Cryptochrome 1 interacts with PIF4 to regulate high temperature-mediated hypocotyl elongation in response to blue light

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Cryptochrome 1 (CRY1) is a blue light receptor that mediates primarily blue-light inhibition of hypocotyl elongation. Very little is known of the mechanisms by which CRY1 affects growth. Blue light and temperature are two key environmental signals that profoundly affect plant growth and development, but how these two abiotic factors integrate remains largely unknown. Here, we show that blue light represses high temperature-mediated hypocotyl elongation via CRY1. Furthermore, CRY1 interacts directly with PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) in a blue light-dependent manner to repress the transcription activity of PIF4. CRY1 represses auxin biosynthesis in response to elevated temperature through PIF4. Our results indicate that CRY1 signal by modulating PIF4 activity, and that multiple plant photoreceptors [CRY1 and PHYTOCHROME B (PHYB)] and ambient temperature can mediate morphological responses through the same signaling component—PIF4.

blue light | cryptochrome | PIF4 | hypocotyl elongation | ambient temperature

Cryptochromes are photolyase-like blue-light receptors first discovered in *Arabidopsis* and later found in all major evolutionary lineages (1–4). *Arabidopsis* cryptochrome 1 (CRY1) and cryptochrome 2 (CRY2) mediate primarily blue-light inhibition of hypocotyl elongation (5) and photoperiodic control of floral initiation (6) via modulation of gene expression. For example, *Arabidopsis* CRY2 undergoes blue light-dependent interaction with CIB1 (CRY2 Interacting bHLH1) to regulate flowering time (7–9). CRYs also suppress the E3 ubiquitin ligase activity of COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1) by forming a complex with SPA1 (SUPPRESSOR OF PHYA-105) and COP1 in a blue light-dependent manner (10–13). COP1 is a RING finger E3 ubiquitin ligase that acts downstream of phytochromes, cryptochromes, and UVR8 (UV Resistance Locus 8) (14, 15) and is responsible for the degradation of various transcription factors in the dark, such as the bHLH transcription factor HFR1 (LONG HYPOCOTYL IN FAR RED1) and the bZIP factor HY5 (12, 16–18). Whether *Arabidopsis* CRY1 undergoes blue light-dependent interaction with transcription factors to regulate hypocotyl elongation is still unknown.

In addition to light, ambient temperature serves as another key environmental cue that affects plant growth and development, but does not induce stress responses to any significant degree (19). Temperature regulates gene expression via chromatin remodeling and also regulation of transcription. It has been demonstrated that H2A.Z histone variant-containing nucleosomes act as thermosensors and mediate temperature induced transcriptome changes (20). PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) is a bHLH transcription factor directly link red light photoreceptor PHYTOCHROME B (PHYB) to light-regulated gene expression

and plant development (21–23). PIF4 also plays a role in sensing high temperature, it not only regulates temperature-mediated floral induction in the short day condition through direct activation of *FT (FLOWERING LOCUS T)* (24), but it also controls high temperature-induced hypocotyl elongation by increasing free indole-3-acetic acid (IAA) concentrations via direct stimulation of *YUC8 (YUCCA8)* or *TAA1 (TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS)* gene expression (25–32).

It was previously reported that red light response was strictly temperature dependent, it promoted hypocotyl extension at 27 °C, whereas repressed hypocotyl elongation at 17 °C or 22 °C (33), but how about blue light? Here, we show that blue light inhibits hypocotyl elongation at both 22 °C and 28 °C, and it represses high temperature-induced hypocotyl elongation through CRY1. More importantly, CRY1 physically interacts with PIF4 in a blue light-dependent manner in plants, to regulate the biosynthesis of auxin in response to elevated ambient temperature. We also show that CRY1 and PIF4 occupy the same promoter regions to repress the transcription activity of PIF4, indicating that CRY1 signal by regulating PIF4 activity. Because PIF4 binds to CRY1 and PHYB to transduce both blue and red light signals, it appears to be the molecular basis for cross-talk between CRY1 and PHYB. Blue light and temperature may also integrate by regulating PIF4.

Significance

Blue light and temperature are two key environmental signals that profoundly affect plant growth and development responses, but how these two abiotic factors integrate remains largely unknown. This study demonstrates a mechanism of multiple photoreceptors and temperature coactions. *Arabidopsis* blue light photoreceptor cryptochrome 1 (CRY1) represses high temperature-induced hypocotyl elongation through PHYTOCHROME-INTERACTING FACTOR 4 (PIF4). CRY1 physically interacts with PIF4 in a blue light-dependent manner to repress the transcription activity of PIF4. Because PIF4 also plays a role in ambient temperature, PIF4 appears to be the molecular basis of cross-talk among blue and red light and ambient temperature signal pathways.

Results

Blue Light Regulates the High Temperature-Mediated Hypocotyl Elongation via CRY1. Unlike red light, all fluence rates of blue light tested repressed hypocotyl extension at both 22 °C and 28 °C (Fig. 1*A*). Furthermore, hypocotyl elongation of WT seedlings in response to elevated temperatures was repressed under blue light conditions, compared with dark, red light, and white light conditions (Fig. 1 *A* and *B* and Fig. S1*A*), indicating that blue light negatively regulates thermomorphogenesis.
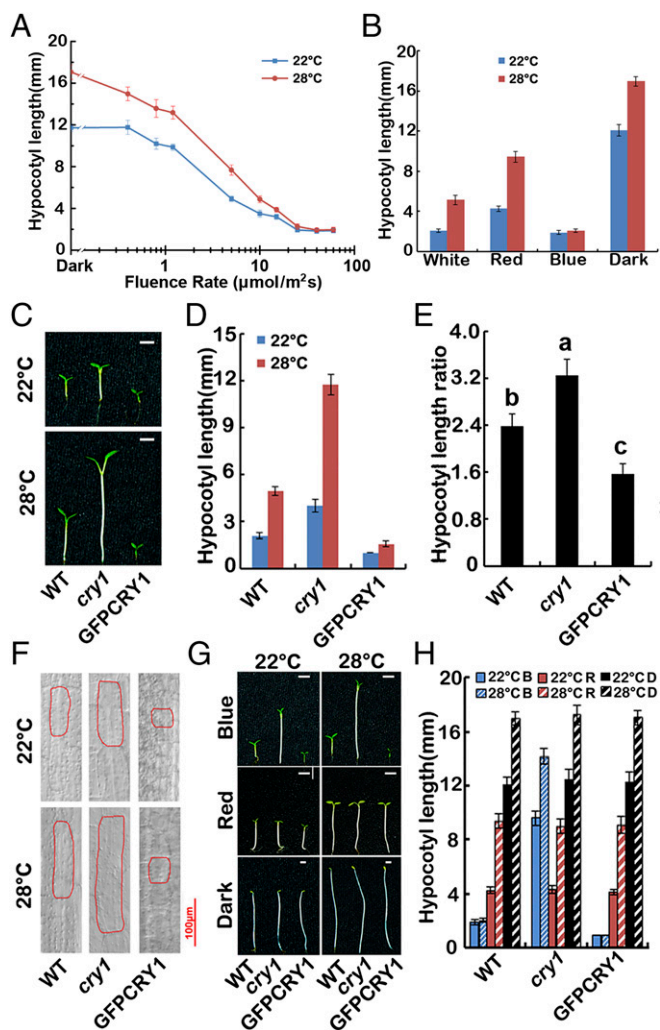

Fig. 1. Blue light inhibits high temperature-induced hypocotyl elongation via CRY1. (*A*) Fluence rate response curves measuring hypocotyl elongation of 4-d-old WT seedlings grown in continuous blue light at 22 °C and 28 °C. (*B*) A phenotypic analysis. Seedlings of the wild genotype were grown under continuous white light, red light, blue light (all 40 μmol·m⁻²·sec⁻¹) or dark conditions in 22 °C or 28 °C for 4 d. The hypocotyl lengths were measured and are shown. (*C–E*) A phenotypic analysis. Seedlings of the indicated genotypes were grown under continuous white light at 22 °C or 28 °C for 4 d. Images of the representative seedlings are shown in *C*, and the hypocotyl lengths of the indicated genotypes were measured and are shown in *D*. Hypocotyl length ratios (28 °C/ 22 °C) of the quantified hypocotyl length in *D* are shown in *E*. The letters "a" to "c" indicate statistically significant differences between ratios for hypocotyl of the indicated genotypes, as determined by Tukey's least significant difference (LSD) test (*P* ≤ 0.01). (*F*) Cell morphologies of representative seedlings of the indicated genotypes. (*G* and *H*) A phenotypic analysis in different light conditions. Seedlings of the indicated genotypes were grown at 22 °C or 28 °C in the dark or continuous blue or red light (40 μmol·m⁻²·sec⁻¹) for 4 d. Images of the representative seedlings are shown in *G*, and the hypocotyl lengths of the indicated genotypes were measured and are shown in *H*. SDs (*n* > 15) are indicated.

CRY1 plays an important role in the inhibition of hypocotyl elongation in response to blue light. It has been shown that blue light and CRYs are required for temperature compensation of the circadian clock (34) and that HFR1 control of hypocotyl growth was temperature dependent in blue light, and proposed that PhyB and CRY1 were critical for controlling growth in high temperature (17). So we investigated the function of CRY1 in hypocotyl elongation in response to ambient temperature changes. Hypocotyl elongation of wild-type (WT), *cry1*, and CRY1 over-expression lines (*35S::GFP-CRY1*) were recorded after 4 d of treatment in continuous white light at 22 °C and 28 °C. The *cry1* mutants exhibited far more hypocotyl elongation at 28 °C than at 22 °C compared with WT (the hypocotyl length ratio of *cry1* 28 °C/ 22 °C is 3.2, WT is 2.4), whereas the GFP-CRY1 overexpression seedlings only elongated modestly at the higher temperature (GFP-CRY1 28 °C/22 °C = 1.6), suggesting CRY1 repressed the high temperature-induced hypocotyl elongation (Fig. 1 *C–E*).

To further confirm this observation, we measured cell lengths in the elongation zone of the hypocotyls. Noticeable cell elongation was observed in WT at 28 °C, whereas cell elongation of *cry1* mutants was more significant at 28 °C than at 22 °C. Cells of the GFP-CRY1 overexpression seedlings only elongated modestly in response to an increase in temperature (Fig. 1*F* and Fig. S1 *E* and *F*). We also examined the effects of the *cry1* mutation on petiole elongation, which was also affected by high temperature. Petiole elongation markedly increased in *cry1* mutants when grown at 28 °C (Fig. S1 *G–I*).

COP1 acts as a central repressor in plant light signaling, and COP1 and CRY1 reside in the same multiprotein complex. Our results indicated that the GUS-COP1 overexpression seedlings were more elongated at 28 °C than 22 °C compared with WT, whereas the *cop1-6* mutant only elongated mildly at the higher temperature (Fig. S1 *B* and *C*), in a manner reminiscent of the GFP-CRY1 over-expression seedlings. Cell-length measurements were also consistent with the hypocotyl elongation data (Fig. S1 *D–F*). Taken together, we conclude that CRY1 serves as a negative regulator of hypocotyl elongation in response to elevated ambient temperature, whereas COP1 serves as a positive regulator.

To investigate whether CRY1 regulates the high temperature-mediated hypocotyl elongation in a blue light-dependent manner, we examined the hypocotyl length of WT, *cry1*, and GFP-CRY1 in continuous blue light, continuous red light, and complete darkness at both 22 °C and 28 °C. In the red light and dark conditions, all had longer hypocotyls at 28 °C, and the hypocotyl ratios (28 °C/22 °C) were almost the same for WT, *cry1* mutant, and GFP-CRY1 (Fig. 1 *G* and *H* and Fig. S1 *K–M*). In contrast, when grown under continuous blue light, *cry1* mutants were more sensitive to temperature changes, whereas GFP-CRY1 was insensitive to temperature changes (Fig. 1 *G* and *H* and Fig. S1 *K–M*). The data presented here suggested that CRY1 repressed high temperature-mediated hypocotyl elongation in a blue light-dependent manner. The transcription level of *CRY1*, and also the protein level of CRY1, showed no significant changes at different temperatures (Fig. S2).

Intriguingly, COP1 also regulated the high temperature-mediated hypocotyl elongation in a light-dependent manner. In the dark, the *cop1-6* mutant was strikingly more elongated at 28 °C than at 22 °C, only mildly elongated in continuous red light, while not elongated at elevated temperatures in continuous blue light. The GUS-COP1 overexpression seedlings were more elongated at 28 °C than 22 °C in either continuous blue or red light, but not in the dark (Fig. S1 *J–M*), indicating that CRY1 may regulate the temperature responses through COP1-dependent and also COP1-independent pathways.

CRY1 Physically Interacts with PIF4 in a Blue Light-Dependent Manner. It was reported that PIF4 and PIF5 were responsible for not only red light but also blue light-regulated hypocotyl elongation (35), and PIF4 plays a prominent role in ambient temperature responses.

We therefore reasoned that CRY1 might regulate the high temperature-induced hypocotyl elongation through PIF4. Our data suggested that CRY1 might regulate temperature responses through a COP1-dependent and also a COP1-independent pathway. For the COP1-dependent pathway, it has been shown that HFR1, which is degraded by COP1, can inhibit PIF4 (17, 36), and that HFR1 controls hypocotyl growth in a temperature-dependent manner in blue light (17). CRY1 suppresses the E3 ubiquitin ligase activity of COP1 to stabilize HFR1 (10–13). Our data also indicated that the *hfr1* mutant was more sensitive to elevated temperature (Fig. S3 *A and B*), and that HFR1 interacted with PIF4 in planta to inhibit the transcription activity of PIF4 (Fig. S3 *C–E*). So that CRY1 might regulate PIF4 via HFR1 to regulate hypocotyl elongation in response to elevated temperature. CRY1 also repressed the transcription of *PIF4*, the expression level of PIF4 was higher in the *cry1* mutant, but lower in the *cop1* mutant (Fig. S4*A*), given that HY5 might regulate the transcription of *PIF4* (32, 37), CRY1-COP1 might regulate *PIF4* transcription via HY5. Many reports have shown that light destabilizes PIFs, whereas PHYs were necessary for the light-induced degradation of PIFs (38, 39); interestingly, CRY1 did not affect the protein stabilization of PIF4 (Fig. S4*B*).

For the COP1-independent pathway, it has been shown that mouse CRYs physically interact with two bHLH proteins, CLOCK and BMAL (40), and *Arabidopsis* CRY2 interacts with bHLH proteins CIBs (7, 8). We investigated the interaction between CRY1 and PIF4 in more detail. The insect cell expressed CRY1 interacted with the *Escherichia coli* expressed PIF4 in an in vitro pull-down assay (Fig. S5*A*), and more CRY1 was pulled down with PIF4 under blue light than under dark (Fig. 2*A*), indicating that CRY1 interacts with PIF4 in a blue light-dependent manner in vitro. CRY1 also interacts with PIF4 in plant cells in the BiFC (bimolecular fluorescence complementation) assays (Fig. 2*B*) (41, 42). As shown in Fig. 2*B*, strong fluorescence was detected in the nuclei of cells cotransformed with cCFP-PIF4 and CRY1-nYFP plasmids, but no fluorescence was detected in cells transformed with the cCFP and CRY1-nYFP or cCFP-PIF4 with nYFP plasmids (Fig. 2*B*). The in vivo interaction of PIF4 and CRY1 was further examined by coimmunoprecipitation (IP). Seedlings were either kept in the dark or exposed to blue light for 20 min (Fig. 2*C*) or 15 min (Fig. S5*B*) or 5, 20, or 60 min time course (Fig. S5*C*) (40 µmol·m^{-2}·sec^{-1}), and then subjected to co-IP analyses. More PIF4 was coprecipitated with CRY1 from seedlings irradiated with blue light than that left in dark. These results argue strongly that blue light stimulates accumulation of the CRY1–PIF4 complex in plant cells. We conclude that PIF4 interacts with CRY1 in vivo, and PIF4 undergoes blue light-dependent physical interactions with CRY1. PIF4 coprecipitated with CRY1 in samples kept at 22 °C and also samples moved to 28 °C, demonstrating that PIF4 interacts with CRY1 in both 22 °C and 28 °C (Fig. S5 *D and E*).

To further study the relationship between CRY1 and PIF4, we investigated genetic interactions between the *CRY1* and *PIF4* genes. As shown in Fig. 2 *D and E*, PIF4-YFP overexpression seedlings exhibited long hypocotyl phenotypes in the red light conditions, whereas blue light repressed the long hypocotyl phenotypes of PIF4-YFP seedlings. Furthermore, blue light suppressed the long hypocotyl phenotype of PIF4-YFP seedlings in a CRY1-dependent manner, because seedlings expressing PIF4-YFP in the *cry1* background showed long hypocotyl phenotypes in both red and blue light conditions. Transgenic seedlings expressed PIF4-MYC and MYC-CRY1 together exhibited a similar phenotype as MYC-CRY1 seedlings under both continuous blue light (Fig. 2*F*) and continuous white light (Fig. S5*F*) conditions, consistent with that blue light suppressed the long hypocotyl phenotype of PIF4-YFP seedlings in a CRY1-dependent manner, the expression level of *PIF4* and *CRY1* genes is shown in Fig. S5 *H and I*. A CRY1-deficient *cry1* mutant was crossed with *pif1345*, resulting *cry1pif4*
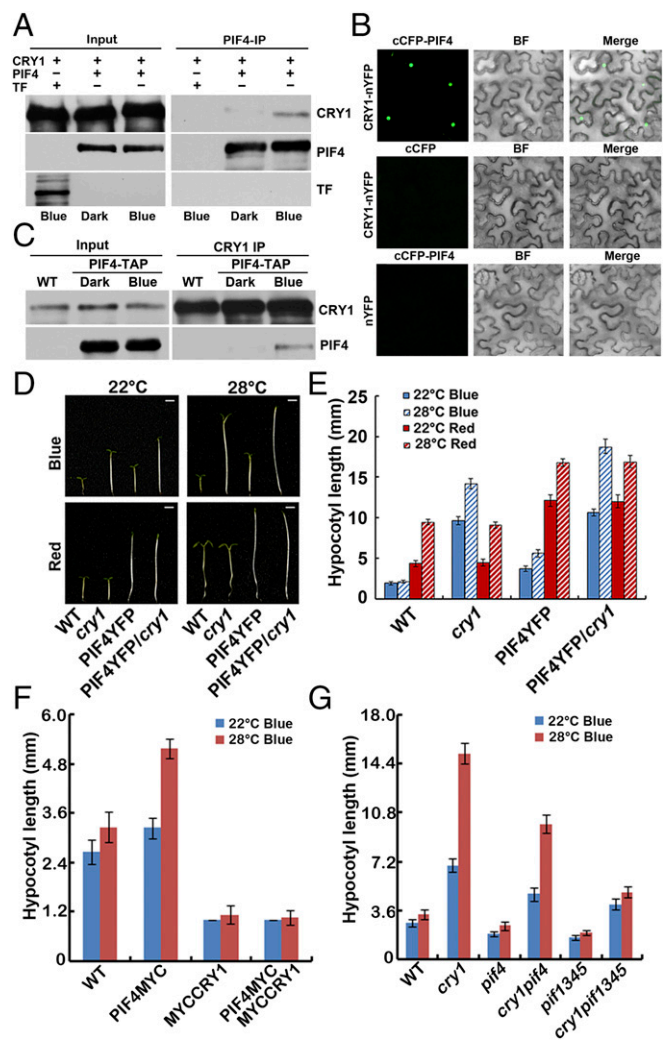

Fig. 2. CRY1 interacts with PIF4 in a blue light-dependent manner. (*A*) In vitro pull-down assays showing blue light-dependent interaction between CRY1 and PIF4. His-TF–tagged PIF4 or His-TF tag were mixed with CRY1 expressed and purified from insect cells (sf9) in the dark condition, and were incubated under blue light (40 µmol·m^{-2}·sec^{-1}) or dark conditions for 30 min, and PIF4 antibody was used for the in vitro pulldown. The products were analyzed by immunoblot probed with the anti-CRY1 (CRY1) or the anti-PIF4 antibody (PIF4) or the anti-His antibody (TF). (*B*) BiFC assays of the in vivo protein interaction. Leaf epidermal cells of *Nicotiana benthamiana* were cotransformated with cCFP-PIF4 and CRY1-nYFP or cCFP and CRY1-nYFP or nYFP and cCFP-PIF4. BF, bright field. Merge, overlay of the YFP and bright-field images. (*C*) Co-IP assays of samples prepared from 6-d-old *35S::PIF4-TAP* seedlings grown in long-day condition (16 light/8 dark), moved to dark for 1 d, then either exposed to blue light (40 µmol·m^{-2}·sec^{-1}) or kept in dark. Total proteins (input) or IP product of anti-CRY1 antibody (CRY1-IP) were probed, in immunoblots, by the anti-CRY1 antibody (CRY1), stripped, and reprobed by the anti-MYC (PIF4-TAP) antibody. (*D–G*) Phenotypic analysis. (*D* and *E*) Seedlings of the indicated genotypes were grown in the 22 °C or 28 °C continuous blue or red light (both 40 µmol·m^{-2}·sec^{-1}) for 4 d. (*F* and *G*) Seedlings of the indicated genotypes were grown in the 22 °C or 28 °C continuous blue light (40 µmol·m^{-2}·sec^{-1}) for 4 d. The hypocotyl lengths of the indicated genotypes were measured and are shown in *F* and *G*. SDs (*n* > 15) are indicated.

and *cry1pif1345*. The high temperature-induced hypocotyl elongation phenotype of *cry1* was partially suppressed in *cry1pif4* but was suppressed in *cry1pif1345* under continuous blue light condition (Fig. 2*G*), which suggested that PIF4 and also other PIFs act downstream of CRY1. It is reported that *pif4* but not *pif1*, *pif3*, *pif5* mutant showed no hypocotyl elongation in response to

higher temperature (25). Our data showed that PIF3 over-expression line was more sensitive to the increased tempera-ture (Fig. S6A). Those results indicate that PIF4 plays a major role in temperature responses, but PIF3 and also maybe other PIFs are also involved in temperature responses. Furthermore, in vitro pull down and also co-IP assays both showed that PIF3 interacted with CRY1 (Fig. S6 B and C), indicating that PIF3 also acts downstream of CRY1. Interestingly, the high temperature-induced hypocotyl elongation phenotype of cry1 was suppressed in both cry1pif4 and cry1pif1345 under white light conditions (Fig. S5G), indicating the cross-talk between different light signaling. cry1pif4 and cry1pif1345 also showed shorter hypocotyl phenotype than cry1 at 22 °C (Fig. 2G and Fig. S5G). These data suggested that PIF4 and PIF3 act downstream of CRY1, and that CRY1 mediates the blue light inhibition of the hypocotyl elongation at 22 °C or in response to elevated temperature at least partially through the PIFs transcription factor.

CRY1 Affects the High Temperature-Induced Elevation of *YUC8* Transcripts and the Free IAA Level. The hormone auxin, or IAA, is fundamental to plant growth and development. It has been reported that plants possess longer hypocotyls because of increased IAA levels when they were grown at elevated temperatures (26, 27, 43, 44). IAA can be formed via tryptophan-dependent and tryptophan-independent pathways (45). PIF4 controls high temperature-in-duced hypocotyl elongation by increasing free IAA levels via direct stimulation of *YUC8* and *TAA1* transcriptions and the resultant increase in YUC8 and TAA1 enzymatic activities (25–28, 30). *IAA19* and *IAA29* are also PIF4 targets (30). Because CRY1 in-teracts with PIF4 in a blue light-dependent manner, we then asked how the CRY1–PIF4 interaction affected the expression of PIF4 target genes. When WT seedlings grown at 22 °C white light con-dition for 4 d were transferred to 28 °C, the transcript abundances of *YUC8* and the auxin response genes *IAA19* and *IAA29* were all elevated. In contrast, the high temperature-induced up-regulation of *YUC8*, *IAA19*, *IAA29* expression was largely abolished in the GFP-CRY1 (Fig. 3A and Fig. S7A). In the *cry1* mutant, the ex-pression levels of *YUC8*, *IAA19*, and *IAA29* were all up-regulated to a larger extent at 28 °C. Blue light repressed the high temperature-mediated hypocotyl elongation, and also the high temperature-me-diated expression increase of *YUC8*, when the WT seedlings grown at 22 °C continuous blue light condition for 4 d were transferred to 28 °C continuous blue light, the transcript abundance of *YUC8* was not significantly elevated, and there was totally no change of *YUC8* expression in the CRY1 overexpression line, whereas in the *cry1* mutant, the expression level of *YUC8* was significantly up-regulated at 28 °C (Fig. S7B). Our results also indicated that the higher temperature did not up-regulate the expression of other *YUC* family genes tested and that CRY1 did not affect the expression of those genes (Fig. S7C). Consistent with that *cry1pif1345* exhibited the same phenotype as *pif1345*, the ex-pression levels of *YUC8*, *IAA19*, and *IAA29* were the same in *cry1pif1345* as in *pif1345* (Fig. 3B).

To examine whether the greatly attenuated elongation re-sponse in GFP-CRY1 overexpression seedlings at high temper-ature is the result of reduced IAA levels, we attempted to chemically rescue the phenotypes by applying exogenous IAA to seedlings. The high temperature-induced hypocotyl elongation phenotype was partially restored to GFP-CRY1 when picloram or IAA was applied under both continuous blue or white light con-ditions (Fig. 3 C and D and Fig. S8 A–E). Consistent with that blue light repressed the elevated temperature-induced hypocotyl elongation and auxin biosynthesis, WT seedlings were more sen-sitive to picloram at 28 °C than at 22 °C under blue light, whereas *cry1* mutant is insensitive to picloram at 28 °C blue (Fig. 3 C and D). Direct measurement of the IAA content of seedlings revealed that WT seedlings grown at 28 °C white light displayed signifi-cantly higher IAA levels compared with levels measured at 20 °C,
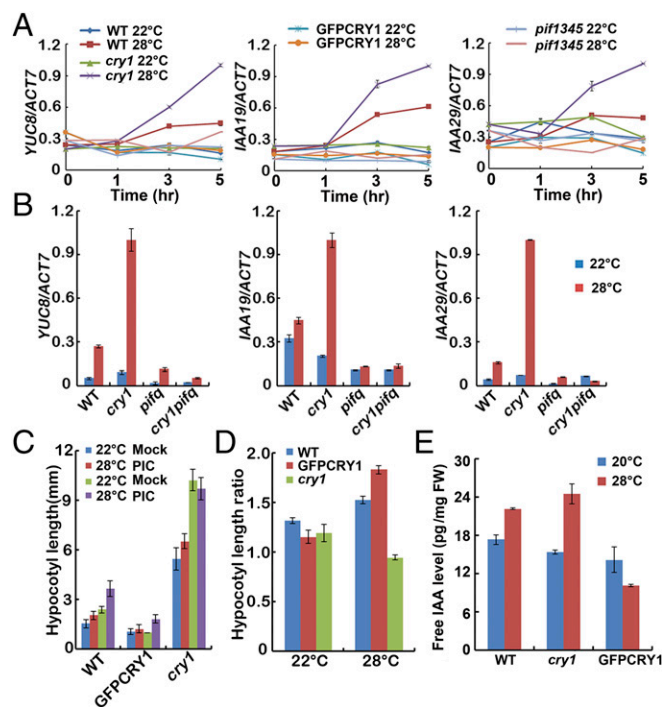

Fig. 3. CRY1 affects the high temperature-induced elevation of *YUC8* transcripts and the free IAA level. (A and B) qPCR results showing high temperature-induced expression patterns of *YUC8*, *IAA19*, and *IAA29* of the indicated genotypes. Four-day-old seedlings grown in 22 °C in continuous white light condition were transferred to 28 °C or were continually placed at 22 °C for a 5-h time course (A) or for only 3 h (B), respectively. (C) Hypocotyl length of 4-d-old WT, GFP-CRY1, and *cry1* seedlings grown in the 22 °C or 28 °C continuous blue (40 μmol·m⁻²·sec⁻¹) conditions, with and without the synthetic auxin picloram. The hypocotyl lengths were measured and are shown in C. SDs (n > 15) are indicated. Hypocotyl length ratios (Picloram/Mock) of the quantified hypocotyl length in C are shown in D. (E) IAA concentrations of 6-d-old WT, *cry1*, and GFP-CRY1 seedlings grown in 20 °C and 28 °C continue white light (40 μmol·m⁻²·sec⁻¹) condition.

and the IAA level increased more in *cry1* mutants grown at 28 °C compared with those grown at 20 °C. In contrast, the elevated levels of IAA were abolished in GFP-CRY1 plants (Fig. 3E).

The auxin-responsive *DR5::GUS* reporter construct was in-troduced into the GFP-CRY1 background, so that we could ana-lyze auxin activity in planta. GUS activity was analyzed in seedlings grown either at 22 °C or 28 °C under continuous white light con-ditions for 4 d. Increased GUS activity was noted in WT grown at 28 °C. In contrast, no significant increase in GUS activity was recorded in GFP-CRY1 transgenic lines at 28 °C (Fig. S8F). Taken together, these data demonstrated that CRY1 affected the tran-scription of PIF4 targets genes and the high temperature-induced IAA increase and also the resultant auxin growth response.

CRY1 Affects the Transcription Activity of PIF4. We hypothesized that because CRY1 undergoes physical interaction with PIF4 and it affects the expression of PIF4 target genes, CRY1 could physically associate with genomic regions that PIF4 bound to. To test this possibility, we performed ChIP-qPCR and ChIP-PCR assays. Both ChIP-qPCR (Fig. 4 A–C) and ChIP-PCR (Fig. S9 B and C) assays showed that, in vivo, CRY1 was associated with the same chromatin region of the *YUC8, IAA19,* and *IAA29* pro-moters as seen for PIF4 (Fig. S9A). The same PCR primer pairs were used for detecting the chromatin binding of PIF4 and CRY1 (Table S1). Consistent with that CRY1 interacts with PIF4 in a blue light-dependent manner, CRY1 associated with the promoters of *YUC8, IAA19,* and *IAA29* genes in a blue

Fig. 4. CRY1 affects the transcription activity of PIF4. (*A*) Diagram depicting the putative promoter of *YUC8, IAA19, IAA29.* (*B* and *C*) Representative results of the ChIP-qPCR assays. Chromatin fragments (~500 bp) were prepared from 7-d-old WT seedlings, immunoprecipitated by the anti-CRY1 antibody, and the precipitated DNA was analyzed by qPCR using the primer pairs indicated. The IP/input ratios are shown with the SDs (*n* = 3). (*D*) Structure of the *YUC8* promoter-driven dual-LUC reporter gene. *YUC8* promoter (−1,635 bp to 0 bp), *35S* promoter, REN luciferase (REN), firefly luciferase (LUC), and T-DNA (LB and RB) are indicated. (*E*–*G*) CRY1 regulates the transcription activity of PIF4. (*E*) Relative reporter activity (LUC/REN) in planta with different effectors expression. Tobacco leaves were transfected with the reporter and the effector (CRY1 only, PIF4 only, or CRY1 and PIF4 together), kept in white light for 3 d. The relative LUC activities normalized to the REN activity are shown (LUC/REN, *n* = 3). (*F* and *G*) Relative reporter activity (LUC/REN) in protoplasts of WT and *cry1* with or without PIF4 expression. *Arabidopsis* protoplasts of WT and *cry1* were transfected with the reporter DNA together with or without PIF4, kept in white light for 8 h (*F*), or kept in dark and blue light (40 μmol·m⁻²·sec⁻¹) for 8 h (*G*). The relative LUC activities normalized to the REN activity are shown (LUC/REN, *n* = 3). (*H*) A hypothetical model depicting CRY1 mediated blue-light regulation of PIF4. The model hypothesizes that in response to blue light, CRY1 interacts with PIF4 to repress the activity of PIF4 promoting transcription of the target gene, and CRY1 can also repress PIF4 through inhibiting COP1-mediated degradation of HFR1.
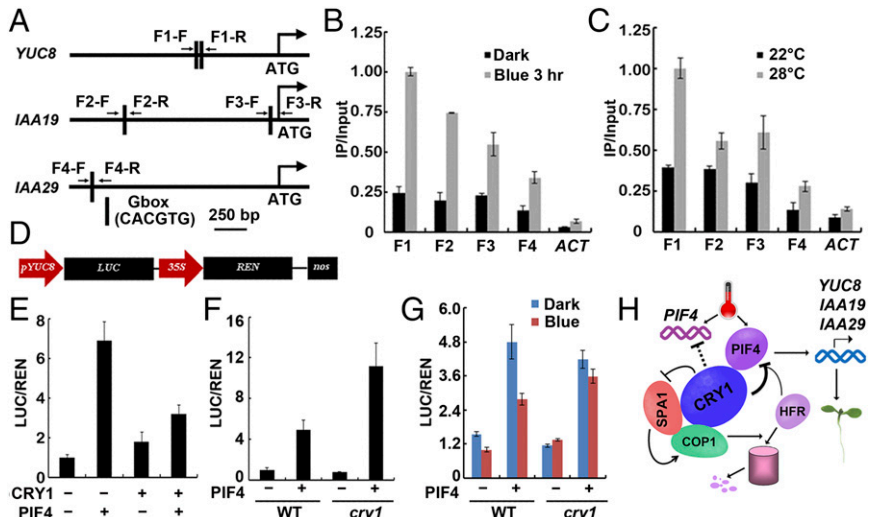
light-dependent manner. Promoter binding of CRY1 was markedly increased with blue light treatment compared with the dark grown control condition (Fig. 4*B* and Fig. S9*B*). Warm temperatures also promoted the association of CRY1 to the *YUC8, IAA19,* and *IAA29* promoters. Promoter associations were greatly enhanced at 28 °C relative to 22 °C especially for *YUC8* (Fig. 4*C* and Fig. S9*C*). Further evidence supporting that CRY1 and PIF4 form a complex to bind to targets promoter came from electrophoretic mobility-shift assays (EMSA) by using PIF4 and CRY1 proteins expressed in vitro. As shown in Fig. S9*D*, PIF4 bound to the G-box containing DNA fragments present in the promoter region of *YUC8,* CRY1 could not bind to the DNA fragments itself but could bind together with PIF4. CRY1 promoter association was significantly decreased in the *pif1345* mutant background (Fig. S9*E*), but was not that significantly affected in the *pif4* mutant background (Fig. S9*F*), consistent with that multiple PIFs acted downstream of CRY1. PIF3 also associated with the *YUC8* promoter (Fig. S9*G*), the same as reported before (46, 47).

CRY1 forms a complex with PIF4 to associate with PIF4 target genes under blue light conditions, and blue light or CRY1 did not affect the DNA binding activity of PIF4 (Fig. S9 *H* and *I*). To analyze whether CRY1 affected the transcription activity of PIF4 on the *YUC8* promoter, a transient transcription assay in tobacco leaves and in protoplasts were used to test PIF4 activity. We used a dual-LUC reporter plasmid that encodes a firefly luciferase (LUC) gene driven by the *YUC8* promoter (−1,635 bp to 0 bp) and a Renilla luciferase (REN) gene driven by the constitutive *35S* promoter (Fig. 4*D*) (7, 8, 48). The *YUC8pro-LUC* reporter was transiently expressed in tobacco leaves together with either CRY1 or PIF4 or both. PIF4 promoted the transcription of the *YUC8* gene, and the expression level of *YUC8 promoter-LUC* was approximately twofold lower when PIF4 was combined with CRY1 than when only PIF4 was infiltrated although the same amount of PIF4 Agrobacteria cells were used (Fig. 4*E*). The reporter was also transiently expressed in WT and *cry1* mutant protoplasts with or without PIF4. The expression level of *YUC8 promoter-LUC* was approximately twofold higher when PIF4 was expressed in *cry1* protoplast than when PIF4 was expressed in WT under white light (Fig. 4*F*). Furthermore, CRY1 repressed the transcription activity of PIF4 in blue light but not in dark (Fig. 4*G*). CRY1 itself did not affect the transcription of the *YUC8 promoter-LUC* construct significantly (Fig. 4 *E*–*G*). Taken together, these data indicated that

CRY1 formed a complex with PIF4 to suppress the transcription activity of PIF4 in a blue light-dependent manner (Fig. 4*H*).

Discussion

Plants evolve with multiple photoreceptors that function by interacting with photoreceptor-specific signaling proteins. Red light photoreceptors Phytochromes interact with PIFs to control red/far red light-regulated gene expression and morphogenesis (21–23). CRY2 interacts with CIBs to regulate the *FT* expression and also photoperiodic flowering (7–9). We show in the present study that CRY1 directly interacts with PIF4 in a blue light-dependent manner to regulate the expression of PIF4 targets and also high temperature-promoted hypocotyl elongation. Our previous studies demonstrate that blue light photoreceptors CRY2 and ZEITLUPE (ZTL) can both regulate CIB1, CRY2 physically interacts with CIB1 in response to blue light to activate its transcription activity (7, 8), whereas ZTL mediates blue-light suppression of CIB1 degradation (9). CIB1 seems to be the molecular basis of cross-talk between CRY2 and ZTL blue light receptors. PIF4 transcription factor interacts with both PhyB and CRY1, and it plays a role in temperature response, so that multiple plant photoreceptors and ambient temperature can mediate plant development through the same signaling component—PIF4. PIF4 appears to be the molecular basis of cross-talk between red and blue light pathways, and also among red and blue light and ambient temperature. It is reported that PhyA interacts with CRY1, and PhyB binds CRY2 (49, 50), so red and blue light may cross-talk at multiple layers to coordinately regulate plant development.

We also showed that CRY1 form a complex with PIF4 to associate with the PIF4 target genes, CRY1 and PIF4, which likely interacted on the nuclear DNA. Furthermore, our transactivation assays revealed that CRY1 repressed the transcription activity of PIF4. This observation is consistent with the CRYs function in animals, where CRYs act as transcriptional repressors (51). Our results suggest that CRY1 directly regulate the activity of PIF4 through physical interaction on the DNA, such that changes in the external environment can rapidly lead to phenotypic changes.

The expression of CRY1 is not temperature regulated (Fig. S2), consistent with the previous result (34). To exclude the possibility that CRY1 inhibits hypocotyl elongation more strongly at 28 °C

Ma et al.

than 22 °C, just because PIF4 is more active at 28 °C, we checked the phenotype of *cry1* in short day condition because PIF4 also has a much bigger role in SD (24). *cry1* mutant was not far more elongated than WT in short day condition (Fig. S9*J*). It was also reported that CRYs are required for temperature compensation of the circadian clock (34). These together suggest CRY1 itself may be involved in temperature response, which need to be further investigated. *cop1-6* mutant is insensitive to the elevated temperature, the same as *pif4*, indicating that COP1 is essential for plant responses to ambient temperature; whether COP1 is involved in ambient temperature signaling independent of PIF4 needs to be further investigated.

Materials and Methods

Plant materials, seedling photothermol assays, immunoblots, the in vitro pull down, BiFC, co-IP, quantitative PCR (qPCR), free IAA measurement, ChIP, EMSA, and dual-LUC assays are as described (7, 8, 12) and in *SI Materials and Methods*.

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