WRKY2 and WRKY10 regulate the circadian expression of \textit{PIF4} during the day through interactions with CCA1/LHY and phyB

Shulei Wang$^{1,3}$, Qingbin Sun$^{1,3}$, Min Zhang$^1$, Chengzhu Yin$^1$ and Min Ni$^{2,*}$

$^1$National Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Taian 271018, China
$^2$Department of Plant and Microbial Biology, University of Minnesota at Twin Cities, Saint Paul, MN 55108, USA
$^3$These authors contributed equally to this article.

*Correspondence: Min Ni (nixxx008@umn.edu)

https://doi.org/10.1016/j.xplc.2021.100265

ABSTRACT

WRKY transcription factors are known mostly for their function in plant defense, abiotic stress responses, senescence, seed germination, and development of the pollen, embryo, and seed. Here, we report the regulatory functions of two WRKY proteins in photomorphogenesis and \textit{PIF4} expression. \textit{PIF4} is a critical signaling hub in light, temperature, and hormonal signaling pathways. Either its expression or its accumulation peaks in the morning and afternoon. WRKY2 and WRKY10 form heterodimers and recognize their target site in the \textit{PIF4} promoter near the MYB element that is bound by CCA1 and LHY under red and blue light. WRKY2 and WRKY10 interact directly with CCA1/LHY to enhance their targeting but interact indirectly with SHB1. The two WRKY proteins also interact with phyB, and their interaction enhances the targeting of CCA1 and LHY to the \textit{PIF4} promoter. SHB1 associates with the \textit{WRKY2} and \textit{WRKY10} loci and enhances their expression in parallel with the \textit{PIF4} expression peaks. This forward regulatory loop further sustains the accumulation of the two WRKY proteins and the targeting of CCA1/LHY to the \textit{PIF4} locus. In summary, interactions of two WRKY proteins with CCA1/LHY and phyB maintain an optimal expression level of \textit{PIF4} toward noon and afternoon, which is essential to sketch the circadian pattern of \textit{PIF4} expression.

Key words: CCA1, LHY, light signaling, PIF4 expression, WRKY2, WRKY10

Wang S., Sun Q., Zhang M., Yin C., and Ni M. (2022). WRKY2 and WRKY10 regulate the circadian expression of \textit{PIF4} during the day through interactions with CCA1/LHY and phyB. Plant Comm. \textbf{3}, 100265.

INTRODUCTION

Light provides the source of energy for the growth and development of plants throughout their entire life cycle. Light is also arguably one of the most important environmental factors that regulate plant growth and development. Light signals are perceived by a complex array of photoreceptors, which are defined by the light wavelength they absorb. The photoreceptors include the red and far-red light-absorbing phytochromes, the ultraviolet A (UVA)/blue-light-absorbing cryptochromes (crys) and phototropins, and the UVB photoreceptor UVR8 (Schäfer and Nagy, 2006). \textit{Arabidopsis thaliana} has five members in the phy family, designated phyA to phyE, that have different and overlapping functions in plant photomorphogenesis (Sharrock and Quail, 1989; Clack et al., 1994; Mathews and Sharrock, 1997; Franklin and Quail, 2010; Sánchez-Lamas et al., 2016). Phytochromes undergo light-induced and photo-reversible switching between two conformers: the biologically inactive red-light-absorbing Pr form and the biologically active far-red-light-absorbing Pfr form (Smith, 2000). The light signal is influenced by light quantity and color, as phytochrome responses are dependent on the proportion of Pfr conformers (Jung et al., 2016; Legris et al., 2016). Photoconversion of phytochromes to Pfr results in their translocation from the cytoplasm into the nucleus and inhibits two classes of photomorphogenesis repressors, the CONSTITUTIVELY PHOTOMORPHOGENIC/DEETIOLATED/FUSCA (COP/DET/FUS) complex and phytochrome interacting factors (PIFs) (Leivar and Quail, 2011; Lau and Deng, 2012; Podolec and Ulm, 2018; Legris et al., 2019).

PIFs belong to a basic helix-loop-helix family of transcription factors (Toledo-Ortiz et al., 2003; Duek and Fankhauser, 2005). Upon red light irradiation, phyB is converted to the biologically active...
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WRKY zinc-finger proteins belong to a superfamily of transcription factors that is restricted to the plant kingdom and probably contains up to 74 members in Arabidopsis (Eulgem et al., 2000). Although the precise regulatory functions of the entire WRKY family are largely unknown, they play important roles in many aspects of plant growth and development (Hussain et al., 2018; Khan et al., 2018). WRKY10, or MIN3 (MINISEED3), is well known for its function in the regulation of seed development (Luo et al., 2005). WRKY10 is expressed in the developing endosperm and the embryo during the early stage of seed development, but not in the late-heart embryo. WRKY10 targets its own promoter and that of HAUK2 (IKU2), a receptor-like protein kinase gene; it interacts with SHB1 after a crosslink and regulates early endosperm proliferation (Zhou et al., 2009; Kang et al., 2013). WRKY2 regulates seed germination and post-germination growth arrested by abscisic acid (ABA) (Jiang and Yu, 2009). WRKY2 also plays an essential role in pollen development (Niewiadomski et al., 2005; Zheng et al., 2018). wrky2 mutant plants exhibited reduced pollen viability, which is associated with decreased pollen germination, pollen tube growth, and male transmission. In addition, WRKY2 directly activates the expression of WOX8 in the zygote and regulates polar organelle localization and asymmetric division of the zygote, its basal daughter cell, and the hypophysis. In wrky2 mutants, the regulation of the apical-basal body axis through an asymmetric division of the polarized zygote is altered (Ueda et al., 2011).

In this study, we report that WRKY10 and WRKY2 are also involved in plant photomorphogenesis. Each of the single knockout wrky10 and wrky2 mutants showed a weak hypocotyl phenotype and moderately reduced PIF4 expression compared with the wild type under red and blue light. The homozygous wrky10 wrky2 mutant is lethal, and the wrky2 wrky10+ mutant showed a strong hypocotyl phenotype and reduced PIF4 expression under red and blue light compared with the wrky10 or wrky2 single mutant. WRKY10 and WRKY2 bind to the promoter of PIF4 near the CCA1 and LHY binding site. The interaction of the two WRKYs with CCA1 or LHY enhances the targeting of CCA1 and LHY to the PIF4 regulatory region. WRKY10 and WRKY2 interact directly with phyB and require it for their function. In addition, SHB1 activates the expression of WRKY10 and WRKY2 when PIF4 is also highly expressed. This feedforward regulatory step maintains sufficient levels of WRKY2 and WRKY10 to enhance PIF4 expression for better plant performance at high light intensity.

RESULTS

WRKY10 and WRKY2 regulate the expression of PIF4 under red and blue light

In early studies, WRKY10 was shown to be expressed in the developing endosperm and early embryo (Zhou et al., 2009). WRKY10 aided the recruitment of SHB1 to its own promoter and that of IKU2 to activate their expression in a W-box-dependent manner (Jiang and Yu, 2009; Kang et al., 2013). However, the expression of WRKY10 was also found in hypocotyls, cotyledons, and roots of pWRKY10::GUS transgenic seedlings, either in the dark or under red light (Figure 1A). To explore whether WRKY10 is involved in photomorphogenesis other than seed development, we
measured the hypocotyl length of the wrky10 knockout mutant under different light conditions (Figure 1B and Supplemental Figure 1A). The wrky10 mutant showed a weak hypocotyl phenotype compared with that of Col under red and blue light but not under far-red light or in the dark. The hypocotyl phenotype of the wrky10 mutant was then tested at a range of red light intensities (Supplemental Figure 1B).

In Arabidopsis, the WRKY transcription factor family probably has up to 74 members with very diverse functions (Eulgem et al., 2000; Noman et al., 2018; Hussaina et al., 2019). To determine whether another WRKY family member functions redundantly with WRKY10 in plant photomorphogenesis, we searched for WRKY10 homologs at the TAIR (http://www.arabidopsis.org/) and NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) sites. WRKY10 was highly homologous to WRKY2, WRKY25, WRKY26, WRKY33, and WRKY34 based on phylogenetic analysis of their full-length protein sequences (Supplemental Figure 1C and 1D). We then identified a T-DNA insertional mutation in each of these homologous genes (Supplemental Figure 2A–2C) and constructed various double mutants with wrky10 (Figures 1B and Supplemental Figure 2D).
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wrky2, each single mutant showed a wild-type hypocotyl phenotype, and the corresponding double mutants with wrky10 showed a wrky10-like hypocotyl phenotype (Supplemental Figure 2D). Similar to WRKY10, WRKY2 was expressed in a wide range of tissues (Figure 1A). The wrky2 mutant also exhibited a weak hypocotyl phenotype under red and blue light, but not under far-red light (Figure 1B and Supplemental Figure 1A).

When wrky2 was crossed with wrky10, a certain proportion of seeds were abnormal, suggesting that the wrky2 wrky10 double homozygous mutant is lethal (Supplemental Figure 2E). To test this hypothesis, we introduced a homozygous wrky2 mutation into the heterozygous wrky10 background based on the fact that wrky10 contains a T-DNA insertion and a linked kanamycin selection marker. In the wrky2 wrky10± segregating population, 75% of the seeds were normal, and the remaining 25% of seeds were presumably aborted (Supplemental Figure 2F).

After obtaining images of their hypocotyl growth under red light at day 4, the wrky2 wrky10± seedlings were transferred to a kanamycin plate, and the surviving individuals were considered to be wrky2 wrky10± seedlings. The hypocotyl length of each individual from the two populations was then measured and plotted (Figure 1C). Compared with that of the wrky10 single mutant, the hypocotyls of the wrky2 wrky10± seedlings were shorter than those of each single mutant under red and blue light but not in the dark (Figure 1B). By contrast, the far-red light phenotype of wrky2 wrky10± was marginal (Supplemental Figure 1A). Furthermore, overexpression of WRKY10 or WRKY2 produced a long hypocotyl phenotype under red and blue light, and a very weak hypocotyl phenotype under far-red light (Figure 1B and Supplemental Figures 1A, 3A, and 3B).

The expression of PIF4 is rhythmic (Zheng et al., 2006; Nozue et al., 2007; Niwa et al., 2009). To further explore the function of WRKY10 and WRKY2 in red and blue light responses, we examined the rhythmic expression of PIF4 in Col and wrky2 wrky10± seedlings after an entrainment cycle of 12-h dark and 12-h red or blue light for 7 days (Figure 1D and 1E). The wrky2 wrky10± seedlings were selected in a kanamycin plate and sampled at zeitgeber time 0 (ZT0) and every 3 h thereafter for 36 h. The presence of kanamycin in the medium did not affect PIF4 expression (Supplemental Figure 3C). The magnitude but not the rhythmic pattern of PIF4 expression was affected in the wrky2 wrky10± seedlings. However, the expression of either WRKY2 or WRKY10 was not rhythmic (Supplemental Figure 3D). The wrky2 wrky10± mutations did not affect the rhythmic expression of the clock genes CCA1 and LHY (Supplemental Figure 3E and 3F).

WRKY2 and WRKY10 formed heterodimers but not homodimers (Figure 1F). If WRKY2 or WRKY10 formed either the functional homodimer or the functional heterodimer, knockout of either WRKY2 or WRKY10 should not have led to a visible hypocotyl phenotype (Figure 1B). In fact, the expression of PIF4 was downregulated moderately in either the wrky2 or wrky10 single mutant and strongly in wrky2 wrky10± seedlings under red and blue light at ZT9 compared with that of the Col wild type (Figure 1G and 1H). By contrast, PIF4 expression was strongly upregulated in 35S::WRKY10:MYC and 35S::WRKY2:MYC overexpression transgenic lines. However, the wrky2 wrky10± seedlings showed only marginal changes in plant thermo responses, such as hypocotyl elongation and PIF4 expression, compared with the Col wild type (Supplemental Figure 3G and 3H).

WRKY10 and WRKY2 bind to the PIF4 promoter

We explored whether WRKY10 and WRKY2 bind to the promoter of PIF4 and directly regulate PIF4 expression. There are three potential WRKY binding elements or W-boxes TTGACC(T/A) in the PIF4 promoter (Hussain et al., 2018; Khan et al., 2018; Mur et al., 2006; Torres et al., 2006; http://meme-suite.org/), and we designated them W1, W2, and W3 (Figure 2A). In a yeast one-hybrid (Y1H) assay, WRKY10 bound to a DNA fragment that contained both the W1 and W2 elements in the PIF4 promoter (Figure 2B). We then synthesized trimeric W1, W2, and W3 elements in tandem repeats. WRKY10 specifically bound to the trimeric W1 element but not to the trimeric W2 or W3 element (Figure 2C). The W1 element is located −845 to −805 bp upstream of the PIF4 start codon. The direct binding of WRKY10 to the W1-box was also confirmed by an electrophoretic mobility shift assay (EMSA) (Figure 2D). A retarded band was observed when WRKY10 was mixed with a biotin-labeled DNA probe that contained the W1-box. The retarded band was effectively competed off with a 10× or greater excess of unlabeled DNA probe. When the W1-box in the DNA probe was mutated, the retarded bands were not competed off (Figure 2D). We next performed ChIP experiments with WRKY10:MYC transgenic lines and anti-MYC antibody. The W12 fragment in the PIF4 promoter was enriched in WRKY10::MYC transgenic plants under red or blue light but not in the dark or under far-red light (Figure 2E). Similar to WRKY10, WRKY2 also bound the W1-box in vitro, and this binding was W1-box specific, as the mutated W1-box failed to compete off the WRKY2-retarded bands (Figure 2F). WRKY2 also directly bound to the promoter of PIF4 in vivo in a red- and blue-light-dependent manner (Figure 2G).

WRKY10 and WRKY2 directly interact with CCA1 and LHY

The binding site for WRKY10 and WRKY2 in the PIF4 promoter is just 226 bp upstream of the MYB binding site for CCA1 and LHY (Sun et al., 2018). It is therefore possible that WRKY10 and WRKY2 interact directly with CCA1 and LHY. We investigated this possibility using a yeast two-hybrid (Y2H) assay. CCA1 and LHY were fused with the GAL4 DNA-binding domain (GBD), and WRKY10 protein was fused with the GAL4 activation domain (GAD). Y2H assays demonstrated a strong interaction of GAD::WRKY10 with GBD::CCA1 or GBD::LHY (Figure 3A). By contrast, GBD::CCA1 or GBD::LHY co-transformed with GAD did not support yeast growth on selection medium or generated only low background activity. GBD::WRKY2 also interacted with GAD::CCA1 or GAD::LHY (Figure 3B).

We next performed a bimolecular fluorescence complementation (BIFC) assay in N. benthamiana leaves to study the interactions of WRKY2/10 with CCA1/LHY in vivo. Strong YFP fluorescent signals were observed in the nuclei when CCA1/YFPN or LHY::YFPN (fused to the N-terminal end of YFP) and WRKY10::YFP C (fused to the C-terminal end of YFP) were co-transformed into N. benthamiana (Figure 3C). No fluorescent
Figure 2. WRKY10 and WRKY2 bind to the PIF4 promoter.

(A) PIF4 promoter region used for Y1H and ChIP-qPCR analysis. M1 indicates MYC binding element 1 for LHY and CCA1; W1, W2, and W3 indicate WRKY-box 1, 2, and 3; and EC indicates LUX binding element. Numbers below indicate fragments used for ChIP-qPCR analysis.

(B and C) Yeast one-hybrid assays for WRKY10 with a region that contains both WRKY-box 1 and 2 (W12, B) or with trimeric WRKY-box 1, 2, or 3 (C) in SD-Leu-Ura plates with or without 300 ng/ml aureobasidin A (AbA) for 3 days.

(D and F) Electrophoretic mobility shift assay (EMSA) for WRKY10 (D) and WRKY2 (F) with wild-type or mutated WRKY-box 1 in the PIF4 promoter. Half μl TnT reaction was used in the binding reaction, and unlabeled oligonucleotides were used as competitors.

(E and G) ChIP analysis of 35S::WRKY10:MYC (E) and 35S::WRKY2:MYC (G) with the PIF4 regulatory region in the dark and under 10 μmol m⁻² s⁻¹ red light, 7 μmol m⁻² s⁻¹ blue light, or 1 μmol m⁻² s⁻¹ far-red light. Error bars represent standard deviations of three biological replicates. *p < 0.05 and **p < 0.01 (Student’s t-test).
signals were detected when CCA1:YFPN or LHY:YFPN was co-transformed with YFPC, or when YFPN was co-transformed with WRKY10:YFPC. To confirm these interactions, we performed co-immunoprecipitation (coIP) assays using the N-terminal MYC-tagged full-length WRKY10 protein (WRKY10-MYC) and the endogenous CCA1 or LHY proteins. Cell extracts were

![Figure 3. WRKY10 and WRKY2 interact with CCA1 or LHY. (A and B) Yeast two-hybrid ONPG assays for interaction between WRKY10 (A) or WRKY2 (B) and CCA1 or LHY. (C) BiFC analysis for YFPN with WRKY10:YFPC, CCA1:YFPN or LHY:YFPN with YFPC, and CCA1:YFPN or LHY:YFPN with WRKY10:YFPC. (E) BiFC analysis for YFPN with CCA1:YFPC or LHY:YFPC, WRKY2:YFPN with YFPC, and WRKY2:YFPN with CCA1:YFPC or LHY:YFPC. Images of Nicotiana leaf epidermal cells were captured 60 h after Agrobacterium transfection. Scale bar corresponds to 10 μm. (D) Co-immunoprecipitation of CCA1 or LHY by WRKY10:MYC in protein extracts prepared from red light-grown transgenic Arabidopsis seedlings with anti-MYC antibody. Protein blots were probed with anti-CCA1 or anti-LHY antibody. (F) Co-immunoprecipitation of CCA1:MBP or LHY:MBP by WRKY2:GST with anti-GST antibody. Protein blots were probed with anti-GST or anti-MBP antibody.](image-url)
prepared from the seedlings and were subjected to immunoprecipitation using anti-MYC antibody. The immunoprecipitated proteins were analyzed in western blots with anti-CCA1 or anti-LHY antibodies. WRKY10-MYC co-immunoprecipitated CCA1 and LHY under red light (Figure 3D) and in the dark (Supplemental Figure 4), WRKY2 also interacted with CCA1 and LHY in BiFC and in vitro coIP assays (Figure 3E and 3F).

We then studied the protein domains of CCA1 and LHY that were involved in their interactions with WRKY10 and WRKY2 using Y2H assays (Supplemental Figure 5A–5E). CCA1 and LHY contain a conserved MYB DNA-binding domain at their N terminus from approximately amino acids 22 to 72 (Sun et al., 2019). We created constructs in which the N-terminal 173 amino acids of CCA1 or LHY and the C-terminal sequence after amino acid 173 were fused to GBD (Supplemental Figure 5A). Full-length CCA1 and LHY interacted with WRKY10 or WRKY2 fused to GAD (Supplemental Figure 5B and 5C). The truncated CCA1 and LHY GBD were then tested against full-length WRKY10 or WRKY2 (Supplemental Figure 5D and 5E). In Y2H assays, WRKY10 interacted with the C terminus of CCA1 and LHY, but not with the MYB DNA-binding domains in their N termini. For an unknown reason, the truncated N terminus of LHY, in contrast to its full length, produced a high background by itself. We also tested the interactions of CCA1 or LHY proteins with two truncated versions of WRKY10 or WRKY2 (Supplemental Figure 5F). WRKY10 contains a long stretch of N-terminal sequence with an unknown function and a short C-terminal WRKY DNA-binding domain. We fused the WRKY10 N-terminal 302 amino acids and its C-terminal 184 amino acids that contain the WRKY domain to GAD. Full-length CCA1 or LHY interacted with either the N terminus or the C terminus of WRKY10 (Supplemental Figure 5G). WRKY2 contains two WRKY DNA-binding domains (Supplemental Figure 5F). We divided the protein in the middle and created an N-terminal and a C-terminal truncation. CCA1 and LHY interacted only with the WRKY2 C terminus (Supplemental Figure 5H).

**WRKY10 and WRKY2 enhance in vitro binding activity of CCA1 and LHY**

WRKY10 and WRKY2 bound to the *PIF4* promoter in vivo and interacted with CCA1/LHY directly (Figures 2 and 3). The next key question was how WRKY10 and WRKY2 interact with CCA1 and LHY in relation to *PIF4* expression. First, the interaction between WRKY10 or WRKY2 and CCA1 or LHY may enhance the DNA-binding activity of CCA1 and LHY. To test this hypothesis, we produced CCA1 and LHY in vitro (Supplemental Figure 6A) and performed EMSA to examine the binding of CCA1 and LHY to the *PIF4* promoter. CCA1 and LHY produced a clearly retarded band with a biotin-labeled probe that contained an M1-box, ATATCT, /C0 595 to /C0 589 bp upstream of the *PIF4* start codon (Figure 4A and 4B), consistent with our previous Y1H assays (Sun et al., 2019). The retarded band was competed off by unlabeled probes, but it was not competed off when the M1 box in the unlabeled probes was mutated from ATATCT to ATTGCT.

To examine whether WRKY10 or WRKY2 influences the DNA-binding activity of CCA1 and LHY, WRKY10 or WRKY2 was produced in the TnT system (Promega, USA) and used for electrophoretic mobility shift assays (Supplemental Figure 6B). In the presence of WRKY10 or WRKY2, the intensity of the retarded band produced by CCA1 or LHY was strongly increased (Figure 4C and 4D). We consistently observed a supershift for
WRKY10 enhances the in vivo DNA-binding activity of CCA1 and LHY

To examine whether WRKY10 or WRKY2 protein indeed influences the binding of CCA1 and LHY to the PIF4 promoter in vivo, we performed ChIP assays with antibodies against CCA1 or LHY in Col wild-type and 35S::WRKY10::MYC or 35S::WRKY2::MYC transgenic plants. The plants were grown under a photoperiod of 12-h dark and 12-h red or blue light for 7 days and were sampled at ZT3, ZT6, and ZT12, or at ZT3 and ZT6. Binding to the PIF4 promoter was enhanced in 35S::WRKY10::MYC and 35S::WRKY2::MYC transgenic plants at ZT3 and ZT6 for CCA1 but only at ZT6 for LHY under either red or blue light (Figure 5A–5F). As controls, ChIP experiments were performed in the cca1 lhy background with antibodies against CCA1 or LHY (Supplemental Figure 7A).

We next performed in vivo transient activation experiments using Arabidopsis leaf protoplasts. For the transient assays, a pPIF4::LUC reporter with a CaMV 35S::REN residing on the same vector and a 35S::WRKY10::MYC or 35S::WRKY2::MYC effector were used (Figure 5G). We delivered the constructs to protoplasts of Wassilewskija (Ws) and the cca1 lhy double mutant in the Ws background. In the wild-type protoplasts, LUC activity was enhanced by more than two-fold when 35S::WRKY2::MYC or 35S::WRKY10::MYC was co-expressed under red or blue light but not in the dark (Figure 5H and 5I). However, such enhancement over pPIF4::LUC expression by WRKY2 or WRKY10 was lost in the cca1 lhy double mutant. Expression of WRKY2 or WRKY10 in Ws and cca1 lhy was very similar in the dark and under red light (Supplemental Figure 7B). Accumulation of WRKY2 or WRKY10 in the protoplasts was not altered in the cca1 lhy double mutant compared with the wild type (Supplemental Figure 7C and 7D).

When the WRKY element of WRKY10 or WRKY2 was mutated from TTGACA to AAAAAA, the expression of pPIF4::LUC was no longer enhanced when protoplasts were transformed with 35S::WRKY2::MYC or 35S::WRKY10::MYC compared with the pPIF4::LUC reporter alone under red light (Figure 5J and 5K). We also overexpressed WRKY2 or WRKY10 driven by its own promoters in pif4 (Figure 5L). The levels of their transgene expression were comparable with or higher than that of the WRKY2 or WRKY10 overexpression lines in the Ws background (Figure 6C and Supplemental Figure 8A and 8B). In contrast to the long hypocotyl phenotype caused by overexpression of WRKY2 or WRKY10 in the Ws background, the promotion of hypocotyl elongation by either WRKY2 or WRKY10 under red, blue, or low blue light was largely diminished in pif4 but was not completely blocked (Figures 5L and 6C, Supplemental Figures 8C and 10C). WRKY2 or WRKY10 may regulate the expression of other genes in addition to PIF4 in the control of hypocotyl elongation. The thermo responses of the WRKY10 or WRKY2 overexpression lines in the pif4 background resembled those of pif4 (Supplemental Figure 8D).

Genetic interaction of WRKY10 and WRKY2 with CCA1/ LHY

To study the genetic relationship between WRKY10 or WRKY2 and CCA1 or LHY, we generated pWRKY10::WRKY10::MYC transgenic lines in Ws. WRKY10 was expressed at a low level (Supplemental Figure 10A). When WRKY10 was driven by its native promoter, over half of the transgenic lines were overexpression lines. The PCR primers detected the expression of both endogenous WRKY10 and the WRKY10 transgene. At least 12 transgenic lines were created, and at least 6 independent lines were examined. For consistency, WRKY2 was also driven by its own promoter (Supplemental Figure 10B). In most of the pWRKY2::WRKY2::MYC lines, WRKY2 was only expressed at a level comparable to that of its endogenous gene. The WRKY10::MYC or WRKY2::MYC transgenes in the Ws background were then crossed to the cca1 lhy background (Figure 6C and 6D). No difference in WRKY10::MYC or WRKY2::MYC expression was detected in Ws.

WRKY10 and WRKY2 interact with SHB1 indirectly

As shown in our previous study, SHB1 also interacts with CCA1 and LHY and positively regulates PIF4 expression (Sun et al., 2019). As WRKY10 and WRKY2 interact with CCA1 or LHY and affect PIF4 expression, a question remains as to whether WRKY10 and WRKY2 also interact with SHB1. We performed coIP assays using MYC-tagged full-length WRKY10 or WRKY2 and GFP-tagged full-length SHB1 in leaf protoplasts from Ws or the cca1 lhy mutant. Protein extracts were prepared from the protoplasts for immunoprecipitation with anti-GFP antibody. The immunoprecipitated proteins were analyzed by western blotting with anti-GFP or anti-MYC antibody. SHB1::GFP co-immunoprecipitated WRKY10::MYC or WRKY2::MYC in protein extracts prepared from Ws but not cca1 lhy mutant leaf protoplasts (Figure 6A, 6B, and Supplemental Figure 9A and 9B). Therefore, the interaction between SHB1 and WRKY10 or WRKY2 requires CCA1 or LHY.

We also performed BiFC assays with Arabidopsis leaf protoplasts to verify an indirect interaction of WRKY10 or WRKY2 with SHB1 in vivo. No fluorescence signals were detected in the nuclei when WRKY10::YFPG and SHB1::YFPH were co-transformed into Arabidopsis leaf protoplasts (Supplemental Figure 9C). Negative controls included WRKY10::YFPG co-transformed with YFPN and YFPG co-transformed with SHB1::YFPN into leaf protoplasts. Both WRKY10::YFPG and SHB1::YFPN fusion proteins were properly expressed in the leaf protoplasts (Supplemental Figure 9D). No signals were detected in BiFC assays when WRKY2::YFPN was co-transformed with SHB1::YFPG into leaf protoplasts (Supplemental Figure 9E and 9F). Therefore, WRKY10 and WRKY2 interact only indirectly with SHB1.

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LHY but not for CCA1 in the presence of WRKY10 or WRKY2 in the EMSA assays. Although both WRKY10 and WRKY2 increase the DNA-binding activity of CCA1, probably through a protein-protein interaction, the reasons for the lack of a supershift for CCA1 in the in vitro assays remain unknown. We next tested whether CCA1 or LHY in turn enhances the DNA-binding activity of WRKY10 or WRKY2. WRKY10 or WRKY2 produced in the TNT reaction bound the W1-box in the PIF4 promoter (Supplemental Figure 6C and 6D). When excess amounts of CCA1 or LHY protein were added to the WRKY10 or WRKY2 binding assay, the affinity of WRKY10 or WRKY2 toward the W1-box was not altered. Therefore, WRKY10 and WRKY2 enhance the DNA-binding activity of CCA1 and LHY, but not vice versa.
or the cca1 lhy mutant (Supplemental Figure 10A and 10B). Overexpression of WRKY10:MYC or WRKY2:MYC in Ws promoted hypocotyl elongation under red or blue light (Figure 6C and Supplemental Figure 10C). By contrast, overexpression of WRKY10:MYC or WRKY2:MYC in cca1 lhy produced a hypocotyl phenotype similar to that of cca1 lhy.

We next examined the expression of PIF4 in Ws, pWRKY2::WRKY2:MYC or pWRKY10::WRKY10:MYC in Ws, cca1 lhy, and pWRKY2::WRKY2:MYC or pWRKY10::WRKY10:MYC in cca1 lhy. PIF4 expression in Ws was relatively low in the dark and was induced by red light (Figure 6D). Red light-induced PIF4 expression was strongly enhanced by WRKY10:MYC.
Figure 6. Genetic interaction of WRKY2 and WRKY10 with CCA1 and LHY.

(A and B) Co-immunoprecipitation of WRKY10:MYC by SHB1:GFP in protein extracts prepared from Arabidopsis Ws (A) or cca1 lhy (B) protoplasts with anti-GFP antibody. Protein blots were probed with anti-MYC antibody.

(C and D) Hypocotyl length (C) and PIF4 expression (D) of pWRKY2::WRKY2:MYC or pWRKY10::WRKY10:MYC transgenic lines in Ws (W) or cca1 lhy (cl).

The corresponding transgenes were introduced from Ws to the cca1 lhy background by genetic crossing. Seedlings were grown in the dark or under 10 μmol m⁻² s⁻¹ red light for 4 days for hypocotyl length measurement and were grown in the dark or transferred from the dark to 15 μmol m⁻² s⁻¹ red light for 3 h. PIF4 expression in each sample was normalized to that of UBQ10 and the wild type. Data are presented as the means ± SE. *p < 0.05 and **p < 0.01 (Student’s t-test).
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overexpression but enhanced only moderately by WRKY2:MYC expression at a level comparable to that of endogenous WRKY2. Blue light-induced PIF4 expression was enhanced moderately by either pWRKY10::WRKY10:MYC overexpression or pWRKY2::WRKY2:MYC expression (Supplemental Figure 10D). The enhancement of PIF4 expression by pWRKY10::WRKY10:MYC or pWRKY2::WRKY2:MYC was impaired in the cca1 lhy mutant under red or blue light (Figure 6D and Supplemental Figure 10D). Therefore, the promotion of PIF4 expression by WRKY10 or WRKY2 accumulation depends on the function of CCA1 and LHY.

We also examined the thermo responses of the WRKY10:MYC transgenic lines in theWs and cca1 lhy backgrounds (Supplemental Figure 10E and 10F). Expression of WRKY10:MYC inWs enhanced hypocotyl elongation at 29°C compared with that of the Ws wild type. Because their hypocotyls were also elongated at 20°C, the ratios of their hypocotyl lengths at 20°C versus 29°C were not changed significantly compared with those of Ws seedlings. Hypocotyl lengths in the two WRKY10:MYC cca1 lhy lines were very similar to that in cca1 lhy. Accumulation of WRKY10 inWs also enhanced PIF4 expression above that of Ws seedlings at 29°C (Supplemental Figure 10F). Because the accumulation of WRKY10:MYC also enhanced PIF4 expression at 20°C, the ratios of PIF4 expression at 20°C versus 29°C were reduced. The expression of PIF4 in WRKY10:MYC cca1 lhy lines was very similar to that in cca1 lhy.

Overexpression of CCA1 by its own promoter created a long hypocotyl phenotype under either red or blue light (Figure 6E). The two transgenes were then crossed to the wrky2 wrky10 background, and expression of the CCA1 transgene was very similar in theWs and wrky2 wrky10 backgrounds (Supplemental Figure 10G). The hypocotyl elongation promoted by CCA1 overexpression appeared to be independent of the wrky2 wrky10 mutations under red or blue light (Figure 6E). We also crossed the cca1 mutation to the wrky10 background and examined whether cca1 wrky10 behaved like cca1 lhy or wrky2 wrky10 (Supplemental Figure 10H). The hypocotyl elongation of cca1 wrky10 was very similar to that of cca1 or the wrky2 single mutant under red light.

WRKY2 and WRKY10 interact with phyB

We next explored whether WRKY2 or WRKY10 interacts with phyB and crys. Considering the very weak hypocotyl phenotype of wrky2 wrky10 under far-red light, we did not pursue the interaction of WRKY2 or WRKY10 with phyA. Interestingly, CCA1 also interacts with phyB preferentially under far-red light, whereas the interaction of LHY with phyB does not show a noticeable difference under red and far-red light (Yeom et al., 2014). The physiological significance and the detailed mechanism of this interaction remain to be elucidated. To explain the hypocotyl phenotype of wrky2 wrky10 under red light, we performed the following interaction assays in the dark and under red light. In Y2H assays, WRKY2 and WRKY10 interacted with the phyB N terminus (621 amino acids) in the dark and under red light, with PIF3 protein as a positive control (Figure 6F). WRKY2 and WRKY10 did not interact with the cry1 N terminus or with full-length cry2, with TOE1 protein as a positive control (Supplemental Figure 11A and 11B). We then performed coIP experiments of MYC-tagged WRKY2 and WRKY10 with phyB in the dark and under red light, as WRKY2 and WRKY10 have only a minor function under far-red light (Figure 6G). WRKY2:MYC or WRKY10:MYC interacted with phyB in the dark and under red light, but they had a consistently higher affinity toward phyB under red light.

We next tested whether the interaction of phyB with WRKY2 or WRKY10 affected the binding of WRKY2 or WRKY10 to the target site. In Y1H assays, WRKY2 and WRKY10 GAD fusions bound their target sites in the dark and under red light (Supplemental Figure 11C). Addition of LexA:phyB-N621 did not enhance the activity of WRKY2 or WRKY10 toward its binding site at a biologically significant level in the dark or under red light. The interaction of phyB with either WRKY2 or WRKY10 did not affect the interaction of the two WRKY proteins with CCA1 or LHY (Supplemental Figure 11D). However, the association of CCA1 or LHY with the target site was significantly reduced in the phyB mutant under red light (Figure 6H). The underlying mechanisms remain unknown, and the two WRKY proteins and phyB may work together in a unique way to enhance the targeting of CCA1 and LHY to the PIF4 locus.

SHB1 upregulates the expression of WRKY10 and WRKY2

Interestingly, SHB1 also upregulated the expression of WRKY10 and WRKY2 at ZT0, ZT3, and ZT6, but not at ZT12, in a gain-of-function shb1-D mutant under both red and blue light (Figure 7A–7D). To study whether SHB1 directly regulates the expression of the two WRKY genes, we performed ChiP analysis with SHB1:MYC transgenic lines in which SHB1:MYC was driven by its native promoter. SHB1:MYC was associated with a region that spans the first and second exons of the WRKY10 region that spans the first and second exons at ZT0, ZT3, and ZT6, but not at ZT12, under red or blue light (Figure 7E and 7G). SHB1:MYC was also detected at the proximal region of the WRKY2 promoter and a region that spans its first and second exons at ZT0, ZT3, and ZT6, but not at ZT12, under red or blue light (Figure 7F and 7H). This reveals another regulatory loop by which SHB1 ensures that sufficient WRKY10 and WRKY2 proteins accumulate to enhance targeting of CCA1 and LHY to the PIF4 locus. The recruitment of SHB1:MYC to the two WRKY

(E) Hypocotyl lengths of Col and pCCA1::CCA1:MYC in Col or wrky2 wrky10 in the dark or under 10 μmol m−2 s−1 red light for 4 days. *p < 0.05 (Student’s t-test).
(F) Interaction of the phyB N terminus (PhyB N-621) with WRKY2, WRKY10, or PIF3 in SD/-Thu/sX-Gal medium in the presence of 10 μM phycocyanobilin (PCB) in the dark or under 30 μmol m−2 s−1 red light.
(G) Co-immunoprecipitation of phyB by WRKY10:MYC or WRKY2:MYC in protein extracts prepared from transgenic seedlings grown in the dark. Protein extracts were kept in the dark or treated with 60 μmol m−2 s−1 red light for 10 min. Protein blots were probed with anti-phyB or anti-Myc antibody.
(H) ChiP analysis of CCA1 or LHY binding to the PIF4 promoter in Col or phyB in the dark or after a transfer from dark to 30 μmol m−2 s−1 red light for 3 h. Enrichment of DNA fragments was quantified by qPCR and normalized to that of UBQ10 and the wild type. Error bars represent standard deviations of three biological replicates. *p < 0.05 (Student’s t-test).
DISCUSSION

Day-length information alters the expression patterns of PRRs and EC, which make a large contribution to day-length-dependent photoperiodic hypocotyl growth (Li et al., 2020). Among the PIFs, PIF4 and PIF5 play a major role in photoperiodic hypocotyl growth. Their expression oscillates with a peak after dawn and then decreases gradually (Nusinow et al., 2011). In the evening, the EC complex represses the expression of PIF4 and PIF5. PRR5 and TOC1 coordinate with the EC complex, directly bind the promoters of PIF4 and PIF5, and inhibit their expression (Li et al., 2020). TOC1 and PRR5 also interact with and inactivate PIF4 to suppress thermomorphogenic growth in the evening (Zhu et al., 2016). In addition, ELF3 interacts with PIF4 in an EC-independent manner and prevents PIF4 from activating its transcriptional targets (Nieto et al., 2015). During the day, CCA1 and LHY bind the PIF4 promoter and recruit SHB1 to promote the expression of PIF4 (Sun et al., 2019). Their combined activity upregulates PIF4 expression in response to changing light intensity and temperature around noon and in the afternoon.

Here, we report that two WRKY family transcription factors, WRKY10 and WRKY2, also participate actively in this regulatory process. WRKY10 and WRKY2 bind their target element in the

loci did not involve CCA1 and LHY (Supplemental Figure 12A and 12B).

Figure 7. SHB1 positively regulates WRKY10 and WRKY2 expression.

(A–D) Expression of WRKY10 or WRKY2 in Ws and the gain-of-function shb1-D mutant under rhythmic 15 μmol m⁻² s⁻¹ red (A and B) or 7 μmol m⁻² s⁻¹ blue (C and D) light at ZT0, ZT3, ZT6, or ZT12.

(E–H) ChIP analysis of SHB1:MYC association with the WRKY10 or WRKY2 loci under rhythmic 15 μmol m⁻² s⁻¹ red (E and F) or 7 μmol m⁻² s⁻¹ blue (G and H) light at ZT0, ZT3, ZT6, or ZT12. Enrichment of DNA fragments was quantified by qPCR and normalized to that of UBQ10 and the wild type. Error bars represent standard deviations of three biological replicates.

(I) A model for the functional interaction of WRKY2 or WRKY10 with CCA1/LHY and phyB in the regulation of PIF4 expression in the dark and under light. SHB1 also imposes a forward regulatory loop to ensure sufficient WRKY10 and WRKY2 accumulation under increasing light intensity toward noon and in the afternoon.
**PIF4 promoter** close to the MYB element bound by CCA1 and LHY. After a few turns of the DNA double helix, WRKY10 and WRKY2 may be on the same face in a major groove adjacent to the major groove bound by CCA1 and LHY. The WRKY10 full-length protein or the WRKY2 C terminus directly contacts the CCA1 and LHY domains outside of their MYB DNA-binding domains (Supplemental Figure 5). Their interactions either aid the recruitment of CCA1 and LHY or enhance their DNA binding affinity to the PIF4 locus. In the proposed model, CCA1 and LHY in turn recruit SHB1 to the PIF4 locus to achieve a high level of PIF4 expression (Figure 7I). Our model also incorporates an SHB1 feedforward loop that upregulates the expression of WRKY2 and WRKY10 during the day.

As shown by ChIP-PCR analysis, WRKY2 and WRKY10 bind the PIF4 promoter only under red or blue light but not in the dark (Figure 2E and 2G). By contrast, WRKY10 protein appears to accumulate abundantly in the dark (Figure 3D and Supplemental Figure 4A and 4B). The EC complex peaks at dusk and binds the PIF4 promoter to repress its transcription in the evening and thereafter (Nusinow et al., 2011). The binding site for CCA1 and LHY is very close to the EC binding site in the PIF4 promoter (Sun et al., 2019). As previously proposed, occupation of a nearby binding site by the EC complex may interfere with the binding of CCA1 and LHY to the PIF4 promoter. The WRKY2 and WRKY10 binding site is also adjacent to the CCA1/LHY and EC binding sites in the PIF4 promoter. Occupation by the EC complex and its associated proteins may interfere with the binding of not only CCA1 and LHY but also WRKY2 and WRKY10 to the PIF4 promoter.

The wrky2 wrky10± mutant shows a strong hypocotyl phenotype under red or blue light but not under far-red light (Figure 1B and Supplemental Figure 1A). The function of these genes under red light can be partially explained by their interaction with phyB (Figures 6F–6H and 7I). The mechanisms for specific involvement of WRKY10 and WRKY2 downstream of blue light receptors remain unknown and are likely to involve other as yet unidentified components.

Over-accumulation of WRKY10 produces little significant change in hypocotyl elongation and abiotic stress responses. A number of WRKY family members also have distinct functions in plant growth and development. WRKY10 regulates seed development (Zhou et al., 2009), WRKY2 regulates seed germination, pollen development and polar organelle localization, and asymmetric division of the embryo (Niewiadomska et al., 2005; Jiang and Yu, 2009; Ueda et al., 2011; Zheng et al., 2018). Other WRKY transcription factors, such as WRKY36, interact with UVR8 and promote hypocotyl elongation by inhibiting the expression of HY5 (Yang et al., 2018), WRKY36, WRKY54, and WRKY70 were also identified as potential phytochrome B-interacting transcription factors in luciferase complementation imaging assays in N. benthamiana leaves in the dark and under red light (Dong et al., 2020). In this study, both WRKY10 and WRKY2 play important roles in the regulation of PIF4 expression. SHB1 targets to the WRKY10 and WRKY2 loci and activates their expression during the period of peak PIF4 expression. However, how SHB1 is recruited to the two WRKY loci remains unknown. Because SHB1 is not a DNA-binding protein, this recruitment may involve other transcription factors. In summary, we discovered an integrated network of circadian regulation and light signaling involving two WRKY proteins that maintain an optimal expression level and a rhythmic expression pattern of PIF4. PIF4 is a critical cellular signaling hub that regulates not only plant photomorphogenic responses but also many other plant physiological processes.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

*A. thaliana* Col-0 andWs plants were used as wild-type ecotypes. The mutant lines wrky10, cca1 lhy, and pif4 were described previously (Zhou et al., 2009; Sun et al., 2019). wrky2 and wrky34 were a gift from Professor Yanru Hu. The mutant lines acquired from the SALK collection were as follows: wrky25, SALK_136966; wrky26, SALK_063388; and wrky33, SALK_006603. Double mutants of wrky10 with other wrky mutants were generated by crossing wrky10 to the respective wrky mutant and were PCR genotyped.

Seeds were sterilized and sown on 1/2 MS (Murashige and Skoog) medium with 0.8% agar but without sucrose and stratified for 3 days at 4°C in the dark. For most light experiments and ChIP assays, seedlings were grown in darkness or under continuous far-red (1 μmol m⁻² s⁻¹), red (10 μmol m⁻² s⁻¹), or blue (7 μmol m⁻² s⁻¹) light for 4 days or as otherwise indicated. For low blue light analysis, seedlings were grown under 15 μmol m⁻² s⁻¹ white light for 5 days and then transferred to 0.7 μmol m⁻² s⁻¹ blue light for 4 days. For the wrky2 wrky10± segregation population, seedlings in the wrky2 background were transferred to 50 mg/l kanamycin medium for an additional 7 days after hypocotyl images were acquired. The seedlings were genotyped for kanamycin resistance linked to the wrky10 locus under 30 μmol m⁻² s⁻¹ white light. Their respective hypocotyl lengths were measured based on their genotypes using NIH ImageJ software. For circadian expression analysis, Arabidopsis seedlings were entrained for 7 days under a photoperiod of 12 h red or blue light and 12 h dark, then released to continuous red or blue light for various numbers of days, as described in the respective figure legends.

**Plasmid construction**

A 1433-bp genomic fragment upstream of the WRKY10 start codon or a 3415-bp genomic fragment upstream of the WRKY2 start codon was PCR amplified from Arabidopsis Col genomic DNA and cloned into the TOPO vector. The fragments were then recombined into pMD163 to generate the pWRKY10::GUS or pWRKY2::GUS expression vector. For 35S::WRKY10::MYC or 35S::WRKY2::MYC, the WRKY10 or WRKY2 genomic coding sequence was PCR amplified with the primer pair WRKY10-CD5-F and WRKY10-CD5-R or WRKY2-CD5-F and WRKY2-CD5-R. The fragments were cloned into the TOPO vector and recombined into the pEarleyGate 203 vector. A 3678-bp genomic fragment

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that contained a 1433-bp sequence upstream of the WRKY10 start codon and the downstream coding region or a 5734-bp genomic fragment that contained a 3415-bp sequence upstream of the WRKY2 start codon and the downstream coding region was PCR amplified from Arabidopsis Col genomic DNA. The fragment was cloned into the TOPO vector and recombined into pMD107 to generate the pWRKY10::MYC or pWRKY2::WRKY2:MYC expression vector. A 3561-bp genomic fragment that contained an 851-bp sequence upstream of the CCA1 start codon and the downstream coding region was PCR amplified from Arabidopsis Col genomic DNA. The fragment was cloned into the TOPO vector and recombined into the pEarley Gate 303 vector to generate pCCCA1::CCA1::MYC. For Y2H or Y1H assays, cDNAs of WRKY2, WRKY10, WRKY10-N, WRKY10-C, WRKY2-N, and WRKY2-C were cloned into pGADT7, and cDNAs of WRKY2, WRKY10, CCA1, LHY, CCA1-N, CCA1-C, LHY-N, LHY-C, and CNT1 were cloned into pGBK7, cDNAs of WRKY2, WRKY10, and PIFl3 were cloned into pB42AD.

GUS assays

Four-day-old pWRKY10::GUS or pWRKY2::GUS seedlings were evacuated and submerged in GUS staining solution (2 mM K3Fe(CN)6, 2 mM K4Fe(CN)6, 1 mg ml⁻¹ x- Gluc, 10 mM EDTA, 0.1% Triton X-100, 100 mg ml⁻¹ chloramphenicol, 50 mM Na2HPO4-NaH2PO4 [pH 7.0]) at 37 °C for 12 h. The stained seedlings were then decolorized and fixed in 70% (v/v) ethanol at room temperature and photographed.

Quantitative RT-PCR

Total RNA was extracted from 5-day-old seedlings with the Universal Plant Total RNA Extraction Kit (BioTeke), SuperScript II Reverse Transcriptase (Invitrogen) was used to synthesize cDNA from the RNA with an oligo(dT) primer. Quantitative real-time PCR was performed using TransStart Tip Green qPCR SuperMix (TransGen Biotech) on a QuantStudio 6 Flex Real-Time PCR system. Transcript levels were normalized to those of the UBQ10 gene and are shown relative to the expression levels in the wild type. Three biological replicates and three technical replicates per biological replicate were used. The primers for RT-PCR analysis are listed in Supplemental Table S1.

Y1H and Y2H assays

Y1H assays were performed as described previously (Foo et al., 2016). In brief, a 146-bp DNA fragment –948 to –802 upstream of the PIF4 start codon that contained the W1- and W2-boxes was PCR amplified and cloned into the Kpn1 and Xhol sites of the pPAbAi vector (Clontech). Three tandem copies of the putative WRKY10 binding elements W1, W2, or W3 were also synthesized as oligonucleotides and ligated into the Kpn1 and Xhol sites of the pPAbAi vector (Clontech). Each repeat of the element had a unique 5' flanking sequence on either side of the core elements. The pPAbAi vectors harboring the constructs were linearized by digestion with BstBI and integrated into the genome of the Y1H Gold strain. The full-length coding sequence of WRKY10 or WRKY2 was PCR amplified from cDNA made from Arabidopsis Col total RNA and cloned into the pGADT7 vector. The empty pGADT7 vector was used as a negative control. GAD:WRKY10 and GAD:WRKY2 or empty GAD vector was subsequently transformed into the yeast strain that contained the pPAbAi constructs. Yeast cells were grown for 3 days on SD/-Leu-Ura selection plates and then transferred to selection plates that contained ABA at the indicated concentrations. To determine the effect of phyB on the binding of WRKY2 or WRKY10 to their target site, yeast strain EGY48 was co-transformed with W12-pLacZ and GAD, GAD:WRKY2, GAD:WRKY10, pLexA, or pLexA::phyB:GUS1 in various combinations. Yeast cells were grown for 3 days on SD/-His-Leu-Ura selection plates, and an ONPG assay was performed as described in a recent study (Li et al., 2021). The transformed cells were grown on SD/-Trp-His-Ado selection plates, and an ONPG assay was performed as described in a recent study (Li et al., 2021).

EMSA assay

The coding region of WRKY10 with an added MYC tag was inserted into the Xho1 and Kpn1 sites of the pTNT vector. The coding region of WRKY2 was cloned into the TOPO vector and was then recombined into pDEST17. Both proteins were synthesized in the TnT in vitro transcription/translation system (Promega). The coding region of CCA1-LHY was inserted into the Xho1 and Kpn1 sites of the pMAL-c5x vector. The two MBP-tagged proteins were produced in E. coli host-strain BL21 (DE3) cells. Half a liter of culture was used for protein purification, and the recombinant proteins were eluted in a final volume of 2 ml. The DNA probes were synthesized with oligonucleotides that contained the putative DNA-binding elements and labeled with biotin. Binding reactions contained 10 mM Tris–HCl (pH 7.5), 150 mM KCl, 1 mM DTT, 2.5% (v/v) glycerol, 0.05 mg ml⁻¹ poly (di-dC), 1 mM ZnCl2, and 0.05% NP-40. Reactions were incubated on ice for 30 min and loaded on native 5% polyacrylamide gels in 0.5x TBE buffer. Gels were run in 0.5x TBE buffer and electro-blotted to Hybond-N+ nylon membrane (Amersham). Detection of the biotin-labeled complex was performed using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) according to the manufacturer’s protocol. Increasing amounts of non-labeled oligonucleotides were used in the competition experiments.

ChIP assay

ChIP assays were performed following a procedure described previously (Bowler et al., 2004; Sun et al., 2019). In brief, 2-g samples of 35S::WRKY10::MYC or 35S::WRKY2::MYC seedlings grown in the dark or under 15 μmol m⁻² s⁻¹ red or blue light were first crosslinked for 30 min in 1% formaldehyde under vacuum. The samples were ground to powder in liquid nitrogen, and the chromatin complexes were isolated with nuclear lysis buffer (50 mM Tris–HCl [pH 8.0], 10 mM EDTA, 0.5% SDS, 0.1 mM PMSF, and 1x protease inhibitor) and sonicated to DNA fragments between 0.2 and 1 kb. The sonicated chromatin complex was then immunoprecipitated with rabbit anti-MYC polyclonal antibody A01704 (GenScript, Piscataway, NJ). Immunoprecipitated samples were sequentially washed with low salt wash buffer (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 20 mM Tris–Cl [pH 8.0]), high salt wash buffer (600 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 20 mM Tris–Cl [pH 8.0]), and TE buffer (10 mM Tris–Cl [pH 8.0] and 1 mM EDTA). DNA fragments were eluted with elution buffer (1% SDS and 0.1 M NaHCO3) at 65 °C and precipitated. The precipitated
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**DNA fragments were analyzed using ChIP-qPCR. The fold enrichment of the specific chromatin fragment was normalized to the UBO10 gene and the control without added antibodies. To examine the influence of WRKY10, WRKY2, or phyB on the association of CCA1 or LHY with their target site, anti-CCA1 R1234-3 (Abiocode, Agoura Hills, CA) or anti-LHY R3095-2 (Abiocode) antibodies were used in the ChIP experiments. To examine the association of SHB1:MYC with the WRKY10 and WRKY2 loci, the pSHB1::SHB1:MYC construct in the pEarley Gate 303 vector was used to generate transgenic plants. Anti-MYC antibodies were used in the ChIP experiments as described above. All primers used for ChIP-qPCR are listed in Supplemental Table S1.**

**BIFC assay**

For WRKY10 and CCA1 or LHY BIFC experiments, the genomic coding regions of WRKY10 and CCA1 or LHY were PCR amplified and cloned into the binary vectors pSPYCE-3SS and pSPYNE-3SS, respectively (Foo et al., 2016). Either pSPYNE::CCA1 or pSPYNE::LHY was co-transformed with pSPYCE::WRKY10 into N. benthamiana leaves by agro-infiltration together with the silencing suppressor P19 vector. Their interactions in N. benthamiana leaves were imaged 3 days after infiltration using a confocal microscope (Leica TCS SPS II, Buffalo Grove, IL). For WRKY2 and CCA1 or LHY BIFC assays, the genomic coding regions of WRKY2 and CCA1 or LHY were PCR amplified and cloned into the binary vectors pSPYNE-3SS and pSPYCE-3SS, respectively. For the BIFC assay of WRKY10 or WRKY2 with SHB1, the SHB1 genomic coding region was cloned into the pSPYNE-3SS or pSPYCE-3SS vector.

**ColP assays**

Transgenic seedlings of 3SS::WRKY10:MYC or 3SS::WRKY2:MYC were used for colP assays. Total proteins were isolated with an IP buffer (10% glycerol, 25 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, 2 mM b-mercaptoethanol, 1 mM PMSE, and 1 X protease inhibitors). The protein extracts were centrifuged at 13 000 rpm for 20 min, and the supernatants were incubated with anti-MYC antibody (Abcam, Cambridge, MA) for 2 h at 4°C and then with protein A/G agarose beads sc-2003 (Santa Cruz, CA) for another 2 h at 4°C. After incubation, beads were washed three times with IP buffer. Finally, 10% input and immunoprecipitated proteins were boiled in 30 ml 2X SDS buffer, separated by 8% SDS–PAGE, and detected in western blots using anti-CCA1 R1234-3 (Abiocode) or anti-LHY R3095-2 (Abiocode) antibodies. For WRKY2:GST and CCA1:MBP or LHY:MBP in vitro colP, WRKY2:GST and CCA1:MBP or LHY:MBP proteins were expressed in E. coli BL21(DE3). Recombinant WRKY2:GST or GST proteins were immobilized on glutathione-Sepharose 4B beads (GE Healthcare, 17-0756-01) in 1X phosphate-buffered saline buffer with 0.1% Nonidet P-40 for 2 h and then incubated with CCA1:MBP or LHY:MBP extracts for 2 h at 4°C. The beads were washed three times with 1X PBS containing 0.1% Nonidet P-40 and processed for SDS–PAGE and western blot analysis using an anti-GST (TransGen Biotech, HT601-01) or anti-MBP (TransGen Biotech, HT701-01) antibody.

For WRKY10:MYC or WRKY2:MYC and SHB1:GFP colP, vectors containing the three constructs driven by the 3SS CaMV promoter or the SHB1 native promoter, respectively, were co-transformed into ArabidopsisWs or ca1 lhy protoplasts. Anti-GFP antibody ab1218 (Abcam) was used for immunoprecipitation, and protein blots were probed with anti-MYC antibody HT101-01 (TransGen Biotech). For colP of phyB by WRKY2 or WRKY10, 3SS::WRKY10:MYC or 3SS::WRKY2:MYC seedlings were homogenized in extraction buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 0.1% (v/v) NP-40, 1 mM PMSE, 1X MG132, and 1X EDTA-free protease inhibitor cocktail in darkness with a dim green light. The tubes that contained the protein extracts were placed horizontally on ice and received a 10-min red light treatment (60 μmol m–2 s–1). Anti-MYC magnetic beads (MBL, MO47-11) were used for immunoprecipitation, and protein blots were probed with anti-phyB (Abiocode, R1244-2) antibodies.

**Transient trans-activation assay**

Arabidopsis mesophyll protoplast isolation and transformation were performed as described previously (Yoo et al., 2007). 3SS::WRKY10:MYC or 3SS::WRKY2:MYC was used as an effector construct. A pGreen II-08000-LUC vector that contained pPIF4::LUC and 3SS::REN was used as a reporter construct. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA). Relative luciferase activity was normalized to REN activity as a LUC/REN ratio for each biological sample. Experiments were repeated three times, and each biological replicate was represented by three technical replicates.

**Protein isolation and western blot**

To detect the accumulation of tagged proteins in transient assays with protoplasts in Supplemental Figure 7C and 7D, total proteins were extracted from protoplasts by boiling in 2X SDS loading buffer at 95°C for 10 min. The tagged proteins were detected with anti-MYC (TransGen Biotech) and anti-Actin11 CW0092 (CWBIO, China) antibodies. To detect the accumulation of WRKY10 or WRKY2 and SHB1 fusion proteins in Supplemental Figure 9D and 9F, BIFC experiments, total proteins were isolated from leaf protoplasts with buffer that contained 50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1% SDS, 1 mM PMSE, and 1X protease inhibitors (Roche). WRKY10 or WRKY2 and SHB1 fusion proteins were immunoprecipitated from the protein extracts with polyclonal anti-MYC A01704 (GenScript) or anti-HA A00168 (GenScript) antibodies. Protein blots were probed with monoclonal anti-MYC (TransGen Biotech) or anti-HA CW0092 (CWBIO, China) antibodies.

**ACCESSION NUMBERS**

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers AT2G46830 (CCA1), AT1G01060 (LHY), AT2G43010 (PIF4), AT4G25350 (SHB1), AT5G56270 (WRKY2), and AT1G55600 (WRKY10).

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at Plant Communications Online.

**FUNDING**

This work was supported by Shandong Agricultural University, China, SDA-2014.

**AUTHOR CONTRIBUTIONS**

S.W. and M.N. conceived and designed the research plan. S.W., Q.S., S.W., M.N. performed the experiments. S.W. and M.N. wrote the manuscript.

**ACKNOWLEDGMENTS**

We thank Professor Yanru Hu from Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences for wrky2 and wrky34 seeds and the ABRC for wrky25, wrky26, and wrky33 seeds. The LexA:phyB-N621 construct was kindly provided by Professor Jigang Li from China Agricultural University. We declare no conflicts of interest.

Received: August 27, 2021
Revised: September 21, 2021
Accepted: November 5, 2021
Published: November 12, 2021

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