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Breakthrough Report

Light-Response Bric-A-Brack/Tramtrack/Broad proteins mediate cryptochrome 2 degradation in response to low ambient temperature

Libang Ma [1,2], **Xu Li** [1], **Zhiwei Zhao** [1], **Yuhao Hao** [1], **Ruixin Shang** [1,2], **Desheng Zeng** [1,2] and **Hongtao Liu** [1,*,†]

1 National Key Laboratory of Plant Molecular Genetics, CAS Center for Excellence in Molecular Plant Sciences, Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai 200032, China

2 University of Chinese Academy of Sciences, Shanghai 200032, China

*Author for correspondence: htliu@cemps.ac.cn

†Senior author.

L.M. and H.L. conceived the project. L.M. performed most of the experiments, X.L. performed the Co-IP and ubiquitination assay. Z.Z., Y. H., R.S., and D.Z. provided material or made some of the constructs. L.M. and H.L. analyzed the data and wrote the manuscript.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plcell) is: Hongtao Liu (htliu@cemps.ac.cn).

Abstract

Cryptochromes (crys) are photolyase-like blue-light receptors first discovered in *Arabidopsis thaliana* and later identified in all major evolutionary lineages. Crys are involved in not only blue light responses but also in temperature responses; however, whether and how cry protein stability is regulated by temperature remains unknown. Here, we show that cry2 protein abundance is modulated by ambient temperature and cry2 protein is degraded under low ambient temperature via the 26S proteasome. Consistent with this, cry2 shows high levels of ubiquitination under low ambient temperatures. Interestingly, cry2 degradation at low ambient temperatures occurs only under blue light and not under red light or dark conditions, indicating blue-light-dependent degradation of cry2 at low ambient temperature. Furthermore, low ambient temperature promotes physical interaction of Light-Response Bric-a-Brack/Tramtrack/Broad (LRB) proteins with cry2 to modulate its ubiquitination and protein stability in response to ambient temperature. LRBs promote high-temperature-induced hypocotyl elongation by modulating the protein stability of cry2 protein. These results indicate that cry2 accumulation is regulated by not only blue light but also ambient temperature, and LRBs are responsible for cry2 degradation at low ambient temperature. The stabilization of cry2 by high temperature makes cry2 a better negative regulator of temperature responses.

Introduction

Cryptochromes are photolyase-like blue-light receptors that were first identified in Arabidopsis (*Arabidopsis thaliana*) and later in all major evolutionary lineages (Cashmore, 1997; Lin, 2002; Sancar, 2003). Arabidopsis cryptochrome 1 (cry1) and cryptochrome 2 (cry2) primarily mediate blue-light-mediated inhibition of hypocotyl elongation (Ahmad and Cashmore, 1993) and photoperiodic control of floral initiation (Guo et al., 1998). Blue-light-activated crys undergo oligomerization to become active dimers or tetramers that interact with signaling proteins such as CRY2 INTERACTING

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bHLH1 (CIB1) or PHYTOCHROME INTERACTING FACTOR4 (PIF4) to transduce signals (Sang et al., 2005; Liu et al., 2008, 2020; Ma et al., 2016, 2020; Pedmale et al., 2016; Wang et al., 2016; Shao et al., 2020).

The abundance and activity of plant crys are regulated by phosphorylation, ubiquitination, and proteolysis (Wang and Lin, 2020). PHOTOREGULATORY PROTEIN KINASES 1–4 (PPK1–4) positively regulate cry activity (Shalitin et al., 2002; Wang et al., 2015; Liu et al., 2017). Photoexcited cry2 proteins become polyubiquitinated and subsequently degraded by the 26S proteasome (Yu et al., 2007). CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) is a RING finger E3 ubiquitin ligase that acts downstream of phytochromes (phys), crys, and UV RESISTANCE LOCUS 8 (UVR8; Ang and Deng, 1994; Christie et al., 2012). The COP1-related protein SUPPRESSOR OF PHYTOCHROME A (SPA1) interacts with COP1 to positively regulate COP1 activity, whereas crys and phys suppress the E3 ubiquitin ligase activity of COP1 by forming a complex with SPA1 and COP1 in a light-dependent manner (Deng et al., 1991; Lian et al., 2011; Liu et al., 2011; Zuo et al., 2011). COP1 is involved in regulating cry2 degradation in response to blue light (Lin et al., 1998; Yu et al., 2007; Liu et al., 2016); it is unclear whether any other E3 ubiquitin ligases are involved in this process.

In addition to light, temperature is a key environmental cue affecting plant growth and development, even within a range that does not induce stress responses to any significant degree (Wigge, 2013; Casal and Balasubramanian, 2019). Temperature regulates gene expression via chromatin remodeling and activation of transcription. Nucleosomes containing the H2A.Z histone variant act as thermosensors to mediate temperature-induced transcriptome changes (Kumar and Wigge, 2010). The bHLH transcription factor PIF4 directly links the red-light photoreceptor phytochrome B (phyB) to light-regulated gene expression and plant development (Castillon et al., 2007). PIF4 also plays a role in high ambient temperature responses, not only regulating temperature-mediated floral induction under short-day conditions through direct activation of *FLOWERING LOCUS T (FT*; Kumar et al., 2012), but also controlling high-temperature-induced hypocotyl elongation by increasing free indole-3 acetic acid (IAA) concentrations via direct stimulation of *YUCCA8 (YUC8)* or *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA1)* expression (Koini et al., 2009; Stavang et al., 2009; Franklin et al., 2011; Nomoto et al., 2012; Oh et al., 2012; Sun et al., 2012; Delker et al., 2014; Lee et al., 2014; Nieto et al., 2014).

The evening complex, comprising EARLY FLOWERING 3 (ELF3), ELF4, and LUX ARRYTHMO (LUX), is a core component of the plant circadian clock (Nusinow et al., 2011) and acts as a temperature-responsive transcriptional repressor for regulating growth (Box et al., 2015). A prion-like domain in ELF3 functions as a thermosensor in Arabidopsis (Gaillochet et al., 2020). Warm temperature also selectively enhances translation of *PIF7* messenger RNA by changing its secondary structure (Lee et al., 2020). Multiple photoreceptors are also involved in temperature responses, with phyB identified as a thermosensor (Jung et al., 2016; Legris et al., 2016; Hahm et al., 2020). Blue light and crys inhibit the hypocotyl elongation promoted by high ambient temperature (Ma et al., 2016), and regulate temperature compensation of the circadian clock (Gould et al., 2013), temperature-dependent internode elongation (Mazzella et al., 2000), and maintenance of plant biomass (Foreman et al., 2011). Whether and how CRY accumulation is regulated by ambient temperature remains unknown.

Here, we show that cry2 accumulation is modulated coordinately by light and temperature. Light-Response Bric-a-Brack/Tramtrack/Broad (LRB) proteins interact physically with cry2 to modulate its protein stability in response to ambient temperature.

Results

Ambient temperature regulates cry2 accumulation

Ambient temperature does not affect the transcription of *CRY* genes (Gould et al., 2013; Ma et al., 2016); however, it was unclear whether and how cry accumulation was regulated by ambient temperature. We therefore investigated whether ambient temperature affects cry2 accumulation. Wild-type (WT) Arabidopsis plants grown at 22°C under white light for 6 days were transferred to 28°C for various periods or moved to 16°C for 1 day before transfer to 28°C for various periods. Levels of cry2 protein increased markedly (>2.5-fold) within 4 h of 28°C treatment (Figure 1, A–C). Plants grown at 22°C under white light were also transferred to 16°C for various periods or moved to 28°C for 1 day and then transferred to 16°C for various periods. Cry2 accumulated to high levels in plants at 28°C, but levels decreased markedly (about two-fold) within 4 h of plants being transferred from 28°C to 16°C or from 22°C to 16°C (Figure 1, A–C). WT seedlings grown under continuous white light for 12 h at 28°C/12 h at 16°C also showed increased cry2 accumulation at 28°C but decreased cry2 accumulation at 16°C (Figure 1, D–E). Taken together, our results establish that the levels of cry2 accumulation are positively regulated by increased temperature.

Cry2 is degraded under low ambient temperature via the 26S proteasome

We next examined the possible mechanisms underlying the temperature regulation of cry2 accumulation. Given that light- and ubiquitin/26S proteasome-dependent proteolysis is a common mechanism associated with many light signaling proteins (Wang and Lin, 2020), we examined levels of a Green Fluorescent Protein (GFP)-cry2 fusion in plants transferred from 28°C to 16°C or 22°C to 16°C in the presence or absence of the 26S proteasome inhibitor MG132, using transgenic seedlings expressing *CRY2pro::GFP-CRY2*. As expected, cry2 abundance decreased markedly at 16°C in the absence of MG132 (Figure 2, A–C). However, in the presence of MG132 (50 μM), cry2 abundance did not decrease at 16°C (Figure 2, A–C), suggesting that the decrease in cry2 levels at low ambient temperature results from

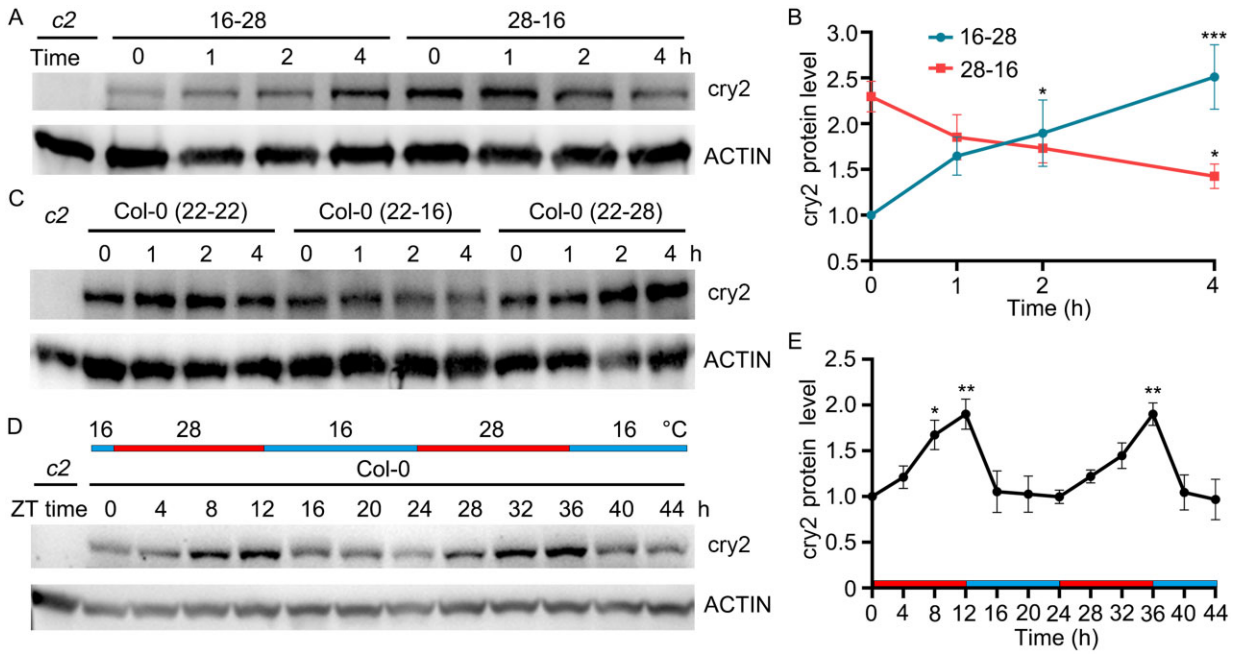

Figure 1 Ambient temperature regulates CRY2 accumulation. A, CRY2 protein accumulates at 28°C while levels decrease at 16°C. Six-day-old Col-0 seedlings grown at 22°C under white light were pretreated at 16°C or 28°C for 24 h and then transferred to 28°C or 16°C for the indicated periods. Immunoblot with *cry2* (c2) as a negative control. ACTIN was used as a loading control. B, Quantification of protein levels in (A) with four biological replicates are shown. Error bars represent SE ($n=4$). Asterisks indicate a significant difference compared with the protein level at time 0 (*$P<0.05$; **$P<0.001$; ***$P<0.0005$; Tukey's multiple comparisons test). C, Cry2 protein accumulates at high temperature and decreases at low temperature. Seven-day-old Col-0 seedlings grown at 22°C under white light were transferred to 28°C or 16°C or maintained at 22°C for the indicated periods. Immunoblot with *cry2* (c2) mutants as a negative control. ACTIN was used as a loading control. D, Cry2 protein levels under continuous white light 16°C/28°C temperature cycle conditions. Col-0 seedlings were sampled at day 7 and day 8. E, Quantified protein levels in (D) with three biological replicates are shown. Error bars represent SE ($n=3$). Asterisks indicate a significant difference compared with the protein level at time 0 (*$P<0.05$; **$P<0.001$; ***$P<0.0005$; Dunnett's multiple comparisons test).

proteolysis of cry2 by the 26S proteasome. The treatment of seedlings with a protein synthesis inhibitor in eukaryotes cycloheximide (CHX; 10 μM), which reduces the overall protein expression, failed to prevent the decrease in cry2 levels in response to decreased ambient temperature (Figure 2, A–C). Taken together, our results establish that cry2 is degraded by the 26S proteasome under low ambient temperature, while high ambient temperature suppresses cry2 protein degradation. Interestingly, cry2 was degraded when seedlings were transferred from 28°C to 16°C only under blue light and not under red light or dark conditions (Figure 2, D–E). These results indicate that cry2 degradation at low ambient temperature is blue-light dependent; only blue-light-activated cry2 is degraded in response to low temperature.

LRBs are involved in regulating CRY2 degradation in response to low temperature

LRB1, LRB2, and LRB3 recruit the red-light photoreceptor phytochrome B (phyB) and PHYTOCHROME INTERACTING FACTOR 3 (PIF3) in response to light signals, promoting the ubiquitination and subsequent co-degradation of both substrates in vivo by the 26S proteasome pathway (Li et al., 2020). We tested whether LRBs affect the stability of cry2 in response to low temperature by first analyzing the protein stability of cry2 in *lrb1 lrb2 lrb3* mutants. The cry2 protein levels decreased when WT plants were transferred from 28°C to 16°C. The *lrb1 lrb2 lrb3* mutant plants contained more cry2 than WT plants, and there was no significant decrease in cry2 in *lrb1 lrb2 lrb3* mutants when plants were transferred from 28°C to 16°C (Figure 3, A and B). Therefore, LRBs are involved in regulating the degradation of cry2 at low ambient temperature. Consistent with this, cry2 had high levels of ubiquitination at 16°C under white light, but much reduced ubiquitination at 28°C under white light. The ubiquitination level of cry2 was significantly reduced in *lrb1 lrb2 lrb3* mutants compared with the WT at 16°C under white light; ubiquitination of cry2 under blue light was used as a control (Figure 3, C and D). These results demonstrate that LRBs are involved in regulating the ubiquitination and degradation of cry2 in response to low ambient temperature.

Ambient temperature does not affect the transcription of LRBs (Figure 3, E–G). However, transgenic lines overexpressing *LRB1* or *LRB2* (harboring the *35S:LRB1-GFP* or *35S:LRB2-GFP* constructs, respectively) revealed that LRB1 and LRB2 levels increase significantly within 1 h of the transition from 16°C to 28°C (Figure 3, H–I). Six-day-old seedlings grown at 22°C under white light were moved to 16°C or 28°C for 1 day and then transferred to 28°C or 16°C for various periods. LRB1 and LRB2 were abundant in plants at 28°C, but protein levels

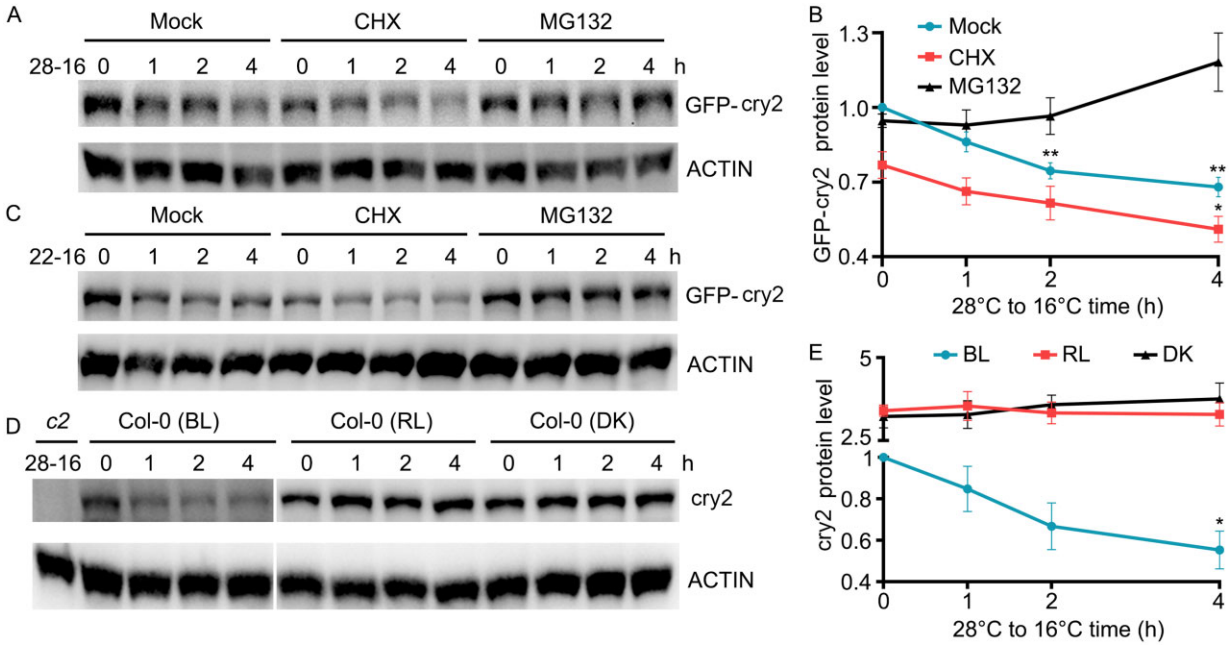

Figure 2 Cry2 protein is degraded at low ambient temperature via the 26S proteasome. A, MG132 inhibits the degradation of GFP-cry2 protein. Six-day-old *CRY2pro::GFP-CRY2* seedlings grown at 22°C under white light were pretreated at 28°C for 24 h and then treated with 10-μM CHX (protein biosynthesis inhibitor) or 50-μM MG132 (26S proteasome inhibitor) for 1 h before transfer to 16°C for the indicated periods. ACTIN was used as a loading control. B, Quantification of protein levels in (A) with four biological replicates is shown. Error bars represent SE ($n=4$. Asterisks indicate a significant difference compared with the protein level at time 0 (*$P<0.05$; **$P<0.001$; ***$P<0.0005$; Dunnett's multiple comparisons test). C, MG132 prevents a decrease in GFP-cry2 protein levels at 16°C. Seven-day-old *CRY2pro::GFP-CRY2* seedlings grown at 22°C under white light were treated with 10-μM CHX or 50-μM MG132 for 1 h and then transferred to 16°C for the indicated periods. ACTIN was used as a loading control. D, Cry2 protein is degraded in a blue light-dependent manner when plants are transferred from 28°C to 16°C. Six-day-old Col-0 seedlings grown at 22°C under white light were pretreated at 28°C under blue light (BL), red light (RL), or dark (DK) conditions for 24 h and then transferred to 16°C for the indicated period. Immunoblot with c2 (*cry2*) as a negative control. ACTIN was used as a loading control. (E) Quantification of protein levels in (D) with four biological replicates is shown. Error bars represent SE ($n=4$). Asterisks indicate a significant difference compared with the protein level at time 0 (*$P<0.05$; **$P<0.001$; ***$P<0.0005$; Dunnett's multiple comparisons test).

decreased markedly within about 4 h of plants being transferred from 28°C to 16°C (Figure 3, H–I). These results indicate that LRB1 and LRB2 accumulation is regulated by ambient temperature and that cry2 degradation in response to low ambient temperature is not because of greater accumulation of LRB protein under low ambient temperature.

CRY2 interacts physically with LRBs

LRBs are involved in regulating the ubiquitination and degradation of cry2 in response to low ambient temperature. To decipher the underlying mechanism, we tested the interaction between cry2 and LRBs. In an in vitro pull-down assay, cry2 was pulled down by LRB1, LRB2, and LRB3 (Figure 4A), indicating that these proteins physically interact in vitro. LRBs also interacted with cry2 in plant cells in bimolecular fluorescence complementation (BiFC) assays (Figure 4B). We detected strong fluorescence in the nuclei of cells co-transformed with cry2-cYFP and nYFP-cLRB1, nYFP-cLRB2, or nYFP-cLRB3 plasmids (Figure 4B), in contrast to cells transformed with individual plasmids, or the flavin-deficient mutant $CRY2^{D387A}$-cYFP (Liu et al., 2008) and nYFP-cLRB1/2/3, suggesting that the fluorophore reconstitutes upon cry2-cYFP–nYFP-cLRB interaction (Figure 4B). $CRY2^{D387A}$ does not interact with LRBs, indicating that the cry2–LRBs interactions are dependent on the chromophores of the cry2 photoreceptor. We also performed co-immunoprecipitation (co-IP) analyses in transgenic Arabidopsis seedlings expressing GFP-tagged LRB1 or LRB2. Cry2 co-precipitated with LRB1 or LRB2 (Figure 4C), indicating that both LRB1 and LRB2 interact with cry2 in planta.

To determine whether ambient temperature affects the interaction between cry2 and LRBs, we examined cry2-LRB1 and cry2-LRB2 complex formation at 28°C and 16°C using co-IP assay. In this experiment, seedlings were pre-treated with the proteasome inhibitor MG132 at 28°C for 1 h to block the degradation of cry2 and LRB promoted by low ambient temperature. Seedlings were then transferred to 28°C or 16°C for 4 h before being subjected to co-IP analyses. More cry2 co-precipitated with LRB1 and LRB2 at 16°C than at 28°C (Figure 4D), indicating that low ambient temperature stimulates accumulation of the cry2-LRB1/LRB2 complex in plant cells.

LRBs regulate hypocotyl elongation in response to ambient temperature

Blue light and cry1 are required for inhibiting hypocotyl elongation promoted by high ambient temperature via

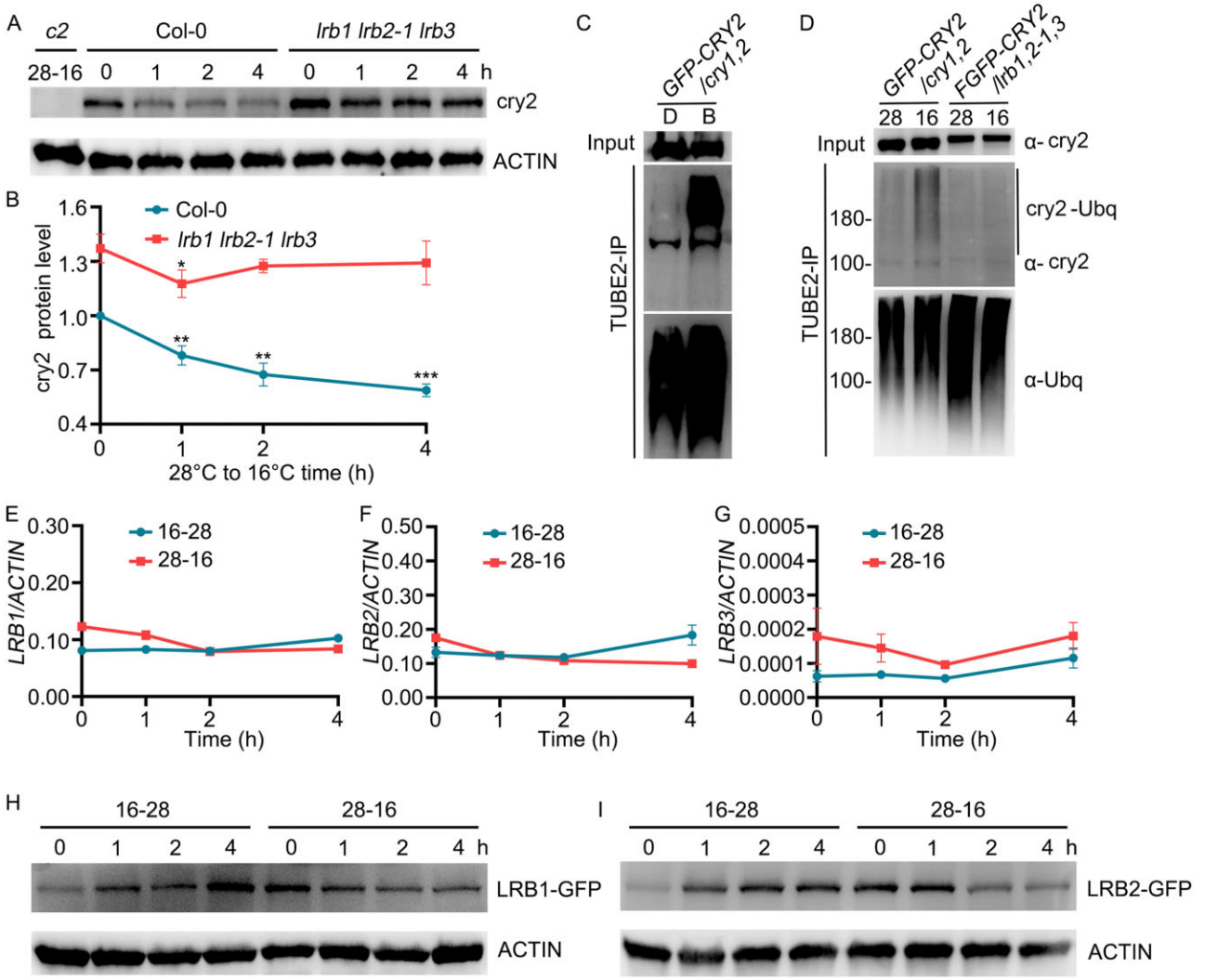

Figure 3 LRBs are involved in regulating cry2 degradation in response to low temperature. A, LRBs are responsible for the degradation of cry2 in response to low ambient temperature. Six-day-old Col-0 and *lrb1 lrb2-1 lrb3* seedlings grown at 22°C under white light were pretreated at 28°C for 24 h and then transferred to 16°C for the indicated periods. Immunoblot with *cry2* (c2) as a negative control. ACTIN was used as a loading control. B, Quantification of protein levels in (A) with three biological replicates is shown. Error bars represent SE ($n = 3$). Asterisks indicate a significant difference compared with the protein level at time 0 (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0005$; Dunnett's multiple comparisons test). C, D, Ubiquitination of GFP-cry2 under blue light (C) and at 16°C (D). Ten-day-old seedlings grown at 22°C under white light were preincubated in the dark (C) or at 28°C (D) for 24 h and then treated with 50-μM MG132 for 1 h. Half of the seedlings were moved to blue light (C) or 16°C (D) and the rest were maintained in the dark (C) or at 28°C (D). Seedlings were collected 2 h later for Tandem Ubiquitin Binding Entities (TUBE2)-IP. E–G, Expression of *LRB1* (E), *LRB2* (F), and *LRB3* (G) following 16°C to 28°C and 28°C to 16°C temperature transitions. Six-day-old Col-0 seedlings grown at 22°C under white light were pretreated at 28°C or 16°C for 24 h and then transferred to 16°C or 28°C for the indicated periods. Error bars represent SE of three biological replicates ($n = 3$). H, I, Protein levels of LRB1 (H) and LRB2 (I) following 16°C to 28°C and 28°C to 16°C temperature transitions. Six-day-old LRB1-GFP and LRB2-GFP seedlings grown at 22°C under white light were pretreated at 28°C or 16°C for 24 h and then transferred to 16°C or 28°C for the indicated periods.

repressing PIF4 (Ma et al., 2016). Both cry1 and cry2 interact with PIFs (Ma et al., 2016; Pedmale et al., 2016), suggesting that cry2 is also involved in hypocotyl elongation promoted by ambient temperature. To test this hypothesis, we investigated the function of cry2 in hypocotyl elongation in response to changes in ambient temperature. Hypocotyl elongation of WT, *cry2*, and *35S::GFP-CRY2* seedlings was recorded after 5 days of treatment at 16°C or 28°C under 12-h blue light/12-h dark conditions. *cry2* mutants exhibited greater hypocotyl elongation at 28°C than at 16°C compared with the WT, while *GFP-CRY2* overexpressing seedlings only elongated modestly at the higher temperature, indicating that blue-light-activated cry2 also represses hypocotyl elongation induced by high temperature (Figure 5, A and B). We analyzed the hypocotyl elongation of WT seedlings and *lrb1 lrb2 lrb3* mutants after 5 days of treatment under 12 h different fluence rate of blue light/12-h dark conditions at 16°C or 28°C and also after 7 days of treatment under 12-h

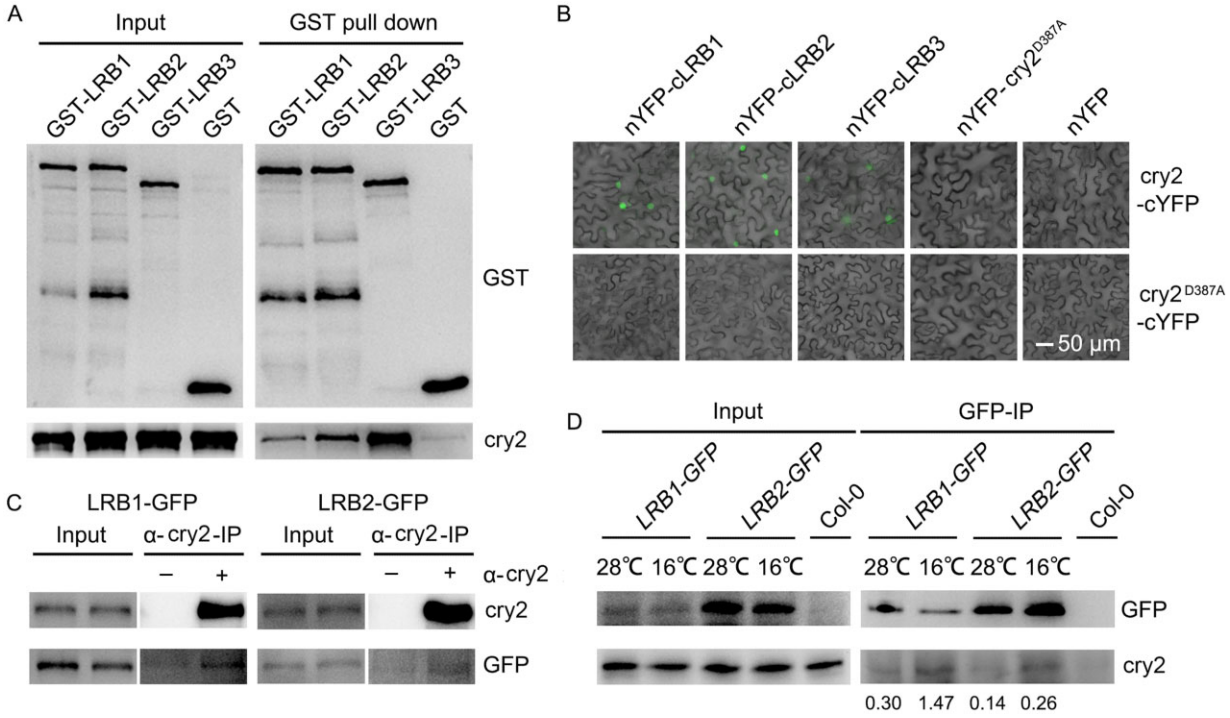

Figure 4 Cry2 physically interacts with LRBs. A, In vitro pull-down assays show that cry2 interacts with LRB1, LRB2, and LRB3. B, BiFC assays show that cry2 interacts with cLRB1 (aa 241–561), cLRB2 (aa 243–561), and cLRB3 (aa 189–505). C, co-IP assays show that cry2 interacts with LRB1 and LRB2 in vivo. Ten-day-old LRB1-GFP, LRB2-GFP, or Col-0 seedlings grown at 22°C under white light were treated with 50-μM MG132 for 1 h and then collected for co-IP. D, Co-IP assays reveal that interactions between CRY2 and LRBs are affected by temperature. Ten-day-old LRB1-GFP and LRB2-GFP seedlings grown at 22°C under white light were pretreated at 28°C for 24 h and then treated with 50-μM MG132 for 1 h. Half of the seedlings were moved to 16°C and the rest were maintained at 28°C. Seedlings were collected 4 h later for co-IP. The numbers below the gels denote the precipitated CRY2 ratio calculated by $cry2^{IP}$-background/LRB^{IP}-background.

white light/12-h dark conditions at 16°C, 22°C, or 28°C. *lrb1 lrb2 lrb3* mutants were insensitive to ambient temperature changes, only elongating modestly at the higher temperature, suggesting that LRBs repress high temperature-induced hypocotyl elongation (Figure 5, A–E), consistent with the phenotype of *CRY2* overexpression lines and accumulation of cry2 in *lrb1 lrb2 lrb3*. Furthermore, *lrb1 lrb2 lrb3 cry2* mutants showed a similar phenotype as the *cry2* mutant, consistent with LRBs modulating the protein stability of cry2 in response to ambient temperature (Figure 5, A–E). In low blue light, *lrb1 lrb2 lrb3* mutants have a reduced response to high temperature (Figure 5B), while at high white light, the high temperature response is fully absent (Figure 5D). We think that is because there is red light in white light, and LRBs are also involved in the degradation of phyB and PIF3 (Ni et al., 2014).

Discussion

Protein accumulation of the blue-light photoreceptor cry2 is modulated by blue light. Blue-light-dependent phosphorylation and oligomerization of Arabidopsis cry2 trigger rapid ubiquitination and subsequent degradation of cry2 in the nucleus (Ahmad et al., 1998; Yu et al., 2007, 2009). Blue-light-dependent cry2 degradation requires the flavin chromophore; the $cry2^{D387A}$ mutant protein that fails to bind flavin adenine dinucleotide no longer undergoes blue-light-dependent degradation (Liu et al., 2008). Blue light and crys are involved in ambient temperature responses. They are required for temperature compensation of the circadian clock (Gould et al., 2013), temperature-dependent internode elongation (Mazzella et al., 2000), maintenance of plant biomass (Foreman et al., 2011), and inhibiting hypocotyl elongation promoted by high ambient temperature (Ma et al., 2016). Ambient temperature does not affect the transcription of *CRY* genes (Gould et al., 2013; Ma et al., 2016), although whether and how cry protein accumulation is regulated by ambient temperature was previously unknown. Here, we showed that cry2 levels are modulated by ambient temperature, with cry2 protein being degraded at low ambient temperature via the 26S proteasome. Cry2 protein accumulation is modulated by not only blue light but also ambient temperature. Light and temperature change in concert with daily and seasonal cycles, and cry2 mediates both blue light and ambient temperature signals to coordinately regulate plant development. It is intriguing that ambient temperature modulates cry2 accumulation in a blue-light-dependent manner. Only blue light activates cry2 degradation at low ambient temperature.

The RING finger E3 ubiquitin ligase COP1 is a key component of light signal transduction. Crys and phys suppress the E3 ubiquitin ligase activity of COP1 by forming a complex with SPA1 and COP1 in a light-dependent manner (Deng et

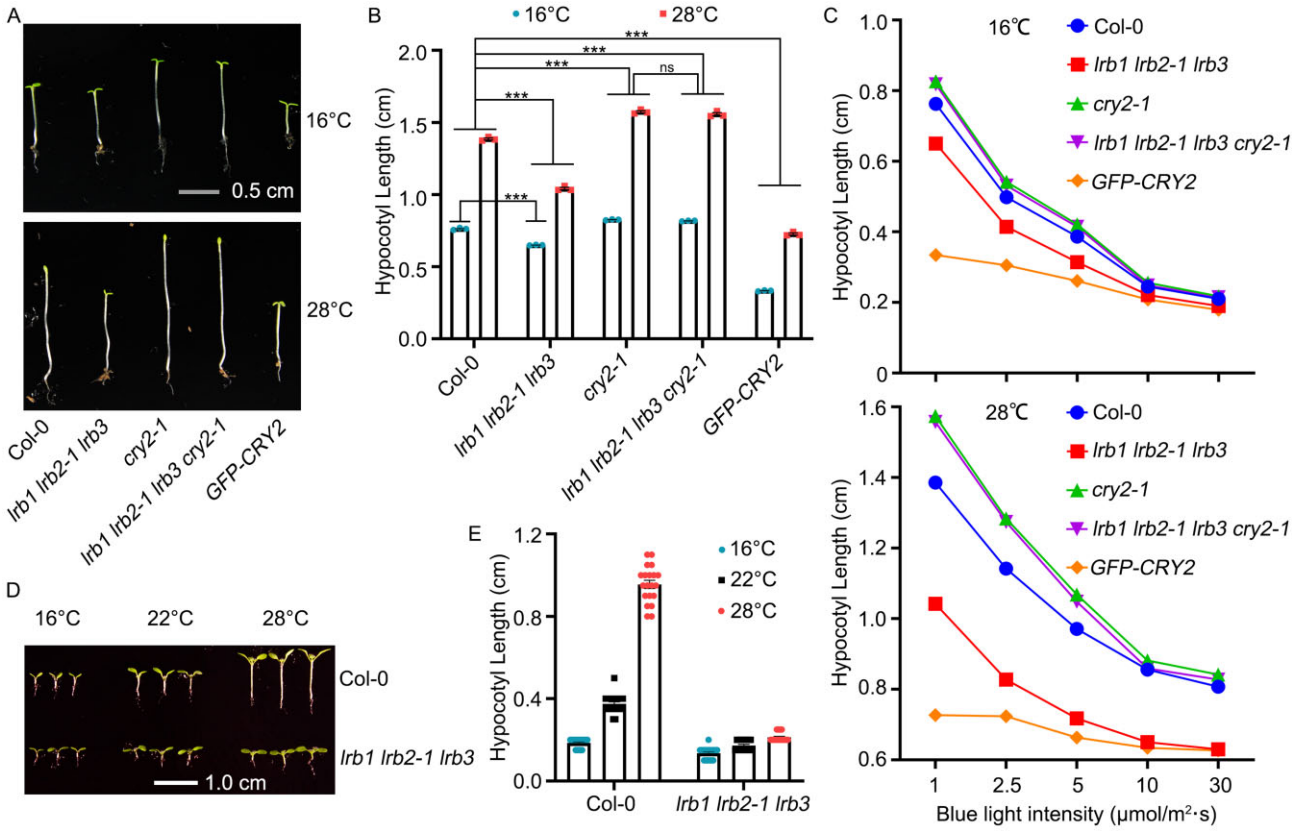

Figure 5 LRBs regulate hypocotyl elongation in response to temperature. A, Hypocotyl phenotypes of 6-day-old Col-0, *lrb1 lrb2-1 lrb3*, *cry2-1*, *lrb1 lrb2-1 lrb3 cry2-1*, and GFP-CRY2 seedlings grown at 16°C or 28°C under low blue light (12-h blue light, 12-h dark, 1 $\mu mol \cdot m^{-2} \cdot s^{-1}$) conditions. B, Quantification of hypocotyl lengths of the genotypes indicated in (A). Error bars represent the SE ($n = 20$, three biological repeats). Asterisks indicate statistically significant differences between the genotypes indicated, as determined using Dunnett's multiple comparisons test (***$P < 0.0005$). (C) Fluence rate response curves measuring hypocotyl elongation of 6-day-old seedlings of indicated genotypes grown in long day blue light (12-h blue light, 12-h dark) at 16°C and 28°C. Asterisks indicate statistically significant differences between the *lrb1 lrb2-1 lrb3* and Col-0 (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0005$; Dunnett's multiple comparisons test). D, Hypocotyl phenotypes of 7-day-old Col-0 and *lrb1 lrb2-1 lrb3* seedlings grown at 16°C, 22°C, or 28°C under white light (12-h light, 12-h dark, 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). E, Quantification of hypocotyl lengths of the genotypes indicated in (D). Error bars represent the SE ($n = 20$).

al., 1991; Lian et al., 2011; Liu et al., 2011; Zuo et al., 2011). COP1 is associated with cry2 degradation, which is partially impaired in *cop1* weak mutant alleles (*cop1-4 and cop1-6*; Wang et al., 2001; Yang et al., 2001; Shalitin et al., 2002). Here, we showed that LRB E3 ubiquitin ligases are involved in regulating the protein stability of cry2 in response to ambient temperature. Very recently, it was reported that LRBs physically interact with photoexcited and phosphorylated cry2 to facilitate polyubiquitination and degradation of cry2 in response to blue light (Chen et al., 2021). Cry2 is therefore controlled by two types of E3 ligase, COP1 and LRBs, and LRBs regulate the degradation of cry2 in response to not only blue light but also ambient temperature. In mammals, the E3 ubiquitin ligases FBXL3 and FBXL21 promote ubiquitination and degradation of crys to control the period of the circadian clock (Yoo et al., 2013). These results demonstrate that multiple E3 ligases are needed to regulate cry activity in both plants and mammals.

LRB1 and LRB2 protein levels are also regulated by ambient temperature. LRB1 and LRB2 protein levels decrease markedly when plants are transferred from 28°C to 16°C. LRBs are responsible for cry2 degradation in response to low ambient temperature, but LRB1 and LRB2 protein levels do not increase but decrease under low ambient temperature, suggests the existence of a regulatory loop.

LRBs are responsible for the degradation of cry2 protein in response to low temperature then they may have stronger biological impacts at low temperature. Our results indicate that LRBs repress high-temperature-induced hypocotyl elongation but not that LRBs show a stronger biological impact at low temperature. We think there are several explanations: 1. LRBs are responsible for the degradation of cry2 protein in response to not only low temperature but also blue light (Chen et al, 2021). Figure 3, A and B shows that there are more cry2 protein in *lrb1 lrb2-1 lrb3* triple mutants than in the WT even at 28°C, though the difference between WT and *lrb1 lrb2-1 lrb3* is much more dramatic at 16°C than at 28°C. Cry2 represses warm temperature promoted hypocotyl elongation as GFP-CRY2 overexpression seedlings only elongated modestly at the higher temperature, similar to

cry1 (Ma et al., 2016). There are more cry2 protein in *lrb1 lrb2-1 lrb3* triple mutants than in the WT especially at 16°C, while cry2 protein inhibits high temperature promoted hypocotyl elongation, so the higher cry2 level effect at 16°C may be overcame by cry2 inhibition of high temperature promoted hypocotyl elongation at 28°C. LRBs and cry2 ensure proper growth in response to ambient temperature change to make sure that hypocotyl would not be too long in high temperature and not too short in low temperature. Warm temperature promotes hypocotyl elongation, cry2 accumulates in response to higher temperature and represses warm temperature promoted hypocotyl elongation. LRBs degrade cry2 protein in response to low temperature so as to release the repression of hypocotyl elongation by cry2; 2. LRBs are not the only E3 that affect cry2 degradation in low temperature; 3. LRBs may affect the degradation of other proteins that affect hypocotyl elongation in response to temperature.

In summary, our results demonstrate that ambient temperature modulates the protein stability of cry2, and LRBs are involved in regulating the degradation of cry2 at low ambient temperature. The stabilization of cry2 by high temperature makes cry2 a better negative regulator of temperature responses.

Materials and methods

Plant materials and growth conditions

We used the *A. thaliana* Columbia (Col-0) accession as the WT. The *cry2-1*, *cry1 cry2 35S:GFP-CRY2*, and *lrb1 lrb2-1 lrb3* lines have been described previously (Yu et al., 2007, 2009; Li et al., 2020; Ni et al., 2014). The *CRY2* promoter (1,176-bp sequence before the ATG) and full-length *CRY2* coding sequences were cloned into the pEGAD-GFP vector (Liu et al., 2008) (*CRY2pro:GFP-CRY2*), and transformed into the *cry1 cry2* mutants. The *lrb1 lrb2-1 lrb3 cry2-1* quadruple mutant was generated by genetic crosses and combined phenotyping and genotyping of F_2 progeny.

All seeds were surface-sterilized in 75% ethanol for 10 min, washed once with 100% ethanol and dried on sterilized filter paper, then sown on half-strength Murashige and Skoog medium supplemented with 0.8% agar and 1% sucrose. Plates were then stratified for 4 days in the dark at 4°C before transfer to a Percival growth chamber (E-22LEDX for white, blue, or red light, Percival Scientific, Perry, IA, USA) under defined light and temperature conditions. The lights used were white light (LED light, 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$), blue light (LED light 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$), red light (LED light 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Immunoblot analysis

Seeds were grown for 6 days in 22°C continuous white light conditions before being moved to 16°C or 28°C defined light conditions for 24 h, then seedlings were exposed to a defined temperature treatment for various times before collection. For CHX and MG132 treatment, 10-μM CHX or 50-μM MG132 were used 1 h before temperature treatment. After treatment, we collected seedlings and prepared whole protein extracts with extraction buffer (25.2-g glycerol, 0.02-g bromophenol blue, 4-g SDS, 20-mL 1M Tris-HCl [pH 6.8], 3.1-g DTT, to 50-mL ddH_2O). Equal protein amounts were loaded and separated on a 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel and transferred to Pure Nitrocellulose Blotting Membrane (P/N66485, PALL, USA). We then probed the membrane with anti-cry2 antibody (produced by Youke, China, 1:3,000 dilution for immunoblot), anti-GFP antibody (11814460001, Roche, Switzerland, 1:3,000 dilution for immunoblot), or anti-ACTIN antibody (D191048, Sangon, China, 1:3,000 dilution for immunoblot) as loading control.

Quantitative reverse transcription polymerase chain reaction analysis

We performed all quantitative reverse transcription polymerase chain reaction (RT-qPCR) expression analyses as described in Ma et al. (2016). Briefly, we isolated total RNAs using a RNAiso Plus kit (Takara Bio). We synthesized first-strand cDNAs from 500 ng of starting total RNA using a Prime Script RT Reagent Kit with a genomic DNA Eraser (Takara Bio). We then used SYBR Premix Ex Tag (Takara Bio) and the MX3000 Real Time PCR System (Stratagene) for the qPCR reactions. We used *ACTIN7* (At5g09810) as an internal control. After initial denaturation of the first-strand cDNAs, we executed the following PCR program: denaturation at 95°C for 30 s, and a two-step PCR for 40 cycles (95°C for 5 s, 60°C for 20 s per cycle) with fluorescence read at the end of each cycle. A dissociation program was performed after the reaction. The biological replicates represent three independent experiments. Three technical replicates were performed for each reaction.

Ubiquitination assay

For ubiquitination assay, 22°C continuous white light grown 10-day-old transgenic seedlings expressing *35S::GFP-CRY2* were pretreated in dark or 28°C white light for 24 h then treated with 50-μM MG132 for 1 h, half of the seedlings were moved into blue or 16°C white light for 2 h. GFP-cry2 protein was extracted by immunoprecipitation with magnetic Tandem Ubiquitin Binding Entities (TUBE2) beads (UM402M, LifeSensors, USA). Proteins were loaded and separated on a 4%–12% pre-cast gel (180-8004H, Tanon, China) and transferred to PVDF Blotting Membrane (10600023, GE Healthcare, USA) and probed by anti-cry2 (produced by Youke, China, 1:3,000 dilution for immunoblot) or anti-ubiquitin antibody (ab7254, Abcam, UK, 1:3,000 dilution for immunoblot).

In vitro pull-downs

We performed in vitro pull-down protein–protein interaction assays as previously described (Liu et al., 2008, 2013; Ma et al., 2016; Liang et al., 2018; Yang et al., 2018), with the following modifications. We cloned the full-length coding sequences of *LRB1*, *LRB2*, or *LRB3* into the pGEX4T-1 vector. The proteins were produced in *Escherichia coli* BL21 strain

and purified by using Glutathione Sepharose 4B (17-0756-01, GE Healthcare, USA). Cry2 protein was produced in insect cells and purified by using Ni-NTA Agarose (R901-15, Invitrogen, USA). Equal amounts of GST-LRB1, GST-LRB2, GST-LRB3, or GST and cry2 were incubated in XB buffer (20-mM Tris [pH 8.0], 150-mM NaCl, 0.2% Triton X-100, 1-mM EDTA, 1-mM PMSF) with glutathione beads (17-0756-01, GE Healthcare, USA). Unbound proteins were removed by washing with washing buffer (50-mM Tris [pH 8.0], 200-mM NaCl, 0.05% Triton X-100, 1-mM EDTA), after that we eluted bound proteins and analyzed the resulting eluates by immunoblot analysis using anti-GST antibody (G081, Abmart, USA, 1:3,000 dilution for immunoblot) or anti-CRY2 antibody (produced by Youke, China, 1:3,000 dilution for immunoblot).

BiFC

The BiFC assay was based on that described previously with slight modifications (Ma et al., 2016), constructs for expression of cLRB1 (aa 241–561), cLRB2 (aa 243–561), cLRB3 (aa 189–505), and cry2, cry2^{D387A} fused to the C- or N-terminus of YFP were transformed into *Agrobacterium* strain GV3101 (with pSoup-P19). Overnight cultures of agrobacteria were collected by centrifugation, resuspended and mixed in MES buffer (10-mM $MgCl_2$, 10-mM MES, 100-μM acetosyringone) to 0.6 OD_{600}, then incubated at room temperature for 2 h before infiltration. Agrobacteria suspensions in a 1-mL syringe (without the metal needle) were carefully press-infiltrated manually onto healthy leaves of 3-week-old *Nicotiana benthamiana*. Plants were left under long day (white light, 16-h light/8-h dark) conditions for 3 days after infiltration.

co-IPs

The co-IP procedure has been described previously (Liu et al., 2008, 2013; Ma et al., 2016; Liang et al., 2018; Yang et al., 2018). Briefly, seedlings from the genotypes Col-0, LRB1-GFP, LRB2-GFP were grown for 10 days in continuous white light conditions, then treated with 50-μM MG132 for 1 h and collected for co-IP. Tissues were ground in liquid nitrogen and then homogenized in binding buffer (20-mM HEPES [pH 7.5], 40-mM KCl, 1-mM EDTA, 1% [v/v] Triton X-100, 1-mM PMSF). Protein extracts were incubated at 4°C for 10 min, before centrifugation at 14,000*g* for 10 min. The supernatants were collected and mixed with 35 μL of anti-CRY2-IgG-coupled protein A Sepharose (17-5280-04, GE healthcare, Sweden) or 35 μL anti-GFP agarose beads (KTSM1301, Alpalife, Shenzhen, China), incubated at 4°C for 30 min, and washed three times with washing buffer (20-mM HEPES [pH 7.5], 40-mM KCl, 1-mM EDTA, 0.1% [v/v] Triton X-100). We eluted bound proteins from the affinity beads with 4× SDS–PAGE sample buffer and analyzed the eluates by immunoblotting with anti-CRY2 antibody (produced by Youke, China, 1:3,000 dilution for immunoblot) and anti-GFP antibody (11814460001, Roche, Switzerland, 1:3,000 dilution for immunoblot) to detect the target proteins.

Phenotypic analysis of Arabidopsis

Col-0, *lrb1 lrb2-1 lrb3*, *cry2*, *lrb1 lrb2-1 lrb3 cry2*, and *35S:GFP-CRY2* seeds were sown on soil and stratified in the dark at 4°C for 4 days before transfer to a Percival growth chamber (E-30LEDL3, Percival Scientific, Perry, IA, USA). Seedlings grown in 16°C or 28°C blue light (12-h light/12-h dark, 1, 2.5, 5, 10, 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$) conditions for 5 days were analyzed.

Accession numbers

Sequence data for genes described in this article can be found in The Arabidopsis Information Resource (TAIR) under the following accession numbers: *CRY2* (At1g04400), *LRB1* (At2g46260), *LRB2* (At3g61600), *LRB3* (At4g01160), *COP1* (At2g32950), *HY5* (At5g11260), *CRY1* (At4g08920).

Supplemental data

The following materials are available in the online version of this article.

Supplemental Table S1. Primers used in this study.

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Conflict of interest statement. None declared.

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