

Cryptochrome 1 (CRY1) and CRY2 are blue-light photoreceptors discovered in Arabidopsis (Ahmad and Cashmore, 1993; Hoffman et al., 1996; Cashmore et al., 1999). CRY1 is primarily responsible for inhibition of hypocotyl elongation under blue light, while CRY2 mainly controls photoperiodic flowering (Guo et al., 1998). Other studies have suggested many roles of CRYs, including regulations of stomata opening, guard cell development, and leaf senescence (Mao et al., 2005; Kang et al., 2009; Meng et al., 2013). However, the function of CRYs in regulation of branching is largely unknown.

CRYs perceive and transmit blue-light signals to downstream genes and ultimately influence plant development. Previous studies demonstrated that CRYs regulated expression of downstream genes primarily through two pathways: the CRY2–CIBs pathway and the CRYs–COP1/SPA1 pathway (Liu et al., 2008a, 2011; Lian et al., 2011; Zuo et al., 2011). Both CRY1 and CRY2 inhibit the activities of E3 ubiquitin ligase COP1 in a blue-light-dependent manner to protect transcription factors, including Long Hypocotyl 5 (HY5), Long Hypocotyl in Far-Red 1 (HFR1) and CONSTANT (CO), to regulate expression of downstream genes required for photomorphogenesis and photoperiodic flowering (Osterlund et al., 2000; Duek et al., 2004; Liu et al., 2008b).

Phytochrome interaction factors (PIFs) are basic helix-loop-helix transcriptional factors that regulate downstream gene expression through binding to the G-box (CACGTG) motif within the promoter region to repress photomorphogenesis in dark conditions (Martinez-Garcia et al., 2000; Leivar and Quail, 2011). The light-activating form of PhyB (Pfr) can bind PIF1, PIF3, PIF4, PIF5, and PIF7, which then undergo phosphorylation and subsequent degradation by 26S protease (Ni et al., 1998; Huq and Quail, 2002; Shen et al., 2005, 2007; Leivar and Quail,
PhyB mediates shade-avoidance response (SAR) in PIF4-, PIF5-, and PIF7-dependent manner (de Wit et al., 2016). Under low red light to far-red light (R:FR) conditions, PhyB transforms from active Pfr to the inactive Pr form and causes accumulation of PIF4 and PIF5 to regulate expression of downstream genes (Lorrain et al., 2008). Several auxin signaling genes are proven to be PIF4 and PIF5 direct targets (Hornitschek et al., 2012; Sun et al., 2013). In the pif4pif5 double mutant, auxin-induced hypocotyl elongation and gene expression were reduced (Hornitschek et al., 2012). The role of PIFs in SAR has been addressed by many phenotype changes, including hypocotyl, stem, and petiole elongation and leaf hyponasty (Ballare, 1999; Casal, 2012). However, few studies have shown that PIFs participate in light signal pathways to regulate branch number.

PIFs integrate the crosstalk of light signal transduction pathways mediated by various photoreceptors. CRY1 and CRY2 regulate low-blue-light-induced SAR partially dependent on the direct interaction with PIF4 and PIF5, which is reminiscent of low R:FR-induced SAR mediated by PhyB (Keller et al., 2011; de Wit et al., 2016; Pedmale et al., 2016). Moreover, CRY1 interacts directly with PIF4 to regulate temperature-mediated hypocotyl elongation (Ma et al., 2016). Despite the knowledge about CRYs direct interaction with PIF4 in regulating downstream gene expression, the mechanism of how cryptochromes regulate PIF4 transcription is still unknown. In this study, we showed the increased branching phenotype of the cry1 mutant is associated with the elevated expression level of the PIF4 gene. Moreover, PIF4 protein targets to the G-box motif within its own promoter to activate transcription. Mutation in CRY1 released the blocking of the positive feedback loop of PIF4 transcription, which may partially explain the increased branching phenotype of the cry1 mutant.

**RESULTS**

**Mutation in CRY1 Increases Rosette Branch Number**

The red-light photoreceptor PhyB regulates branching by perceiving R:FR ratio in Arabidopsis, and the phyb mutant shows constitutive shade-avoidance syndromes including elongated hypocotyl and reduced branching (Finlayson et al., 2010; Krishna Reddy and Finlayson, 2014). Cryptochromes have also been revealed to regulate SARs by perceiving low-blue-light signal (de Wit et al., 2016; Xu et al., 2017). To test whether CRY1 is involved in the regulation of branching, we made a comparison between the wild type (WT) and a fast-neutron mutagenized cry1 mutant cry1-304 (Bruggemann et al., 1996). The plants were grown under long-day (LD) conditions (16 h light/8 h dark) at low density (1 plant/pot) to avoid the influence of overlapping growth. The rosette branch numbers were counted on the 15th day post anthesis (DPA). The result showed that the cry1 mutant produced more branches than normal (Figure 1A and 1B). To confirm the function of CRY1 in branching regulation, we constructed the expression vector 35S:CRY1 and transformed it into the cry1 mutant.
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To test the influence of planting density on branching, we planted the cry1 mutant in three densities (1, 4, or 8 plants per pot). The branch number of both WT and cry1 mutant reduced gradually with the increase of planting density, implying that shade signaling is not impaired in the absence of CRY1. Moreover, the cry1 mutant displayed significantly more branching phenotype than WT at all three planting densities, despite the degree of difference decreasing at higher density (Figure 1D). We further tested the influence of light intensity. The cry1 mutant and the WT plants were grown under three light intensities (40, 70, or 120 μmol m⁻² s⁻¹). The result showed that both genotypes produced more branches under the high light density than under the low light density. Moreover, the cry1 mutant plants constitutively produced more branches than WT (Figure 1E). These results suggested that CRY1-mediated inhibition of branching is a process partially influenced by planting density and light intensity.

CRY1 Represses the Transcription of PIF4 Gene under Blue Light

To interrogate the mechanism of how CRY1 inhibits branching, we tested the expression profiles of known or putative branching-related genes (Supplemental Figure 1). Consistent with the increased branching phenotype of cry1, the mRNA level of BRANCHED2 (BRC2, an integrator and suppressor of branching signal) was downregulated in the absence of CRY1 (Finlayson et al., 2010; Casal, 2012). Interestingly, loss of CRY1 function elevated the transcription levels of the PIF4 and PIF5 genes that participate in SAR to promote hypocotyl elongation (Lorrain et al., 2008; Pedmale et al., 2016). Given that the circadian clock regulates the rhythmic expressions of PIF4/5 to achieve diurnal control of hypocotyl elongation, we surmised that CRY1 may also modulate PIF4/5 expression to influence branching. To test this hypothesis, we compared the expression pattern of PIF4 in WT and the cry1 mutant under LD conditions and then under continuous blue- or red-light conditions. The result demonstrated that the rhythmic expression pattern was not changed, but the mRNA level of PIF4 in the cry1 mutant was overall higher than normal during the 24-h diurnal period. Notably, PIF4 mRNA reverted to normal level in the cry1 mutant as in WT under continuous red-light condition. In contrast, the PIF4 mRNA level was constitutively higher in the cry1 mutant than in WT under continuous blue-light conditions (Figure 2A and 2B). These results suggested that CRY1 inhibits the transcription of the PIF4 gene in a blue-light-dependent manner.

Loss-of-Function Mutant of PIF4/5 Rescues the Increased Branching Phenotype of the cry1 Mutant

To test whether the more branching phenotype of the cry1 mutant is due to the upregulation of PIF4/5, we crossed the cry1, pif4, and pif5 mutants to obtain the cry1pif4 double mutant and the cry1pif4pif5 triple mutant. The result showed that the pif4 mutant displayed moderately reduced branching phenotype compared with WT (Figure 3A). Moreover, the increased branching phenotype of the cry1 mutant partially reverted in the absence of PIF4 and was completely rescued in the loss of both PIF4 and PIF5, demonstrating that the pif4pif5 mutant is epistatic to the cry1 mutant in the genetic pathway regulating branching. Given that the PIF4 and PIF5 genes are close homologs among the seven PIFs in Arabidopsis, they seem to be functionally

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redundant in tuning branch number. We further made the 35S:PIF4-FLAG construct and obtained multiple transgenic lines in WT background. Those PIF4 overexpression lines produced significantly more branches than WT but slightly fewer branches than the cry1 mutant (Figure 3B and 3C, two representative lines #6 and #8 are shown), suggesting that the increased branching phenotype of the cry1 mutant is likely due to the increased expression of both PIF4 and PIF5.

PIF4 Binds to Its Own Promoter

Previous studies have shown that PIF4 binds to the G-box motif (CACGTG) in the promoters of target genes (Huq and Quail, 2002). We analyzed the promoter of PIF4 and found a G-box motif upstream of its start codon. Therefore, we surmised that PIF4 may target its own promoter. To test this possibility, we performed a chromatin immunoprecipitation (ChIP) experiment using the PIF4-FLAG overexpression line #6 and the anti-FLAG antibody to investigate the binding ability of PIF4 to four different regions (a, b, c, d) upstream of the transcription start site of the PIF4 gene (Figure 4A). The result showed that PIF4 selectively interacts with the regions b and c, containing or close to the G-box motif (Figure 4B). We further tested whether PIF4 directly binds to its own promoter in vitro by using an electrophoretic mobility shift assay (EMSA). The full-length PIF4 was fused to glutathione S-transferase (GST) for EMSA. The probes were biotin-labeled 60-bp fragments containing the G-box motif designed from the PIF4 promoter. Figure 4C shows that PIF4 strongly bound to the probes, which could be efficiently competed off by the excesses of unlabeled probes but not by the probes mutated in the G-box motif, suggesting that PIF4 can bind to its own promoter by recognizing the G-box motif.

CRY1 Acts as a Repressor in the PIF4 Self-Activated Transcriptional Loop

To test whether the binding of PIF4-FLAG protein to the PIF4 promoter could activate the transcription of the endogenous PIF4 gene, we designed five primer pairs to amplify primary or mature transcript to distinguish the endogenous and exogenous PIF4 expressions (Figure 4D). Among them, P0 was designed for checking the mature transcript and the other four primer pairs (P1, P2, P3, and P4) were for primary transcript. All of these primers were designed before the T-DNA insertion site in the pif4 mutant to ensure that the primary transcripts of PIF4 were detectable in the pif4 mutant (Figure 4F and Supplemental Figure 2), implying that the absence of PIF4 protein impairs the transcription of PIF4 gene. Interestingly, the endogenous primary transcripts of PIF4 were significantly upregulated in the cry1 mutant but not in the PIF4 overexpression lines, suggesting a saturation activity of PIF4 protein possibly held by CRY1 and that the absence of CRY1 may release the activity of PIF4 protein to activate the transcription of PIF4 gene. To test this hypothesis, we performed a dual-luciferase reporter assay in tobacco leaves.

Figure 3. The Abundant Branching Phenotype of cry1 Mutant Was Suppressed by pif4/pif5 Mutant.

(A) Representative images of indicated plants on the 10th DPA.

(B) Statistical analysis of the rosette branch number of each genotype as shown in (A). Data are means ± SE (n ≥ 9). Comparisons between the values of WT and indicated line were performed by Student’s t-tests (*P < 0.05, **P < 0.01).

(C) Western blot to confirm the overexpression of PIF4-flag protein in the indicated transgenic lines. The blot was probed by the anti-FLAG antibody. Ponceau S staining of Rubisco protein was used as a loading control.
PIF4 promoter and the Renilla luciferase (REN) gene was driven by the constitutive 35S promoter. Relative reporter activity (LUC/REN) was calculated as the indicator of PIF4’s ability to activate its own promoter. A revised PIF4 promoter with a mutated G-box motif was used as a negative control (Figure 5A). The pEGAD-35S:PIF4-GFP and pEGAD-35S:CRY1-GFP vectors were constructed as the effective factors to co-transform with the reporter vector into the leaves of Nicotiana benthamiana. The result shows that PIF4 exhibited strong transcription activity on the PIF4 promoter but failed to activate the mutated promoter (Figure 5B). Moreover, the co-transfection of CRY1 with PIF4 repressed the reporter activity, consolidating that CRY1 inhibits PIF4 expression through impairing the PIF4 self-activated transcription loop.

CRY1 Inhibits the Binding of PIF4 with the PIF4 Promoter

To obtain an insight into how CRY1 represses PIF4 protein to activate the PIF4 gene transcription, we inspected whether CRY1 interacts with the PIF4 promoter.

Figure 4. CRY1 Represses the Transcriptional Activation Activity of PIF4 on the PIF4 Gene.

(A) Schematic diagram of PIF4 promoter. The regions tested for the binding of PIF4 by ChIP-qPCR assay are shown. (B) ChIP-qPCR assay using the WT and PIF4-FLAG overexpression line 35S:PIF4 #6. The samples were prepared from 7-day-old seedlings grown under LD conditions and immunoprecipitated by the anti-FLAG antibody. The precipitated DNA was analyzed by qPCR using the primer pairs indicated in (A). The bindings to ACT2 and YUC8 were used as the negative and positive control, respectively. The data are means ± SE (n = 3). **P < 0.01 (Student’s t-test). (C) EMSA showing the interaction of PIF4 with the G-box motifs present in the PIF4 promoter. The GST protein was incubated with the labeled probe to serve as a negative control; mutated probes were used as a negative control. Ten- and 20-fold excesses of unlabeled or mutated probes were used for competition. Mu, mutated probe in which the G-box motif 5’-CACGTG-3’ was replaced with 5’-AACGTG-3’. (D) Diagram depicting the genomic region of PIF4 gene. The boxes represent the exons. The inverted triangle indicates the insertion site of T-DNA in pif4 mutant. (E and F) The mRNA level of PIF4 gene in each indicated genotype analyzed by qRT-PCR using primer pairs amplifying P0 (E) and P1–P4 (F) as indicated in (D). Samples were 7-day-old seedlings grown under LD conditions and collected at the 6th hour of the light period. Data are means ± SE from three independent biological replicates (each with two technical replicates). Comparisons between the values of WT and indicated line were performed by Student’s t-tests (*P < 0.05, **P < 0.01).
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Figure 5. CRY1 Represses PIF4 to Activate the PIF4 Promoter.
(A) Schematic illustration of the dual-luciferase reporter construct, which contains two reporter genes: REN (Renilla luciferase), driven by the 3S promoter, and LUC (firefly luciferase), driven by the WT PIF4 promoter (−2472 bp to 0 bp) or modified PIF4 promoter with mutated G-box, respectively.
(B) Upper panel: relative reporter activity (LUC/REN) when co-expressed with the different effector. Tobacco leaves were transfected with the reporter and the effector (CRY1, PIF4, or both CRY1 and PIF4), kept in the dark for 15 h, and exposed to continuous white light for 3 days. The value of WT PIF4 promoter with no effector was arbitrarily set to 1. Data are means ± SE (n = 3). **P < 0.01 (Student’s t-test). Lower panel: western blot showed the expression of protein in the indicated tobacco leaves probed by the anti-GFP antibody. Ponceau S staining of Rubisco protein was used as a loading control. Triangle depicts the CRY1-GFP proteins. Arrow depicts the PIF4-GFP proteins.

(C) EMSA shows that GST-CRY1 interferes with GST-PIF4 binding to the DNA probes derived from the PIF4 promoter in vitro. Biotin-labeled probes were incubated with a fixed amount of GST-PIF4 and an increasing amount of GST-CRY1 or GST. Mutated probes were used as negative control. Mu, mutated probe in which the G-box motif 5'-CACGTG-3' was replaced with 5'-AAGCGT-3'.

can affect PIF4 binding ability in vitro. The EMSA result shows that GST-CRY1 could efficiently block GST-PIF4 from binding to the G-box-containing probes (Figure 5C). Next, we tested whether the loss-of-CRY1 function could affect the binding ability of PIF4 to its own promoter in vivo. Multiple PIF4-FLAG overexpression lines in cry1 background were generated and two lines expressing comparable or slightly less transgenic proteins in comparison with the lines in WT background were selected for further investigation (Figure 6A). First, we measured the branch number and found that the two PIF4-FLAG overexpression lines in the cry1 mutant background produced significantly more branches than the two PIF4-FLAG overexpression lines in WT background (Figure 6B). Second, we compared the affinity of PIF4-FLAG protein with that of the PIF4 promoter in the cry1 mutant and WT plants by ChIP. The results demonstrated that the binding ability of PIF4-FLAG protein to the PIF4 promoter was significantly enhanced in the cry1 mutant compared with WT (Figure 6C). Taken together, these results suggested that CRY1 represses PIF4 transcription at least partially by blocking PIF4 protein to bind with its own promoter.

DISCUSSION

CRY1 is the first member of the cryptochromes originally discovered in Arabidopsis (Ahmad and Cashmore, 1993). CRY1 mediates a dozen blue-light responses including inhibition of hypocotyl elongation, entrainment of the circadian clock, stomata opening, and programmed cell death (Yu et al., 2010; Xiong et al., 2019). However, its functions in regulating plant architecture remain largely unknown, probably because such a phenotype is subtle or undetectable in certain genetic backgrounds or growth conditions. In this study, we found that the cry1 mutant in the col-4 ecotype background displayed significant more rosette-branching phenotype than the WT plants grown under low planting density. Despite the fact that the increased branching phenotype of the cry1 mutant is less pronounced under higher planting density and lower light intensity, the difference is constitutively significant under all our experimental regimes, suggesting that CRY1 may regulate branching through a divergent pathway of SARs. Although BRC2 expression is reduced in the cry1 mutant (Supplemental Figure 1) and there is a G-box motif (−1234 bp to −1229 bp, numbered relative to the start codon) in the BRC2 promoter, our ChIP results detected no binding signal of PIF4 to the BRC2 promoter (Supplemental Figure 3), implying that the BRC2 gene may not be under the direct regulation of PIF4 and CRY1.

Recent studies and our results showed that CRY1 and CRY2 physically interact with PIF4/5 to mediate low-blue-light-induced hypocotyl elongation (Supplemental Figure 4) (de Wit et al., 2016; Pedmale et al., 2016). Here, we show that mutations in PIF4/5 are epistatic to the cry1 mutant in the regulation of branching. PIFs are the key growth-promoting transcriptional factors mediating shade-induced or circadian gating of hypocotyl growth. The mRNA level of PIF4/5 shows a typical rhythmic expression pattern, peaking in the middle of the day and diminishing in the early evening. The expression profiles of PIF4/5 are strictly controlled by the circadian clock and photoreceptors at both transcriptional and post-transcriptional levels in response to the change of ambient light conditions. For example, the circadian clock evening complex (composed of ELF3, ELF4, and LUX) regulates the proper expression of PIF4/5 under diurnal conditions by repressing the PIF4/5 transcription in the early evening (Nusinow et al., 2011). The red-/far-red-light receptor PhyB controls the turnover of PIF4/5 proteins during the day. These regulations underline the molecular basis for the rhythmic expression of PIF4/5 in gating hypocotyl elongation at dawn. However, it remains unclear how the PIF4/5 transcriptions are sharply elevated in the early day (Nusinow et al., 2011). Here, we show that PIF4 targets its own promoter to form a self-activated transcriptional feedback loop, which may partially solve this puzzle (Figure 6D). Moreover, the mRNA expression level of
PIF4 is significantly upregulated in the absence of blue light and the loss of CRY1 function (Figure 2), demonstrating a key role of CRY1 in inhibiting growth by repressing PIF4 transcription during the light period of diurnal cycles.

The inhibition of PIF4 expression by CRY1 may be achieved by at least two mechanisms. First, CRY1 directly represses the transcription activity of PIF4 by physical interaction with PIF4 (Ma et al., 2016). Second, CRY1 may block PIF4 from binding to the PIF4 promoter as shown in this study (Figures 5C and 6C). It has been proposed that the physical interaction between CRY1 and PIF4 could provide specificity to affect positive or negative transcription activity dependent on target genes (Supplemental Figure 5) (Pedmale et al., 2016). This regulating specificity is probably determined by the chromosomal microenvironment surrounding specific target genes or by temporal and spatial cues within different cells, the molecular basis of which requires elucidation by further experiments.

**METHODS**

**Plants Materials and Growth Conditions**

All the Arabidopsis plants used in this study were in Columbia-4 ecotype background. The cry1-304 mutant was previously reported (Bruggemann et al., 1996; Mockler et al., 1999); the pif4 (CS66043) and pif5 (SALK_087012C) mutants were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/abrc/). Double and triple mutants were generated by genetic crossing. Plants were grown in LD conditions (16 h light/8 h dark) at 22°C for general growth and seed harvesting. For phenotypic analysis, the surface-sterilized seeds were planted on half-strength Murashige and Skoog (1/2 MS) growth medium containing 1% (w/v) sucrose with 0.6% (w/v) agar, stratified at 4°C in darkness for 4 days, grown at 22°C for 5 days, and transferred to soil. The rosette branch numbers were measured on the 10th or 20th DPA as described previously (Finlayson et al., 2010; Zhang et al., 2014).

**Western Blot Analyses**

The 7-day-old seedlings were harvested, frozen by liquid nitrogen, ground into powder, and suspended in 0.44 M sucrose, 2.5% dextran T40, 20 mM HEPES–KOH (pH 7.4), 10 mM MgCl2, 0.5% Triton X-100, 5 mM DTT, 1 mM PMSF, 40% glycerol, 8% SDS, 0.02% bromophenol blue, and 6.2% DL-dithiothreitol (DTT). Protein extractions were incubated at 98°C for 5 min, separated on 10% SDS–polyacrylamide gels, and transferred to a nitrocellulose membrane. Protein was visualized using the anti-CRY1 antibody as described previously (Liu et al., 2011) or the anti-FLAG antibody (Abmart). The membrane was stained by Ponceau S or stripped followed by reprobing using the anti-Actin antibody to indicate relative loadings. Quantification of proteins was performed using ImageJ software (http://rsb.info.nih.gov/ij/).

**Quantitative Real-Time PCR**

Total RNA was extracted from 7-day-old seedlings by TRNzol reagent (Tiangen). cDNAs were synthesized by EasyScript First-Strand cDNA Synthesis SuperMix (TransGen). Quantitative real-time PCR (qRT-PCR) was performed using LightCycler 480 (Roche) and SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara). Gene expression levels were normalized to the ACTIN2 gene. Primers are listed in Supplemental Table 1.

**Chromatin Immunoprecipitation Assays**

Three grams of 7-day-old seedlings was crosslinked under vacuum in formaldehyde solution (0.4 M sucrose, 10 mM Tris [pH 8.0], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1% formaldehyde) for 15 min, two times. Crosslinking was stopped under vacuum for 5 min after adding glycine to a final concentration of 200 mM. The seedlings were rinsed with water, frozen in liquid nitrogen, ground to a fine powder at low temperature, and suspended in the ice-cold Honda buffer (0.44 M sucrose, 1.25% Ficoll, 2.5% dextran T40, 20 mM HEPES–KOH [pH 7.4], 10 mM MgCl2, 0.5% Triton X-100, 5 mM DTT, 1 mM PMSF, 10 mM MgCl2, 0.5% Triton X-100, 5 mM DTT, 1 mM PMSF, 40% glycerol, 8% SDS, 0.02% bromophenol blue, and 6.2% DL-dithiothreitol [DTT]). Protein extracts were incubated at 98°C for 5 min, separated on 10% SDS–polyacrylamide gels, and transferred to a nitrocellulose membrane. Protein was visualized using the anti-CRY1 antibody as described previously (Liu et al., 2011) or the anti-FLAG antibody (Abmart). The membrane was stained by Ponceau S or stripped followed by reprobing using the anti-Actin antibody to indicate relative loadings. Quantification of proteins was performed using ImageJ software (http://rsb.info.nih.gov/ij/).

**Figure 6. Mutation in CRY1 Elevates the Binding of PIF4 to Its Own Promoter.**

(A) Western blot showed the expression of PIF4-FLAG protein in the indicated transgenic lines probed by the anti-FLAG antibody. Ponceau S staining of Rubisco protein was used as a loading control.

(B) Statistical analysis of the rosette branch number of each genotype. Data are means ± SE (n ≥ 9). The letters above the bars indicate significant differences (P < 0.05) as determined by one-way ANOVA analysis.

(C) ChIP–qPCR assay using the PIF4-FLAG overexpression line 35S::PIF4#6 and 35S::PIF4#4/ cry1. The samples were prepared from 7-day-old seedlings grown under LD conditions and immuno-precipitated by the anti-FLAG antibody. The precipitated DNA was analyzed by qPCR using the primer pair amplifying fragment b as indicated in Figure 4A. The binding to ACT2 was used as a negative control. The data are means ± SE (n = 3). **P < 0.01 (Student’s t-test).

(D) The proposed working model. In the WT plant, CRY1 protein represses the transcriptional activation activity of PIF4 as well as the binding ability of PIF4 to its own promoter. Mutation of CRY1 released the self-activating feedback loop of PIF4, enhancing the transcription of PIF4 and downstream genes to promote rosette branching.
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and protease inhibitor cocktail). Samples were precipitated by centrifugation with 2000 g at 4°C for 20 min, resuspended in the ice-cold Honda buffer, and precipitated by centrifugation three times. The precipitates were suspended in ice-cold Nuclei Lysis Buffer (50 mM Tris–HCl [pH 8.0], 10 mM EDTA, 1% SDS, 1 mM PMSF, protease inhibitor cocktail) and sheared by sonication for 15 s every 1 min, six times. Samples were diluted 10 times with ice-cold ChIP Dilution Buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl [pH 8.0], 167 mM NaCl) to obtain the final chromatin solution. Dynabeads Protein A (Invitrogen, 10002D) were washed by ice-cold binding/washing buffer (150 mM NaCl, 20 mM Tris–HCl [pH 8.0], 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM PMSF) three times. Every 60 µl of Dynabeads was then suspended in 100 µl of ice-cold binding/washing buffer, mixed with 2 ml of chromatin solution and 8 µl of anti-FLAG antibody (Invitrogen, MA1-91878), and incubated (turned up and down six times per minute) at 4°C for 16 h. The Dynabeads were then washed with ice-cold binding/washing buffer five times and TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA) two times (1 min per time), then suspended in 10% Chelex and boiled at 99°C for 10 min. Samples were incubated with Proteinase K at 43°C for 1 h and boiled at 95°C for 10 min. The supernatants were collected by centrifugation for qRT-PCR using primers listed in Supplemental Table 1.

Dual-Luciferase Reporters Assay

A transient dual-luciferase assay to test the transcriptional activity of PIF4 was performed as previously described using N. benthamiana plants (Zhang et al., 2014). The reporter vector, pGreen-PIF4ProLuc, encodes two luciferases, the Renilla Luciferase (REN) controlled by the constitutive 35S promoter, and the firefly luciferase (LUC) controlled by the wide-type PIF4 promoter or the mutated PIF4 promoter with the mutant G-box (AACGTG) of the CYC1 promoter. The effector vectors pEGAD-35S-PIF4-GFP and pEGAD-35S:CRY1-GFP were constructed by cloning the CDS of PIF4 and CRY1 into the vector pEGAD-35S:GFP, respectively. The pEGAD-35S:GFP construct was used as the negative control. The reporter vector was co-transformed into Agrobacterium (strain AGL0) with the helper plasmid, pSoup-p19. The effector vectors (including the negative control) were respectively transformed into Agrobacterium. Cultures of agrobacteria (OD600, 0.8–1.0) were collected by centrifugation, resuspended in infiltration buffer (0.15 mM acetoxyribose, 10 mM MgCl2, 10 mM 2-(N-morpholine)-ethanesulfonic acid [MES]–NaOH [pH 5.6]), and incubated at room temperature for 3–4 h before infiltration. The infiltration buffer mixture containing reporter vector and effector vector (at a ratio of 1:4) was infiltrated into the leaves of N. benthamiana (about 1 month old). The infiltrated plants were kept in darkness for about 15 h and grown in continuous white light for 3 days. The dual-luciferase assay was performed using the Dual-luciferase Reporter Assay System (Promega). In brief, leaves infected by agrobacteria were frozen by liquid nitrogen and suspended in Passive Lysis buffer. Every 7-µl suspension was mixed with 35 µl of Luciferase Assay buffer; the LUC activity was measured using a luminometer (Berthold). Next, 35 µl of Stop and Glow buffer was added to quench the firefly luciferase and measure the LUC activity. Three biological replicates were prepared.

EMSA

Full-length CDSs of CRY1 and PIF4 were PCR amplified and cloned into pGEX-4T vector. The recombinant GST fusion proteins were expressed in Escherichia coli BL21 (DE3) cells and purified to homogeneity using an amylose resin column. Biotin-labeled probes were incubated with GST fusion proteins at room temperature for 15 min, and free and bound probes were separated via PAGE. Mutated PIF4 probes, in which the G-box motif 5’-CACGTG-3’ was replaced with 5’-AACGTG-3’, were used as negative controls. Probes used for EMSA are listed in Supplemental Table 1.

Luciferase Complementation Imaging Assay

The firefly luciferase complementation imaging assay was performed with N. benthamiana leaves. Primers used for vector construction are shown in Supplemental Table 1.

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Supplemental Table 1. The constructs were introduced into Agrobacterium tumefaciens strain GV3101. GV3101 carrying the indicated constructs were cultured in Luria–Bertani (LB) medium at 28°C overnight and transferred to fresh LB medium with 10 mM MES [pH 5.6] and 40 µM acetoxyribose (1:100 ratio, v/v) for 16 h. The culture was pelleted and resuspended in 10 mM MgCl2 containing 0.2 mM acetoxyribose to a final concentration of OD600 = 1.5. Bacteria were kept at room temperature for at least 3 h without shaking. For co-transformation, equal volumes of Agrobacterium suspensions carrying the indicated constructs were infiltrated into N. benthamiana leaves. After infiltration, plants were incubated at 22°C for 3 days under 16 h light/8 h dark before measuring LUC activity. Leaves were sprayed with 0.5 mM luciferin and placed in the dark for 5 min before luminescence detection.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at Plant Communications Online.

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AUTHOR CONTRIBUTIONS

B.L. and J.L. designed the research. H.Z., L.X., H.L., X.L., G.Y., and T.Z. performed the experiments. H.Z., L.X., and T.Z. collected the phenotypic data. H.Z., L.X., and H.L. analyzed data. B.L. and J.L. wrote the manuscript.

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