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Research

Arabidopsis cryptochrome 1 undergoes COP1 and LRBs-dependent degradation in response to high blue light

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Summary

- *Arabidopsis* cryptochrome 1 (CRY1) is an important blue light photoreceptor that promotes photomorphogenesis under blue light. The blue light photoreceptors CRY2 and phototropin 1, and the red/far-red light photoreceptors phytochromes B and A undergo degradation in response to blue and red light, respectively. This study investigated whether and how CRY1 might undergo degradation in response to high-intensity blue light (HBL).
- We demonstrated that CRY1 is ubiquitinated and degraded through the 26S proteasome pathway in response to HBL. We found that the E3 ubiquitin ligase constitutive photomorphogenic 1 (COP1) is involved in mediating HBL-induced ubiquitination and degradation of CRY1. We also found that the E3 ubiquitin ligases LRBs physically interact with CRY1 and are also involved in mediating CRY1 ubiquitination and degradation in response to HBL.
- We further demonstrated that blue-light inhibitor of cryptochromes 1 interacts with CRY1 in a blue-light-dependent manner to inhibit CRY1 dimerization/oligomerization, leading to the repression of HBL-induced degradation of CRY1.
- Our findings indicate that the regulation of CRY1 stability in HBL is coordinated by COP1 and LRBs, which provides a mechanism by which CRY1 attenuates its own signaling and optimizes photomorphogenesis under HBL.

Introduction

Light, as one of the most important environmental signals, profoundly regulates plant growth and development during the whole life span from seed germination to flowering (Fankhauser & Chory, 1997; Deng & Quail, 1999; Kami *et al.*, 2010; Yadav *et al.*, 2020). Plants sense both quantitative and qualitative dynamic light changes, and they fine-tune their status of growth and development accordingly via multiple photoreceptors. These include the red/far-red light receptors phytochromes (PHYs) phyA to phyE, the blue light receptors cryptochromes (CRYs) CRY1 and CRY2 and phototropins (PHOT1 and PHOT2), and the ultraviolet (UV)-B light receptor UVR8 (Ahmad & Cashmore, 1993; Briggs & Christie, 2002; Quail, 2002; Rizzini *et al.*, 2011; Liu *et al.*, 2020). Among them, CRYs are shown to regulate a broad spectrum of physiological processes, including photomorphogenesis, flowering, circadian rhythm, and stomatal opening and development (Ahmad & Cashmore, 1993; Guo *et al.*, 1998; Somers *et al.*, 1998; Toth *et al.*, 2001; Mao *et al.*, 2005; Liu *et al.*, 2008; Kang *et al.*, 2009). CRYs are also present in other organisms, from bacteria to humans. CRYs regulate the circadian rhythm through serving as photoreceptors in *Drosophila melanogaster* or integral components of the circadian clock in mammals, and also provide navigation during long-distance migration of migratory butterflies and birds through sensing the Earth's magnetic field (Emery *et al.*, 1998; Kume *et al.*, 1999; Gegear *et al.*, 2010).

The *Arabidopsis* genome encodes two homologous CRYs: CRY1 and CRY2. CRY1 promotes photomorphogenesis in low, medium, and high intensities of blue light (Ahmad & Cashmore, 1993; Lin *et al.*, 1996), whereas CRY2 mainly enhances photomorphogenesis under low-intensity blue light (Lin *et al.*, 1998). Moreover, CRY2 acts as the primary blue light photoreceptor to promote floral initiation under a long-day photoperiod (Guo *et al.*, 1998). CRY1 and CRY2 comprise a photolyase-related N-terminal domain (also known as CNT1 and CNT2) (Sancar, 1994, 2003; Yang *et al.*, 2000). However, CRYs lack photolyase activity and are characterized by distinguishing C-terminal domains (CCT1 and CCT2, also known as CCE, CRY C-terminal Extension) (Lin *et al.*, 1995; Yang *et al.*, 2000; Yu *et al.*, 2010). Both CRY1 and CRY2 interact with constitutive photomorphogenic 1 (COP1) through their C-terminal domain (Wang *et al.*, 2001; Yang *et al.*, 2001), a RING-finger E3 ubiquitin ligase that interacts with and targets the degradation of a set

of transcription factors, such as HY5 and CONSTANS (CO), to regulate photomorphogenesis and flowering (Deng *et al.*, 1992; Osterlund *et al.*, 2000; Jang *et al.*, 2008; Liu *et al.*, 2008). CRY1 and CRY2 also interact with SUPPRESSOR OF PHYA-105 1 (SPA1) (Lian *et al.*, 2011; Liu *et al.*, 2011; Zuo *et al.*, 2011), the enhancer of COP1 E3 ligase activity that interacts with COP1 (Seo *et al.*, 2003). The interactions of CRY1 and CRY2 with SPA1 dissociate COP1 from SPA1 and enhance the interaction of CRY2 with COP1, respectively, thus repressing the E3 ligase activity of COP1 and promoting protein accumulation of HY5 and CO (Lian *et al.*, 2011; Liu *et al.*, 2011; Zuo *et al.*, 2011). Phytochromes and UVR8 also physically interact with COP1 to mediate red/far-red and UV-B light signaling, respectively (Wang *et al.*, 2001; Yang *et al.*, 2001; Seo *et al.*, 2004; Favory *et al.*, 2009; Jang *et al.*, 2010). The N-terminal domain of CRYs is known to mediate CRYs' dimerization/oligomerization (Sang *et al.*, 2005; Yu *et al.*, 2007b; Ma *et al.*, 2020a; Shao *et al.*, 2020), and BIC1/2 function as inhibitors to suppress CRY2 dimerization/oligomerization in a blue-light-dependent manner (Q. Wang *et al.*, 2016; Ma *et al.*, 2020b). It has been shown that CRY1 N-terminus is able to mediate blue light signaling independent of its C-terminus (He *et al.*, 2015) and that CRY1 interacts with Aux/IAA and ARF6/8 proteins and BES1/BIM1 through its N-terminus to regulate auxin and brassinosteroid signaling, respectively (Wang *et al.*, 2018; Xu *et al.*, 2018; Mao *et al.*, 2020). Moreover, CRY1 and CRY2 interact with PIFs to mediate responses to low levels of blue light or high temperature (Ma *et al.*, 2016; Pedmale *et al.*, 2016).

The excitation of photoreceptors by light not only increases their biological activity but can also lead to their degradation and, thus, inactivation. For example, high intensities of light irradiation or prolonged illumination can lead to degradation of the photoreceptors to reduce light signaling and optimize the status of plant growth and development. It has been demonstrated that blue light induces the degradation of CRY2, and that COP1 and the E3 ubiquitin ligases Light-Response Bric-a-Brack/Tramtrack/Broads (LRBs; LRB1–3) mediate this process (Shalitin *et al.*, 2002; Chen *et al.*, 2021). Prolonged red light irradiance also triggers the degradation of phyA and phyB. While COP1 is required for phyA degradation, both COP1 and LRBs are shown to mediate phyB degradation (Seo *et al.*, 2004; Jang *et al.*, 2010; Ni *et al.*, 2014). High-intensity blue light (HBL) also induces the degradation of PHOT1, and the Cullin3-related E3 ubiquitin ligase NPH3 is involved in this process (Roberts *et al.*, 2011). It has been demonstrated that both CRY1 and CRY2 undergo blue-light-dependent phosphorylation (Shalitin *et al.*, 2002, 2003), and that only the phosphorylated CRY2 is biologically active and undergoes degradation through the 26S proteasome (Yu *et al.*, 2007a). However, CRY1 is stable under the relatively low fluence rates of blue light, under which CRY2 efficiently undergoes degradation (Lin *et al.*, 1998). Whether HBL might induce CRY1 degradation is unknown.

In this study, we show by biochemical studies that CRY1 is ubiquitinated and degraded through the 26S proteasome-dependent pathway in response to HBL. Through examination of CRY1 ubiquitination and degradation in the COP1 loss-of-function mutant background, we demonstrate that COP1 is responsible for mediating CRY1 ubiquitination and degradation in response to HBL. In addition to COP1, the E3 ligases LRBs interact with CRY1 and are also involved in mediating CRY1 ubiquitination and degradation in HBL. We show by protein–protein interaction studies that CRY1 physically interacts with BIC1 in a blue-light-dependent manner, and that BIC1 inhibits CRY1 dimerization/oligomerization, which is required for HBL-induced degradation of CRY1. These results indicate that COP1 and LRBs act as E3 ligases to mediate CRY1 ubiquitination and degradation under HBL, and that dimerization/oligomerization of CRY1 is required for its degradation in response to HBL. Our study reveals a regulatory mechanism by which CRY1 homeostasis is regulated by high blue light. This mechanism enables plants to reduce CRY1 signaling under high blue light and fine-tune their growth and developmental status.

Materials and Methods

Plant material and growth conditions

All *Arabidopsis* plants used were of the Columbia ecotype, and Columbia (Col-0) seeds of *Arabidopsis* were used as the wild-type (WT). The *cry1*, *cry1 cry2*, *lrb1 lrb2-2 lrb3* (*lrb123*), *cop1-4*, *lrb1 lrb2-2 lrb3 cop1-4* (*lrb123 cop1*) and *bic1 bic2* mutants, and transgenic lines overexpressing Myc-tagged CRY1 in *cry1* mutant background (*Myc-CRY1-OX*) were described previously (Sang *et al.*, 2005; Q. Wang *et al.*, 2016; Chen *et al.*, 2021). *Myc-CRY1-OX* transgenic lines were introgressed into WT or *cop1-4* mutant background by genetic crossing, to generate *Myc-CRY1-OX*/WT or *Myc-CRY1-OX/cop1* plants, respectively, which were confirmed by phenotypic analyses and/or western blot analysis.

Imbibed seeds were kept at 4°C for 3 d and grown on Murashige & Skoog (MS) nutrient medium plus 2% sucrose with 0.8% agar at 22°C under white light (120 μmol m^{-2} s^{-1}). Experiments involving blue, red, and far-red light illuminations were described previously (Wang *et al.*, 2018).

Western blot assay

Arabidopsis protein extracts for western blot assays were prepared and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane as described previously (W. Wang *et al.*, 2016). The membrane was blocked with 5% skimmed milk in phosphate-buffered saline–Tween solution. After probed with primary and secondary antibodies, the membrane was incubated in enhanced chemiluminescence solution and detected by a Tanon 5200 luminescent imaging workstation (Tanon Science & Technology Co. Ltd, Shanghai, China). The primary antibodies used in this study included: anti-CCT1 (1 : 1000) and anti-CCT2 antisera (1 : 500) (Sang *et al.*, 2005), anti-Flag (F3165; Sigma), anti-maltose-binding protein (anti-MBP; E8032S; NEB, Ipswich, MA, USA), anti-ubiquitin (3936S; CST, Boston, MA, USA), anti-Myc (05-724; Millipore), anti-tubulin (T6074; Sigma), anti-82 kDa heat shock protein (anti-HSP82; Abcam, Cambridge, UK), and

anti-green fluorescent protein (anti-GFP; M20004L; Abmart, Berkeley Heights, NJ, USA) antibodies. Rabbit IgG HRP-linked (NA934; Cytiva, Shanghai, China) was used as the secondary antibody (1 : 20 000) to detect the anti-CCT1 and anti-CCT2 antisera. Mouse IgG HRP-Linked (A0168; Sigma) was used (1 : 10 000) to detect the anti-tubulin, anti-GFP, anti-MBP, anti-ubiquitin, anti-HSP82, anti-Myc, and anti-Flag antibodies.

Yeast two-hybrid assay

The complementary DNA (cDNA) fragments of *BIC1* were cloned into the BD vector, and the fragments of *CRY1*, *CNT1*, and *CCT1* were cloned into the AD vector. The GAL4 yeast two-hybrid assays were performed as described previously (Wang *et al.*, 2018).

Split luciferase complementation assay in tobacco

The DNA sequences encoding CRY1 and the C-termini of LRB1 (cLRB1, AA 246–561), LRB2 (cLRB2, AA 248–561), and LRB3 (cLRB3, AA 193–505) were amplified and then cloned into pCambia1300-nLUC vector and pCambia1300-cLUC vector, respectively, and split luciferase complementation (split-LUC) assays were performed as described previously (Du *et al.*, 2020). For the split-LUC experimental analysis of the interaction of CRY1 with BIC1, *35S:CRY1-nLUC* and *35S:cLUC-BIC1* were co-expressed in tobacco leaves. For the split-LUC assays to determine the effect of BIC1 on the dimerization/oligomerization of CRY1, *35S:BIC1-YFP* or *35S:GUS-YFP* (Wang *et al.*, 2018) constructs were co-expressed in the presence of *35S:CRY1-nLUC* and *35S:cLUC-CRY1* (*YFP*, yellow fluorescent protein; *GUS*, β-glucuronidase). Luminescence intensities were detected using Tanon image software after the treatment.

Pull-down assays with proteins expressed in *Escherichia coli*

The construction of pCold-TF-CNT1 and pCold-TF-CCT1 was described previously (Du *et al.*, 2020). The fragments of *BIC1* were cloned into pMAL-c2X vectors (NEB). Pull-down assays were performed as described previously (Xu *et al.*, 2016). Prey protein MBP-BIC1 was detected by anti-MBP antibody, and bait proteins His-TF, His-TF-CNT1, and CCT1 were visualized by Coomassie brilliant blue staining.

Pull-down assays with *Arabidopsis* protein extracts

For the blue-light-specific CRY1–BIC1 interaction assay, MBP-BIC1 bait proteins were first incubated with 10 µl MBP magnetic beads for 1 h and then washed three times with lysis buffer. The protein extracts from *Myc-CRY1-OX* seedlings were used as prey, and the seedlings were adapted to darkness for 4 d and then remained in darkness for 1 h or exposed to blue light (50 µmol m^{-2} s^{-1}), red light (50 µmol m^{-2} s^{-1}), or far-red light (10 µmol m^{-2} s^{-1}) for 1 h. The procedures for the assays were described previously (Du *et al.*, 2020).

Co-immunoprecipitation assay

For the co-immunoprecipitation (Co-IP) assays of blue-light-dependent CRY1–BIC1 and CRY1–LRBs interactions, one half of the etiolated seedlings overexpressing Flag-GFP-tagged BIC1, LRB1, and LRB2 (*FGFP-BIC1-OX*, *FGFP-LRB1-OX*, and *FGFP-LRB2-OX*) were exposed to blue light (30 or 100 µmol m^{-2} s^{-1}) for 15 min and the other half were kept in darkness for 15 min. Co-IP assays were performed as described previously (Lian *et al.*, 2011). For the Co-IP assays of the effects of BIC1 on CRY1 dimerization/oligomerization, proteins were extracted from dark-adapted *Myc-CRY1-OX*/WT seedlings exposed to blue light (30 µmol m^{-2} s^{-1}) or kept in darkness for 20 min. The extracts were immunoprecipitated with 20 µl anti-Myc agarose beads (Sigma) in the presence of MBP or MBP-BIC1 for 2 h.

Precipitate polyubiquitinated proteins from seedlings

For the assays of the effects of COP1 on CRY1 ubiquitination, etiolated *Myc-CRY1-OX* and *Myc-CRY1-OX/cop1* seedlings were exposed to high blue light (100 µmol m^{-2} s^{-1}) for 1 h. For the assays of the effects of LRBs on CRY1 ubiquitination, dark-adapted WT and *lrb123* seedlings were treated with 50 µM MG132 in liquid MS for 4 h and then exposed to high blue light (100 µmol m^{-2} s^{-1}) for 3 h. Total proteins were extracted and determined by Bradford assay, and then equal amounts of total protein in 1 ml of lysis buffer were incubated with 20 µl Tandem Ubiquitin Binding Entities 2 (TUBE2) agarose beads (UM402; LifeSensors Inc., Malvern, PA, USA) at 4°C for 2 h. The immunoprecipitates were washed three times with lysis buffer and eluted into 20 µl 2× loading buffer and then subjected to western blot analysis with anti-Myc antibody or anti-CCT1 antiserum.

Cell-free degradation assay

For the assay of ATP-dependent CRY1 degradation, total proteins from etiolated WT seedlings exposed to high blue light (100 µmol m^{-2} s^{-1}) for 15 min were extracted with degradation buffer (50 mM Tris hydrochloride, pH 7.5; 150 mM sodium chloride; 10% glycerol; 0.2% Triton X-100; 1 mM Pefabloc, 1× EDTA-free Protease Inhibitor Cocktail (Roche), 150 µM cycloheximide, 10 mM magnesium chloride, 1 mM dithiothreitol). The total protein concentration was determined by Bradford assay. Samples were incubated at 22°C in a ThermoMixer® (Eppendorf, Hamburg, Germany) after 5 mM ATP was added immediately or not. For the assay of MBP-COP1-induced CRY1 degradation, MBP and MBP-COP1 expressed in *Escherichia coli* (Rosetta; Weidi Biotechnology Co. Ltd, Shanghai, China) were incubated with amylase resin (NEB) at 4°C for 1 h, and then fully washed with lysis buffer five times and eluted with lysis buffer plus 10 mM maltose at 4°C for 30 min. The elution was treated by Amicon Ultra 15 according to the manufacturer's instruction. Then, 500 ng purified MBP and MBP-COP1 were added in extracts from etiolated WT seedlings exposed to high

blue light (100 μmol m^{-2} s^{-1}) for 15 min in the presence of 5 mM ATP. The reaction was stop with 5× loading buffer. Samples were boiled at 100°C for 5 min and subjected to western blot analysis with anti-CCT1 antiserum.

Quantitative reverse transcription PCR

Total RNAs were isolated with RNAprep Plant kit (Tiangen) and then were reverse-transcribed to cDNA using an iScript cDNA Synthesis kit (Bio-Rad). *ACT2* was used as an internal control for quantitative reverse transcription PCR (qRT-PCR). qRT-PCR analysis was described previously (Wei *et al.*, 2021). The primers used are listed in Supporting Information Table S1.

Results

CRY1 undergoes degradation in response to high blue light irradiation

Given the previous demonstrations that prolonged red light and blue light induce degradation of phyA, phyB, and CRY2 (Lin *et al.*, 1998; Seo *et al.*, 2004; Jang *et al.*, 2010), and that relatively low-intensity blue light does not induce the degradation of CRY1, we explored whether CRY1 would be degraded under high-intensity blue light. To do this, we performed western blot analyses with anti-CCT1 and anti-CCT2 antisera to examine the endogenous CRY1 and CRY2 levels, respectively, in the etiolated WT seedlings treated with different fluence rates of blue light. The results showed that, consistent with a previous study (Yu *et al.*, 2007a), CRY2 was phosphorylated and degraded in seedlings exposed to a low fluence rate of blue light (1 μmol m^{-2} s^{-1}) (Fig. 1a), and that medium (10 μmol m^{-2} s^{-1}) or high (100 μmol m^{-2} s^{-1}, HBL) fluence rates of blue light trigger rapid degradation of CRY2 (Fig. 1b). However, CRY1 was barely phosphorylated and degraded in response to low or medium blue light irradiation (Fig. 1a–c). Interestingly, HBL irradiation clearly induced phosphorylation and degradation of CRY1 (Fig. 1b,c). *In vitro* calf intestine phosphatase treatment assay confirmed that the slow migrating proteins induced by HBL were phosphorylated CRY1 (Fig. S1a). We further analyzed whether high red light (HRL) and high far-red light (HFRL) would affect CRY1 stability. As showed in Fig. 1(d), CRY1 was degraded in response to HBL, but not to HRL or HFRL. These results indicate that CRY1 undergoes degradation under high blue light. To exclude the possibility that the decrease in CRY1 protein level might result from downregulation of *CRY1* transcripts by high blue light, we performed qRT-PCR to analyze the transcript levels of *CRY1*. The results showed that *CRY1* transcripts did not decrease; rather, they increased slightly in the etiolated seedlings exposed to HBL for the times indicated (Fig. S1b). Furthermore, we analyzed the effects of HBL on the stability of Myc-CRY1 fusion protein with the dark-grown seedlings overexpressing CRY1 tagged by Myc (*Myc-CRY1-OX*) exposed to HBL, which shows a fully etiolated phenotype similar to WT in the dark but exhibits a hypersensitive response to blue light (Sang *et al.*, 2005). The results showed that HBL also induced Myc-CRY1 phosphorylation and degradation (Fig. S1c, d). Taken together, these results demonstrate that CRY1 undergoes degradation in response to high blue light.

High blue light induces CRY1 ubiquitination and degradation through the 26S proteasome

It is shown that red and blue-light-triggered degradation of phyA, phyB, CRY2, and PHOT1 proceeds through the 26S proteasome pathway (Seo *et al.*, 2004; Yu *et al.*, 2007a; Jang *et al.*, 2010; Roberts *et al.*, 2011). To explore whether CRY1 might be degraded through the 26S-proteasome-dependent pathway under HBL, we performed western blot assays to analyze the endogenous CRY1 levels with anti-CCT1 antiserum using the dark-grown WT seedlings pretreated with the 26S proteasome inhibitor MG132 and then exposed to HBL. The results showed that HBL triggered CRY1 phosphorylation, but it hardly induced CRY1 degradation in the seedlings treated with MG132 (Fig. 2a,b), indicating that CRY1 is degraded through the 26S proteasome pathway. To further confirm the 26S-proteasome-pathway-dependent degradation of CRY1 under HBL, we performed a cell-free degradation assay in the presence or absence of ATP, which is required for ubiquitin-proteasomal proteolysis (Wang *et al.*, 2009). The results showed that, in the absence of ATP, CRY1 was hardly degraded, whereas CRY1 was clearly degraded upon 15–60 min incubation in the presence of ATP (Fig. 2c). Taken together, these results demonstrate that CRY1 is degraded through the 26S proteasome pathway under high blue light.

Given that protein ubiquitination is generally coupled with degradation through the 26S proteasome and that CRY1 is degraded through the 26S proteasome (Fig. 2a–c), we explored whether CRY1 would be ubiquitinated *in vivo* upon HBL irradiation. We first performed an immunoprecipitation (IP) assay with *Myc-CRY1-OX* seedlings either adapted in darkness or exposed to HBL, and then we detected ubiquitinated Myc-CRY1 with anti-ubiquitin antibody. Protein blot assay with anti-Myc antibody showed that the smeared putative ubiquitinated Myc-CRY1 was detected in the immunoprecipitates of the *Myc-CRY1-OX* seedlings exposed to HBL, but not in those adapted in the dark (Fig. S2). A further protein blot assay with anti-ubiquitin antibody confirmed that HBL irradiation, but not dark adaptation, strongly induced the ubiquitination of Myc-CRY1 (Fig. 2d). To further confirm whether CRY1 ubiquitination would be induced by HBL, we precipitated polyubiquitinated proteins using TUBE2 (Fig. 2e) to detect the polyubiquitinated Myc-CRY1 in *Myc-CRY1-OX* seedlings either adapted in darkness or exposed to low and high blue light with anti-Myc antibody. The results showed that Myc-CRY1 was highly polyubiquitinated in HBL, with being more pronounced with MG132 application (Fig. 2e). By contrast, Myc-CRY1 was not polyubiquitinated in the dark or low blue light. The Myc-GUS control protein was not polyubiquitinated in HBL. Taken together, these results demonstrate that high blue light stimulates CRY1 ubiquitination *in vivo*.

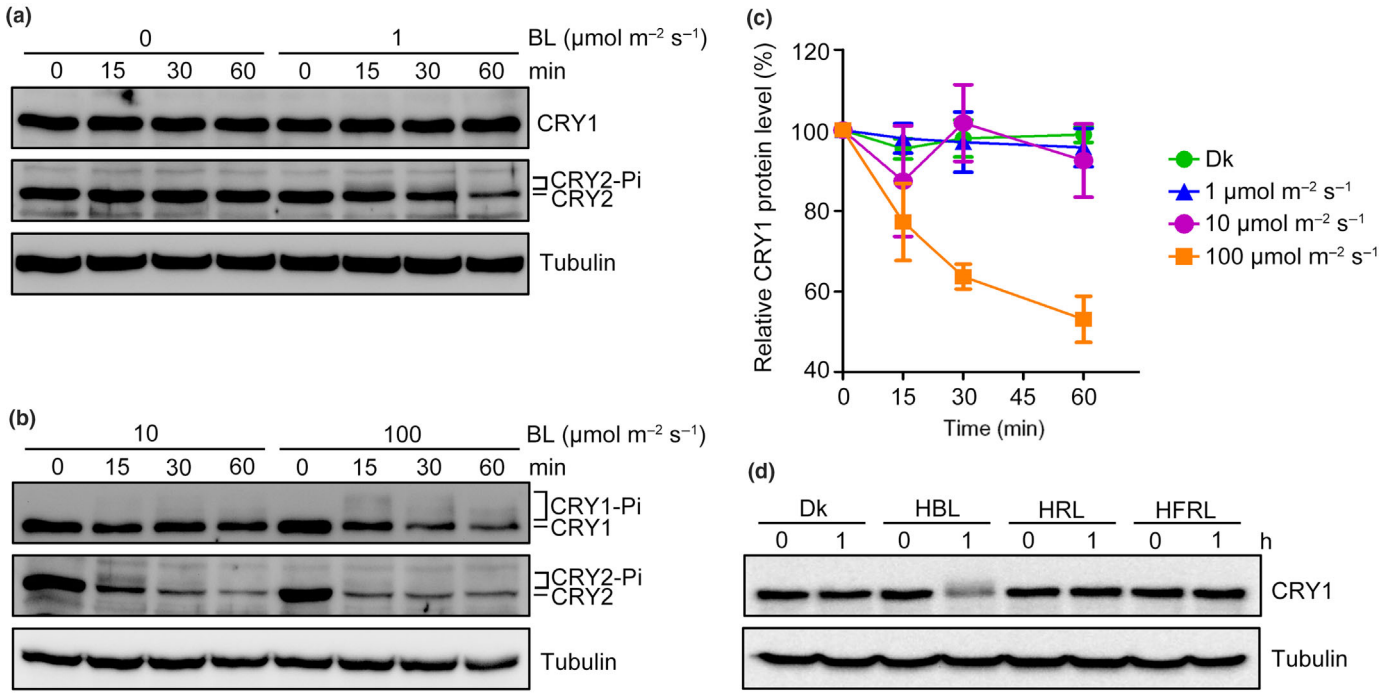

Fig. 1 Cryptochrome 1 (CRY1) undergoes degradation in response to high blue light irradiation. (a–c) Western blot assay results showing *Arabidopsis* CRY1 and CRY2 degradation patterns in different intensities of blue light. (a, b) Six-day-old etiolated wild-type (WT) seedlings were exposed to the intensities of blue light (BL) and time indicated. The endogenous CRY1 and CRY2 in this and other figures were detected with anti-CCT1 and anti-CCT2 antisera, respectively. Tubulin in this and other figures served as loading control. CRY1-Pi and CRY2-Pi, phosphorylated CRY1 and CRY2, respectively. (c) The CRY1 protein levels of three biological repeats in (a) and (b) were normalized to tubulin and quantified using ImageJ. Data in (c) are mean ± SD ($n = 3$). (d) Western blot assay showing high blue-light-specific CRY1 degradation in *Arabidopsis*. Six-day-old etiolated WT seedlings were kept in darkness (Dk) or exposed to high blue light (HBL, 100 μmol m^{-2} s^{-1}), high red light (HRL, 100 μmol m^{-2} s^{-1}), and high far-red light (HFRL, 10 μmol m^{-2} s^{-1}) for 1 h.

COP1 is involved in mediating the ubiquitination of CRY1 in high blue light

It is known that COP1 and its E3 ubiquitin ligase enhancer SPAs are responsible for light-induced degradation of CRY2, phyA, and phyB (Shalitin *et al.*, 2002; Seo *et al.*, 2004; Jang *et al.*, 2010). To examine whether COP1 would be involved in the regulation of HBL-induced degradation of CRY1, we analyzed the endogenous CRY1 levels of *cop1* mutant exposed to HBL for different lengths of time. The results showed that, in contrast to WT, prolonged HBL irradiation hardly induced CRY1 degradation in *cop1* mutant (Fig. 3a,b). To further evaluate whether COP1 would be involved in ubiquitinating CRY1 *in vivo*, we generated *cop1* mutant plants expressing Myc-CRY1 (*Myc-CRY1-OX/cop1*) by genetic crossing and analyzed Myc-CRY1 ubiquitination with TUBE2. The results showed that HBL-induced ubiquitination of Myc-CRY1 was greatly compromised in *cop1* mutant (Fig. 3c). To determine whether COP1 would promote the degradation of the phosphorylated or nonphosphorylated CRY1, we employed cell-free degradation assays with MBP-COP1 fusion protein expressed in *E. coli* in the presence of ATP. The results showed that COP1 mainly promoted the degradation of the phosphorylated CRY1 (Figs 3d, S3). Taken together, these results suggest that COP1 mediates HBL-induced CRY1 ubiquitination and degradation.

LRBs physically interact with CRY1 to mediate high blue-light-induced ubiquitination and degradation of CRY1

Given that LRBs interact with phyB and CRY2 to promote their ubiquitination and degradation in red light and blue light, respectively (Christians *et al.*, 2012; Ni *et al.*, 2014; Chen *et al.*, 2021), and that CRY1 and phyB share the same downstream factors such as COP1/SPAs, PIFs, Aux/IAAs, ARFs, BES1/BIM1, and AGB1 to mediate light signaling (Wang *et al.*, 2001, 2018; Yang *et al.*, 2001; Huq & Quail, 2002; Lian *et al.*, 2011, 2018; Liu *et al.*, 2011; Zuo *et al.*, 2011; Lu *et al.*, 2015; Ma *et al.*, 2016; Pedmale *et al.*, 2016; Wu *et al.*, 2018; Xu *et al.*, 2018, 2019; Mao *et al.*, 2020), we asked whether LRBs might also interact with CRY1 to mediate high-blue-light-induced degradation of CRY1. Our first attempt to confirm the interactions of CRY1 with LRBs by split-LUC assays in tobacco leaves transiently expressing CRY1 tagged with N-terminus of luciferase (CRY1-nLUC), together with the full-length LRB1 or LRB2 fused to cLUC, failed. As the C-terminal BACK-containing domain of LRBs (cLRB1, cLRB2, and cLRB3) is known to mediate the interactions with their target proteins, we then performed split-LUC assays with cLRBs fused to cLUC (cLUC-cLRBs). The results demonstrated that the luciferase activity was reconstituted when cLUC-cLRB1 or cLUC-cLRB2 or cLUC-cLRB3 was expressed with CRY1-nLUC, indicating interactions of CRY1 with LRBs (Figs 4a,b, S4). To examine

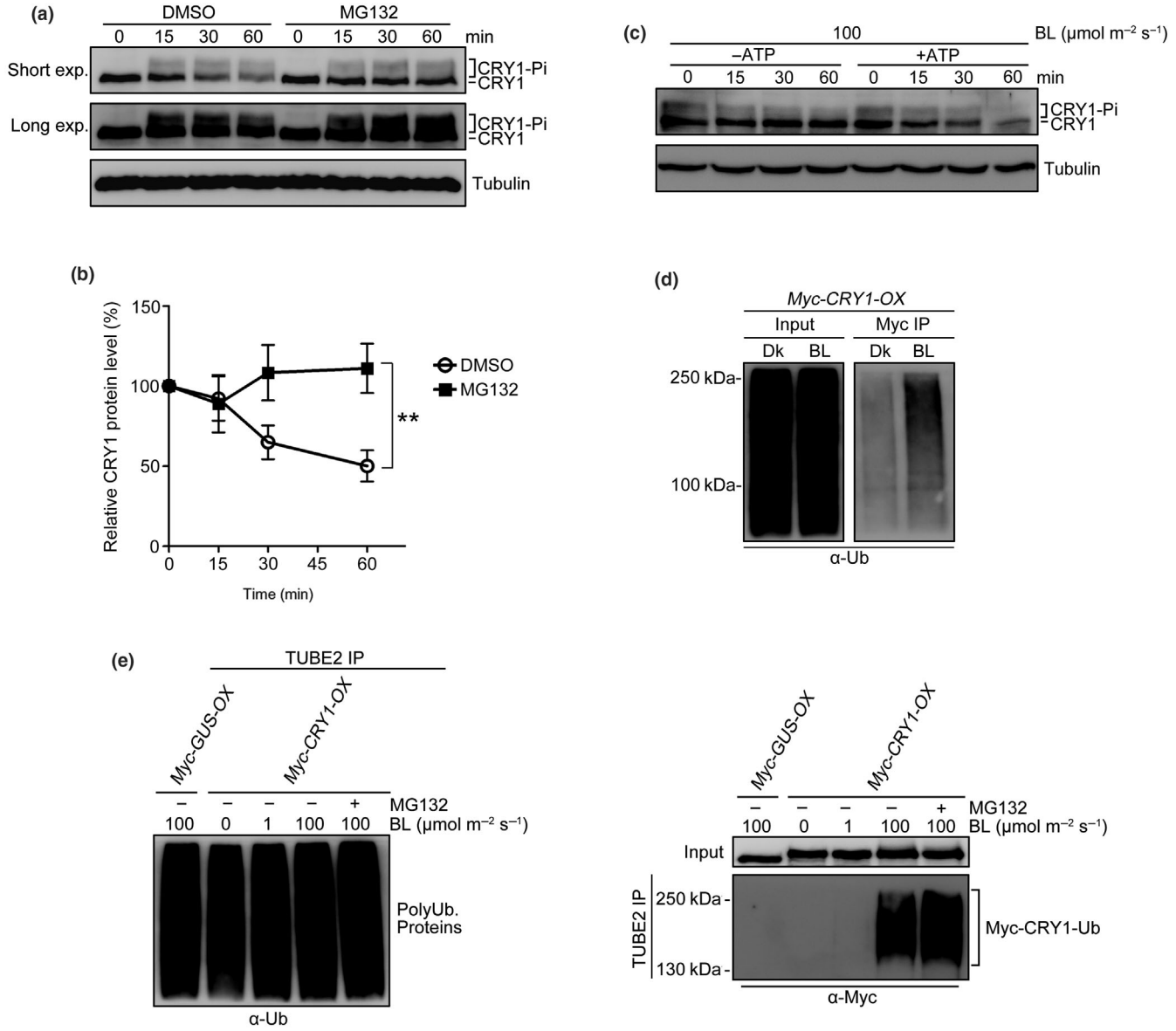

Fig. 2 High blue light (HBL) induces cryptochrome 1 (CRY1) ubiquitination and degradation through the 26S proteasome. (a, b) Western blot assay results showing inhibition of HBL-induced CRY1 degradation by the 26S proteasome inhibitor MG132 in *Arabidopsis*. Six-day-old etiolated wild-type (WT) seedlings were pretreated with mock (dimethyl sulfoxide, DMSO) or 50 μM MG132 in liquid Murashige & Skoog for 4 h in darkness and then exposed to HBL (100 μmol m^{-2} s^{-1}) for the time indicated. Short exp., short exposure time; Long exp., long exposure time. BL, blue light; CRY1-Pi, phosphorylated CRY1. (b) The CRY1 protein levels of three biological repeats were normalized to tubulin and quantified using ImageJ. Data in (b) are mean ± SD ($n = 3$) (Student's *t*-test; **, $P < 0.01$). (c) Cell-free degradation assay showing ATP-dependent degradation of CRY1. Extracts from 6-d-old etiolated WT seedlings exposed to HBL (100 μmol m^{-2} s^{-1}) for 15 min were treated with or without 5 mM ATP for the time indicated at room temperature. (d) Immunoprecipitation (IP) assay showing HBL-induced CRY1 ubiquitination in *Arabidopsis*. Six-day-old etiolated *Myc-CRY1-OX* seedlings were kept in darkness (Dk) or exposed to HBL (100 μmol m^{-2} s^{-1}) for 1 h, followed by IP with anti-Myc agarose beads, and then analyzed by western blots using anti-ubiquitin (Ub) antibody. (e) Precipitation of ubiquitinated proteins showing HBL-specific CRY1 ubiquitination in *Arabidopsis*. Six-day-old etiolated *Myc-GUS-OX* (negative control) and *Myc-CRY1-OX* seedlings with or without MG132 pretreatment were exposed to the indicated intensities of blue light for 1 h. Total polyubiquitinated proteins were precipitated using Tandem Ubiquitin Binding Entities 2 (TUBE2) and then analyzed by western blots using anti-Ub and anti-Myc antibody.

the interactions in *Arabidopsis*, we performed Co-IP assays with the etiolated *FGFP-LRB1-OX* and *FGFP-LRB2-OX* seedlings (Chen *et al.*, 2021) adapted in darkness or exposed to high blue light. The results showed that both LRB1 and LRB2 were co-immunoprecipitated by the endogenous CRY1 in the extracts prepared from *FGFP-LRB1-OX* and *FGFP-LRB2-OX* seedlings exposed to HBL, but not from those adapted in the dark (Fig. 4c,d). These results suggest that LRBs interact with CRY1 in a blue-light-dependent manner. To evaluate whether LRBs-CRY1 interactions might affect CRY1 degradation, we analyzed the endogenous CRY1 levels in *lrb123* mutant seedlings exposed to HBL for different lengths of time. The results

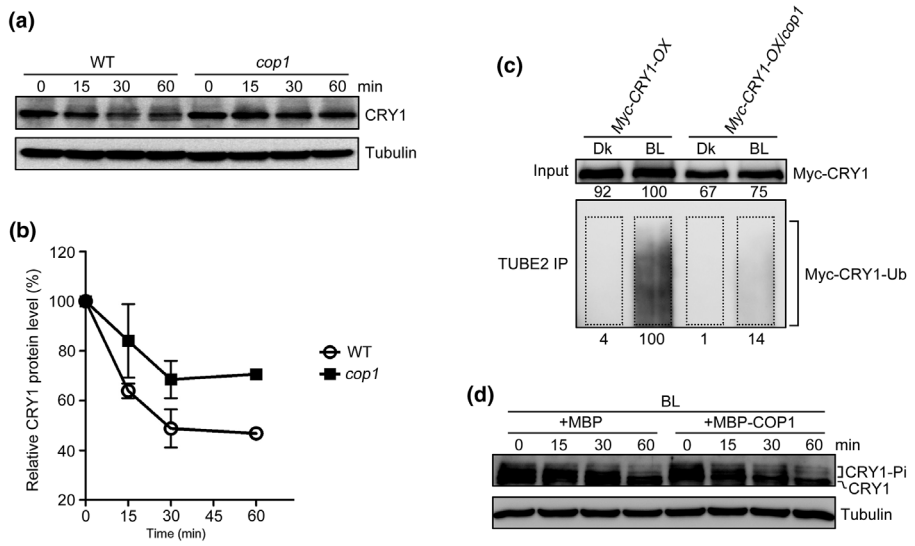

Fig. 3 Constitutive photomorphogenic 1 (COP1) is involved in mediating the ubiquitination of cryptochrome 1 (CRY1) in high blue light. (a, b) Western blot assay results showing involvement of COP1 in CRY1 degradation in *Arabidopsis*. Six-day-old etiolated wild-type (WT) and *cop1* seedlings were exposed to high blue light (HBL, 100 μmol m^{-2} s^{-1}) for the time indicated. (b) The CRY1 protein levels of three biological repeats in (a) were normalized to tubulin and quantified using ImageJ. Data in (b) are mean ± SD ($n = 3$). (c) Precipitation of ubiquitinated proteins showing that COP1 is responsible for HBL-induced CRY1 ubiquitination in *Arabidopsis*. Six-day-old etiolated *Myc-CRY1-OX* and *Myc-CRY1-OX/cop1* seedlings were exposed to HBL (100 μmol m^{-2} s^{-1}) for 1 h. Total polyubiquitinated proteins were precipitated using Tandem Ubiquitin Binding Entities 2 (TUBE2) and then analyzed by western blots using anti-Myc antibody. The western blot results were quantified using ImageJ, and the relative intensities are shown below each lane. BL, blue light; Dk, darkness; IP, immunoprecipitation. (d) Cell-free degradation assay showing COP1 stimulation of CRY1 degradation. Extracts from 6-d-old etiolated WT seedlings exposed to HBL (100 μmol m^{-2} s^{-1}) for 15 min were treated with maltose-binding protein (MBP) or MBP-COP1 in the presence of 5 mM ATP for the time indicated. CRY1-Pi, phosphorylated CRY1.

showed that, in contrast to WT, HBL irradiation hardly induced CRY1 degradation in *lrb123* mutant (Fig. 4e,f). To further evaluate whether LRBs would be involved in mediating CRY1 ubiquitination *in vivo* to induce CRY1 degradation, we precipitated polyubiquitinated proteins using TUBE2 to detect the polyubiquitinated CRY1 in WT and *lrb123* mutant plants either adapted in darkness or exposed to HBL with anti-CCT1 antiserum. As shown in Fig. 4(g), HBL-induced ubiquitination of CRY1 was greatly compromised in *lrb123* mutant. Taken together, these results indicate that LRBs interact with CRY1 in a blue-light-dependent manner and mediate HBL-induced CRY1 ubiquitination and degradation.

Given that COP1 and LRBs are involved in mediating CRY1 degradation in HBL, we asked whether they might work independently. We therefore analyzed CRY1 degradation in the *lrb123 cop1* quadruple mutant exposed to HBL and found that CRY1 degradation was more dramatically inhibited in *lrb123 cop1* mutant than in *cop1* or *lrb123* mutant (Fig. S5). These results indicate that COP1 and LRBs act additively in regulating HBL-induced CRY1 degradation.

LRBs promote hypocotyl elongation in blue light

Given the demonstrations that LRBs interact with CRY1 to mediate CRY1 ubiquitination and degradation in HBL, we asked whether LRBs might be involved in regulating hypocotyl elongation in blue light. To test this postulation, we analyzed the hypocotyl phenotype of *lrb123* mutant seedlings in darkness and in low, medium, and high intensities of blue light. The results showed that *lrb123* mutant hypocotyls were as long as WT in the dark but significantly shorter than WT under low, medium, or high blue light (Fig. 5a,b). These results indicate that LRBs promote hypocotyl elongation in blue light.

CRY1 physically interacts with BIC1 in a blue-light-dependent manner

Given the previous demonstration that BIC1 interacts with CRY2 and represses CRY2 dimerization/oligomerization in a blue-light-dependent manner and that CRY1 binds to BIC1 *in vitro* (Q. Wang *et al.*, 2016; Ma *et al.*, 2020b), we asked whether BIC1 might also interact with CRY1 to inhibit CRY1 dimerization/oligomerization and affect HBL-induced degradation of CRY1 in *Arabidopsis*. To test this possibility, we first evaluated whether BIC1 would interact with CRY1. We carried out GAL4 yeast two-hybrid assays in darkness and in blue light by co-transforming yeast cells with the bait construct expressing GAL4 binding domain fused to BIC1, together with prey construct expressing CRY1, CNT1, or CCT1. The results showed that both CRY1 and CNT1 interacted with BIC1 in darkness and blue light, whereas CCT1 did not (Fig. 6a,b). We then performed *in vitro* pull-down assays with the bait His-TF-CNT1 and His-TF-CCT1 fusion proteins and the prey MBP-BIC1 fusion protein, and found that MBP-BIC1 was pulled down by His-TF-CNT1, but not by His-TF-CCT1 or the His-TF control (Fig. 6c).

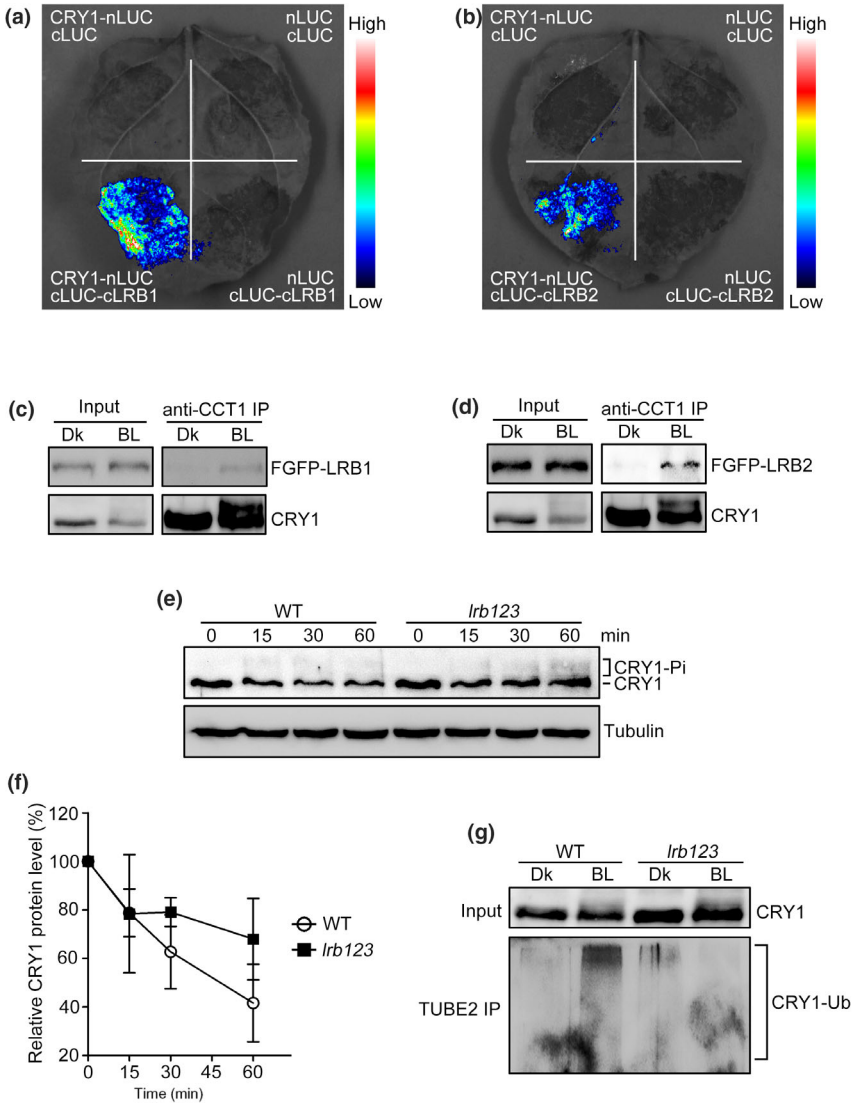

Fig. 4 Light-Response Bric-a-Brack/Tramtrack/Broads (LRBs) physically interact with cryptochrome 1 (CRY1) to mediate high blue-light (HBL)-induced ubiquitination and degradation of CRY1. (a, b) Split-LUC assays showing interactions of CRY1 with LRB1 and LRB2 in tobacco leaves. CRY1-nLUC was expressed with cLUC-cLRB1 (AA 246–561) or cLUC-cLRB2 (AA 248–561). The empty vectors were used as negative controls. (c, d) Co-immunoprecipitation (Co-IP) assays showing interactions of CRY1 with (c) LRB1 and (d) LRB2. Six-day-old etiolated *FGFP-LRB1-OX* and *FGFP-LRB2-OX* seedlings were kept in darkness or exposed to HBL (100 μmol m^{-2} s^{-1}) for 15 min, followed by immunoprecipitation (IP) with anti-CCT1 antiserum. The IP (CRY1) and Co-IP signals (LRB1 and LRB2) were detected by western blots probed with anti-CCT1 antiserum and anti-Flag antibody, respectively. BL, blue light; Dk, darkness. (e, f) Western blot assay results showing that LRBs are involved in HBL-induced CRY1 degradation in *Arabidopsis*. Six-day-old etiolated wild-type (WT) and *lrb123* seedlings were exposed to HBL (100 μmol m^{-2} s^{-1}) for the time indicated. CRY1-Pi, phosphorylated CRY1. (f) The CRY1 protein levels of three biological repeats in (e) were normalized to tubulin and quantified using ImageJ. Data in (f) are mean ± SD ($n = 3$). (g) Precipitation of ubiquitinated proteins showing that LRBs are responsible for HBL-induced CRY1 ubiquitination in *Arabidopsis*. Six-day-old etiolated WT and *lrb123* seedlings with MG132 pretreatment were exposed to HBL (100 μmol m^{-2} s^{-1}) for 3 h. Total polyubiquitinated proteins were precipitated using Tandem Ubiquitin Binding Entities 2 (TUBE2) and then analyzed by western blots using anti-CCT1 antiserum.

To examine whether CRY1 would interact with BIC1 *in vivo*, we performed a split-LUC assay by transiently expressing CRY1-nLUC, together with cLUC-BIC1, in tobacco leaves. The results demonstrated that the luciferase activity was reconstituted when cLUC-BIC1 and CRY1-nLUC were co-expressed, indicating interactions of CRY1 with BIC1 (Fig. 6d). Furthermore, we performed a semi-*in-vivo* pulldown assay using MBP-BIC1 fusion protein as bait and *Arabidopsis* protein extracts as prey, which were prepared from *Myc-CRY1-OX* seedlings adapted in the dark and exposed to blue, red, or far-red light. The results showed that MBP-BIC1 pulled down Myc-CRY1 from *Myc-CRY1-OX* seedlings exposed to blue light, but not from those either adapted in darkness or exposed to red or far-red light (Fig. 6e), indicating blue-light-specific interaction of CRY1 with BIC1. To confirm whether CRY1 might interact with BIC1 in *Arabidopsis*, we performed Co-IP assays with etiolated *FGFP-BIC1-OX* seedlings (Q. Wang *et al.*, 2016) adapted in darkness or exposed to blue light. As shown in Fig. 6(f), BIC1 was co-immunoprecipitated by

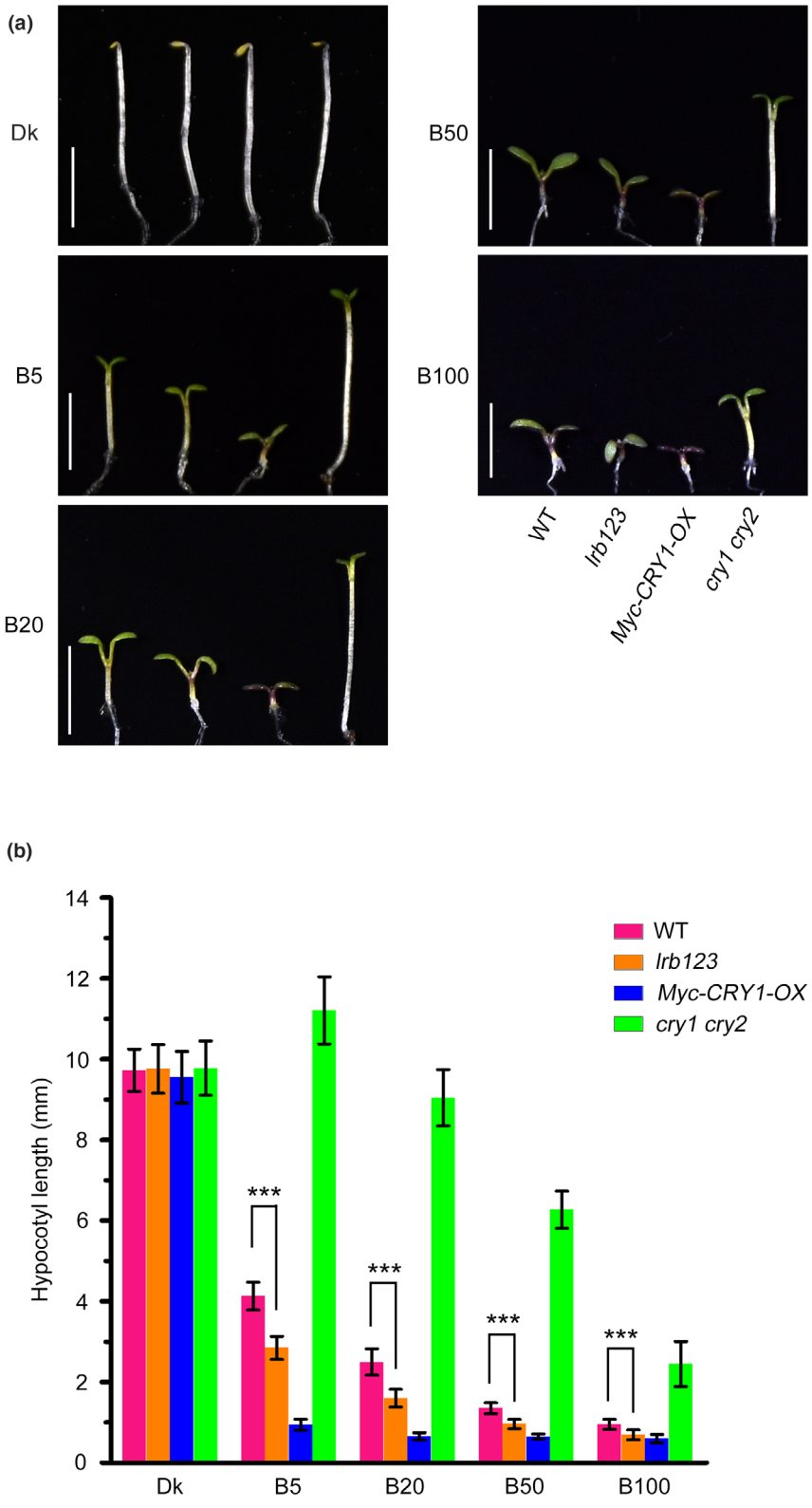

Fig. 5 Light-Response Bric-a-Brack/Tramtrack/Broads (LRBs) promote hypocotyl elongation in blue light. (a, b) The wild-type (WT), *cry1 cry2*, *lrb123*, and *Myc-CRY1-OX* *Arabidopsis* seedlings grown in darkness (Dk), low blue light (B5, 5 μmol m^{-2} s^{-1}), middle blue light (B20, 20 μmol m^{-2} s^{-1}), and high blue light (B50 and B100, 50 and 100 μmol m^{-2} s^{-1}) for 6 d were photographed (a), and hypocotyl lengths were measured (b). Bars, 5 mm. Data in (b) are mean ± SD ($n > 23$) (Student's *t*-test; ***, $P < 0.001$). *cry*, cryptochrome; Dk, darkness.

the endogenous CRY1 in the protein extracts prepared from *FGFP-BIC1-OX* seedlings exposed to blue light, but not from those adapted in the dark. Taken together, these results demonstrate that CRY1 physically interacts with BIC1 in a blue-light-dependent manner.

BIC1 inhibits CRY1 dimerization/oligomerization in blue light

To explore whether BIC–CRY1 interaction might affect CRY1 dimerization/oligomerization, we first performed split-LUC

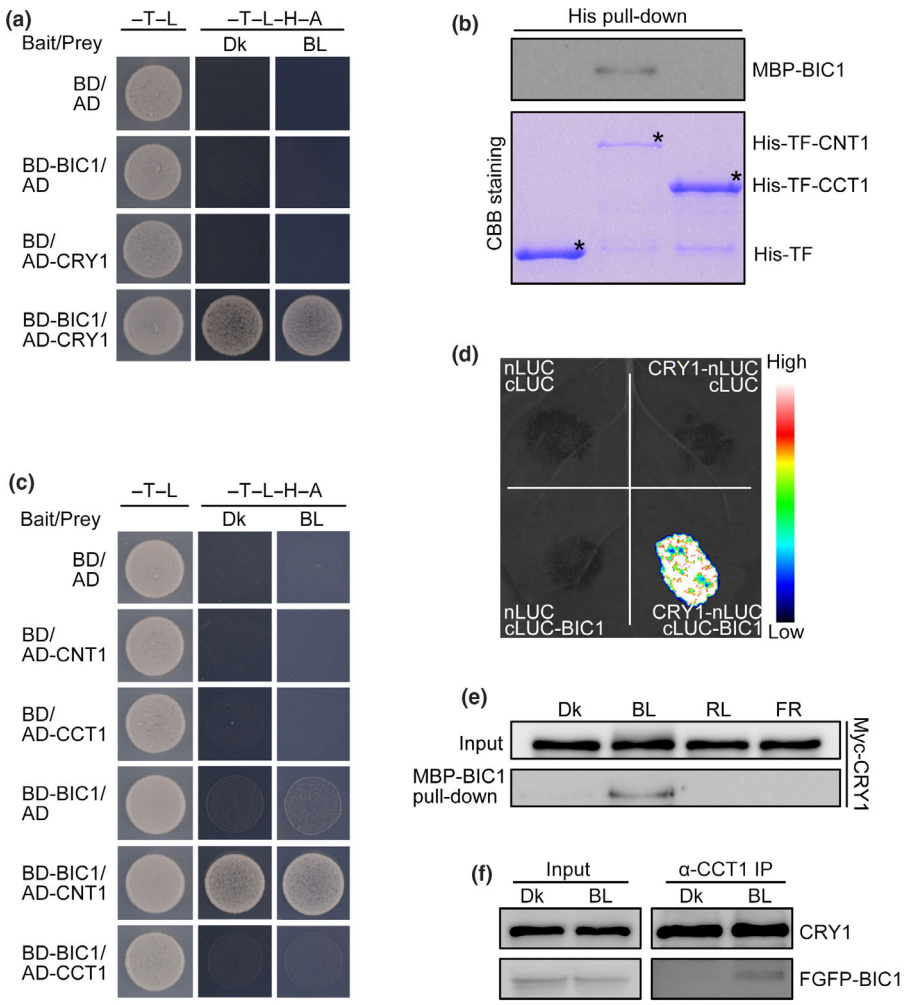

Fig. 6 Cryptochrome 1 (CRY1) physically interacts with BIC1 in a blue-light-dependent manner. (a, b) Yeast two-hybrid assay showing interactions of BIC1 with (a) CRY1 and (b) CNT1. Yeast cells co-expressing the indicated combinations of constructs were grown on SD−T−L or SD−T−L−H−A medium in continuous darkness or blue light (30 μmol m^{-2} s^{-1}). AD, GAL4 DNA-activation domain; BD, GAL4 DNA-binding domain; BL, blue light; Dk, darkness. (c) His pull-down assay showing interaction of CNT1 with BIC1. His-TF, His-TF-CNT1 and -CCT1 served as baits, and maltose-binding protein (MBP)-BIC1 served as prey. Asterisks denote input proteins of His-TF, His-TF-CNT1 and His-TF-CCT1 stained with Coomassie brilliant blue (CBB). (d) Split-LUC assay showing interaction of CRY1 with BIC1 in tobacco leaves. CRY1-nLUC was expressed with cLUC-BIC1. The empty vectors were used as negative controls. (e) Semi-*in-vivo* MBP pull-down assay showing blue light-specific interaction of CRY1 with BIC1. MBP-BIC1 served as bait. Prey were the protein extracts prepared from *Myc-CRY1-OX Arabidopsis* seedlings that were dark adapted and exposed to blue light (BL, 50 μmol m^{-2} s^{-1}), red light (RL, 50 μmol m^{-2} s^{-1}), or far-red light (FR, 10 μmol m^{-2} s^{-1}) for 1 h. (f) Co-immunoprecipitation (Co-IP) assay showing blue-light-dependent interaction of CRY1 with BIC1 in *Arabidopsis*. Six-day-old etiolated *FGFP-BIC1-OX* seedlings were kept in darkness or exposed to blue light (30 μmol m^{-2} s^{-1}) for 15 min, followed by immunoprecipitation (IP) with anti-CCT1 antiserum. The IP (CRY1) and Co-IP signals (BIC1) were detected by western blots probed with anti-CCT1 antiserum and anti-Flag antibody, respectively.

assays with tobacco leaves transiently co-expressing CRY1-nLUC and cLUC-CRY1 that were adapted in the dark or exposed to blue light. The results showed that the luciferase activity was detected when CRY1-nLUC was co-expressed with cLUC-CRY1 in blue-light-exposed tobacco leaf, but not in the dark (Fig. S6). We then performed split-LUC assays again with tobacco leaves expressing CRY1-nLUC and cLUC-CRY1 plus BIC1-YFP or GUS-YFP control protein that were adapted in the dark or exposed to blue light. The results demonstrated that significantly less luciferase activity was detected in tobacco leaves co-expressing CRY1-nLUC and cLUC-CRY1 in the presence of BIC1-YFP than in the presence of GUS-YFP under blue light, and that luciferase activity was hardly detected when CRY1-nLUC was co-expressed with cLUC-CRY1 in darkness regardless of the presence of BIC1-YFP (Fig. 7a–c). Western blot analyses indicated that CRY1-nLUC, cLUC-CRY1, BIC1-YFP and GUS-YFP were basically expressed at similar levels in the samples tested (Fig. 7d). Next, we performed Co-IP assays to further determine the capacity of interaction between Myc-CRY1 and the endogenous CRY1 with the protein extracts prepared from dark-grown *Myc-CRY1-OX*/WT seedlings with or without blue light irradiation in the presence of MBP-BIC1 fusion protein or MBP. The results demonstrated that, in the dark, Myc-CRY1 hardly interacted with CRY1 in the presence of either MBP or

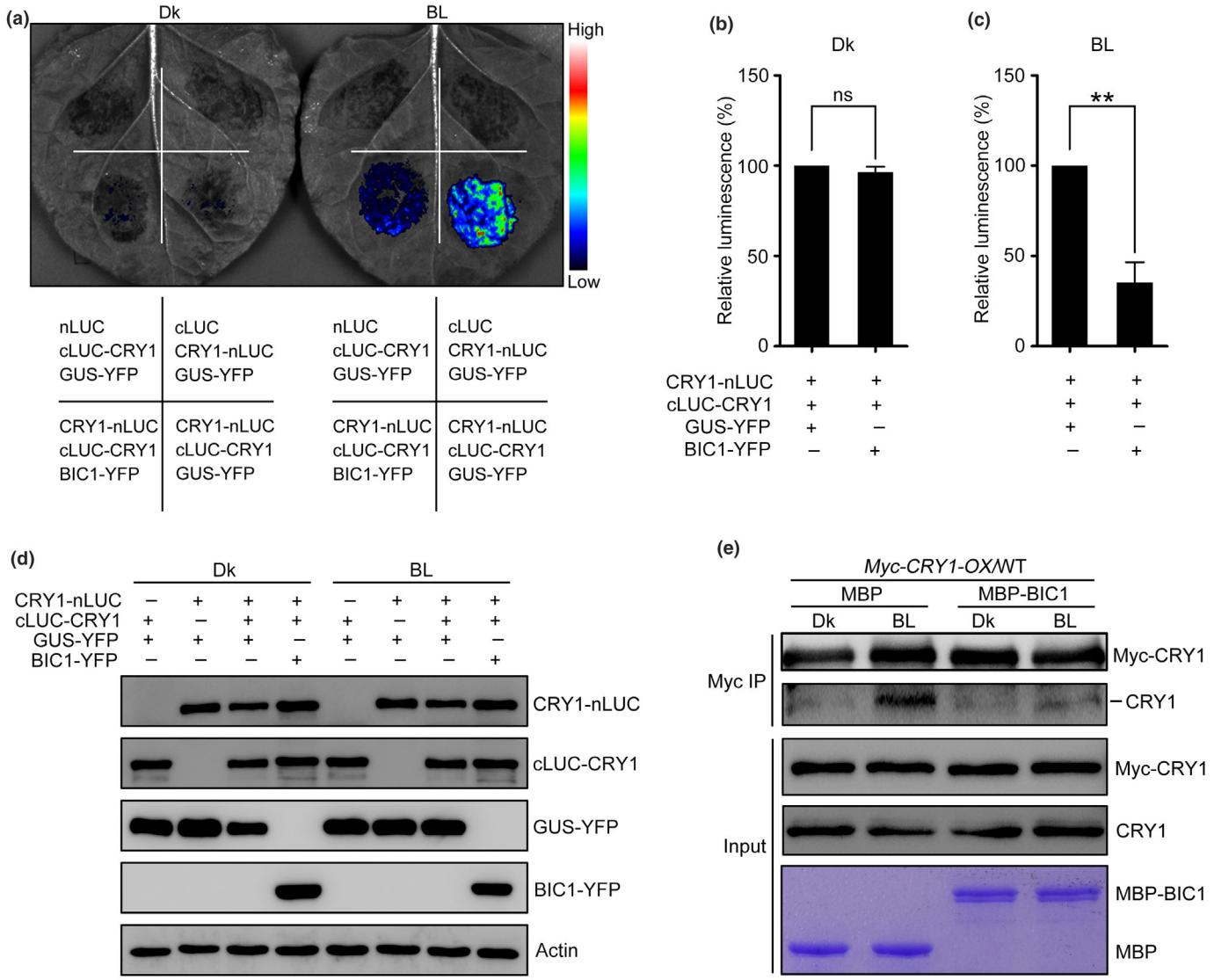

Fig. 7 BIC1 inhibits cryptochrome 1 (CRY1) dimerization/oligomerization in blue light. (a–d) Split-LUC assays showing BIC1 inhibition of blue-light-induced CRY1 dimerization/oligomerization in tobacco leaves. BIC1-yellow fluorescent protein (YFP) and β-glucuronidase (GUS)-YFP (negative control) were transiently co-expressed together with CRY1-nLUC and cLUC-CRY1, respectively. (a) Tobacco leaves, adapted in darkness for 1 d, were exposed to blue light (50 μmol m^{-2} s^{-1}) or kept in darkness for 30 min, and then luminescence intensities were detected. Dk, darkness; BL, blue light. (b, c) Luminescence intensities of different combinations of three biological replicates were quantified; ns, no significant differences (Student's *t*-test; **, $P < 0.01$). (d) Protein levels of different combinations in (a) were detected by western blots probed with anti-GFP antibody and anti-CCT1 antiserum, respectively. Actin served as loading control. (e) Co-immunoprecipitation (Co-IP) assay showing BIC1 inhibition of blue light-induced CRY1 dimerization/oligomerization. The extracts from dark-adapted *Myc-CRY1-OX*/WT *Arabidopsis* seedlings exposed to blue light (30 μmol m^{-2} s^{-1}) or kept in darkness for 20 min were immunoprecipitated with anti-Myc agarose beads in the presence of maltose-binding protein (MBP) or MBP-BIC1, respectively. The immunoprecipitation (IP; Myc-CRY1) and Co-IP signals (CRY1) were detected by western blots probed with anti-Myc antibody and anti-CCT1 antiserum, respectively.

MBP-BIC1, whereas, upon blue light irradiation, Myc-CRY1 interacted with CRY1 much more strongly in the presence of MBP than in the presence of MBP-BIC1 (Fig. 7e). Taken together, these results indicate that BIC1 represses CRY1 dimerization/oligomerization in blue light.

BIC1 inhibits high blue-light-induced CRY1 degradation

To determine whether BIC1-inhibited dimerization/oligomerization of CRY1 might affect HBL-induced degradation of CRY1, we first analyzed the CRY1 degradation pattern in *bic1 bic2* mutant and *FGFP-BIC1-OX* seedlings exposed to HBL for different lengths of time. The results showed that 15–60 min HBL irradiation clearly induced CRY1 phosphorylation and degradation in *bic1 bic2* mutant, but not in *FGFP-BIC1-OX* seedlings (Fig. 8a,b). We then analyzed CRY1 protein levels in *bic1 bic2* mutant and *FGFP-BIC1-OX* seedlings grown in continuous darkness or under low or high blue light. We found that CRY1 accumulated at similar levels in dark-grown WT, *bic1 bic2*, and *FGFP-BIC1-OX* plants (Fig. 8c). However, though much less

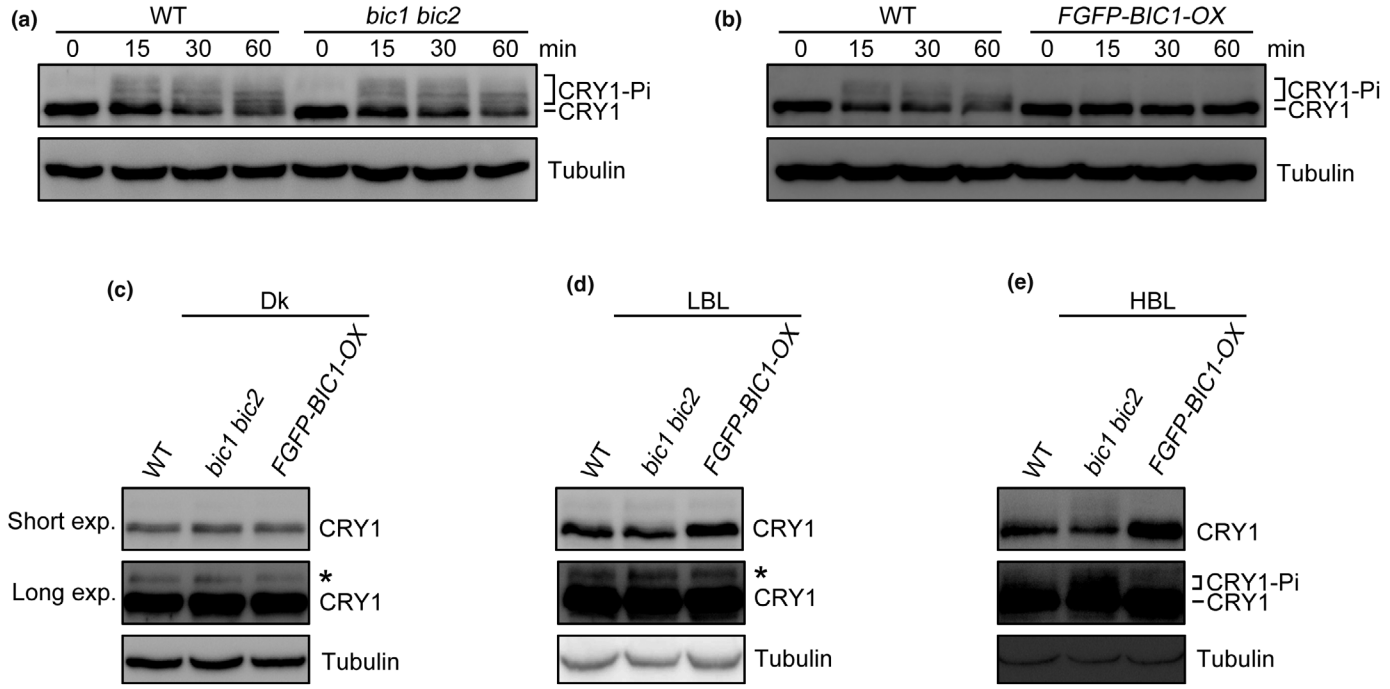

Fig. 8 BIC1 inhibits high blue-light (HBL)-induced cryptochrome 1 (CRY1) degradation. (a) Western blot assay showing similar HBL-induced CRY1 degradation pattern in wild-type (WT) and *bic1 bic2 Arabidopsis* seedlings. Six-day-old etiolated WT and *bic1 bic2* mutant seedlings were exposed to HBL (100 μmol m^{-2} s^{-1}) for the time indicated. CRY1-Pi, phosphorylated CRY1. (b) Western blot assay showing inhibited HBL-induced CRY1 degradation in *FGFP-BIC1-OX Arabidopsis* seedlings. Six-day-old etiolated WT and *FGFP-BIC1-OX* seedlings were exposed to HBL (100 μmol m^{-2} s^{-1}) for the time indicated. (c–e) Western blot assays showing CRY1 degradation patterns in WT, *bic1 bic2* mutant, and *FGFP-BIC1-OX Arabidopsis* seedlings grown in (c) darkness (Dk), (d) continuous low blue light (LBL, 5 μmol m^{-2} s^{-1}), and (e) high blue light (HBL, 50 μmol m^{-2} s^{-1}) for 6 d. Asterisks indicate a band nonspecifically recognized by anti-CCT1 antiserum. Short exp., short exposure time; Long exp., long exposure time.

CRY1 protein accumulated in *bic1 bic2* than in WT plants grown in HBL (Fig. 8e), much more CRY1 accumulated in *FGFP-BIC1-OX* than in WT plants grown in either low or high blue light (Fig. 8d,e). Moreover, we found that more phosphorylated CRY1 protein accumulated in *bic1 bic2* than in WT, whereas less phosphorylated CRY1 was accumulated in *FGFP-BIC1-OX* than in WT plants in high blue light but not in the dark or low blue light (Fig. 8c–e). These results suggest that BIC1-inhibited CRY1 dimerization/oligomerization may lead to the repression of CRY1 phosphorylation and degradation in high blue light.

Discussion

CRY1 undergoes the 26S proteasome-dependent degradation in response to high blue light

The previous studies have reported that *Arabidopsis* red/far-red light photoreceptors phyB and phyA and blue light photoreceptors CRY2 and PHOT1 are degraded upon red light irradiation and blue light irradiation, respectively (Lin *et al.*, 1998; Seo *et al.*, 2004; Jang *et al.*, 2010; Roberts *et al.*, 2011). As the key blue light photoreceptor promoting photomorphogenesis (Ahmad & Cashmore, 1993; Lin *et al.*, 1996), CRY1 is shown to be stable in relatively low blue light (Lin *et al.*, 1998). Whether high blue light may affect CRY1 stability remains unknown. In this study, we found that CRY1 protein is degraded upon high blue light irradiation (Fig. 1b,c), but not upon high red or far-red light irradiation (Fig. 1d). It is well established that CRY2 is phosphorylated immediately upon even low blue light (Yu *et al.*, 2007a). However, compared with CRY2, CRY1 is phosphorylated in response to high blue light (100 μmol m^{-2} s^{-1}) but is hardly phosphorylated in response to low (1 μmol m^{-2} s^{-1}) or medium (10 μmol m^{-2} s^{-1}) blue light (Fig. 1a,b). We show, by cell-free degradation assay, that only the phosphorylated CRY1 induced by high blue light largely undergoes degradation (Fig. 3d), which is consistent with the previous demonstrations that only the phosphorylated CRY2 and phyA, but not the nonphosphorylated CRY2 and phyA, are predominantly subject to degradation in response to blue and red light, respectively (Saijo *et al.*, 2008; Liu *et al.*, 2017).

We show by protein expression studies using the 26S proteasome inhibitor MG132 that high blue-light-induced CRY1 degradation proceeds through the 26S proteasome pathway (Fig. 2a,b). This conclusion is further supported by cell-free degradation assays showing that only in the presence of ATP is CRY1 degraded in extracts from WT seedlings exposed to HBL (Fig. 2c). We also determined CRY1 ubiquitination *in vivo* upon high blue light irradiation by first performing an IP assay with *Arabidopsis* seedlings overexpressing Myc-CRY1 either adapted in darkness or exposed to high blue light, finding that the Myc-CRY1 was ubiquitinated under high blue light only (Fig. 2d).

Further, a TUBE2 assay confirmed that Myc-CRY1 was highly polyubiquitinated in high blue light but not in the dark or low blue light (Fig. 2e). It is known that, before CRY2, phyA, and PIFs are ubiquitinated and degraded through the 26S proteasome in blue or red light, they must first of all undergo phosphorylation (Al-Sady *et al.*, 2006; Yu *et al.*, 2007a; Saijo *et al.*, 2008; Shen *et al.*, 2008; Liu *et al.*, 2017). Based on the previous studies and our results, we propose that CRY1 may also first of all be phosphorylated and then subject to ubiquitination and degradation.

COP1 and LRBs are involved in mediating high blue-light-induced CRY1 ubiquitination and degradation

COP1 is an E3 ubiquitin ligase that ubiquitinates a number of substrates, such as HY5, CO, LAF1, HFR1, and phyA (Osterlund *et al.*, 2000; Seo *et al.*, 2003, 2004; Duek *et al.*, 2004; Jang *et al.*, 2005, 2008; Liu *et al.*, 2008). As CRY1 physically interacts with COP1 (Wang *et al.*, 2001; Yang *et al.*, 2001), we explored whether COP1 might be involved in mediating high blue-light-induced ubiquitination of CRY1. We found that CRY1 was hardly ubiquitinated in *cop1* mutant in high blue light (Fig. 3c). We tried very hard to perform an *in vitro* ubiquitination assay to determine ubiquitination of CRY1 by COP1 using MBP-COP1, E1, E2, and ubiquitin, but failed. This might be due to lack of photoreceptor activity of CRY1 purified from *E. coli*, which is essential for blue-light-induced phosphorylation and degradation of CRY1 (Fig. 3d). It is interesting to note that CRY1, CRY2, and some other substrates of COP1 possess a kind of conserved VP peptide motif, through which COP1 interacts with (Holm *et al.*, 2001; Lau *et al.*, 2019; Ponnu *et al.*, 2019). On the one hand, CRY1 and CRY2 may inhibit the E3 ubiquitin ligase activity of COP1 on its substrates by physically displacing the substrates from COP1 (Lau *et al.*, 2019; Ponnu *et al.*, 2019). On the other hand, both CRY1 and CRY2 could be the potential substrates of COP1. Given the establishment that the outcome of the interactions of both CRY1 and CRY2 with COP1 and SPA1 is suppression of COP1 E3 ligase activity, our results suggest that COP1-mediated high blue-light-induced CRY1 ubiquitination and degradation might be a negative feedback for maintaining E3 activity of COP1 (Lian *et al.*, 2011; Liu *et al.*, 2011; Zuo *et al.*, 2011). In view of these results, it is of interest to postulate that COP1 may maintain the homeostasis of multiple photoreceptors to optimize light signaling and plant growth in different light conditions.

The E3 ubiquitin ligases LRBs were first identified as negative regulators of red light signaling, which mediate phyB/D and PIF3 degradation in red light (Christians *et al.*, 2012; Ni *et al.*, 2014). LRBs were not considered to be involved in blue light signaling as *lrb12* double mutant shows no significant hypocotyl phenotype in blue light (Christians *et al.*, 2012). However, the most recent study has shown that LRBs also mediate CRY2 ubiquitination and degradation in blue light (Chen *et al.*, 2021). Here, we also show that LRBs interact with and mediate CRY1 degradation in high blue light (Fig. 4), which gives an insight into how LRBs may desensitize blue light signaling to prevent plants from undergoing too much enhanced photomorphogenesis. As the endogenous CRY1 level may not be high enough, although COP1 and LRBs act additively in mediating the degradation of CRY1 (Fig. S5) and CRY2 (Chen *et al.*, 2021), COP1-mediated polyubiquitinated CRY1 in the *lrb123* mutant may be too little to be detected (Fig. 4g). The present study indicates that the homeostasis of CRY1 is likely under strict and complicated proteolysis control in high blue light by more than one E3 ubiquitin ligase. In mammals, two F-box E3 ubiquitin ligases, FBXL3 and FBXL21, are shown to mediate CRY1 ubiquitination and degradation and regulate the period of the circadian clock (Busino *et al.*, 2007; Siepka *et al.*, 2007; Yoo *et al.*, 2013). These studies, together with our results, demonstrate that different E3 ligases-coordinated degradation of the CRY photoreceptor may be evolutionarily conserved from plants to mammals, which constitutes a similar mechanism to 'slow down' blue light signaling to that found in phytochrome photoreceptors-mediated red light signaling (Seo *et al.*, 2004; Jang *et al.*, 2010).

BIC1 interacts with CRY1 to inhibit its dimerization/oligomerization and degradation in response to high blue light

BIC1 and BIC2 were first identified as the negative regulators of CRY2 signaling, and it is shown that CRY2 physically interacts with BIC1 (Q. Wang *et al.*, 2016) and BIC1 binds to CRY1 *in vitro* (Ma *et al.*, 2020b). Overexpression of BIC1 leads to a long hypocotyl phenotype similar to *cry1 cry2* double mutant under blue light. In this study, we carried out a series of biochemical assays to evaluate the direct interaction of CRY1 with BIC1, including yeast two-hybrid, *in vitro*, and semi-*in-vivo* pull-down, split-LUC, and Co-IP. The combined results from these assays demonstrate that CRY1 interacts with BIC1 through its N-terminus in a blue-light-dependent manner (Fig. 6). Given the demonstrations that CRY1 N-terminus mediates dimerization/oligomerization of CRY1 (Sang *et al.*, 2005) and that BIC1 inhibits dimerization/oligomerization of CRY2 (Q. Wang *et al.*, 2016; Ma *et al.*, 2020b), we explored whether BIC1 would regulate CRY1 dimerization/oligomerization. Our results suggest that BIC1 represses CRY1 dimerization/oligomerization in a blue-light-dependent manner (Fig. 7). Given that BIC1 represses CRY2 degradation dependent on its dimerization/oligomerization (Q. Wang *et al.*, 2016; Ma *et al.*, 2020b; Shao *et al.*, 2020), we also evaluated the involvement of BIC1/2 in regulating high blue-light-induced degradation of CRY1 using *bic1 bic2* mutant and *FGFP-BIC1-OX* seedlings. Our results suggest that BIC1/2 mainly inhibit CRY1 degradation under high blue light (Fig. 8b, e). Interestingly, analyses of CRY1 phosphorylation in *bic1 bic2* mutant and *FGFP-BIC1-OX* plants under high blue light indicate that BIC1 acts to inhibit the phosphorylation of CRY1 in high blue light (Fig. 8e). Taken together, our results suggest that BIC1 interacts with CRY1 to repress its dimerization/oligomerization, phosphorylation, and degradation in high blue light.

In sum, this study suggests that the homeostasis of CRY1 is coordinated by COP1, LRBs, and BICs under high blue light. This conclusion is further supported by our qRT-PCR assays

with *lrb123*, *cop1* and *bic1 bic2* mutants indicating that CRY1 activity was enhanced in these mutants, as the expression of CRY1-inhibited genes promoting cell elongation, such as *XTH17*, *IAA29* and *PRE1* (Mao *et al.*, 2021), was upregulated by COP1, LRBs, and BICs (Fig. S7). Previous studies have demonstrated that *Arabidopsis* COP1 preferentially degrades the phosphorylated CO, HFR1 and PIF1 (Duek *et al.*, 2004; Sarid-Krebs *et al.*, 2015; Zhu *et al.*, 2015) and that COP1 associates preferentially with the phosphorylated CRY1 and CRY2 (Holtkotte *et al.*, 2017). Moreover, it has been shown that, in mouse, AMPK-mediated CRY1 phosphorylation stimulates the binding of FBXL3 to CRY1, targeting it for ubiquitin-mediated degradation (Lamia *et al.*, 2009). Based on these studies and our results, we postulate that COP1 and LRBs may preferentially interact with the phosphorylated CRY1 to promote its ubiquitination and degradation, leading to a decrease in CRY1 levels (Fig. S8) and subsequent inhibition of CRY1 signaling and photomorphogenesis. On the other hand, BIC1 interacts with CRY1 to repress its dimerization/oligomerization. The monomerized CRY1 may not be able to undergo blue-light-induced phosphorylation, and thus may have no or less capacity to interact with COP1 or LRBs, leading to the inhibition of ubiquitination and degradation of CRY1 and to accumulation of a pool of CRY1 monomers. As soon as blue light intensity decreases, these CRY1 monomers may dimerize/oligomerize promptly to mediate blue light signaling and promote photomorphogenesis. Therefore, the coordination of CRY1 homeostasis by COP1, LRBs and BICs constitutes a strictly regulated mechanism that efficiently and accurately controls CRY1 levels (Fig. S8) and optimizes plant photomorphogenesis according to the blue light intensities. This mechanism may allow plants to avoid excessively inhibited hypocotyl/stem elongation under high light and to enhance their competitiveness with the neighboring plants for capturing sunlight to promote their growth and development when light intensity becomes normal.

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Author contributions

LM, WW and H-QY designed the experiments. LM, JZ, GY, PX, XC, SD, FX, LJ, SZ, XW, YL, HC, ZM, TG and SK performed the experiments. LM, WW and H-QY wrote the paper.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 The degradation of CRY1 in high blue light.

Fig. S2 IP assay showing CRY1 ubiquitination in high blue light.

Fig. S3 CBB staining showing purified MBP and MBP-COP1.

Fig. S4 Split-LUC assay showing interaction of CRY1 with LRB3 in tobacco.

Fig. S5 Western blot assays showing that COP1 and LRBs act additively in regulating CRY1 degradation in high blue light.

Fig. S6 Split-LUC assay showing blue light-induced CRY1 dimerization/oligomerization in tobacco.

Fig. S7 qRT-PCR analyses showing up-regulation of CRY1-inhibited genes promoting cell elongation by COP1, LRBs and BICs.

Fig. S8 A model illustrating how COP1 and LRBs mediate high blue light-induced CRY1 degradation.

Table S1 All the primers (Sequences of oligos) used in this study.

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