Regulation of *Arabidopsis* photoreceptor CRY2 by two distinct E3 ubiquitin ligases

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Cryptochromes (CRYs) are photoreceptors or components of the molecular clock in various evolutionary lineages, and they are commonly regulated by polyubiquitination and proteolysis. Multiple E3 ubiquitin ligases regulate CRYs in animal models, and previous genetics study also suggest existence of multiple E3 ubiquitin ligases for plant CRYs. However, only one E3 ligase, Cul4COP1/SPAs, has been reported for plant CRYs so far. Here we show that Cul³LRBs is the second E3 ligase of CRY2 in *Arabidopsis*. We demonstrate the blue light-specific and CRY-dependent activity of LRBs (Light-Response Bric-a-Brack/Tramtrack/Broad 1, 2 & 3) in blue-light regulation of hypocotyl elongation. LRBs physically interact with photoexcited and phosphorylated CRY2, at the CCE domain of CRY2, to facilitate polyubiquitination and degradation of CRY2 in response to blue light. We propose that Cul⁴COP1/SPAs and Cul³LRBs E3 ligases interact with CRY2 via different structure elements to regulate the abundance of CRY2 photoreceptor under different light conditions, facilitating optimal photoresponses of plants grown in nature.

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CRYs are photolyase-like flavoproteins that act as photo-receptors in plants or core components of the molecular clock in mammals1–3. Regulation of CRY abundance is an important mechanism to control cellular photo-responses and chrono-responses. For example, two cullin 1 family E3 ubiquitin ligases, SCF{FBXL3} and SCF{FBXL21}, regulate ubiquitination and degradation of mCRYs to govern the circadian clock in mammals4–8. Similarly, a cullin 1-based E3 ubiquitin ligase receptor, Jetlag, and a cullin 4-based E3 ubiquitin ligase receptor, Brwd3, bind to dCRY to regulate ubiquitination of Tim (Timeless) and dCRY in Drosophila9,10.

Arabidopsis cryptochrome 2 (CRY2) is one of the best studied plant CRYs that mediate blue light inhibition of cell elongation and photoperiodic promotion of floral initiation11,12. These physiological activities of CRY2 are regulated by at least three blue light-dependent mechanisms. First, CRY2 undergoes blue light-dependent oligomerization to become active tetramers13–17. The BIC1 and BIC2 (Blue-light Inhibitors of Cryptochromes 1 and 2) proteins interact with photoexcited CRY2 to negatively regulate CRY2 photolyse-mization in light, whereas the active CRY2 homooligomers may also undergo thermal relaxation to become inactive monomers in the absence of light14–17. Second, the activity of CRY2 homooligomers is positively regulated by protein phosphorylation reactions catalyzed by four related protein kinases PPKs (Photoregulatory Protein Kinases 1–4)18–20. Third, the photoexcited and phosphorylated CRY2 proteins undergo polyubiquitination catalyzed by the E3 ubiquitin ligase Cul4COP1/SPAs and subsequently degraded by the 26S proteasome21,22. Like animal CRYs, the abundance and overall cellular activity of plant CRYs are regulated by phosphorylation, ubiquitination, and proteolysis3. However, only a cullin 4 family E3 ubiquitin ligase, Cul4COP1/SPA3, is presently known to regulate ubiquitination and degradation of plant CRYs3, raising the question how the highly conserved CRYs are differentially regulated in different evolutionary lineages.

Under natural light conditions, plants rely on the coaction of blue light receptors CRYs and the red/far-red light receptors phytochromes phys23,24 to achieve the optimal photoreponses25. Mechanistically, the CRY-phy coaction could be achieved by different photo-receptors physically coexisting with the same signaling proteins, such as bHLH transcription factors, Phytochrome Interacting Factors (PIF1–8)26, Photoregulatory Protein Kinases (PPPK1–4)20,27, the substrate receptors and co-receptors of the Cul4COP1/SPA3 E3 ligases, COP128 and SPA29. For example, CRY2 interacts with PPKs, which catalyze blue light-dependent phosphorylation of CRY2 to positively regulate the functions, polyubiquitination, and degradation of CRY220. On the other hand, PPKs interact with the phyB-PIF3 complex to negatively regulate the function of phyB by the so-called “mutually assured destruction” mechanism27,30. According to this hypothesis, the photoexcited phyB interacts with PIF3, recruiting PPKs to phosphorylate PIF3; phosphorylated PIF3 interacts with the substrate receptors of the Cul3LRBs, Light-Response Bric-a-Brack/Tramtrack/Broad (LRB 1–3), which are the substrate receptors of the Cul3LRBs E3 ligases that catalyze polyubiquitination and degradation of PIF3 that also leads to phyB degradation27.

Arabidopsis genome encodes three LRBS, LRB1, LRB2, and LRB330,31. LRB1 and LRB2 share higher homology and higher levels of mRNA expression than LRB3, and they act redundantly to suppress phyB-dependent photoresponses31. It remains unclear whether Cul3LRBs play roles in the CRY-dependent signaling process in addition to its important function regulating PIF3 ubiquitination and phyB-dependent red-light responses.

**Results**

LRBs are required for the CRY-dependent blue light responses. We have previously shown that the blue light-dependent ubiquitination and proteolysis of CRY2 is diminished but not completely abolished in the cop1 null mutant32, suggesting the existence of another E3 ubiquitin ligase in addition to Cul4COP1/SPAs. We analyzed CRY2 complexes isolated from transgenic Arabidopsis plants overexpressing GFP-CRY2, using the immunoprecipitation-mass spectrometry (IP-MS)20, and identified LRB1 and LRB2 as CRY2-associated proteins in a blue light-dependent manner (Supplementary Table 1). We analyzed flowering time of the lrb1lrb2lrb3 triple mutant. The lrb123 triple mutant showed slightly delayed flowering in long day, and it appears somewhat additive to the delay-flowering of the cry1cry2 mutant (Supplementary Fig. 1). However, given the known role of LRBS as regulators of phytochrome abundance and functions30,31, and the antagonistic as well as redundant functions of phytochromes and cryptochromes in the control of flowering time, it is technically difficult to clarify the exact roles of LRBS in the CRY-dependent control of flowering time by only the genetic analyses. We then examined whether LRBS play a role in blue light-dependent regulation of seedling development. As reported previously30, the lrb123 triple mutant showed no apparent abnormality when grown in darkness or far-red light, but it exhibited a dramatic short-hypocotyl phenotype when grown in continuous red light (Fig. 1a, b). The lrb123 triple mutant also showed short-hypocotyl phenotype when grown in either long-day or short-day photoperiods illuminated with white light (Fig. 1a, b). These results are consistent with the established function of LRBS regulating red light-dependent activities of phyb30,31. When grown in continuous blue light, the cry1cry2 mutant developed long hypocotyl phenotype as previously reported32, whereas the lrb123 showed hypocotyls modestly shorter than that of the wild-type seedings (Fig. 1c, d). The short hypocotyl phenotype of lrb123 mutant in blue light can be rescued by constitutively overexpressing the LRB2 protein in the lrb123 mutant (Supplementary Fig. 2). This defective phenotype of the lrb123 mutant seedlings (6-day-old) is more apparent in seedlings grown in blue light of the light intensities higher than 10 μmol m−2 s−1 (Fig. 1d), which is consistent with the previous report that showed hardly distinguished phenotype of the wild-type and the lrb12 double mutant (4-day-old) grown in continuous blue light with the light intensities of 10 μmol m−2 s−1 or lower31. These results argue for a light intensity-dependent function of LRBS in blue light. To examine the functional relationship between CRYs and LRBS, we prepared the cry1cry2lrb123 quintuple mutant and compared the hypocotyl phenotype of the quintuple mutant with its parents (Fig. 1e, f). When grown under continuous blue light, the cry1cry2 mutant and the lrb123 mutant exhibit long or short hypocotyl phenotype, respectively. However, the cry1cry2lrb123 quintuple mutant shows the long hypocotyl phenotype indistinguishable from that of the cry1cry2 mutant parent in blue light (Fig. 1e, f), but not in other light conditions (Fig. 1a, b). This result demonstrates that the function of LRBS in blue light is dependent on the activity of the blue-light receptor CRYs. Comparing with the lrb123 parent, the lrb123cop1 quadruple mutant shows de-etiolated phenotype of the cop1 parent in darkness but a slight additive effect of the lrb123 and cop1 mutants in blue light (Fig. 1g, h). Taken together, these results argue that LRBS regulate plant development by controlling not only the red-light-dependent activity of phytochromes but also the blue-light-dependent activity of cryptochromes.

LRBs are required for the blue light-dependent CRY2 degrada-tion. We next tested how LRBS regulate cryptochrome activity by examining the blue light-dependent degradation of CRY2 in...
the lrb123 mutants. Results shown in Fig. 2 demonstrate that LRBs are required for the blue light-induced degradation of CRY2. The CRY2 protein level decreased by at least 70% (in 30 min) to 85% (in 60 min) in etiolated wild-type seedlings transferred to blue light (30 μmol m\(^{-2}\) s\(^{-1}\)) (Fig. 2a, b). In contrast, levels of the CRY2 protein exhibited little change in one lrb123 triple mutant allele (lrb1lrb2-1lrb3) or <50% decrease in the second lrb123 triple mutant allele (lrb1lrb2-2lrb3) under the same condition (Fig. 2a, b). This result indicates that lrb1lrb2-1lrb3 is a slightly stronger allele in comparison to lrb1lrb2-2lrb3. We next compared the blue light-induced CRY2 degradation in lrb123, cop1, and lrb123cop1 quadruple mutant in seedlings grown in continuous dark or blue light. CRY2 shows similar abundance in the wild-type and both lrb123 triple mutant alleles grown in continuous dark or blue light. CRY2 shows higher abundance in the wild-type and both lrb123 triple mutant alleles grown in continuous blue light, whereas the level of CRY2 is higher than that of the wild-type in the cop1 mutant grown in continuous blue light (Fig. 2c, d). Results of this experiment indicate that COP1, but not LRBs, determines abundance of the CRY2 protein in seedlings exposed to prolonged blue light. We hypothesize that LRBs are responsible for the rapid blue light response of CRY2 proteolysis but not the prolonged photoreponse of CRY2 proteolysis, whereas COP1 is required for the prolonged photoreponse of CRY2 proteolysis. This difference may be explained, at least partially, by the respective roles of Cul3LRBs and Cul4COP1/SPA ligases in CRY2 signal transduction. COP1 is a major signaling molecule of CRY2\(^{33-35}\), in addition to its role as a E3 ligase of CRY2\(^{18}\), but LRBs are not known to play a direct role in the CRY2 signaling transduction. It is conceivable that the CRY2 suppression of COP1 activity in the early stage of light exposure, including COP1-dependent CRY2 ubiquitination, may be partially overcome by some feedback regulations during prolonged light exposure. We further tested the hypothesis that LRBs and COP1 may regulate CRY2 degradation under different light conditions, by comparing the photoreponsive CRY2 proteolysis in etiolated seedlings transferred to blue light from 30 min to 14 h (Fig. 2e, f). In etiolated seedings exposed to blue light, CRY2 degradation shows clear defect in the lrb123 triple mutants within 2 h of light exposure, but the cop1 mutant exhibits more pronounced defect in CRY2 degradation during prolonged light exposure. These results are consistent with the observations that more CRY2 accumulated in the cop1 mutant in continuous blue light, but not in the lrb123 mutant alleles (Fig. 2c, d). Importantly,
little CRY2 degradation was detected in the *lrb123cop1* quadruple mutant for both short or long exposure of blue light (Fig. 2c–f). We concluded that LRBs and COP1 are both required for the blue light-induced CRY2 degradation.

**LRBs interact with photoexcited and phosphorylated CRY2.**

Given that a substrate receptor of E3 ubiquitin ligases must physically interact with the substrate, we examined the CRY2/LRB interaction (Fig. 3), using the co-immunoprecipitation (co-IP) assays of proteins co-expressed in the heterologous HEK293T (Human Embryo Kidney 293T) cells as we previously reported. Because we have previously shown that the blue light-induced phosphorylation of CRY2 is required for CRY2 ubiquitination and degradation, and that PPK1 is one of the four related protein kinases that specifically phosphorylate photoexcited CRY2, we tested CRY2/LRB interaction in the presence of the wild-type PPK1 in response to blue light in HEK293T cells. Results of this experiment show that LRB1 and LRB2 preferentially interact with phosphorylated CRY2 in HEK293T cells co-expressing PPK1, but only when cells were exposed to blue light (Fig. 3a, b). LRB2 failed to interact with the photo-insensitive CRY2*D387A* mutant that does not bind the FAD (Flavin Adenine Dinucleotide) chromophore, which is consistent with the light dependence of CRY2/LRB interaction. LRB2 failed to interact with CRY2 in light-treated HEK293T cells co-expressing the catalytically inactive PPK1*D267N* (Fig. 3d), which is consistent with the phosphorylation dependence of CRY2/LRB interaction. These results support a hypothesis that LRBs directly and specifically interact with photoexcited and phosphorylated CRY2.

We next examined the CRY2/LRB interaction in plant cells, using the BiFC (Bimolecular Fluorescence Complementation) assay in Arabidopsis leaves and split-luciferase assay in tobacco (*Nicotiana benthamiana*) leaves (Fig. 4b, c). Results of the BiFC assay show strong BiFC signals between CRY2 and LRB1 or CRY2*D387A* and LRB2 (Fig. 4a). In contrast, no BiFC signal was detected between the photo-insensitive CRY2*D387A* and LRB1 or CRY2*D387A* and LRB2 (Fig. 4a). These results clearly demonstrate...
the direct interaction of CRY2 and LRB proteins in vivo. Consistently, the split-luciferase assays suggest the direct interaction between CRY2 and LRBs that contains deletion of the BTB domain (Fig. 4b, c). Given the common affinity of BTB to Cullin 3 (Cul3)31,38, this experiment argues that the CRY2/LRB interaction is independent from BTB domain of LRBs or LRB/Cullin interaction. CRY2/LRB complexes are also detected by the co-IP (co-immunoprecipitation) assays in transgenic Arabidopsis seedlings stably co-expressing Flag-GFP-tagged CRY2 (FGFP-CRY2) and Myc-LRB1 or FGFP-CRY2 and Myc-LRB2 (Fig. 4d, e). The co-IP assays detected increasing amount of the CRY2/LRB complexes in etiolated seedlings exposed to blue light or in seedlings grown in LD and continuous white light (Fig. 4d, e). These results support a hypothesis that LRBs complex with CRY2 in a light-dependent manner and that LRBs act as the substrate receptors of Cul3 LRBs for the blue light-dependent CRY2 ubiquitination. It is noticed that the extent of photo-promotion of the accumulation of CRY2/LRB complexes in Arabidopsis seedlings is not as strong as that detected in light-treated heterologous HEK293T cells (Fig. 4d, e). These results are consistent with the notions that CRY2 oligomerization is the primary photoreaction of the CRY2 photoreceptor and that LRBs interact with CRY2 to facilitate CRY2 degradation in vivo, but not in heterologous HEK293T cells.

LRBs and COP1 are required for the photoresponsive polyubiquitination of CRY2. To test whether the Cul3LRBs and Cul4COP1/SPAs E3 ubiquitin ligases catalyze blue light-dependent CRY2 ubiquitination, we performed three independent experiments. In the first two experiments, we prepared transgenic plants overexpressing FGFP-CRY2 in the wild-type (WT), the strong (lrb1lrb2-1lrb3) or weak (lrb1lrb2-2lrb3) alleles of the lrb123 triple mutant (Fig. 5a, b, d), and the cop1 mutant backgrounds (Fig. 5c, d). In the third experiment, we prepared double-tagged transgenic lines that overexpress FGFP-CRY2 with either the Myc-tagged LRB (Myc-LRB1, Myc-LRB2) or the Myc-tagged COP1 (Myc-COP1) (Fig. 5e). In the third experiment, we prepared double-tagged transgenic lines that overexpress FGFP-CRY2 with either the Myc-tagged LRB (Myc-LRB1, Myc-LRB2) or the Myc-tagged COP1 (Myc-COP1) (Fig. 5e).

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blue light-treated wild-type background seedlings (Fig. 5a–c). However, little ubiquitinated CRY2 was detected in etiolated seedlings or in blue light-treated lrb123 or cop1 mutant background seedlings (Fig. 5a–c). In the other two experiments, FGFP-CRY2 were immunoprecipitated by the GFP-trap beads, and the level of polyubiquitination of FGFP-CRY2 was analyzed by immunoblots probed against anti-ubiquitin antibody (Fig. 5d, e). In etiolated seedlings exposed to blue light, a high level of ubiquitinated FGFP-CRY2 was detected in the wild-type seedlings overexpressing FGFP-CRY2. In contrast, a lower level of ubiquitinated FGFP-CRY2 was detected in lrb1lrb2-2lrb3 weak allele, whereas almost no ubiquitinated FGFP-CRY2 was detected in the lrb1lrb2-1lrb3 strong allele or the cop1 mutant seedlings transgenically overexpressing FGFP-CRY2 (Fig. 5d). In comparison to FGFP-CRY2 overexpressed in the wild-type background, the blue light-induced polyubiquitination of FGFP-CRY2 is enhanced in
Fig. 4 LRBs interact with CRY2 in vivo. a Confocal microscopic images showing BiFC signals of indicated protein pairs transiently expressed in Arabidopsis leaves. The Hoechst 33342 (which is used to stain the nuclei) and GFP (BiFC) signals are shown. The relative intensity of CRY2/LRB interaction was presented as BiFC ratio, calculated as BiFC ratio = [GFP intensity]nuclei / [Hoechst 33342 intensity]nuclei. BiFC ratio (mean ± SD), total number of quanitified images and nuclei are shown blow the images. CRY2P532L, photo-insensitive CRY2, is used as the negative control. Scale bars, 10 μm. b Split-luciferase complementation assays showing the interactions of LRB1 and CRY2 or LRB2 and CRY2 in tobacco. Indicated split-LUC protein pairs were transiently co-expressed in N. benthamiana with FGFP-PPK1. LRB1ΔBTB, LRB1 with deletion of BTB domain (143-212 aa); LRB2ΔBTB, LRB2 with deletion of BTB domain (145-250 aa). c Quantification of split-luciferase complementation assays shown in (b). Data was shown as mean ± SD (n = 3 individual experiments). d–e Co-immunoprecipitation assays showing the interactions of LRB1 and CRY2 (d) or LRB2 and CRY2 (e) in Arabidopsis. 7-day-old seedlings expressing Myc-LRB or co-expressing FGFP-CRY2 and Myc-LRB were grown in the dark and then treated with blue light (100 μmol m⁻² s⁻¹) for the indicated time, or grown in long days (LD, 16 h light/8 h dark) and continuous white light (cWL). Plant extracts were immunoprecipitated by anti-Flag and anti-Myc antibodies, respectively. Co-IP experiments of 7-day-old seedlings co-expressing FGFP-CRY2 and Myc-CRY2 were performed in parallel as positive controls. The relative interaction intensity of CRY2/LRB or CRY2/CRY2 is calculated by [Co-IP intensity] / [IP intensity] and shown blow the immunoblots. The above experiments were repeated twice with similar results.

COP1 and LRBs interact with different structural elements of CRY2. We wish to understand different mechanisms governing the activities of COP1 and LRBs with respect to CRY2 ubiquitination. Most presently known light-dependent CRY2-interacting proteins interact with the photon-sensing PHR domain of CRY2. However, COP1 is one of the few exceptions. COP1 binds to the CCE domain of CRY2 regardless of light in vitro or in yeast cells, although COP1 binds to CRY2 in the light-dependent manner in vivo. Like COP1, LRB2 also interacts with the CCE domain of CRY2 in heterologous HEK293T cells (Fig. 3e, f). However, in contrast to COP1 that interacts with CRY2s regardless of light in vitro or in yeast cells, LRBs preferentially interact with phosphorylated CRY2 in the blue light-dependent manner in HEK293T cells (Fig. 3a–d). Given that analyses of molecular interactions of plant proteins in heterologous yeast cells, HEK293T cells, or in vitro are essentially equivalent, we reasoned that CRY2 may interact with COP1 or LRBs via different structural elements and/or respond to the photoresponsive CRY2 conformation changes differently.

We investigated this question by analyses of the CRY2P532L mutation that is impaired at the VP motif (Fig. 6a). The VP motif is a conserved COP1-binding motif of many substrates of the Cul4COP1/SPAs E3 ligase, including CRY2. We previously reported that the CRY2P532L mutation impaired at the VP motif (Fig. 6a) loses all the physiological activities tested but retains many photochemical properties of CRY2, including blue light-induced oligomerization, phosphorylation, ubiquitination, and degradation. We first compared the relative activity of FGFP-CRY2 and FGFP-CRY2P532L, using the hypocotyl inhibition assay in transgenic plants expressing the respective recombinant protein at similar levels in the crylcriy2 mutant background (Supplementary Fig. 3a). Transgenic seedlings expressing FGFP-CRY2P532L showed no obvious activity mediating light inhibition of hypocotyl elongation under all light intensities of blue light tested (Fig. 6b, Supplementary Fig. 3b). This result confirmed our previous report that CRY2P532L is a loss-of-function mutation. We then compared the kinetics of blue light-induced degradation of FGFP-CRY2 and FGFP-CRY2P532L, using the same transgenic lines (Fig. 6c, d). In transgenic seedlings, the mutant recombinant protein FGFP-CRY2P532L is degraded in response to blue light as we previously reported, although it appears to degrade slower than its wild-type counterpart (Fig. 6c, d). The apparent half-life of FGFP-CRY2P532L is more than twice that of FGFP-CRY2 (Fig. 6d). When co-expressed with PPK1 in HEK293T cells, both the wild-type CRY2 and the CRY2P532L mutant protein exhibits clear blue light-dependent phosphorylation (Fig. 6e, f). In HEK293T cells co-expressing SPA1, COP1 interacts with CRY2 in the light-dependent manner with both phosphorylated and unphosphorylated CRY2, whereas neither the photo-insensitive CRY2D387A mutant nor the COP1-insensitive CRY2P532L mutant interact with COP1 or SPA1 (Fig. 6e). LRB2 preferentially interacts with photoexcited and phosphorylated CRY2, as well as the photoexcited and phosphorylated CRY2P532L mutant to the similar extent (Fig. 6f). These results suggest that CRY2P532L remains photoresponsive in vivo and that CRY2P532L undergoes blue light-dependent ubiquitination and degradation in vivo (Fig. 6c, d). Since the CRY2P532L mutant does not interact with COP1, these results confirm that COP1 and LRB2 interact with different structural elements of CRY2. Taken together, results of our analyses of the CRY2P532L mutant further supports the hypothesis that Cul3LRBs is the second type of E3 ligase required for blue light-dependent CRY2 ubiquitination and degradation.

The notion that Cul3LRBs and Cul4COP1/SPAs regulate CRY2 predominantly in the early or prolonged state of light exposure, respectively, seems inconsistent with the kinetics of CRY2P532L degradation in vivo (Fig. 6c, d). Because the CRY2P532L interacts with LRB2 but not COP1 (Fig. 6e, f), its degradation is expected to mimic that of the wild-type CRY2 in the cop1 mutant (Fig. 2). Instead, the kinetics of CRY2P532L degradation is more like that of the wild-type CRY2 in the lrb123 triple mutant (Fig. 2). This seemingly perplexing phenomenon may be explained by the unusual photochemical characteristics of the CRY2P532L mutant. Photoexcited CRY2P532L monomers exhibit increased affinity among themselves in comparison to the wild-type CRY2 (Fig. 6g, h) without obvious change of the rate or affinity in the dark-reversion reaction (Fig. 6i, j). Because photooigomerization is necessary for all light-dependent reactions of CRY2, including photoreponsive ubiquitination and degradation, it is conceivable that the unusual photochemical characteristics of the CRY2P532L mutant may change its relationship with various regulators in vivo, including LRBs or 26S proteosomes, to alter its degradation kinetics in the unexpected manner. Taken together, the results of our experiments argue that two types of substrate receptors, COP1 and LRBs, interact with CRY2 via distinct structural elements of CRY2 to enable the blue light-induced CRY2 ubiquitination and degradation by the respective E3 ubiquitin ligases, Cul3LRBs and Cul4COP1/SPAs.
Fig. 5 LRBs and COP1 are both required for CRY2 ubiquitination. Immunoblots showing the ubiquitination of FGFP-CRY2 in indicated genotypes. 7-day-old etiolated seedlings constitutively expressing FGFP-CRY2 in indicated genotypes were pretreated with MG132 and kept in the dark or exposed to 30 μmol m\(^{-2}\) s\(^{-1}\) of blue light for 5, 10, and 15 min. The blue light treated samples (5, 10, and 15 min) were pooled together while performing immunoprecipitation for (a–e). a–c Total ubiquitinated proteins were purified by TUBE2-conjugated beads. Immunoprecipitated proteins were analyzed by immunoblots probed with anti-ubiquitin antibody (α-Ubq) or anti-CRY2 antibody (α-CRY2). The extent of CRY2 ubiquitination was shown below the immunoblots, calculated as [CRY2-Ubq intensity]\(^{\text{IP}}\)/[Ubq intensity]\(^{\text{IP}}\). d–e FGFP-CRY2 proteins were purified with GFP-trap beads. Immunoprecipitated proteins were analyzed by immunoblots probed with anti-ubiquitin antibody (α-Ubq), anti-Flag antibody (α-Flag) or anti-Myc antibody (α-Myc) for detecting epitope-tagged proteins. The extent of CRY2 ubiquitination is calculated as [CRY2-Ubq intensity]\(^{\text{IP}}\)/[Flag intensity]\(^{\text{IP}}\) with the short exposure immunoblots and shown below the immunoblots. S. exp or L. exp: short or long chemiluminescence exposures of immunoblots. D, dark treatment; B, blue light treatment. The above experiments were repeated at least twice with similar results.
Discussion

In this study, we demonstrate that, in addition to Cul4COP1/SPAs, the E3 ubiquitin ligases Cul3LRBs is required for blue light regulation and function of CRY2 (Fig. 7a). Cul3LRBs appears capable of targeting photoexcited and phosphorylated CRY2 for a rapid ubiquitination and degradation, whereas Cul4COP1/SPAs is responsible for the sustained ubiquitination and degradation of CRY2 in plants under prolonged light exposure. The differential regulation of CRY2 turnover in response to different light conditions appears necessary for the optimal photoresponses of plants grown in nature. LRBs and COP1 interact with different structural elements of CRY2, which explain, at least partially, the different kinetic features of the
LRB- and COP1-dependent CRY2 degradation in response to blue light. Photoresponsive expression of LRBs may also play a role in the light regulation of CRY2 turnover. Blue light positively regulates LRB1 and LRB2 protein expression (Fig. 7b–e). The abundance of recombinant LRB proteins in transgenic plants expressing LRB1 or LRB2 driven by either the respective native promoters or the ACT2 constitutive promoter increase in response to blue light. Because Cul4COP1/SPA-dependent ubiquitination and 26S proteasome pathway is best known for degradation of photo-signaling proteins in darkness, it is tempting to speculate that Cul4COP1/SPA may play a role in the regulation of the LRB3-mediated light inhibition of Cul4COP1/SPA may lead to increased LRB protein accumulation, accelerated turnover of not only photoexcited CRY2 but also photoexcited phyB, and sustained overall photosensitivity of plants grown in nature (Fig. 7a). However, this hypothesis remains to be tested directly.

LRBs and COP1 apparently interact with CRY2 differently: LRBs interacts with photoexcited and phosphorylated CRY2, whereas COP1 interacts with CRY2 regardless of its phosphorylation states. This result would be consistent with CRY2 interacting with COP1 or LRBs via different structural elements. Photoexcitation of CRYs may result in different conformational changes and interactions with different proteins. First, photoexcited CRYs may undergo initial conformational change associated with CRY oligomerization, which have been directly shown by several recent structural studies of the PHR domain of CRY2. Second, light-induced phosphorylation of CRYs may result in electrostatic repelling of the PHR and CCE domains of CRYs and open conformation of CRYs, which has been deduced from the conventional structure-function studies. However, technical difficulties, including the intrinsically disordered nature of the CCE domain of CRY2, has prevented a direct test of conformational changes of the CRY2 holoprotein. Because COP1 interacts with CRY2 regardless of CRY2 phosphorylation, whereas LRBs primarily interacts with phosphorylated CRY2, we propose that the first type of conformational change is primarily responsible for the photoresponsive interaction of COP1 and CRY2, whereas the second type of conformational change of CRY2 is primarily responsible for the photoresponsive interaction of LRBs and CRY2. These hypotheses, however, remain to be further tested.

It is interesting that plants evolved with two distinct E3 ubiquitin ligases to regulate a CRY photoreceptor by different modes of interaction under different light conditions. In mammals, two related Cul1-based E3 ligases, SCFβTr1 and SCFγTr2, act to promote ubiquitination and degradation of CRYs in the nucleus and cytoplasm, respectively, to control the period of the circadian clock. In Drosophila, Jetlag of a Cul1-based E3 ligase and Brwd3 of a Cul4-based E3 ligase regulate ubiquitination and degradation of dCRY-dependent circadian rhythm. Our finding that Cul3LRB or Cul4COP1/SPA catalyzes CRY2 ubiquitination in different light conditions, to promote rapid or prolonged degradation of dCRY, respectively, highlights the importance of photoreceptor turnover in the regulation of photosensitivity of plants. Altogether, these results appear to argue for the similar selection pressure to maintain multiple mechanisms regulating the abundance of CRYs is evolutionarily conserved for not only circadian rhythms in animals but also photomorphogenesis in plants. Our finding that Cul3LRB or Cul4COP1/SPA catalyzes CRY2 ubiquitination in different light conditions, to promote rapid or prolonged degradation of dCRY, respectively, highlights the importance of photoreceptor turnover in the regulation of photosensitivity of plants. Altogether, these results appear to argue for the similar selection pressure to maintain multiple mechanisms regulating the abundance of CRYs during evolution of both animal and plant lineages. The complex mechanism regulating CRY2 turnover is likely evolved to optimize photomorphogenesis under natural light conditions. In this regard, it is particularly interesting that Cul3LRB has been previously shown to be the E3 ligase of PIF3 and crucial for the function of phyB and phytochrome-dependent photoresponses. Our finding that Cul3LRB also catalyzes blue light-dependent ubiquitination of CRY2 to regulate blue light-dependent photomorphogenesis argues for a previously unrecognized mechanism toward a better understanding of how plants respond to light in the nature white light conditions.

Methods
Plasmid construction. All plasmids used in this study were generated by In-Fusion Cloning methods (https://www.takarabio.com/products/cloning/in-fusion-cloning). The sequences subcloned into plasmids were verified by Sanger sequencing. The mammalian cell expression vectors used in this study were made with pQCMV-Flag-GFP (Supplementary Fig. 4). pQCMV-GFP (Supplementary Fig. 4), and pCMV-Myc90, pQCMV-Flag-GFP vector was modified from the commercial plasmid pEGFP-N1 (Clontech), which allows the inserted gene expressed under
Fig. 7 Blue light positively regulates LRBs protein abundance in plants. a A hypothetic model depicting the degradation of CRY2 by LRBs and COP1 E3 ubiquitin ligases. CRY2 exists as inactive monomers in darkness (grey). Upon blue light exposure, CRY2 undergoes photoexcitation, oligomerization, and phosphorylation to become photoactive. Photoactivated CRY2 then were targeted by two distinct E3 ubiquitin ligases, LRBs, and COP1, for degradation. Minus sign in a circle, indicates negative charge; dashed line, indicates a hypothetical mechanism. b–e Representative immunoblots (top) and a quantitative analysis (bottom) showing the expression of recombinant LRB proteins in transgenic plants driven by native promoters (b, d) or the ACT2 constitutive promoter (c, e). Seedlings were grown in darkness or continuous blue light (50 μmol m⁻² s⁻¹) for 5, 6, or 7 days. Proteins were extracted and subjected to western blot analysis. LRBs and HSP90 were detected with anti-Flag and anti-HSP90 antibodies, respectively. HSP90 is used as a loading control. Data were shown as mean ± SD (n = 3 individual immunoblots). The above experiments were repeated at least three times with similar results.
control of the CMV promoter and fused with dual in-frame tags of Flag and GFP. pQCMV-GFP vector was further modified from pQCMV-Flag-GFP by removing the coding sequences of cDNA or plasmids made before by PCR, the purified PCR products were then subcloned into SpeI/KpnI-digested pQCMV-Flag-GFP vector through a fusion between a 2x Flag and GFP tags. The pQCMV-Myc-LRB2 plasmids were prepared by cloning the CDS of the genes into the BamHI site of pCMV-Myc-vector. For plasmids expressing HA-PPK1 and HA-PPK1D267N, the coding sequences of PPK1 and PPK1D267N were PCR-amplified from plasmids made before by PCR using primers, of which a 2x HA coding sequence was attached to the N’ end of forward primer, and introduced into SpeI/KpnI-digested pQCMV-GFP vector by infusion. The gene-specific primers for constructs used for expressing recombinant proteins in HEK293T cells are listed in Supplementary Table 2.

The BiFC constructs were built with the vectors described previously32,33. The coding sequences of LRBI (AT2G46620), LRBJ (AT3G16000), and LRBJD34,35 were PCR-amplified and cloned into pDONR/Zeo entry vector through BP reaction (Gateway™ Clonase II Enzyme mix, cat. # 11790920, Invitrogen). The entry constructs were then introduced into the destination vector pX-nYF (N-terminus of YFP fused to C-terminus of genes) or pCF-P (N-terminus of GFP fused to N-terminus of genes) by LR reaction (Gateway™ LR Clonase II Enzyme mix, cat. # 11791020, Invitrogen). The gene-specific primers of Gateway cloning for constructs used for BiFC assays are listed in Supplementary Table 2.

The split-LUC constructs were made with the vectors described previously32. CR2 were fused to the C-terminus of cLUC (the C-terminus of Luciferase). LRBIΔTβδ were generated by inserting two fragments, 1-432 bp and 751-1683 bp of LRBI coding sequence through the same method as making LRBIΔTβδ, LRBIΔTβδ and LRBIΔTβδ were then fused to the N-terminus of nLUC (the N-terminus of Luciferase). The primers for constructs used for Split-Luc assays are listed in Supplementary Table 2.

For immunoblot signals detected by enhanced conventional chemiluminescent (ECL) method, membranes were blocked with 5% non-fat milk in PBST for 1 h, blotted with anti-CRY2 primary antibody (1:3000, homemade, produced in rabbit)36 in PBST for 1.5 h, washed 8 min x 3 times with PBST, blotted with anti-rabbit-HRP (1:10000, cat # A300086, ThermoFisher) secondary antibody in PBST for 1.5 h, washed 5 min x 3 times with PBST and then incubated with ECL solution for X-ray film development. The membranes were then stripped with stripping buffer [0.2 M glycine, pH 2.5] and re-probed with anti-HSP98 (1:10000, cat # A8510099-31-P1, Beijing Protein Innovation) primary antibody and anti-Mouse-AP (1:50000, cat # A1340, ThermoScientific) secondary antibody. The antibody from Orizya sativa can also recognize Arabidopsis HSP90 protein34. Three independent blots were performed in parallel for quantification analysis. The quantification of protein intensity of ECL immunoblots was performed by ImageJ.

For immunoblot signals detected by Odyssey CLX Infrared Imaging System (Li-COR), membranes were blocked with 0.5% casein in PBS for 1 h, blotted with anti-CRY2 (1:3000) and anti-HSP98 (1:10000) mixed primary antibodies in PBST for 0.5% casein for 1.5 h, washed 8 min x 3 times with PBST, blotted with Donkey anti-rabbit 790 (1:15000, cat # A11374, ThermoFisher) and Donkey anti-mouse 680 (1:15000, cat # A10338, Thermofisher) secondary antibodies in PBST for 1.5 h, washed 8 min x 3 times with PBST and then incubated with Odyssey ECL solution for X-ray film development. The membranes were then stripped with stripping buffer [0.2 M M glycine, pH 2.5] and re-probed with anti-HSP98 (1:10000, cat # A8510099-31-P1, Beijing Protein Innovation) primary antibody and anti-Mouse-AP (1:50000, cat # A1340, ThermoScientific) secondary antibody. The antibody from Orizya sativa can also recognize Arabidopsis HSP90 protein34. Three independent blots were performed in parallel for quantification analysis. Quantification of signals were processed with Image Studio Lite software. CRY2 degradation curves were indicated by CRY2 (B/D) ratio, calculated as CRY2 (B/D) = [CRY2/HSP90]blow / [CRY2/HSP90]blew, and analyzed with one phase decay of nonlinear regression.

Plant materials and growth conditions. All mutants used in this study are in the Arabidopsis thaliana Columbia ecotype background. The cry1cry2 double mutant was described as previously36. The lrb1lrb2lrb3lrb4lrb5 cry2-1 double mutant alleles of COP1 as previously described31. The cry1cry2lrb4lrb5lrb6lrb7 cry1cry2 double mutants were gifts from Dr. Peter Quail and as described previously30,31. The cry1cry2cry2rdr6 cry1cry2cry2 mutants were gifts from Dr. Peter Quail and as described previously30,31. LRB2·ΔΔ was generated with two fragments, 1-432 bp and 751-1683 bp of LRB2 coding sequence with 15 bp overlaps, by infusion cloning method. The genotypes of all mutants were verified by PCR using primers listed in Supplementary Table 2.

For routine maintenance, etiolated seedlings grown on MS medium containing 3% sucrose were treated with 16 h light / 8 h dark condition (22°C). For hypocotyl phenotype analysis, seedlings were grown on MS plates with 3% sucrose at 20–22°C for 6 days under different light conditions. Light-emitting diode (LED) was used to obtain monochromatic light (blue light, peak at 465 nm, half-bandwidth of 25 nm; red light, peak 660 nm, half-bandwidth of 20 nm; far-red light, peak 735 nm, half-bandwidth of 21 nm). For hypocotyl phenotype analysis, seedlings were grown on MS plates with 3% sucrose for 7 days, then subjected to 100 μmol m−2 s−1 blue light for the indicated time. For immunoprecipitation of polyubiquitylated proteins, 7-day-old etiolated seedlings grown on MS medium containing 3% sucrose were treated with 50 μM MG132 (Cat # S62619, Selleck) in liquid MS in the dark overnight with genistein, and then moved to 30 μmol m−2 s−1 blue light for 5, 10, and 15 min before harvest.

Blue-light-induced CRY2 degradation assays. For endogenous CRY2 degradation analysis, seedlings were grown in the dark for 7 days and then treated with 30 μmol m−2 s−1 of blue light for indicated time. For pFGFP-CRY2 and pFGFP-CRY2P532L degradation analysis, seedlings were treated with 100 μmol m−2 s−1 of blue light. All proteins were homogenized by Tissuelyser (Qiagen, Germany) and total proteins were extracted in equal tissue volume of protein extraction buffer [120 mM Tris pH 6.8, 100 mM EDTA, 4% w/v SDS, 10% v/v beta-mercaptoethanol, 5% v/v Glycerol, 0.05% v/v Bromophenol blue53, heated at 100°C for 8 min, centrifuged at 16000 rcf for 10 min, and analyzed by western blot. Proteins were separated in 10% SDS-PAGE and transferred to nitrocellulose membrane.

For immunoblot signals detected by enhanced conventional chemiluminescent (ECL) method, membranes were blocked with 5% non-fat milk in PBST for 1 h, blotted with anti-CRY2 primary antibody (1:3000, homemade, produced in rabbit53) in PBST for 1.5 h, washed 8 min x 3 times with PBST, blotted with anti-rabbit-HRP (1:10000, cat # A300086, ThermoFisher) secondary antibody in PBST for 1.5 h, washed 5 min x 3 times with PBST and then incubated with ECL solution for X-ray film development. The membranes were then stripped with stripping buffer [0.2 M M glycine, pH 2.5] and re-probed with anti-HSP98 (1:10000, cat # A8510099-31-P1, Beijing Protein Innovation) primary antibody and anti-Mouse-AP (1:50000, cat # A1340, ThermoScientific) secondary antibody. The antibody from Orizya sativa can also recognize Arabidopsis HSP90 protein34. Three independent blots were performed in parallel for quantification analysis. The quantification of protein intensity of ECL immunoblots was performed by ImageJ.

Protein expression and co-immunoprecipitation in HEK293T cells. Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) FBS, 100 μg/mL streptomycin and 100 μg Penicillin in, humidified 5% (v/v) CO2 air at 37°C. Cells were seeded at a density of approximately 8 × 103 cells/6-cm plate and the transfection were carried out with a calcium phosphate precipitation protocol. Briefly, different combination of plasmid DNA (2~5 μg) and pCambia330120, which allows the interested genes to be expressed under Actin2 promoter and fuse with 2x Flag and GFP tags, were transfected into HEK293T cells using lipofectamine 2000 (Invitrogen), centrifuged at 16000 rcf for 10 min, and analyzed by Odyssey CLX by Image Studio Lite software. Three independent blots were performed in parallel for quantification analysis. Quantification of signals were processed with Image Studio Lite software. CRY2 degradation curves were indicated by CRY2 (B/D) ratio, calculated as CRY2 (B/D) = [CRY2/HSP90]blow / [CRY2/HSP90]blew, and analyzed with one phase decay of nonlinear regression.

Expression level of FGFP-CRY2 and FGFP-CRY2P532L were used for analysis. For br123 mutant blue-light hypersensitivity phenotype rescue experiments, FGFP-LRB1 (Supplementary Fig. 5) and FGFP-LRB2 were induced in 25 μM Glufosinate-ammonium (cat # CP6420, Bomei Biotechnology) and analyzed with one phase decay of nonlinear regression.
times. Proteins were competed from the beads with 35 μl 3XFlag peptide solution [500 ng/μl in 1% Brij buffer] for 30 min at room temperature with mixing. Elution was transferred to a new tube, mixed with 3×SDS buffer, denatured at 100 °C for 4 min and subjected to western blot analysis. Western blots were performed as described above. Primary antibodies used here were anti-Flag (1:1500, cat # F1804, Sigma), anti-Myc (1:5000, cat # 05-724, Millipore), and anti-HA (1:1000, cat # 12205, Roche), and secondary antibodies were used anti-Mouse-HRP (1:10000, cat # 31430, ThermoFisher) and anti-Rabbit-HRP (1:10000, cat # 31460, ThermoFisher).

Bimolecular fluorescence complementation (BiFC) assay. BiFC assays in Arabidopsis plants were performed as previously described methods with minor modifications37. Briefly, Agrobacteria AGL0 transformed with BiFC plasmids were grown in LB medium with 40 μM Acetosyringone and 1% Glucose in 28 °C shaker overnight. Agrobacteria were centrifuged at 5000 rcf for 10 min, washed once with washing buffer [10 mM MgCl2, 100 μM Acetosyringone] and resuspended in infiltration buffer [1/4 MS pH 6.0, 1% sucrose, 100 μM Acetosyringone, and 0.01% Silwet L-77] to 0.5 of OD600. The agro cultura were infiltrated into 3–4 weeks old Arabidopsis leaves with syringe. Each BiFC assay was performed with at least 3 independent leaves infiltrated for each. Plant leaves were dried before and after signal detecting with Tanon-5200. The quantification of fluorescence intensity was performed by ImageJ.

Immuno precipitation of ubiquitinated proteins in Arabidopsis. 7-day-old etiolated seedlings were treated with 50 μM MG132 in liquid MS in the dark overnight with gentle shaking, and then treated with 30 μmol μl−1 light blue for 5, 10, and 15 min before harvest. For each immuno precipitation assay, about 3 g of seedlings were used. Seedlings were ground in liquid nitrogen and homogenized in 1.5x cold IP buffer (without inhibitors). Total ubiquitinated proteins were eluted with 2XSDS sample buffer, denatured at 100 °C for 4 min and subjected to western blot analysis. Anti-ubiquitin antibody (a Ubq, cat # 14-6078-80, ThermoFisher) was used to detect total ubiquitinated proteins, and homemade anti-CRY2 antibody was used to detect CRY2 protein.

For GFPG-CRY2 protein purification, the cleared plant lysate were incubated with 50 μl GFPG-agarose beads (homemade or cat # gta-20, Chromotek) and rotated at 4 °C for 2 h. Beads were pelleted and washed four times with ice-cold IP buffer (without inhibitors). Total ubiquitinated proteins were eluted with 2XSDS sample buffer, denatured at 100 °C for 4 min and subjected to western blot analysis. Anti-Flag antibody was used to detect GFPG-CRY2 (IP) fusion protein, and anti-ubiquitin antibody was used to detect ubiquitinated CRY2.

Statistics and reproducibility. For Fig. 1b, n = 16 for WT (dark), n = 15 for WT (red), n = 17 for WT (FR), n = 14 for WT (LD), n = 17 for WT (SD), n = 22 for cry1cry2 (FR), n = 17 for cry1cry2 (LD), n = 20 for cry1cry2/2lrb2-2lrb3 (red), n = 25 for cry1cry2/2lrb2-2lrb3 (SD), n = 17 for cry1cry2/2lrb2-2lrb3 (dark), n = 18 for cry1cry2/2lrb2-2lrb3 (FR), n = 17 for cry1cry2/2lrb2-2lrb3 (LD), n = 17 for cry1cry2 (FR), n = 20 for cry1cry2 (SD), n = 20 for cry1cry2 (dark), n = 20 for cry1cry2/2lrb2-2lrb3 (dark), n = 20 for cry1cry2/2lrb2-2lrb3 (FR), n = 20 for cry1cry2/2lrb2-2lrb3 (LD)

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Author contributions

Y.C. and X.H. conducted most of the experiments. S.L. and T.S. performed some biochemical experiments in HEK293T. H.H. and H.R. prepared some genetic materials. S.L. and Z.G. helped with biFC experiments. X.W. and D.L. provided critical feedback. J.W. provided mass-spectrometry supports. C.L. and Q.W. conceived the project, designed the experiments, and wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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