

# Transcription factors BBX11 and HY5 interdependently regulate the molecular and metabolic responses to UV-B

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## Abstract

UV-B radiation acts as a developmental cue and a stress factor for plants, depending on dose. Activation of the transcription factor ELONGATED HYPOCOTYL 5 (HY5) in a UV RESISTANCE LOCUS 8 (UVR8)-dependent manner leads to the induction of a broad set of genes under UV-B. However, the underlying molecular mechanisms regulating this process are less understood. Here, we use molecular, biochemical, genetic, and metabolomic tools to identify the B-BOX transcription factor B-BOX PROTEIN 11 (BBX11) as a component of the molecular response to UV-B in *Arabidopsis thaliana*. *BBX11* expression is induced by UV-B in a dose-dependent manner. Under low UV-B, *BBX11* regulates hypocotyl growth suppression, whereas it protects plants exposed to high UV-B radiation by promoting the accumulation of photo-protective phenolics and antioxidants, and inducing DNA repair genes. Our genetic studies indicate that *BBX11* regulates hypocotyl elongation under UV-B partially dependent on *HY5*. Overexpression of *BBX11* can partially rescue the high UV-B sensitivity of *hy5*, suggesting that *HY5*-mediated UV-B stress tolerance is partially dependent on *BBX11*. *HY5* regulates the UV-B-mediated induction of *BBX11* by directly binding to its promoter. *BBX11* reciprocally regulates the mRNA and protein levels of *HY5*. We report here the role of a *BBX11*-*HY5* feedback loop in regulating photomorphogenesis and stress tolerance under UV-B.

## Introduction

Plants are often exposed to the solar UV-B radiation. While a low dose of UV-B acts as a morphogenetic cue and promotes the accumulation of beneficial metabolites, high UV-B irradiation induces stress and hampers plant growth (Galvão and Fankhauser, 2015; Jenkins, 2017; Shi and Liu, 2021; Podolec et al., 2021a, 2021b, 2021c). In plants, the photoreceptor UV RESISTANCE LOCUS 8 (UVR8) is known to perceive both

UV-B and short-wave UV-A radiation (Kliebenstein et al., 2002; Brown et al., 2005; Favory et al., 2009; Rizzini et al., 2011; Christie et al., 2012; Tilbrook et al., 2013; Jenkins, 2014; Rai et al., 2020, 2021; Podolec et al., 2021a, 2021b, 2021c). UVR8 exists as a dimer in the ground state which upon absorption of UV-B photons, monomerizes to activate UV-B signaling (Kaiserli and Jenkins, 2007; Heijde et al., 2013; Qian et al.,

2016). The UVR8 monomer binds competitively to the E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) via its VP motif to inhibit targeting its substrates for degradation (Oravecz et al., 2006; Favory et al., 2009; Huang et al., 2013; Yin et al., 2015, 2016; Hayes et al., 2017; Lau et al., 2019; Ren et al., 2019; Ponnu and Hoecker, 2021). The UVR8–COP1 complex formation also stabilizes the transcription factor ELONGATED HYPOCOTYL 5 (HY5) that regulates the expression of several downstream genes involved in UV-B protection (Ulm et al., 2004; Oravecz et al., 2006; Favory et al., 2009; Huang et al., 2012; Binkert et al., 2014). UVR8 is reverted back to its inactive dimer form by the action of REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 (RUP1) and RUP2 (Gruber et al., 2010; Heijde and Ulm, 2013; Podolec et al., 2021a, 2021b, 2021c). UVR8 also directly binds to some transcription factors like WRKY36, BES1-INTERACTING MYC-LIKE1 (BIM1)/BRASSINAZOLE-RESISTANT 2 (BES1), and MYB73/77 to inhibit their DNA binding and transcriptional regulation (Liang et al., 2018; Yang et al., 2018; Liang et al., 2020; Yang et al., 2020). PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) and PIF5 are other transcription factors that are inactivated under UV-B (Tavridou et al., 2020). In the absence of UV-B, WRKY36 inhibits the transcription of HY5 (Yang et al., 2018). However, under UV the binding of UVR8 to WRKY36 leads to the release of this inhibition leading to HY5-mediated signaling and stress tolerance (Yang et al., 2018).

A high dose of UV-B leads to the activation of a UV-B acclimation response leading to UV-B tolerance. Protection against UV-B in plants involves the accumulation of several metabolites which act as sunscreen compounds and shield plants from UV-B radiation (Yoshiyama et al., 2013). These include several flavonoids, pigments, and other phenolics that absorb UV radiation to enhance cellular protection (Hectors et al., 2014; Liang et al., 2020). UV-B induces the expression of genes like CHALCONE ISOMERASE (*CHI*) and CHALCONE SYNTHASE (*CHS*) that are involved in the synthesis of the photoprotective pigments anthocyanins (Shi and Liu, 2021). In addition to the protective metabolites, plants also mitigate UV-B stress by activating their DNA repair mechanisms to amend UV-B DNA damage (Podolec et al., 2021a, 2021b, 2021c). Protection of the photosynthetic apparatus is an integral part of UV-B acclimation and tolerance across several photosynthetic organisms, including algae (Allorent et al., 2016). UVR8 plays a crucial role in activating the phenylpropanoid pathway and synthesizing reactive oxygen species (ROS) scavenging metabolites under UV-B (Brown and Jenkins, 2008; Hideg et al., 2013). The transcription factor HY5 plays an important role in the activation of several genes that are involved in the biosynthesis of phenolic acids, anthocyanins, and induction of the DNA repair pathway (Ulm et al., 2004; Brown and Jenkins, 2008; Binkert et al., 2014).

In addition to HY5, several other transcription factors are regulated by UV-B. Notable among them are members of the B-BOX PROTEIN (BBX) family of transcriptional regulators. Recently, BBX20, BBX21, and BBX22 were identified as

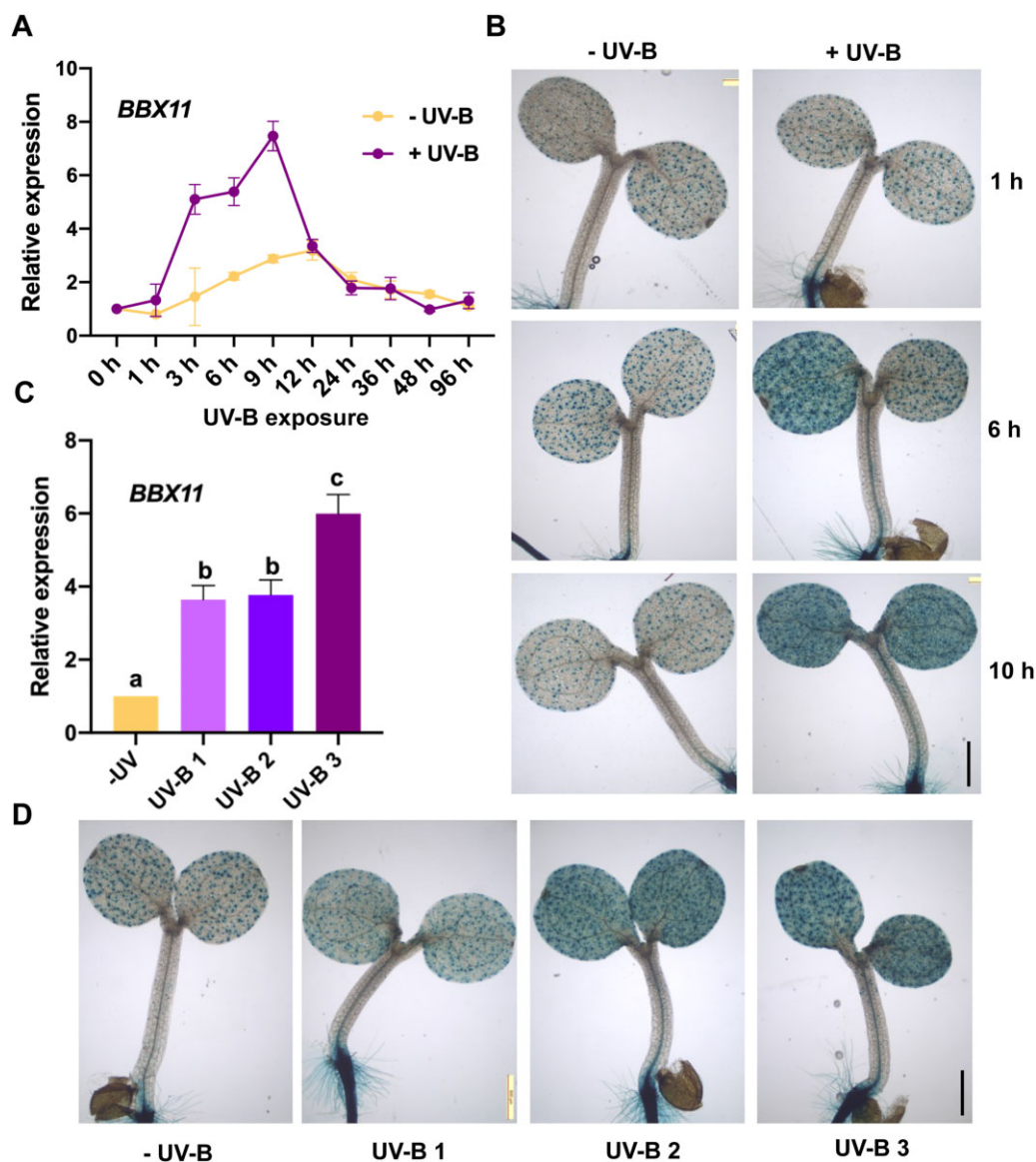
cofactors of HY5 that render transcriptional activation potential to HY5, which lacks an activation domain (Bursch et al., 2020). BBX proteins are zinc-finger transcription factors that play a myriad of roles in plant developmental processes including photomorphogenesis, flowering, thermomorphogenesis, and shade avoidance (Crocco and Botto, 2013; Gangappa and Botto, 2014; Vaishak et al., 2019; Xu, 2019; Yadav et al., 2020). A small subset of BBX proteins have been found to play roles in UV-B signaling and stress tolerance (Jiang et al., 2012; Bai et al., 2014; Yadav et al., 2019a, 2019b). BBX24 is a negative regulator of UV-B photomorphogenesis (Jiang et al., 2012). UV-B induces both BBX24 mRNA and protein, and the accumulated protein binds to HY5 to inhibit its transcriptional activity (Jiang et al., 2012). BBX31, which codes for a microprotein acts as a positive regulator of UV-B signaling and protection (Yadav et al., 2019a). Under UV-B, BBX31 positively regulates photomorphogenesis independent of HY5, whereas it promotes UV-B stress tolerance in an HY5-dependent manner (Yadav et al., 2019a). RUP2 delays flowering under UV-B by physically binding with CONSTANS, which happens to be BBX1, and inhibiting its association with the promoter of FLOWERING LOCUS T (Arongaus et al., 2018). A number of other UV-B-induced BBX proteins have been identified and are predicted to function in UV-B signaling (Lyu et al., 2020).

Here, we identify the role of BBX11 in the regulation of UV-B signaling and stress tolerance. UV-B induces *BBX11* expression in a dose-dependent manner. BBX11 regulates hypocotyl elongation under UV-B partially dependent on HY5. Under high fluence UV-B, BBX11 promotes the accumulation of phenolic compounds and induces photoprotection genes to enhance UV-B stress tolerance. Our data indicate that HY5 directly binds to the promoter of *BBX11* and positively regulates its expression under UV-B. We propose here a BBX11-HY5 feedback loop regulating UV-B photomorphogenesis and stress tolerance.

## Results

### *BBX11* expression is induced by UV-B

Microarray data suggest that the Arabidopsis BBX coding gene, *BBX11*, is induced by various fluence rates ( $7\text{ W/m}^2$  and  $3\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ ) of UV-B (Ulm et al., 2004; Brown and Jenkins, 2008). These reports prompted us to investigate if BBX11 plays any role in UV-B signaling. To validate the UV-B-mediated induction of *BBX11*, we performed RT-qPCR analysis of *BBX11* expression in 5-day-old Columbia-0 (Col-0) seedlings grown in constant white light and exposed to supplemental UV-B (+ UV-B) ( $2.2\ \text{W/m}^2$ ) or not (–UV-B) for 0–96 h (Figure 1A). Under the control conditions, *BBX11* transcript level marginally increased over this time period; however, UV-B treatment strongly upregulated the expression level of *BBX11*. *BBX11* mRNA steadily increased after 3 h of UV-B treatment and peaked to about eight-fold upregulation around 9 h (Figure 1A). In order to determine if the UV-B-mediated induction of *BBX11* is UVR8 dependent, we checked the expression of *BBX11* in *uvr8-6*. Under UV-B



**Figure 1** *BBX11* expression is induced by UV-B. A, RT-qPCR analysis of *BBX11* expression in 5-day-old Col-0 seedlings grown in constant white light and exposed to supplemental UV-B 2 ( $2.2 \text{ W/m}^2$ ) (+ UV-B) or not (–UV-B) for the indicated time period. GAPDH was used as internal control. Error bars represent SEM,  $n = 3$  (B) Histochemical staining of 5-day-old p*BBX11*:GUS seedlings grown in constant white light and exposed to supplemental UV-B (+ UV-B) or not (–UV-B) for the indicated durations, Scale bar- 500  $\mu\text{m}$ . In (A and B) fluence of UV-B used was  $2.2 \text{ W/m}^2$  (C) RT-qPCR analysis of *BBX11* expression in 5-day-old Col-0 seedlings grown in constant white light and exposed to supplemental UV-B of different doses UV-B 1 ( $0.91 \text{ W/m}^2$ ), UV-B 2 ( $2.2 \text{ W/m}^2$ ), UV-B 3 ( $3.9 \text{ W/m}^2$ ), or not (–UV) for 6 h. GAPDH was used as internal control. Error bar represents SEM,  $n = 3$ , Statistical groups indicated by letters were determined by one-way ANOVA, with Tukey's test  $P \leq 0.05$ . D, Histochemical staining of 5-day-old p*BBX11*:GUS seedlings grown in constant white light and exposed to supplemental UV-B 1, UV-B 2, UV-B 3, or not (–UV) for 10 h before staining. Scale bar: 500  $\mu\text{m}$ .

1 ( $0.9 \text{ W/m}^2$ ), the mRNA levels of *BBX11* in Col-0 seedlings are upregulated three- to four-fold after 3 and 8 h of exposure to UV-B (Supplemental Figure S1A). Under UV-B, *BBX11* transcript levels were reduced in *uvr8-6*, suggesting that the UV-B-mediated induction of *BBX11* is at least partially dependent on UVR8 (Supplemental Figure S1A). Under  $2.2 \text{ W/m}^2$  of UV-B, *BBX11* expression in *uvr8-6* was reduced at 3 h but comparable to Col-0 at 8 h (Supplemental Figure S1B). This suggests that at higher fluence and longer exposure, the UV-B-mediated induction of *BBX11* might

involve factors other than UVR8. We further examined if *BBX12* and *BBX13*, which are structurally similar to *BBX11* and belong to the same clade, are also induced by UV-B. *BBX12* and *BBX13* mRNA levels, unlike *BBX11*, did not show a strong upregulation when irradiated with supplemental UV-B 2 ( $2.2 \text{ W/m}^2$ ) as compared to their levels in (–UV-B) conditions (Supplemental Figure S1C).

In order to determine the spatio-temporal expression pattern of *BBX11* in response to UV-B, we performed histochemical staining of 5-day-old p*BBX11*:GUS seedlings grown

in constant white light and exposed to supplemental UV-B (+ UV-B) or not (–UV-B) for 1, 6, and 10 h (Figure 1B). Under UV-B, speckled GUS staining was detected in the cotyledons as reported earlier for cWL-grown *pBBX11:GUS* seedlings (Figure 1B; Job and Datta, 2021). Low staining was also detected in the hypocotyl vasculature whereas high GUS staining was seen in the hypocotyl root junction (Figure 1B). Consistent with our RT-qPCR data, staining in the cotyledons was substantially higher in plants treated with UV-B for 6 and 10 h (Figure 1B). Next, we quantified the relative expression of *BBX11* under various fluence levels of UV-B (0.9, 2.2, and 3.9 W/m<sup>2</sup>) (Figure 1C). For the convenience of the readers, we have denoted various fluence levels of UV-B used in different experiments throughout the manuscript as follows: UV-B 1 (near 1 W/m<sup>2</sup>), UV-B 2 (2–2.5 W/m<sup>2</sup>), UV-B 3 (3.1–4.8 W/m<sup>2</sup>). The hypocotyl elongation experiments were performed under UV-B 1, whereas in the stress experiments plants were exposed to UV-B 3. The biochemical and gene expression analysis were performed using seedlings treated with the intermediate UV-B 2. Our results indicate a gradual increase in the expression levels as the dose of UV-B is elevated (Figure 1C). We further checked the spatial expression of *BBX11* under the different fluences. We found enhanced GUS staining in the cotyledons with the increase in the UV-B irradiation, indicating that UV-B induces *BBX11* expression in a dose-dependent manner (Figure 1D).

### BBX11 regulates hypocotyl growth suppression under UV-B

The low dose of UV-B inhibits hypocotyl elongation in *Arabidopsis* (Jenkins, 2017). In order to investigate if *BBX11* has any role to play in UV-B-mediated photomorphogenesis, we characterized the loss and gain of function mutants of *BBX11*. For loss of function mutants, we used the T-DNA insertion mutant *bbx11-1* and the clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 mutants *bbx11-2* and *bbx11-3* generated by us and described previously (Job and Datta, 2021). We also characterized OE1, OE2, and OE3, three independent lines overexpressing *BBX11*, also reported in our previous study (Job and Datta, 2021). Seedlings were grown for 5 days under low fluence cWL (9 μmol m<sup>-2</sup> s<sup>-1</sup>) without UV-B (–UV-B) and their hypocotyl lengths compared with seedlings grown under cWL with UV-B 1 supplementation (+ UV-B) (Figure 2, A–C). As previously reported, under cWL *bbx11* showed elongated hypocotyl length and *BBX11*-OE showed short hypocotyl length compared to Col-0 (Figure 2B). Under UV-B, hypocotyl growth was reduced in seedlings of all three genotypes. However, *bbx11-1* showed enhanced suppression of hypocotyl length, whereas OE3 showed less inhibition in hypocotyl growth under UV-B relative to Col-0 (Figure 2, A–C). This suggests that *BBX11* regulates hypocotyl growth suppression under UV-B.

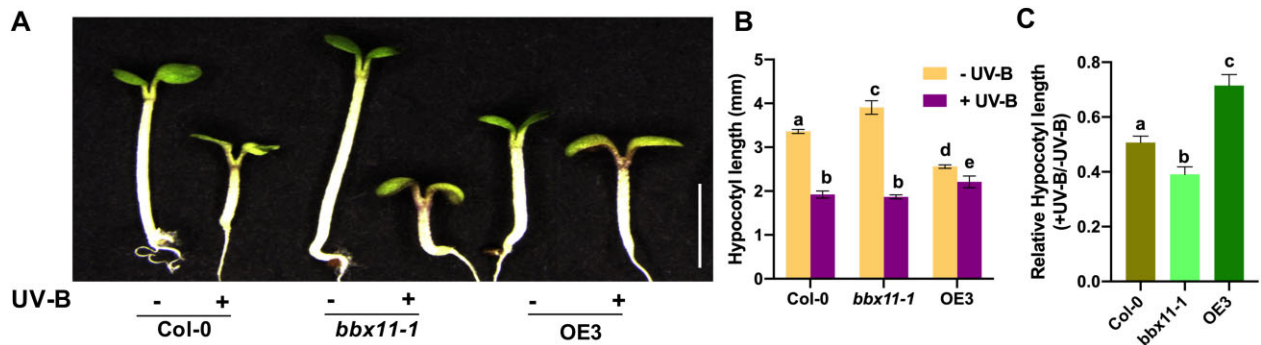
### BBX11 provides protection against UV-B stress

Since higher UV-B irradiation elevates *BBX11* expression, we asked if *BBX11* plays any role in high UV-B stress tolerance

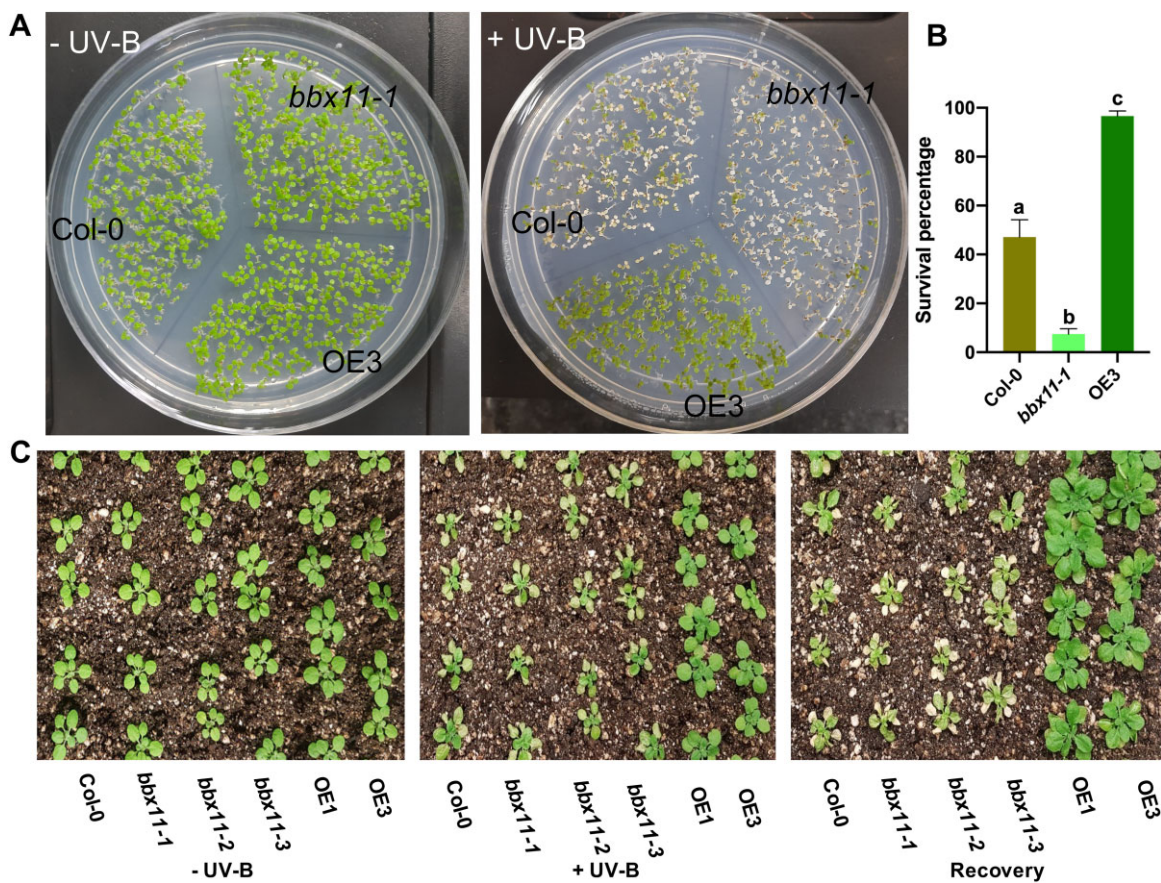
in plants. Four-day-old Col-0, *bbx11-1*, and OE3 seedlings grown on plates under constant white light were irradiated with supplemental UV-B 3 (+ UV-B) or not (–UV-B) for 30 h and allowed to recover under white light for 1 day before imaging (Figure 3A). We estimated the survival percentage of seedlings by counting seedlings in which the cotyledons remained green and did not bleach under UV-B radiation (Figure 3B). The survival percentage in Col-0 was close to 50%, while in *bbx11-1* it was <10% and in OE3 almost 95% of seedlings survived (Figure 3B). This suggests that *BBX11* protects plants from the damage caused by high dose of UV-B. Similar results were obtained in another independent experiment where seedlings on plates were exposed to high UV-B radiation for 20 h (Supplemental Figure S3A). In another experiment, Col-0, *bbx11-1*, and OE3 seedlings were initially acclimatized by growing them under constant white light supplemented with UV-B 1 for 5 days. These seedlings were then irradiated with UV-B 3 for another 24 h followed by recovery under white light for 1 day before imaging. Here, we did not find substantial difference in the stress tolerance phenotype between the three genotypes (Supplemental Figure S3B). In order to investigate the effect in older plants, we treated plants grown in the soil under white light for 18 days with supplemental UV-B 3 (+ UV-B) or not (–UV-B) for 3 days and allowed them to recover under white light for 5 days. After the recovery period, the Col-0 and *bbx11-1* plants showed signs of bleaching, with slightly more chlorotic patches in the mutant compared to the wild-type (Figure 3C). However, the OE3 plants showed enhanced tolerance to bleaching (Figure 3C). Together, these data suggest that *BBX11* promotes tolerance to high doses of UV-B in plants.

### BBX11 promotes accumulation of photo-protective phenolic compounds

To determine if the enhanced tolerance of plants overexpressing *BBX11* could be due to elevated levels of known or unknown protective metabolites we compared the metabolome of UV-B treated and untreated Col-0, *bbx11-1*, and OE3 plants. Untargeted metabolic profiling of 18-day-old plants grown under constant white light and exposed to 0, 24, and 96 h of supplemental UV-B radiation captured 379 metabolic features showing differential accumulation in the three genotypes. Partial least squares-discriminant analysis revealed that the metabolomes of Col-0, *bbx11-1*, and OE3 plants were altered differently after exposure to UV-B radiation for 24 and 96 h (Supplemental Figure S4A). We used comprehensive cluster analysis to visualize the response of metabolites under white light (0 h) and the two durations of UV-B treatments (24 and 96 h) in the three genotypes as a heat map (Figure 4A). The top 25 metabolites showing maximum variability among the genotypes in the absence (0 h) or presence of UV-B for 24 and 96 h were identified by this approach (Figure 4A). These include several amino acids, sugars, organic acids, carboxylic, fatty acids, and phenolic acids. OE3 substantially accumulated the intermediate



**Figure 2** BBX11 regulates UV-B mediated hypocotyl growth inhibition. A–C, Representative images (A), quantification of hypocotyl lengths (B) and relative hypocotyl lengths (C) of 5-day-old seedlings of the indicated genotypes grown in constant white light supplemented with UV-B 1 (+) or not (–). Scale bar in (A) indicates 2 mm. In (B) and (C), error bars represent  $SEM$ ,  $n = 3$ . Statistical groups indicated by letters were determined by two-way ANOVA, with Sidak's multiple comparison test,  $P \leq 0.05$ .



**Figure 3** BBX11 protects plants from UV-B radiation. A, 4-day-old Col-0, *bbx11-1*, and OE3 seedlings grown on plates under constant white light were treated with supplemental UV-B 3 (+ UV-B) or not (–UV-B) for 30 h and allowed to recover under white light for 1 day before imaging. B, quantification of seedling survival percentage, calculated as mentioned in methods. Error bars represent  $SEM$ ,  $n = 4$ , statistical groups indicated by letters were determined by one-way ANOVA, with Tukey's test  $P \leq 0.05$ . C, Plants of the indicated genotypes were grown in soil under long-day conditions (16/8 h) in white light for 18 days and exposed to supplemental UV-B 3 (+ UV-B) or not (–UV-B) for 3 days and allowed to recover under white light for 5 days before being photographed.

metabolites of the phenylpropanoid biosynthesis pathway under UV-B treatment (Figure 4A). The accumulation of phenolics in Col-0, *bbx11-1*, and OE3 genotypes showed much variation after 96 h of UV-B treatment (Figure 4A). The relative accumulation of phenolic acids, for example,

shikimic acid, sinapinic acid, cinnamic acid, vanillic acid, coumaric acid, ferulic acid, caffeic acid, hydroxybenzoic acid, and benzoic acid, was significantly higher in OE3 (Figure 4B; Supplemental Figure S4B). These phenolics play a crucial role in conferring UV-B stress tolerance. In our *pBBX11:GUS*



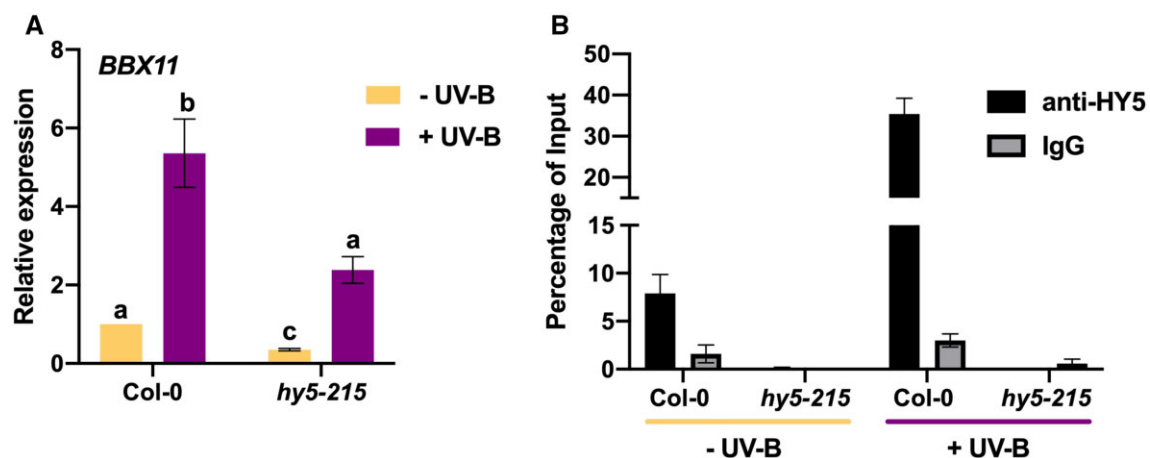
identified as one of the genes that is induced by UV-B in WT seedlings but not in the *hy5* mutant (Brown and Jenkins, 2008). In order to verify the HY5-dependent induction of *BBX11* under UV-B, we checked the transcript levels of *BBX11* in Col-0 and *hy5-215* (Figure 5A). While there was five-fold upregulation in the mRNA levels of *BBX11* in the WT seedlings after UV-B treatment, we could find just over two-fold upregulation in *hy5* seedlings (Figure 5A). This suggests that HY5 positively regulates the expression of *BBX11* under UV-B. HY5 has been previously shown to physically bind to the *BBX11* promoter fragment containing the G-box variant, in seedlings grown under white light (Zhao et al., 2020; Job and Datta, 2021). In order to verify whether similar binding also happens under UV-B, we performed Chromatin Immunoprecipitation (ChIP) followed by qPCR on seedlings grown under WL +/- UV-B and compared the enrichment of HY5 on the *BBX11* promoter (Figure 5B). Our results indicate that HY5 binding on the *BBX11* promoter in Col-0 seedlings is almost five-fold enriched under + UV-B conditions (Figure 5B). Such enrichment was not seen when chromatin from *hy5* seedlings (negative control) was used for a similar experiment (Figure 5B). We also did not find any enrichment of HY5 on a fragment from the 3'-untranslated region of *BBX11* (Supplemental Figure S5A). All these data together suggest that under UV-B, the binding of HY5 to the promoter of *BBX11* is increased, which leads to the enhanced expression of *BBX11*.

### BBX11 and HY5 regulate UV-B photomorphogenesis and stress tolerance in a partially interdependent manner

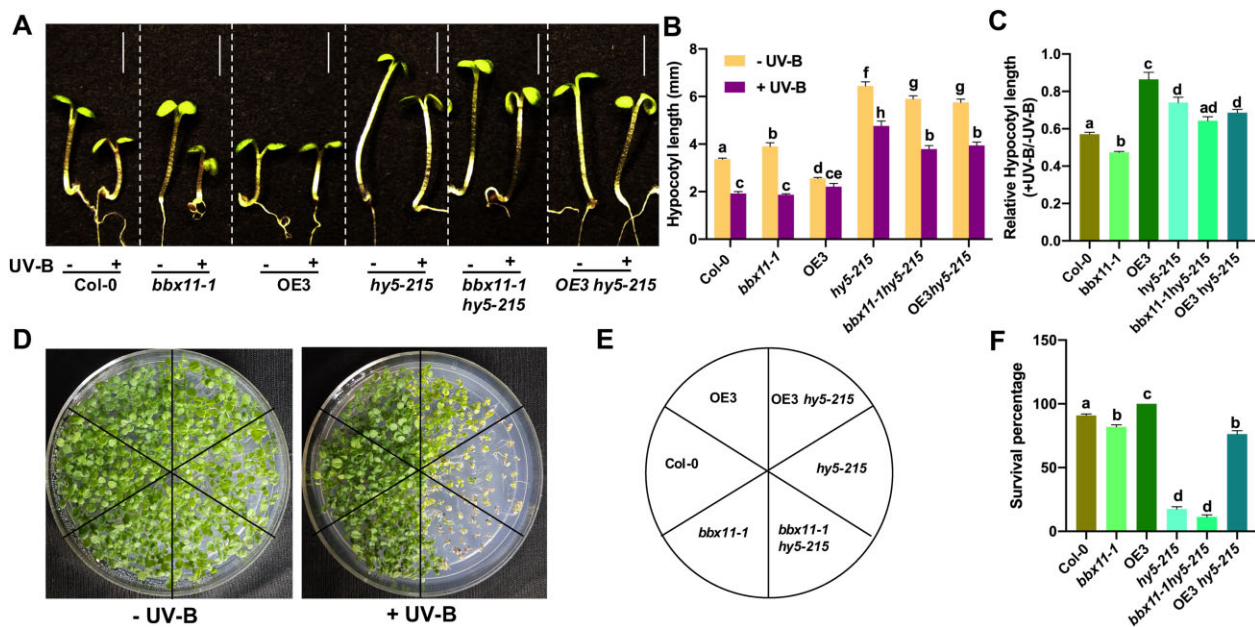
HY5 positively regulates photomorphogenesis and stress tolerance under UV-B (Brown and Jenkins, 2008; Binkert et al., 2014). Our results have indicated that *BBX11* also

regulates UV-B photomorphogenesis and stress tolerance. In order to understand the genetic interaction between *BBX11* and *HY5*, we characterized *bbx11-1hy5-215* and OE3*hy5-215* grown under UV-B. In our experimental conditions, the average hypocotyl length of *bbx11* seedlings under UV-B was around 1.9 mm, whereas it was around 4.8 mm for *hy5* (Figures 2 and 6, A–C). The hypocotyl length of *bbx11-1hy5-215* seedlings under UV-B was around 3.8 mm (Figure 6B). The relative hypocotyl length in + UV-B/-UV-B for *bbx11-1hy5-215* was almost similar to *hy5-215* (Figure 6C). The OE3*hy5-215* seedlings grown in + UV-B conditions, exhibited hypocotyl length intermediate between OE3 and *hy5-215* (Figure 6B), however, the relative hypocotyl length of OE3*hy5-215* was similar to *hy5-215* (Figure 6C). This suggests that *BBX11* regulates hypocotyl elongation under UV-B partially dependent on HY5.

To investigate the genetic interaction between *BBX11* and *HY5* to regulate UV-B stress tolerance we irradiated 5-day-old white light (WL)-grown seedlings of all genotypes indicated in Figure 6E with UV-B-3 for 20 h following which seedlings were allowed to recover for 10 days before imaging (Figure 6, D–F). Under these conditions, the survival % was about 90% in Col-0. *bbx11-1* showed reduced survival, whereas OE3 exhibited enhanced survival compared to WT (Figure 6F). *hy5-215* and *bbx11-1hy5-215* seedlings showed <10% survival under the UV-B stress. However, under these conditions the survival percentage of OE3*hy5-215* seedlings enhanced to around 75%. This suggests that *BBX11* might be partially dependent on HY5 to provide protection against UV-B stress. The incomplete rescue of the *hy5* phenotype by *BBX11* OE also suggests that there might be other



**Figure 5** HY5 regulates the UV-B-mediated induction of *BBX11* by directly binding to its promoter. A, RT-qPCR analysis of *BBX11* expression in 5-day-old Col-0 and *hy5-215* seedlings grown in constant white light and exposed to supplemental UV-B 2 (+) or not (-) for 6 h. GAPDH was used as internal control. Error bars represent SEM,  $n = 3$ . Statistical groups indicated by letters were determined by one-way ANOVA, with Tukey's test,  $P \leq 0.05$  (B) ChIP-qPCR analyses of HY5 binding to the *BBX11* promoter in vivo. Fourteen-day-old Col-0 and *hy5-215* seedlings grown in constant white light and exposed to supplemental UV-B 3 (+) or not (-) for 3 h before crosslinking. DNA-protein complexes were immunoprecipitated using antibodies against HY5 (anti-HY5) and rabbit IgG as negative control (IgG). ChIP DNA was quantified by RT-qPCR with primers specific to the previously known HY5-binding site. Error bars represent SD,  $n = 2$ .



**Figure 6** BBX11 acts partially independent of HY5 to regulate photomorphogenesis and stress tolerance under UV-B. A–C, Representative images (A) quantification of hypocotyl lengths (B) and relative hypocotyl lengths (C) of 5-day-old seedlings of the indicated genotypes grown in constant white light supplemented with UV-B 1 (+) or not (–). Scale bars in (A) indicate 2 mm. In (B) and (C), error bars represent  $SEM$ ,  $n = 3$ . Statistical groups indicated by letters were determined by one-way ANOVA, with Tukey’s test (multiple comparison for (B)),  $P \leq 0.05$  (D, E) 5-day-old seedlings of the indicated genotypes as shown in (E) grown in constant white light were exposed to supplemental UV-B 3 (+ UV-B) or not (–UV-B) for 20 h and allowed to recover under white light for 10 days before imaging. F, quantification of seedling survival percentage, calculated as mentioned in methods. Error bars represent  $SEM$ ,  $n = 3$ . Statistical groups indicated by letters were determined by one-way ANOVA, with Tukey’s test,  $P \leq 0.05$ .

targets of HY5 regulating the UV-B acclimation and stress tolerance response.

### Overexpression of BBX11 induces photoprotection and DNA damage repair genes

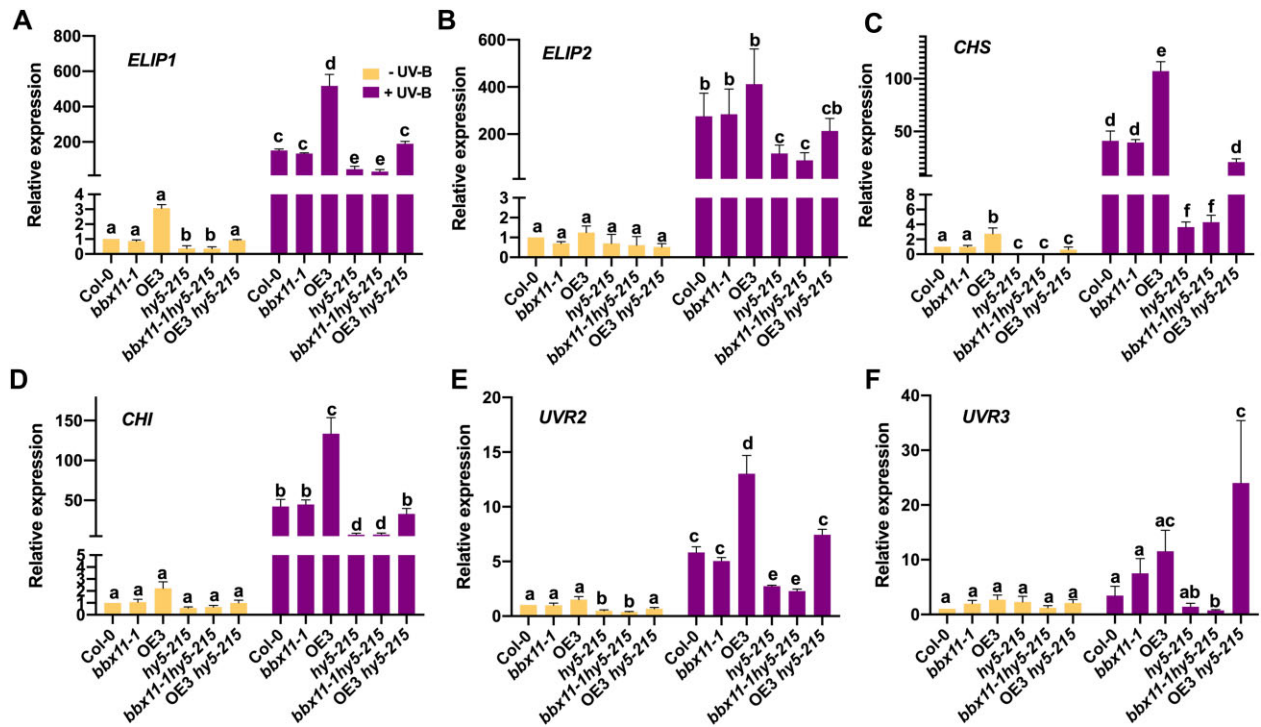
Since overexpression of BBX11 could rescue the bleaching phenotype of *hy5* under moderate UV-B stress, we hypothesized that BBX11 might act downstream of HY5 in the same pathway and regulate similar photoprotective genes. HY5 is known to regulate the *EARLY LIGHT-INDUCIBLE PROTEIN (ELIP)* genes which code for the early light-induced proteins that prevent photooxidative damage in plants. HY5 also induces the expression of genes involved in the synthesis of anthocyanins such as *CHI*, *CHS*, and *FLAVANONE 3-HYDROXYLASE (F3H)*. Additionally, the *UVR2* and *UVR3* gene coding for DNA damage repair protein is activated by HY5 under UV-B. The mRNA expression levels of all these genes under UV-B were downregulated in *hy5* as reported earlier (Figure 7, A–F). The transcript level of the six genes were upregulated in the BBX11 overexpressing line OE3, while it was similar to WT in *bbx11-1* and almost equal to *hy5-215* in the *bbx11hy5-215* double mutant (Figure 7, A–F). Interestingly, in plants overexpressing BBX11 in the *hy5-215* background the mRNA levels of *ELIP1*, *CHS*, *CHI*, *UVR2*, and *UVR3* were enhanced compared to the levels detected in *hy5-215*. The transcript level of *F3H* was upregulated in OE3 compared to Col-0 but mRNA levels in OE3*hy5-215* were similar to that in *hy5-215* (Supplemental Figure S6). The

upregulation of these photoprotective genes can contribute to the enhanced tolerance to UV-B seen in OE3*hy5-215* seedlings. This suggests that the overexpression of BBX11 can induce photoprotection and DNA damage repair genes to enhance UV-B stress tolerance.

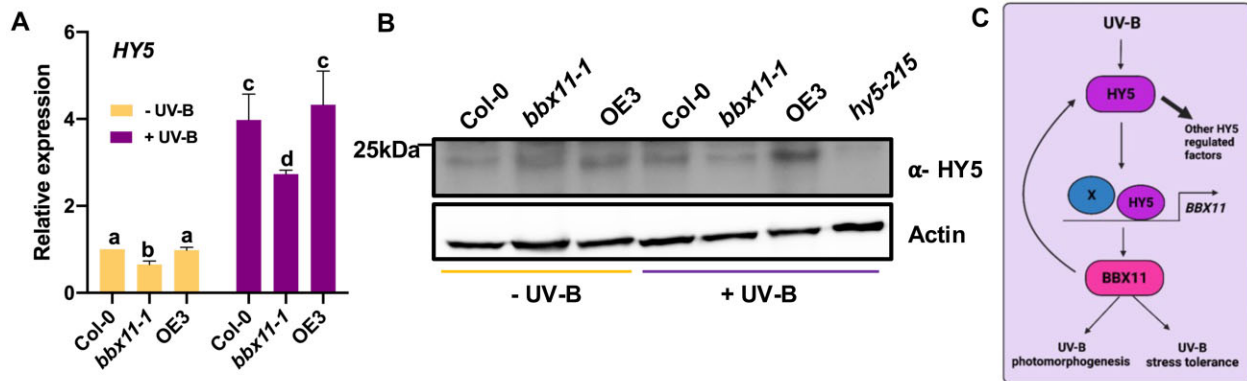
### The HY5-BBX11 feedback loop regulates UV-B photomorphogenesis and stress tolerance

Previously, we found that HY5 activates BBX11 transcription by binding to its promoter under UV-B (Figure 5). Under UV-B stress conditions (UV-B 3), BBX11 acts downstream of HY5 to promote stress tolerance (Figure 6, D–F). However, OE3*hy5-215* seedlings exhibit photomorphogenesis phenotypes similar to *hy5* (Figure 6, A and B). This suggests that HY5 can also act downstream of BBX11. Together these indicate that a feedback loop between HY5 and BBX11 might be operating under UV-B to modulate responses. Since several BBX proteins are known to regulate transcription, we asked if BBX11 might regulate HY5 transcription under UV-B. We quantified the relative expression of HY5 in Col-0, *bbx11-1* and OE3 exposed to supplemental UV-B 2 (+ UV-B) or not (–UV-B). The HY5 transcript level in *bbx11-1* was reduced compared to WT under both conditions, suggesting that BBX11 might modulate HY5 transcription (Figure 8A). The HY5 mRNA levels were not significantly different between the WT and OE3 in –UV-B as well as +UV-B conditions (Figure 8A). We further went on to check if BBX11 modulates HY5 protein accumulation under UV-B. Our





**Figure 7** BBX11 induces photoprotection and DNA damage repair genes under UV-B. A–F, RT-qPCR analysis of ELIP1 (A) ELIP2 (B), CHS (C), CHI (D), UVR2 (E), and UVR3 (F) in 5-day-old seedlings grown in constant white light and exposed to supplemental UV-B 2 (+) or not (–) for 4 h. GAPDH was used as internal control. Error bars represent SEM,  $n = 3$ . Statistical groups indicated by letters were determined by one-way ANOVA, with Tukey's test,  $P \leq 0.05$ .



**Figure 8** HY5-BBX11 feedback loop regulates photomorphogenesis and stress tolerance under UV-B. A, qRT-PCR analysis of HY5 expression in 5-day-old seedlings of the indicated genotypes grown in constant white light and exposed to supplemental UV-B 2 (+) or not (–) for 4 h. GAPDH was used as internal control. Error bars represent SEM,  $n = 3$ . Statistical groups indicated by letters were determined by one-way ANOVA, with Tukey's test,  $P \leq 0.05$ . B, Immunoblot analysis of HY5 and actin (loading control) levels in Col-0, *bbx11-1*, and OE3 seedlings exposed to 6 h of supplemental UV-B 2 (+ UV-B) or not (–UV-B). C, Working model indicating the role of an HY5-BBX11 feedback loop in regulating UV-B photomorphogenesis and stress tolerance. “X” indicates an unknown factor that might regulate BBX11 transcription in addition to HY5 under UV-B.

Western blot data indicate that HY5 protein accumulation is suppressed in *bbx11-1* under UV-B (Figure 8B). On the other hand there is enhanced accumulation of HY5 in OE3 in presence of UV-B (Figure 8B). Such a difference in the accumulation of HY5 protein between the loss and gain of function mutants of *BBX11* was not evident in –UV-B conditions. Overall, our data suggest that under UV-B, *BBX11* might be regulating the transcriptional and protein levels of

HY5, thereby forming a feedback loop to regulate plant responses under UV-B (Figure 8C).

## Discussion

### BBX11 regulates UV-B photomorphogenesis

*BBX11* has been previously characterized as a positive regulator of photomorphogenesis under red light (Zhao et al.,

2020; Job and Datta, 2021; Liu et al., 2021). BBX11 directly interacts with phytochrome B (phyB) and PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) and promotes phyB-mediated degradation of PIF4 under red light to inhibit hypocotyl elongation (Song et al., 2021). The inhibition of PIF4 activity might suppress hypocotyl elongation in the *BBX11* overexpressing line OE3 in the absence of UV-B (Figure 2, A and B). Here we report the role of BBX11 in regulating UV-B mediated hypocotyl growth inhibition. *bbx11-1* showed enhanced suppression of hypocotyl length while the overexpressor exhibited less inhibition of hypocotyl length under UV-B (Figure 2C). This might suggest that BBX11 negatively regulates UV-B mediated hypocotyl growth inhibition. However, it can be argued that the “enhanced suppression of hypocotyl length” in the mutant can be a result of the elongated hypocotyl of the mutant in –UV-B conditions (Figure 2). Moreover, the enhanced accumulation of flavanols in OE3 could reduce the effective dose of UV-B exposure, which could be responsible for the slightly elongated hypocotyl of this genotype under UV-B (Figure 2, A and B). Based on these arguments it may be concluded that BBX11 promotes UV-B photomorphogenesis and stress tolerance. Previously, another BBX protein BBX31 has been reported to positively regulate photomorphogenesis and stress tolerance under UV-B (Yadav et al., 2019a). BBX24, the third BBX protein known to play a role in UV-B signaling, negatively regulate photomorphogenesis under white light as well as under UV-B (Jiang et al., 2012). Our results also indicate that BBX11 regulates UV-B-mediated photomorphogenesis partially dependent on *HY5*. Although the induction of *BBX11* by UV-B is compromised in *hy5*, it is not completely reduced to the –UV-B levels (Figure 5A). This indicates that there can be *HY5*-independent mechanisms functioning through BBX11. Our *pBBX11:GUS* lines indicated that *BBX11* is strongly expressed in the guard cells of the cotyledons. However upon UV-B treatment for longer durations (6 and 10 h) or high intensity (UV-B 2 and UV-B 3), the blue staining becomes more diffused throughout the cotyledon as indicated in Figure 1, B and D. In the future it would be interesting to study the physiological role of *BBX11* expression in the guard cells both under visible light and UV-B.

### BBX11 protects plants from UV-B radiation

High UV-B irradiation causes stress and leads to UV-B acclimation and tolerance response in plants. The molecular and metabolic response of plants to protect themselves from damaging UV-B radiation involves the induction of genes and accumulation of metabolites offering photoprotection. Plants exposed to high UV-B irradiation often resort to the following strategies: (1) Accumulate photoprotective sunscreen compounds to shield the radiation exposure of cellular DNA and other macromolecules; (2) Detoxify the radiation-induced ROS that causes lipid and protein peroxidation; and (3) Repair damaged DNA. In our study, we found that plants overexpressing BBX11 exhibit enhanced UV-B tolerance. Our metabolomic analysis indicated differential accumulation of several primary and secondary

metabolites in OE3 and *bbx11-1* compared to Col-0. These include amino acids, sugars, and several phenolic acids. Glycine, Alanine, and Valine accumulated at high levels in *bbx11-1*, whereas the levels of Tryptophan and Tyrosine were higher in OE3 after 96 h of UV-B exposure. The two aromatic amino acids might play roles in the synthesis of pigments, hormones, and polyphenolic compounds that help shielding the plants from UV-B radiation. Trehalose showed an increased accumulation in the less tolerant *bbx11-1* mutant. The role of sugars in UV-B acclimation response needs further investigation. Several phenolic acids like shikimic acid, sinapinic acid, coumaric acid, ferulic acid, caffeic acid, and benzoic acid showed higher accumulation in the UV-tolerant OE3 plants. Most of these phenolics play critical roles in the synthesis of cell wall components and act as intermediates in the phenylpropanoid pathway leading to the synthesis of UV-B absorbing flavonoids and pigments. In the future, it might be interesting to study the tissue-specific localization of these sunscreen metabolites. Previously we reported the role of BBX11 in the regulation of protochlorophyllide levels and chlorophyll synthesis during deetiolation. Our gene expression analysis also indicated that the transcript levels of the anthocyanin biosynthesis genes *CHS* and *CHI* are upregulated in plants overexpressing BBX11. We detected reduced NBT staining in OE3 plants indicating the reduced accumulation of superoxides in these lines. The mRNA levels of *ELIP1* and *ELIP2* were also upregulated in OE3 suggesting enhanced photoprotection activities. The increased expression of *UVR2* and *UVR3* might indicate that DNA repair pathways are also activated in plants overexpressing BBX11 contributing to UV-B acclimation and tolerance.

### HY5-BBX11 feedback loop regulates UV-B responses

BBX proteins function as critical cofactors of *HY5* regulating *HY5* activity (Bursch et al., 2020). Some BBX factors physically interact with *HY5* to modulate its transcriptional potential whereas some others form a BBX-*HY5* feedback loop (Job et al., 2018; Zhao et al., 2020). A BBX11-BBX21-*HY5*-positive feedback loop operates under white light to regulate photomorphogenesis (Zhao et al., 2020). We found that *HY5* directly binds to the promoter of BBX11 and activates its expression under UV-B (Figure 5). Furthermore, we detected enhanced *HY5* accumulation in seedlings overexpressing BBX11 under UV-B (Figure 8B). All our results together indicate the operation of a BBX11-*HY5* feedback loop to regulate photomorphogenesis and stress tolerance under UV-B (Figure 8). The loop adds partial interdependence to the partially independent functions of the two transcription factors to regulate UV-B photomorphogenesis and stress tolerance. Under UV-B stress conditions, overexpression of BBX11 in *hy5* could partially rescue the acute bleaching phenotype of *hy5* (Figure 6, D–F). This suggests that BBX11 can at least partially activate the tolerance response even in the absence of *HY5*. BBX11 might regulate a subset of *HY5*-modulated photoprotective genes/metabolites that can provide partial tolerance. However, as *HY5* acts as a master

regulator with several targets, the total repertoire of HY5-regulated genes and metabolites might be required to enhance tolerance. While the accumulation of the metabolites is required for the long-term acclimation response the activation of the genes is generally an early response. The mRNA levels of HY5 peak early (1.5–3 h) after exposure to UV-B radiation (Supplemental Figure S7). During these early hours, *BBX11* transcript levels also increase constantly and reach a peak at around 9 h after initial exposure (Figure 1A). Our results indicate that *BBX11* activates genes like *CHS*, *CHI*, *ELIPs*, *UVR2*, *UVR3* within 4 h after UV-B exposure. This suggests that *BBX11* might be acting as a coactivator of HY5 for activating these genes in response to UV-B exposure. It is interesting to note that under UV-B, the mRNA levels of *BBX11* exhibit only a partial reduction in *hy5*. This suggests the role of some additional factor (X) in regulating *BBX11* transcription under UV-B (Figure 8C). In white light, HY5 and *BBX21* are known to bind directly to the promoter of *BBX11* and positively regulate its transcription. A recent study indicates that *BBX20*, *BBX21*, and *BBX22* might act as functional partners of HY5 during prolonged UV-B treatment (Podolec et al., 2021a, 2021b, 2021c). In the future, it might be interesting to investigate how different BBX proteins partner with HY5 and enable it to multitask under different conditions including UV-B exposure. The 5 times enhanced binding of HY5 on the *BBX11* promoter under UV-B is interesting but lacks molecular explanation at the moment. Any possible role of *UVR8*, *COP1*, and *RUPs* in *BBX11* mediated UV-signaling is also left to be explored. Nonetheless, this work introduces *BBX11* as a key component of plant's molecular armory to respond to UV-B radiation.

## Materials and methods

### Plant material

All overexpression lines and mutants used in this study are in the *Arabidopsis* Col-0 ecotype. The mutants *bbx11-1*, *bbx11-2*, *bbx11-3*, OE1, OE3, *bbx11-1hy5-215*, and OE3*hy5-215* are described previously (Job and Datta, 2021). *hy5-215* and *uvr8-6* have been described in previous studies (Datta et al., 2006; Favory et al., 2009).

### Growth condition and light treatment

Seeds were surface sterilized with 4% w/v bleach for 3 min and washed with sterilized water before sowing on 0.5 × Murashige and Skoog (MS) medium containing 0.8% agar and 1% (w/v) sucrose. Plates were stratified at 4°C for 3 days under dark and illuminated with white light (80 μmol m<sup>-2</sup> s<sup>-1</sup>) for 8 h to induce uniform germination before putting them out under experimental conditions. Plants were grown at 21°C in Percival growth chambers under low fluence constant white light (9 μmol m<sup>-2</sup> s<sup>-1</sup>) unless otherwise stated. UV-B supplementation was provided through the UV-B narrowband tube (TL 20W/01 RS SLV; waveband between 305 and 315 nm and λ<sub>max</sub> 311 nm). Intensity of UV-B radiation was measured using a UV light meter (UVA/UVB 850009;

Sper Scientific, Scottsdale, AZ, USA). The fluence and duration of treatment used in various experiments have been mentioned in the respective experiments.

### Histochemical GUS staining

The *pBBX11:GUS* line has been reported previously (Job and Datta, 2021). Histochemical GUS staining was carried out as described previously (Weigel and Glazebrook, 2002). Briefly, 5-day-old seedlings were kept in 90% acetone on ice and rinsed with GUS staining solution (0.5-M sodium phosphate buffer, 10% Triton X-100, 100-mM potassium Ferrocyanide, 100-mM potassium Ferriicyanide, and 2-mM X-Gluc). Vacuum infiltration of samples for 30 min under dark on ice was performed following which the seedlings were incubated at 37°C overnight. Seedlings were washed with 20%, 35%, and 50% ethanol, fixed using FAA solution, and kept in 70% ethanol for imaging. The seedlings were documented using Leica (DM2500) microscope.

### ChIP

ChIP assay was done as described previously (Yadav et al., 2019a). Briefly, 14-day old seedlings were treated with the indicated light conditions for 3 h before cross-linking with 1% formaldehyde, and the chromatin was isolated. Purified chromatin was sonicated and precipitated with anti-HY5 (Agresera, Vannas, Sweden; 1:100) or IgG antibody (negative control; Invitrogen, Waltham, MA, USA), washed, and reversed cross-linked. The eluted DNA fragments were used in qPCR with the primers listed in Supplemental Table S1.

### Hypocotyl length measurement

Seeds were sterilized, stratified, and uniformly germinated as indicated above. For hypocotyl length measurements seeds were grown for 5 days under constant white light (9 μmol m<sup>-2</sup> s<sup>-1</sup>) or together with UV-B 1 (0.94 W/m<sup>2</sup>) supplementation. Approximately 30 seedlings of each genotype were photographed with Leica (M165 FC), and hypocotyl length was measured with ImageJ software. Three independent biological replicates (*n* = 3) with at least 30 seedlings each were analyzed for all hypocotyl length assays.

### UV-B stress tolerance

For UV-B stress tolerance analysis, 4- to 5-day-old seedlings grown on 0.5MS without sucrose plates under constant white light (20 μmol m<sup>-2</sup> s<sup>-1</sup>) were treated with supplemental UV-B 3 (4.2 or 4.8 W/m<sup>2</sup>) or not (-UV-B) for the time indicated and allowed to recover under white light for durations as mentioned in respective figure legends before imaging. Seedling survival rate was measured by scoring green, expanded, and healthy seedlings as positive and pale green/bleached seedlings as negative. An average of 50 seedlings per genotype were analyzed per condition/replicate. For UV-B stress analysis in adult plants, plants were grown in soil under long-day conditions (16/8 h) in white light for 18 days and exposed to supplemental UV-B 3 (3.9 W/m<sup>2</sup>) or not (-UV-B) for 3 days and allowed to recover under white light for 5 days before being photographed.

### Metabolite extraction and derivatization for metabolomics using GC–MS (Gas Chromatography–Mass Spectrometry)

For comprehensive metabolomics, 20 mg/mL of lyophilized tissue was used for metabolite extraction as described previously (Yadav et al., 2019a; Lingwan and Masakapalli, 2021). The extraction buffer was prepared in a ratio of 3:1:1 by mixing up prechilled methanol, chloroform, and water, respectively; 0.01-mg/mL ribitol was added to the extraction buffer as an internal standard. The extracted metabolites were subjected to drying using a speed vac. Samples were TMS (TriMethylSilyl) derivatized using methoxamine hydrochloride, Pyridine, and *N*-Methyl-*N*-trimethylsilyl trifluoroacetamide (Lisec et al., 2006). The derivatized samples were subjected to data acquisition using Agilent GC–MS 5977B equipped with 5% phenyl methyl siloxane column. Mass Hunter software was used for all data analysis. The metabolite was identified based on their fragments, retention time, and probability match against the NIST 17 library. Metaboanalyst version 5.0 was used for multivariate statistical analysis (Pang et al., 2021).

### NBT staining

For superoxide anion detection, seedlings were stained in freshly prepared NBT solution (0.2% solution in 50-mM sodium phosphate buffer, pH 7.5) overnight with gentle shaking. The seedlings were washed and decolorized with bleaching solution (3 (ethanol):1 (acetic acid):1 (glycerol)) and imaged using Leica (M165 FC) Microscope.

### RNA isolation and RT-qPCR analysis

For *BBX11*, *BBX12*, *BBX13*, and *HY5* mRNA expression analysis, 5-day-old seedlings grown under white light were treated with supplemental UV-B (2.2 W/m<sup>2</sup>) for the indicated time. For dose-dependent *BBX11* expression, analysis seedlings were grown under white light for 5 days and treated with supplemental UV-B 1 (0.91 W/m<sup>2</sup>), UV-B 2 (2.2 W/m<sup>2</sup>), and UV-B 3 (3.9 W/m<sup>2</sup>) for 6 h before harvesting the samples for RNA isolation. Total RNA was isolated using the TRIzol (Invitrogen, Waltham, MA, USA) method. The samples were immediately frozen in liquid nitrogen after the UV-B treatment. The tissue was grounded into a fine powder, and 1 mL of TRIzol reagent was added immediately and mixed by vortexing. Extraction of total RNA was performed as manufactures protocol. Purified RNA was quantified, and the cDNA library was made using Takara Primescript first-strand cDNA synthesis Kit according to manufactures instructions. Three-fold diluted cDNA was used for RT-qPCR using SYBR Green (Takara Shiga, Japan) in Roche LC 96 qPCR machine. *GAPDH* was used as the internal control.

### Total protein isolation and western blot

Five-day-old seedlings grown under continuous white light (60 μmol m<sup>-2</sup> s<sup>-1</sup>) were treated with supplemental UV-B (3.1 W/m<sup>2</sup>) before freezing in liquid N<sub>2</sub>. The tissue was ground into a fine powder, and total protein was extracted in extraction Buffer (50-mM Tris–HCl pH 6.8, 0.5% SDS (Sodium Dodecyl Sulfate) w/v, 10% glycerol, 150-mM NaCl,

2 M Urea) with 50 μM MG132 (Sigma, St Louis, MO, USA) and 1 × proteinase inhibitor cocktail (Sigma). The protein extract was cleared by centrifugation at 12,000 g for 20 min, and equal amounts of proteins were separated in 12% SDS–PAGE. Anti HY5 (Agresera, Vännäs, Sweden; 1:500) and anti-ACTIN (Sigma; 1:1,000) were used to perform western blot.

### Statistical analysis

All statistical analyses were performed using GraphPad Prism version 8.0 software. Details of the analysis are given in the respective figure legends.

### Accession numbers

*BBX11* (AT2G47890), *HY5* (AT5G11260), *CHS* (AT5G13930), *CHI* (AT3G55120), *ELIP1* (AT3G22840), *ELIP2* (AT4G14690), *UVR2* (AT1G12370), and *UVR3* (AT3G15620).

### Supplemental data

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

**Supplemental Figure S1.** UV-B mediated induction of *BBX11* is partially dependent on *UVR8* whereas *BBX12* and *BBX13* mRNA expression is not induced by UV-B.

**Supplemental Figure S2.** Relative hypocotyl length of loss- and gain-of-function mutants of *BBX11* under UV-B.

**Supplemental Figure S3.** Overexpression of *BBX11* enhances protection against UV-B stress.

**Supplemental Figure S4.** *BBX11* modulates accumulation of photoprotective secondary metabolites.

**Supplemental Figure S5.** *HY5* directly binds to the promoter of *BBX11*.

**Supplemental Figure S6.** Expression of *F3H* is upregulated in OE3 seedlings overexpressing *BBX11*.

**Supplemental Figure S7.** *HY5* is an early UV-B inducible gene.

**Supplemental Table S1.** List of primers used in this study.

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*Conflict of interest statement.* The authors declare that there is no conflict of interest. Some of the results reported here have been incorporated as part of a patent application where S.D. and N.J. are the inventors.

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