Arabidopsis B-box transcription factors BBX20-22 promote UVR8 photoreceptor-mediated UV-B responses

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SUMMARY
Plants undergo photomorphogenic development in the presence of light. Photomorphogenesis is repressed by the E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1), which binds to substrates through their valine–proline (VP) motifs. The UV RESISTANCE LOCUS 8 (UVR8) photoreceptor senses UV-B and inhibits COP1 through the cooperative binding of its own VP motif and photosensing core to COP1, thereby preventing COP1 binding to substrates, including the basic leucine zipper (bZIP) transcriptional regulator ELONGATED HYOCOTYL 5 (HY5). As a key promoter of visible light and UV-B photomorphogenesis, HY5 requires coregulators for its function. The B-box family transcription factors BBX20–BBX22 were recently described as HY5 rate-limiting coactivators under red light, but their role in UVR8 signaling was unknown. Here we describe a hypermorphic bbx21-3D mutant with enhanced photomorphogenesis, carrying a proline-to-leucine mutation at position 314 in the VP motif that impairs the interaction with and regulation by COP1. We show that BBX21 and BBX22 are UVR8-dependently stabilized after UV-B exposure, which is counteracted by a repressor induced by HY5/BBX activity. bbx20 bbx21 bbx22 mutants under UV-B are impaired in hypocotyl growth inhibition, photoprotective pigment accumulation and the expression of several HY5-dependent genes under continuous UV-B, but the immediate induction of marker genes after exposure to UV-B remains surprisingly rather unaffected. We conclude that BBX20–BBX22 contribute to HY5 activity in a subset of UV-B responses, but that additional, presently unknown, coactivators for HY5 are functional in early UVR8 signaling.

Keywords: UVR8, BBX20–BBX22, HY5, COP1, UV-B, photomorphogenesis, signal transduction, Arabidopsis thaliana.

INTRODUCTION
Plants integrate light signals through a number of photoreceptor-initiated signaling pathways (Demarsy et al., 2018; Galvao & Fankhauser, 2015; Kami et al., 2010; Podolec et al., 2021a), which enables optimized growth in changing environments. When exposed to light, including ultraviolet-B (UV-B), seedlings undergo a developmental program termed photomorphogenesis, characterized by features such as hypocotyl growth inhibition, anthocyanin and flavonoid pigment accumulation, and cotyledon expansion (Galvao & Fankhauser, 2015; Kami et al., 2010). In Arabidopsis thaliana, UV-B radiation (280–315 nm) is sensed by the photoreceptor UV RESISTANCE LOCUS 8 (UVR8) that induces a signaling pathway resulting in UV-B acclimation, and thus enhanced UV-B tolerance (Favory et al., 2009; Kliebenstein et al., 2002; Podolec et al., 2021a; Rai et al., 2021; Rizzini et al., 2011). UVR8 is homodimeric in its inactive ground state, but monomerizes and becomes activated upon UV-B photon reception (Rizzini et al., 2011). A valine–proline (VP) motif in activated UVR8 represses the activity of the E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) by competitively interacting with the binding site of COP1 substrates (Cloix et al., 2012; Favory et al., 2009; Lau et al., 2019; Yin et al., 2015).

Prominent among COP1 substrates is ELONGATED HYOCOTYL 5 (HY5), a key photomorphogenesis-promoting basic leucine zipper (bZIP) transcriptional regulator that induces the expression of many UV-B-activated genes (Brown et al., 2005; Oravecz et al., 2006; Ulm et al., 2009; Kliebenstein et al., 2002; Podolec et al., 2021a; Rai et al., 2021; Rizzini et al., 2011).
Upon UV-B exposure, HY5 is transcriptionally induced and the HY5 protein is stabilized (Brown et al., 2005; Favory et al., 2009; Huang et al., 2013; Ulm et al., 2004). However, the HY5 mode of action in UV-B signaling is not fully understood as it lacks a functional transcriptional activation domain (Ang et al., 1998; Burko et al., 2020; Stracke et al., 2010). Nevertheless, HY5 binds the promoters of many UV-B-induced genes and is required for their UV-B-induced expression (Binkert et al., 2014; Brown et al., 2005; Brown & Jenkins, 2008; Oravecz et al., 2006; Stracke et al., 2010; Ulm et al., 2004). Thereby, HY5 promotes UV-B responses such as hypocotyl growth inhibition, anthocyanin and flavonoid pigment accumulation, and ultimately stress acclimation (Brown et al., 2005; Favory et al., 2009; Huang et al., 2012; Oravecz et al., 2006). HY5-regulated genes include those encoding REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 (RUP1) and RUP2, which are crucial negative feedback regulators facilitating UVR8 ground state reversion by redimerization (Gruber et al., 2010; Heijde & Ulm, 2013). In agreement, rup1 rup2 display strong UV-B hypersensitivity, further supported by the phenotype of the UVR8<sup>S101D</sup> mutant variant that is constitutively monomeric <em>in vivo</em> and underlies the enhanced UV-B photomorphogenic phenotype of the <em>uvr8-17D</em> mutant allele (Podolec et al., 2021b).

Recently, the transactivation domain-containing, B-box zinc-finger transcription factors BBX20–BBX22 were characterized as coregulators of HY5 under red light, interacting with HY5 and providing it with transcriptional activity (Bursch et al., 2020). BBX20–BBX22, as well as BBX23, another member of class IV of the B-box family, have been described as positive regulators of visible light photomorphogenesis (Chang et al., 2008; Chang et al., 2011; Datta et al., 2007; Datta et al., 2008; Fan et al., 2012; Wei et al., 2016; Zhang et al., 2017), whereas two other members of the subfamily, namely BBX24 and BBX25, play a repressive role (Crocco et al., 2015; Gangappa et al., 2013; Indorf et al., 2007; Jiang et al., 2012; Yan et al., 2011). In darkness, BBX20–BBX25 are degraded via COP1, whereas they are stabilized in visible light (Chang et al., 2011; Fan et al., 2012; Gangappa et al., 2013; Huang et al., 2022; Indorf et al., 2007; Jiang et al., 2012; Job et al., 2018; Xu et al., 2016; Zhang et al., 2017). BBX21, BBX24 and BBX25 contain experimentally defined COP1-interacting VP motifs, similar to other COP1 targets (Bursch et al., 2020; Holm et al., 2001; Lau et al., 2019; Yan et al., 2011). Moreover, HY5 has been suggested to repress BBX22 accumulation in an unknown manner (Chang et al., 2011). Although the negative regulator BBX24 has been implicated in UV-B signaling by suppressing HY5 activity (Jiang et al., 2012), it is not known whether the class-IV BBX family positive regulators play a role in UVR8-dependent UV-B signaling in Arabidopsis. Functional homologs of class-IV BBX positive regulators in <em>Malus × domestica</em> (apple) have been linked to anthocyanin biosynthesis under UV-B (Bai et al., 2014; Fang et al., 2019), but their regulation and role in UVR8 signaling remain unknown. Recently, class-IV BBX homologs in <em>Solanum lycopersicum</em> (tomato) have been shown to form a module with HY5 to regulate HY5 induction under UV-B (Yang et al., 2022).

In this work, we identified and characterized a gain-of-function mutant of BBX21, namely <em>bbx21-3D</em>, that contains a hypermorphic proline-to-leucine mutation at position 314 (P314L) in its VP motif. Phenotypic analyses suggested that the enhanced visible light and UV-B photomorphogenesis of this mutant is linked to the increased activity of BBX21<sup>P314L</sup> and impaired negative regulation by COP1. We further explored the role of the BBX20–BBX22 subfamily in UV-B photomorphogenesis and found that BBX proteins are regulated at both transcriptional and post-translational levels under UV-B. We uncovered a HY5/BBX-mediated negative feedback mechanism affecting BBX protein stability. Moreover, a <em>bbx20 bx21 bx22</em> mutant showed a lack of flavonoid and anthocyanin accumulation, impaired inhibition of hypocotyl elongation and reduced marker gene expression under continuous UV-B, but not upon short-term exposure to UV-B. Collectively, our work reveals that BBX20–BBX22 play an important role in promoting HY5-mediated UV-B responses.

**RESULTS**

<em>bbx21-3D</em> is a gain-of-function mutant of BBX21 with an enhanced photomorphogenic response

In a hypocotyl length-based ethyl methanesulfonate (EMS) mutant screen for altered UV-B photomorphogenesis (Podolec et al., 2021b), we discovered a mutant with an enhanced photomorphogenic response under white light with supplemental UV-B (mutant <em>bbx21-3D</em> in Figure 1a,b). However, the manifestation of this phenotype under white light in the absence of supplemental UV-B indicated that it was not specific to UV-B (Figure 1a,b). A causative transition mutation in <em>AT1G75540</em> (BBX21) was identified by whole-genome sequencing of a bulk segregant population; this mutation alters the VP motif of BBX21, namely with a hypermorphic proline-to-leucine mutation at position 314, resulting in a <em>bbx21<sup>P314L</sup></em> variant (Figure S1). The mutant allele showed a dominant hypocotyl phenotype (Figure 1c), and hence a hypermorphic <em>bbx21-3D</em> phenotype was named <em>bbx21-3D</em>. <em>BBX21</em> mRNA levels were not elevated in <em>bbx21-3D</em> compared with the wild type (Figure S2). To verify that the enhanced photomorphogenic phenotype of <em>bbx21-3D</em> was caused by the <em>BBX21<sup>P314L</sup></em> mutation, we used CRISPR/Cas9 to knockout <em>BBX21</em> in the <em>bbx21-3D</em> background. Unlike <em>bbx21-3D</em>, a <em>BBX21</em> knockout allele in <em>bbx21-3D</em> showed an elongated hypocotyl phenotype comparable with the <em>bbx21-1</em> null allele as well as a <em>bbx21<sup>KD</sup></em> CRISPR/Cas9 knockout allele generated in the Col-0 wild-type background (Figure S1). These data support...

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that BBX21P314L is the causative mutation for the hypermorphic phenotype of bbx21-3D. To further confirm that the bbx21-3D phenotype is caused by the loss of a functional VP motif, we used CRISPR/Cas9 to mutate the C-terminus of BBX21 in a wild-type background (Figure S1). Indeed, a premature stop codon causing a C-terminal deletion removing the BBX21 VP motif mimicked the short-hypocotyl phenotype of bbx21-3D (Figure S1), supporting the hypothesis that the VP motif is crucial to negatively regulate BBX21 activity.

Whereas bbx21-3D showed an enhanced photomorphogenic response with and without UV-B (+UV-B and –UV-B), the bbx21-1 null mutant exhibited slightly reduced hypocotyl growth inhibition in the presence of UV-B.
(Figure 1a,b). Consistently, anthocyanin accumulation was enhanced in bbx21-3D in both -UV-B and +UV-B conditions, compared with the wild type, but was reduced relative to the wild type under -UV-B conditions in bbx21-1 (Figure 1d). UV-B-induced accumulation of CHALCONE SYNTHASE (CHS), a key enzyme for phenylpropanoid biosynthesis, was also enhanced in bbx21-3D relative to the wild type, whereas it accumulated to lower levels in the bbx21-1 null mutant (Figure 1e). In bbx21-3D, UV-B-induced HY5 activation was comparable with that of the wild type, indicating that the bbx21-3D enhanced photomorphogenic phenotype was not associated with increased HY5 transcript levels (Figure S2). However, HY5 protein accumulation was increased in bbx21-3D compared with the wild type under white light and in response to UV-B (Figure 1f). BBX21P314L thus likely promotes HY5 accumulation at the post-transcriptional level. Moreover, whereas the UV-B-induced expression of RUP2 seemed unaffected by BBX21 (Figure S2), several other UV-B marker genes such as EARLY LIGHT-INDUCIBLE PROTEIN 2 (ELIP2), CHS and FLAVANONE 3-HYDROXYLASE (F3H) showed lower gene activation in bbx21-1 compared with in bbx21-3D (Figure S2). Ultimately, bbx21-3D showed constitutive acclimation to UV-B, as its photosynthetic capacity was less affected after UV-B stress compared with the wild type (Figure 1g). Altogether, these data support a positive regulatory role of BBX21 in the UVR8 signaling pathway.

As BBX21 promotes photomorphogenesis under visible light (Datta et al., 2007; Xu et al., 2016), we tested the bbx21-3D mutant in darkness and in red and blue light. In darkness, bbx21-1 and bbx21-3D seedling phenotypes were comparable to that of the wild type (Figure S3). However, under both red and blue light, bbx21-1 indeed showed a reduced photomorphogenetic phenotype relative to the wild type, whereas bbx21-3D showed an enhanced photomorphogenetic phenotype, including hypocotyl growth inhibition and anthocyanin accumulation (Figure S3). Overall, the contrasting phenotypes of bbx21-3D and bbx21-1 support a positive regulatory role of BBX21 in photomorphogenenic responses to both visible light and UV-B.

**BBX21P314L function requires HY5 and shows increased activity in both a COP1-dependent and a COP1-independent manner**

The VP motif of BBX21 is located close to the C-terminus of the protein (residues 313/314 of the 331-amino-acid protein; Figure S1). This motif presumably mediates the interaction with COP1, leading to polyubiquitination and the proteasomal degradation of BBX21 during skotomorphogenesis (Bursch et al., 2020; Job et al., 2018; Lau et al., 2019; Xu et al., 2016). In agreement, mutation of the VP motif to Ala-Ala (AA) increased the post-translational stability of BBX21 (Bursch et al., 2020), C-terminal deletion including the VP motif mimicked the bbx21-3D phenotype (Figure S1) and the BBX21P314L mutation resulted in hypermorphogenic phenotypes in all light conditions tested (Figures 1 and S3).

We thus tested the interactions of BBX21 and BBX21P314L with HY5 and the C-terminal WD40-domain of COP1 (COP1C340) in yeast two-hybrid (Y2H) assays. Whereas the interaction of BBX21 with COP1C340 was clearly detectable, BBX21P314L interaction with COP1C340 was impaired (Figure 2a). Interestingly, by contrast, the interaction between BBX21P314L and HY5 was strongly enhanced compared with the BBX21–HY5 interaction (Figure 2a). The intrinsic transcriptional activities (‘auto-activation’) of BBX21P314L and BBX21 fused to the LexA binding domain were comparable in the heterologous yeast system (Figure 2b), making it unlikely that the bbx21-3D phenotype is linked to the intrinsically enhanced transcriptional activity of BBX21P314L. Nonetheless, the loss of interaction with COP1, and thus putative stabilization of BBX21P314L, and the enhanced interaction between BBX21P314L and HY5 provide mutually non-exclusive mechanistic explanations for the enhanced photomorphogenic phenotype of bbx21-3D.

We further compared the phenotypes of hy5-215 bbx21-3D (hy5 bbx21-3D) and cop1-4 bbx21-3D (cop1 bbx21-3D) double mutants with their corresponding single mutants. cop1 bbx21-3D showed an enhanced constitutively photomorphogenic phenotype compared with cop1 (Figure 2c–e; see also Figures 3 and S4). This supports the notion that BBX21P314L may exhibit increased activity independently of its protein stabilization through the removal of COP1-mediated degradation. In agreement, HY5 accumulated to higher levels in darkness in cop1 bbx21-3D compared with cop1 (Figure 2f). On the other hand, hy5 bbx21-3D showed no aberrant phenotype in darkness, as was observed for hy5 and bbx21-3D (Figure 2c–e). However, under all light conditions tested, including UV-B (Figure 3) and monochromatic red and blue light (Figure S4), hy5 was epistatic to bbx21-3D, suggesting that the enhanced photomorphogenic phenotype of bbx21-3D is dependent on functional HY5. This is in line with the lack of both a phenotype (Figure 2c–e) and HY5 protein accumulation (Figure 2f) in bbx21-3D in darkness, where COP1 is most active in degrading HY5.

**BBX21 and BBX22 are stabilized in response to UV-B**

The analysis of bbx21 null and hypermorphogenic mutants suggested a role for class-IV BBX proteins, which include BBX20 and BBX22, in UV-B signaling. Whereas BBX20 was apparently not transcriptionally regulated by UV-B, BBX21 transcript levels were slightly repressed by UV-B (Figure 4a; see also Figure S2). By contrast, within 1 h of UV-B exposure, a transient induction of BBX22 expression was observed that was dependent on both UVR8 and HY5 (Figure 4a,b). Beyond transcriptional regulation, BBX20,
Figure 2. The BBX21P314L variant shows higher activity in a both COP1-dependent and -independent manner.

(a) Yeast two-hybrid analysis of the interactions of BBX21 and BBX21P314L with the C-terminal WD40-domain of COP1 (COP1C340) and HY5. Left: growth assay. Right: quantitative β-galactosidase assay. AD, activation domain; BD, DNA binding domain; +H, +His medium (SD/Tri-/-Leu) as transformation control; −H, selective −His medium (SD/Tri-/-Leu/-His); MU, Miller units; ns, non-significant (P > 0.05); ****P < 0.0001. (b) Analysis of the transactivation activity of BBX21 and BBX21P314L fused to the LexA DNA binding domain using a quantitative β-galactosidase assay in yeast. BD, DNA binding domain; MU, Miller units; ns, non-significant (P > 0.05); ****P < 0.0001 (as determined by one-way ANOVAs followed by Tukey’s test for multiple comparisons). (c) Representative images (scale bar: 5 mm). (d) Quantification of hypocotyl lengths (values of independent measurements are shown with mean values indicated by horizontal lines; n > 60). (e) Quantification of anthocyanin concentrations (values of independent measurements, means and SEMs are shown; n = 3) in wild-type (Col-0), bbx21-3D, hy5, hy5 bbx21-3D, cop1 and cop1 bbx21-3D seedlings grown in darkness. (d, e) Shared letters indicate no statistically significant difference between the means (P > 0.05), as determined by one-way ANOVAs followed by Tukey’s test for multiple comparisons. (f) Immunoblot analysis of HY5 and actin (loading control) levels in cop1 hy5, wild-type (Col-0), bbx21-3D, cop1 and cop1 bbx21-3D seedlings grown for 4 days in darkness.

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BBX21 and BBX22 proteins are post-translationally regulated by COP1 (Chang et al., 2011; Fan et al., 2012; Job et al., 2018; Xu et al., 2016). We thus tested BBX protein levels in lines expressing GFP-tagged BBX20, BBX21 and BBX22 under the constitutive cauliflower mosaic virus (CaMV) 35S promoter. GFP-BBX20 showed constitutive protein levels under UV-B (Figure 4c), in agreement with the absence of a conserved VP motif in this protein (Figure S5). By contrast, the transient stabilization of GFP-BBX21 was detectable after 1 h of UV-B (Figure 4d). GFP-BBX22

Figure 3. The bbx21-3D phenotype requires functional HY5.
(a) Representative images (scale bar: 5 mm). (b) Quantification of hypocotyl lengths (values of independent measurements are shown with mean values indicated by horizontal lines; n > 60). (c) Quantification of anthocyanin concentrations (values of independent measurements, means and SEMs are shown; n = 3) in wild-type (Col-0), bbx21-3D, hy5, hy5 bbx21-3D, cop1 and cop1 bbx21-3D seedlings grown in weak white light supplemented with UV-B (+) or not (-). (b, c) Shared letters indicate no statistically significant difference between the means (P > 0.05), as determined by two-way ANOVAs followed by Tukey's test for multiple comparisons.
was strongly stabilized in response to UV-B, with immunoblots revealing two specific bands (Figure 4e), previously reported as representing full-length and truncated forms of GFP-BBX22 (Chang et al., 2011). Full-length GFP-BBX22 levels peaked following approximately 3 h of UV-B exposure, whereas the truncated GFP-BBX22 accumulated for at least up to 9 h of UV-B treatment (Figure 4e). As the truncated GFP-BBX22 presumably lacks the VP motif (Figure S5), its accumulation is likely to be the result of specific proteolytic cleavage following the stabilization of full-length GFP-BBX22. Using antibodies specifically raised against a BBX22199-213 peptide, we observed a similar pattern for endogenous BBX22 in the wild type (Figure 4f). We conclude that BBX21 and BBX22 are transiently stabilized.
under UV-B, consistent with the notion that their COP1-mediated degradation is relieved once UVR8 binds and represses COP1 activity.

**HY5 negatively regulates BBX21 and BBX22 accumulation under white light and UV-B**

To confirm that the observed BBX protein stabilization was indeed dependent on the UVR8 signaling pathway, we analyzed BBX21 and BBX22 levels in different genetic backgrounds. As expected, GFP-BBX21 and GFP-BBX22 protein stabilization under UV-B was absent in uvr8-12 null mutants (Figure 5a,b). This was confirmed for endogenous BBX22 (Figure 5c). Conversely, in comparison with the wild type, endogenous full-length and truncated BBX22 showed strongly enhanced levels in response to UV-B in uvr8-17D and rup1 rup2 (Figure 5c), which are UV-B hypersensitive mutants because of impaired UVR8 inactivation through redimerization (Gruber et al., 2010; Heijde & Ulm, 2013; Podolec et al., 2021b).

Interestingly, in the absence of HY5, strongly enhanced accumulation of GFPPBX21 was observed before, after 1 h and particularly after 6 h of UV-B treatment (Figure 5a). This contrasts with the very transient accumulation of GFP-BBX21 in the wild type at 1 h and particularly after 6 h of UV-B (Figures 4d and 5a). These data suggest that HY5 negatively regulates BBX21 accumulation and is required for the transient nature of BBX21 stabilization under UV-B in the wild type. A similar observation was made regarding BBX22 accumulation, where the absence of HY5 allowed the over-accumulation of BBX22 and shifted the peak of full-length BBX22 stabilization to later time points (Figure 5b,d). It is of note that the overall level of endogenous full-length BBX22 was lower in hy5 hyh, presumably because of the abolished induction of BBX22 gene expression in the absence of HY5 and HYH (Figure 4b) (Chang et al., 2008; Chang et al., 2011). BBX22 physically interacts with the bZIP domain of HY5 (Datta et al., 2008) and, together with BBX20 and BBX21, forms a transcriptional module that promotes photomorphogenesis (Bursch et al., 2020).

We speculated that the repressive effect of HY5 on BBX22 levels may be explained by the co-degradation of HY5 and BBX22 by COP1. However, this was not the case, as BBX22 was degraded normally in a hy5/Pro\textsubscript{Box}::HY5\textsubscript{SN77} line where the COP1-interacting N-terminus of HY5 is deleted and HY5 is thus constitutively stable (Figure 5a) (Ang et al., 1998; Osterlund et al., 2000). On the other hand, BBX22 stabilization was strongly repressed in bbx21-3D and Pro\textsubscript{Box}::GFP-BBX21 lines (Figure 5f). Overall, our data suggest that HY5 together with its BBX21 coactivator induce a repressor of BBX22 (and possibly BBX21) protein stability as part of a negative feedback loop included in the photomorphogenic program.

**BBX20, BBX21 and BBX22 promote UVR8- and HY5-dependent responses**

To determine whether BBX proteins contribute to UVR8- and HY5-dependent, UV-B-induced photomorphogenic responses, we tested the phenotypes of combinatorial mutants, including a bbx20 bbx21 bbx22 triple mutant. As shown previously (Favory et al., 2009; Tavridou et al., 2020), uvr8 mutant seedlings were strongly impaired in UV-B-induced hypocotyl growth inhibition, whereas hy5 showed longer hypocotyls under both –UV-B and +UV-B conditions, with reduced inhibition of hypocotyl elongation under +UV-B conditions (Figures 6a,b and S6). The bbx20 bbx21 bbx22 triple mutant displayed longer hypocotyls in –UV-B conditions, similar to hy5, and an intermediate hypocotyl length between that of the wild type and hy5 under +UV-B conditions (Figures 6a,b and S6). These data indicate that BBX proteins partially regulate the HY5-dependent inhibition of hypocotyl elongation under +UV-B conditions. On the other hand, anthocyanin accumulation was strongly compromised in the bbx20 bbx21 bbx22 triple mutant, resembling hy5 (Figure 6c). Flavonol profiling by high-performance thin layer chromatography (HPTLC) further revealed that bbx20 bbx21 bbx22 was strongly impaired in the UV-B-dependent accumulation of flavonol glycosides (Figure 6d). It is of note that the hy5 bbx20 bbx21 bbx22 quadruple mutant showed a weak additive phenotype for both hypocotyl length and pigment accumulation, suggesting that an HY5-independent activity of BBX proteins exists in addition to the above described BBX-independent activity of HY5 (Figure 6a-c). Related to pigment biosynthesis, UV-B-induced CHS protein accumulation was impaired but not completely abolished in bbx20 bbx21 bbx22, whereas it was undetectable in hy5 (Figures 6e and S6), suggesting that there are additional important regulators of HY5 activity besides BBX20, BBX21 and BBX22. In agreement with reduced CHS accumulation and impaired pigment biosynthesis, the CHS transcript did not accumulate to high levels under prolonged +UV-B conditions in bbx20 bbx21 bbx22, similar to hy5 and unlike UV-B-exposed wild-type seedlings (Figure 6f).

**BBX20, BBX21, and BBX22 promote the expression of some genes under prolonged UV-B exposure, but not their short-term induction after UV-B exposure**

BBX proteins regulate HY5 transcription (Bursch et al., 2020; Xu et al., 2016); however, both HY5 transcript and HY5 protein accumulation after UV-B exposure were not strongly affected in the bbx20 bbx21 bbx22 triple mutant, although HY5 protein levels were overall slightly lower in the mutant (Figure 7a,b). The HY5-dependent induction of UV-B marker genes such as RUP2, CHS and ELIP2 was only weakly, if at all, affected in bbx20 bbx21 bbx22 in response to UV-B (Figure 7c-e). Overall, the induction of transcripts...
was largely independent of BBX20, BBX21 and BBX22, and the residual induction in bbx20 bbx21 bbx22 was HY5 dependent (Figure 7b–e). This was in stark contrast to the impaired induction of CHS after 4 days of continuous +UV-B conditions (Figure 6f). Thus, we checked the expression of other marker genes in continuous −UV-B and +UV-B conditions. Interestingly, in bbx20 bbx21 bbx22, F3H induction under prolonged +UV-B conditions was also impaired, whereas MYB12 and ELIP2 were only partially affected (Figure 8a–c). By contrast, whereas the UV-B...
Figure 6. BBX proteins are necessary for hypocotyl growth inhibition and pigment accumulation under UV-B.
(a) Representative images of wild-type (Col-0), uvr8-12 (uvr8), hy5-215 (hy5), bbx20 bbx21 bbx22 and hy5 bbx20 bbx21 bbx22 mutant seedlings in white light supplemented (+) or not (−) with UV-B. Scale bar: 5 mm. (b) Quantification of hypocotyl length of seedlings as shown in (a). Values of independent measurements and means as horizontal lines are shown (n>60). (c) Anthocyanin concentrations in seedlings as shown in (a). (d) HPTLC analysis of the flavonol glycoside levels in seedlings as described in (a) grown in white light or white light supplemented with UV-B. K-3R-7R, kaempferol-3-O-rhamnoside-7-O-rhamnoside; SG, sinapoyl glucose; K-3G-7R, kaempferol-3-O-glucoside-7-O-rhamnoside; Q-3G-7R, quercetin-3-O-glucoside-7-O-rhamnoside. (e) Immunoblot analysis of CHS and actin (loading control) levels in seedlings as described in (a) that were exposed for 0–9 h to supplemental UV-B, as indicated. (f) RT-qPCR analysis of CHS expression in Col-0, hy5 and bbx20 bbx21 bbx22 seedlings grown for 4 days in white light or white light supplemented with UV-B. (c, f) Values of independent measurements, means and SEMs are shown (c, n=3; f, n=4). (b, c, f) Shared letters indicate no statistically significant difference between the means (P>0.05), as determined by two-way ANOVAs followed by Tukey’s test for multiple comparisons.

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induction of RUP1 and RUP2 remained strongly dependent on HY5. RUP1 and RUP2 transcripts accumulated normally under prolonged +UV-B conditions in bbx20 bbx21 bbx22 (Figure 8d,e), despite reduced HY5 levels (Figure 7a). In summary, our data suggest that BBX20, BBX21 and BBX22 redundantly promote pigment biosynthesis and the
BBX proteins are necessary for the expression of some but not all marker genes in white light conditions constantly supplemented with UV-B. (a–e) RT-qPCR analysis of F3H (a), MYB12 (b), ELIP2 (c), RUP1 (d) and RUP2 (e) expression in wild-type (Col-0), hy5 and bbx20 bbx21 bbx22 seedlings grown for 4 days (96 h) in white light or white light supplemented with UV-B. Values of independent measurements, means and SEMs are shown (n = 4); shared letters indicate no statistically significant difference between the means (P > 0.05), as determined by two-way ANOVA followed by Tukey’s test for multiple comparisons.

(f) Working model for a role of BBX proteins in UV-B photomorphogenesis. Left panel: under weak white light conditions devoid of UV-B, UVR8 is inactive and COP1 targets downstream factors, including HY5 and BBX proteins, and possibly a hypothetical factor X, for degradation. A small residual pool of HY5 and BBX proteins are responsible for the low basal expression of light-induced marker genes and a weak inhibition of hypocotyl elongation. Middle panel: upon exposure of seedlings to UV-B, UVR8 represses COP1 activity. This results in the accumulation of COP1 targets: HY5, BBX proteins, and factor X. HY5 increasingly binds promoters of light-responsive genes, and factor X provides it with transcriptional activity, so that the expression of marker genes is strongly induced. BBX proteins play a minor role in this transcriptional induction. Right panel: in prolonged UV-B conditions, COP1 remains repressed by UVR8, but this is dampened by negative feedback on photoreceptor activity through RUP1/RUP2. HY5 remains highly stable, but BBX protein levels are repressed through the action of a hypothetical repressor (R) that is induced as a part of HY5/BBX-mediated photomorphogenesis. BBX proteins play a role as HY5 coactivators in allowing the sustained expression of genes involved in pigment biosynthesis, whereas other UV-B marker genes are induced by HY5 in a BBX-independent manner. Hypocotyl length inhibition is controlled by both BBX proteins and factor X.

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accumulation of some transcripts, such as CHS and F3H, under prolonged +UV-B conditions. However, BBX20, BBX21 and BBX22 do not seem to regulate the early induction of HY5 and other UV-B marker genes immediately after UV-B exposure, and they play a partial role in the HY5-dependent inhibition of hypocotyl elongation.

DISCUSSION

Upon UV-B perception, nuclear UVR8 promotes photomorphogenesis and acclimation by directly or indirectly modulating the activity of several transcriptional regulators (Favory et al., 2009; Liang et al., 2018; Liang et al., 2019; Podolec et al., 2021a; Qian et al., 2016; Qian et al., 2020; Yang et al., 2018; Yin et al., 2016). These include the bZIP transcriptional regulators HY5 and HYH that control the expression of many genes downstream of UVR8 and both the cryptochrome and phytochrome pathways (Ang et al., 1998; Brown et al., 2005; Burko et al., 2020; Holm et al., 2002; Oravecz et al., 2006; Stracke et al., 2010; Ulm et al., 2004). These photoreceptors inhibit the activity of the COP1/SPA E3 ubiquitin ligase complex, allowing the accumulation of HY5 and HYH followed by the induction of the photomorphogenic program under light (Hoecker, 2017; Podolec & Ulm, 2018). Despite its important and broad function as transcriptional regulator, HY5 itself lacks a transcriptional activation domain (Ang et al., 1998; Burko et al., 2020; Stracke et al., 2010). Recently, BBX20, BBX21 and BBX22 were characterized as HY5 coregulators that provide transactivational activity, allowing transcriptional gene activation in response to both red light and the butenolide molecule karrikin (Bursch et al., 2020; Bursch et al., 2021), but their involvement in UVR8 signaling was unknown.

Here, we uncovered a hypermorphic bbx21 allele in a genetic screen for mutants with enhanced UV-B responses. BBX21 is a promoter of photomorphogenesis that is targeted by COP1 for degradation (Xu et al., 2016). We identified a bbx21-3D mutant containing a P314L mutation, which functionally abolishes the COP1 interaction domain of BBX21. This VP-mutated allele of COP1 substrate allowed us to test the functional relevance of a VP motif in an endogenous context. The bbx21-3D phenotype was largely consistent with that of previously published BBX21 overexpression lines (Job et al., 2018; Xu et al., 2016), supporting the mutation that BBX21P314L is post-translationally stabilized and thus present at elevated levels in the mutant background, as expected for a BBX21 VP mutant (Bursch et al., 2020; Lau et al., 2019; Podolec & Ulm, 2018). Unfortunately, we were not able to raise specific anti-BBX21 antibodies to probe directly for endogenous BBX21P314L levels compared with wild-type BBX21. Interestingly, however, the bbx21-3D mutation enhanced the cop1-4 mutant phenotype, although no interaction between BBX21 and COP1 is expected to occur in this genetic context as the corresponding COP1P282 protein expressed in cop1-4 lacks the VP-interacting WD40 repeat domain (Lau et al., 2019; McNellis et al., 1994). These data suggest that BBX21P314L also exhibits elevated activity independently of its impaired interaction with and regulation by COP1. The additive phenotype of cop1 and bbx21-3D could result from the post-transcriptional stabilization of HY5, and/or a stronger HY5-BBX21P314L interaction, as our yeast interaction data suggest, and thus increased activity of BBX21 as a HY5 coactivator (Bursch et al., 2020).

We found similarities but also interesting differences in the role of BBX proteins in UV-B signaling compared with that under visible light. Regarding the hypocotyl elongation phenotype, hy5 and bbx20 bbx21 bbx22 were comparably elongated under constant white light devoid of UV-B, but differed under constant white light supplemented with UV-B, wherein BBX proteins accounted for only a part of the HY5-dependent response. Interestingly, and similarly to our observations, the HY5-dependent hypocotyl response of seedlings during karrikin signaling relies only partially on BBX20 and BBX21 (Bursch et al., 2021). Furthermore, the BBX-dependent transcriptome accounted only for a fraction of HY5-regulated transcription under red light, suggesting that additional HY5 coregulators are indeed involved in some responses (Bursch et al., 2020). In contrast to the hypocotyl elongation phenotype, BBX proteins were crucial for the UV-B-dependent biosynthesis of phenylpropanoid pigments (flavonols and anthocyanins). Intriguingly, when analyzing the molecular basis of this phenotype, we found that gene expression involved in phenylpropanoid biosynthesis (CHS and F3H) was impaired under prolonged and constant UV-B, but the induction of early UV-B marker genes within 6 h of UV-B exposure was almost completely intact. Similarly, CHS protein accumulation after UV-B exposure was decreased but not abolished in the bbx20 bbx21 bbx22 triple mutant. These data suggest that early and transient HY5 responses to UV-B are BBX20, BBX21 and BBX22 independent, whereas for long-term responses such as pigment biosynthesis, HY5 relies on these BBX proteins as transcriptional coactivators. It is of note that the long-term accumulation of some transcripts, such as RUP1 and RUP2, was also independent of the BBX proteins.

BBX21 and BBX22 were post-transcriptionally stabilized under UV-B in a UVR8-dependent manner. This is consistent with their status as COP1 substrates (Chang et al., 2011; Xu et al., 2016; this work) and how active UVR8 directly inhibits COP1 activity by competitively blocking the COP1 substrate binding site (Lau et al., 2019; Podolec et al., 2021a). Consistent with previous literature on visible light responses (Chang et al., 2011; Job et al., 2018; Xu et al., 2016), we observed a transient stabilization of BBX21 and BBX22, with protein levels decreasing after a few hours of UV-B exposure. This transient stabilization could be linked to an attenuation of UVR8 signaling through the
RUP1- and RUP2-mediated negative feedback loop (Gruber et al., 2010; Heijde & Ulm, 2013; Podolec et al., 2021b; Ren et al., 2019), as supported by extended BBX22 accumulation in uv8-17D and rup1 rup2 backgrounds. Moreover, our data indicate the existence of an additional negative regulator of BBX21 and BBX22 stability. This repressor seems to be linked to HY5 activity, as suggested by the overaccumulation of BBX21 and BBX22 in the hy5 background, and as already reported for BBX22 under visible light (Chang et al., 2011). Conversely, plant lines with enhanced photomorphogenesis (such as bbx21-3D and BBX21 overexpression lines) show lower levels of BBX22 accumulation. Collectively, our data point to an uncharacterized negative feedback mechanism that attenuates photomorphogenesis induced by the HY5/BBX transcriptional module.

We conclude that BBX20, BBX21 and BBX22 play important roles as HY5 coactivators in inducing UV-B responses, such as pigment biosynthesis, and to a lesser degree the inhibition of hypocotyl elongation, mainly under long-term (constant) -UV-B conditions (for our working model, see Figure 8f). Short-term responses to changing UV-B conditions on the other hand (such as a few hours after UV-B exposure) seem to be mostly independent of these BBX proteins, as seen by the normal transcriptional induction of HY5-dependent UV-B marker genes in the bbx20 bbx21 bbx22 triple mutant. This further indicates the existence of a specific HY5 coactivator or multiple coactivators under UV-B, the activation of which is likely to be crucial for early gene activation upon UV-B reception and signaling by the UVR8 photoreceptor. Whether COP1 regulates further HY5 coregulators and whether they are members of the large BBX family remains to be determined.

EXPERIMENTAL PROCEDURES

Plant materials

All lines used in this study are in the A. thaliana Columbia (Col-0) background. The following lines have been described previously: rup1 rup2 (Gruber et al., 2010); cop1-4 (Deng et al., 1992); uv8-12 and uv8-17D (Podolec et al., 2021b); hy5-215 (Oyama et al., 1997); hy5-215 hy2 (Zoulas et al., 2020); bbx21-1 (Datta et al., 2007); bbx22-1 (Chang et al., 2008); bbx20-1, bbx21-1 bbx22-1, bbx20-1 bbx21-1 bbx22-1, hy5-215 bbx20-1 bbx21-1 bbx22-1, Col-0/Pro35S:BBX20 #1 and #2, hy5-215/Pro35S:HY5N777 #27 and hy5-215/Pro35S:BBX21 #2 (Bursch et al., 2020); bbx20-1 bbx21-1 (Bursch et al., 2021); and Col-0/Pro35S:BBX22 #1 and #2, hy5-215/Pro35S:HY5 #15 (Job et al., 2018). Pro35S:GFTrGBPBBX22 lines #1 and #2 were generated in the Col-0 background using a pB7WG2 vector (Karimi et al., 2002) in which the BBX22 coding DNA sequence (CDS) was inserted after cloning into pDONR221 (primers BBX22_attB1_Fw and BBX22_attB2_Rv, Table S1) using Gateway technology (ThermoFisher Scientific, https://www.thermoﬁsher.com). bbx21-3D was identified in a forward-genetic screen based on hypocotyl length under UV-B of an EMS mutagenized Col-0 population (Podolec et al., 2021b), and was back-crossed three times. Combinatorial mutant lines bbx20-1 bbx22-1, hy5-215 bbx21-3D, cop1-4 hy5-215, cop1-4 bbx21-3D, uv8-12/Pro35S:GFTrGBPBBX21 #2, uv8-12/Pro35S:GFTrGBPBBX22 #1 and hy5-215/Pro35S:GFTrGBPBBX22 #1 were generated by crossing and then genotyped by PCR and sequencing.

Generation of BBX21 CRISPR/Cas9-mutated lines

The CRISPR/Cas9 system was used to delete the BBX21 C-terminus in the wild type and to knock out BBX21 in the wild type and in the bbx21-3D mutant. The single guide RNA (sgRNA) directed against the BBX21 sequence were inserted into the pHE401 vector (Wang et al., 2015) using overlapping complementary oligos (Table S1). Arabidopsis plants were then transformed using the floral-dip method (Clough & Bent, 1998). Several independent transgenic events were selected and the BBX21 locus was sequenced in T3 to identify lines with desired mutations.

Growth conditions and light treatments

Seeds were surface sterilized using chlorine gas or ethanol and sown on half-strength MS agar medium (Duchefa, https://www.duchefa-biochemie.com) supplemented with 1% (w/v) sucrose (except for experiments in monochromatic red/blue light, which were performed on media without sucrose). Plates were left for 2 days in the dark at 4°C for stratification. For growth in darkness, plates were exposed for 6 h to approximately 60 μmol m⁻² sec⁻¹ of white light and then transferred to darkness at 22°C for 4 days. For light treatments, plates were grown for 4 days at 22°C in the following light conditions: weak white light (3.6 μmol m⁻² sec⁻¹; L18W/330 tubes; Osram, https://www.osram.com) supplemented or not with narrowband UV-B (1.5 μmol m⁻² sec⁻¹; TL20W/01RS tubes; Philips, https://www.lighting.philips.com), monochromatic red light (150 μmol m⁻² sec⁻¹; floralLEDs in cabinet; CLF Plant Climatics, https://www.plantclimatics.de), monochromatic blue light (50 μmol m⁻² sec⁻¹; floralLEDs in CLF Plant Climatics cabinet). For UV-B stress treatment, plates were irrigated with broadband UV-B (21 μmol m⁻² sec⁻¹; Philips TL20W/12RS tubes).

Hypocotyl length measurements

Hypocotyl length was determined as described previously (Podolec et al., 2021b). In short, seedlings were grown for 4 days in the appropriate conditions and approximately 60 seedlings for each genotype/condition were aligned on an agar plate and scanned. Individual hypocotyls were measured using the NeuronJ plugin of ImageJ (Meijering et al., 2004).

Extraction and quantification of anthocyanins

Anthocyanins were quantified as described previously (Yin et al., 2012). Seedlings were grown for 4 days in the appropriate conditions and approximately 50 mg of seedlings were collected for each genotype and condition. Samples were frozen in liquid nitrogen, ground and 250 μl of extraction buffer (99% v/v methanol, 1% v/v HCl) was added to the samples. Extraction was performed for at least 1 h by incubating the samples on a rotary shaker at 4°C. After centrifugation for 5 min, the absorbance of 150 μl of the clear supernatant was measured at 530 and 655 nm. The following formula was used to calculate the relative quantity of anthocyanins: (A530 – 0.25 × A655) / seedling mass (in mg).

Extraction and visualization of flavonoids

High-performance thin layer chromatography (HPTLC) was used to analyze the flavonol profile, as described previously (Podolec et al., 2021b). In short, exactly 50 mg of seedlings was collected
for each genotype and condition, and the samples were frozen and then ground. A 100-μL volume of extraction buffer (80% (v/v) methanol) was added per sample, and these were then incubated for 10 min at 70°C on a shaker. After a 5 min centrifugation at 15,000 g, supernatants were collected and 10 μL was loaded on silica HPTLC plates. The extracts were then separated for approximately 45 min using a mobile phase (5 mL ethyl acetate, 600 μL formic acid, 600 μL acetic acid glacial, 1.3 mL water). After migration, the plate was dried and sprayed with 1% (w/v) diphenylboric acid 2-aminoethylmerester (DPBA; Roth, https://www.carlotherm.com) solution in 80% (v/v) methanol. The plate was exposed under a 365-nm UV-A lamp to reveal the flavonoid profile.

Measurements of photosynthetic efficiency

The maximum quantum efficiency of photosystem II was measured after dark-adapting plants for 5 min using a Fluorocam (Photon Systems Instruments, https://ipsi.cz) with blue (470 nm) LEDs, calculated as Fv/Fm = (Fm – Fo)/Fm, where Fm is the maximal fluorescence and Fo is the minimal fluorescence in the dark-adapted state.

Protein extraction and immunoblot assays

For all immunoblot assays that include (GFP-I)BBX levels, a previously described buffer (Job et al., 2018) was used: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (w/v) sodium deoxycholate, 0.5% (v/v) Triton X-100, 1 mM, 50 μM MG132 (Sigma-Aldrich, https://www.sigmaaldrich.com), 50 μM ALLN (VWR, https://www.vwr.com) and 50 μM Protease Inhibitor Cocktail (Sigma-Aldrich). For HY5 immunoblots, a previously described buffer (Oravecz et al., 2006) was used: 0.1 mM Tris-HCl, pH 8.0, 50 μM EDTA, 0.25% NaCl, 0.7% (w/v) SDS, 10 mM NaF, 15 mM β-glycerophosphate, 15 mM p-nitrophenyl phosphate, a Complete EDTA-free Protease Inhibitor Cocktail tablet (Roche, https://www.roche.com) and 1 mM DTT. DTT. For CHS immunoblot assays, a previously described phosphate buffer (Arongaus et al., 2018) was used: 50 mM Na-phosphate, pH 7.4, 150 mM NaCl, 10% (v/v) glycerol, 5 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM DTT, 2 mM NaVO4, 2 mM NaF, 1% (v/v) Protease Inhibitor Cocktail (Sigma-Aldrich) and 50 μM of MG132 (Sigma-Aldrich).

In all cases, samples were harvested, frozen, ground and mixed with extraction buffer before centrifugation for 25 min at 15,000 g, 4°C. The clear supernatants were collected and protein concentrations were determined using the Bradford-based Bio-Rad protein assay (Bio-Rad, https://www.bio-rad.com). Samples were then denatured and separated using SDS-PAGE. Proteins were transferred on PVDF membranes (Roth) for 7 min at 20 V using the iBlot dry blotting system (ThermoFisher Scientific), before blocking in TBS-T with milk.

To determine transcript levels by reverse transcription quantitative polymerase chain reaction (RT-qPCR), RNA was extracted using the ReliaPrep RNA Tissue Miniprep System kit (Promega, https://www.promega.com) and treated with DNase according to the manufacturer’s instructions. cDNA synthesis was performed using the TaqMan reverse transcription kit (Applied Biosystems, now ThermoFisher Scientific, https://www.thermofisher.com), with a 1:1 mix of oligo-dT and random hexamer primers. The qPCR reaction was performed using the PowerUp SYBR Green Master Mix reagents (Applied Biosystems, now ThermoFisher Scientific) on a QuantStudio™ 6 System (ThermoFisher Scientific) following the recommended protocol, using the primers listed in Table S1. The ΔΔCt method (Livak & Schmittgen, 2001) was used to calculate expression values, with PP2A as a reference gene. Each experiment was performed by combining between three and six independently made biological replicates. All expression values were normalized within each biological replicate against the untreated wild type, which was set to 1.

Statistical analysis

Statistical analyses were performed using PRISM 9.2.0 (GraphPad, https://www.graphpad.com). One- or two-way analyses of variance (ANOVA) were performed on log-transformed or non-transformed data, followed by Tukey’s test for multiple comparisons, to distinguish statistically different groups (P > 0.05) using different letters. For the analysis of Fv/Fm (Figure 1g), a repeated-measures ANOVA was used. For pairwise comparisons (Figure 4a), unpaired two-tailed Student’s t-tests were used and P-values were represented.

Accession numbers

Sequence data from this work can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AT4G39070 (BBX20), AT1G75540 (BBX21), AT1G78600 (BBX22), AT5G13930 (CHS), AT3G51240 (F3H), AT2G32290 (COP1), AT5G11260 (HY5), AT3G17609 (HYH), AT2G47460 (MYB12), AT4G14690 (ELIP2), AT5G52250 (RUP1), AT5G23730 (RUP2) and AT5G63860 (UVR8).
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AUTHOR CONTRIBUTIONS

RP and RU designed the research. TBW, RP and ML performed the experiments. HJ contributed new tools. TBW, RP and RU analyzed the data and wrote the article. All authors reviewed and approved the final version for publication.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest associated with this work.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. The bbx21-3D phenotype is dependent on functional BBX21 and can be recapitulated by deleting the C-terminal 25 amino acids that include the VP motif.

Figure S2. BBX21 promotes the expression of some marker genes in response to UV-B.

Figure S3. The bbx21-3D phenotype in darkness and in monochromatic red and blue light.

Figure S4. Genetic relationship between bbx21-3D and cop1 and hy5 in monochromatic red and blue light conditions.

Figure S5. Conservation of putative VP motifs in BBX20, BBX21 and BBX22.

Figure S6. Genetic redundancy among BBX20, BBX21 and BBX22.

Table S1. Primers used in this study.

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