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# BBX21 Integrates Brassinosteroid Biosynthesis and Signaling in the Inhibition of Hypocotyl Growth under Shade

Gabriel Gómez-Ocampo[1,†], Carlos D. Crocco[1,†], Jimena Cascales[1,†], Jana Oklestkova[2], Danuše Tarkowská[2], Miroslav Strnad[2], Santiago Mora-Garcia[3], José L. Pruneda-Paz[4], Miguel A. Blazquez[5] and Javier F. Botto[1,*]

[1]IFEVA (CONICET-UBA), Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, Ciudad Autónoma de Buenos Aires C1417DSE, Argentina
[2]Laboratory of Growth Regulators, Palacký University & Institute of Experimental Botany, The Czech Academy of Sciences, Šlechtitelů 27, Olomouc CZ-78371, Czech Republic
[3]Fundación Instituto Leloir, IIBBA-CONICET, Avenida Patricias Argentinas 435, Ciudad Autónoma de Buenos Aires C1405BWE, Argentina
[4]Section of Cell and Developmental Biology, Division of Biological Sciences, University of California San Diego, La Jolla, CA 92093, USA
[5]Instituto de Biología Molecular y Celular de Plantas, CSIC-Universitat Politècnica de València, C/Ingeniero Fausto Elio s/n, Valencia 46022, Spain
[†]Authors contributed equally.

*Corresponding author: Email, botto@agro.uba.ar

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**B-Box-containing zinc finger transcription factors (BBX) are involved in light-mediated growth, affecting processes such as hypocotyl elongation in *Arabidopsis thaliana*. However, the molecular and hormonal framework that regulates plant growth through BBX proteins is incomplete. Here, we demonstrate that BBX21 inhibits the hypocotyl elongation through the brassinosteroid (BR) pathway. BBX21 reduces the sensitivity to 24-*epi*BL, a synthetic active BR, principally at very low concentrations in simulated shade. The biosynthesis profile of BRs showed that two active BR—brassinolide and 28-homobrassinolide—and 8 of 11 intermediates can be repressed by BBX21 under white light (WL) or simulated shade. Furthermore, BBX21 represses the expression of *CYTOCHROME P450 90B1 (DWF4/CYP90B1)*, *BRASSINOSTEROID-6-OXIDASE 1 (BR6OX1, CYP85A1)* and *BR6OX2 (CYP85A2)* genes involved in the BR biosynthesis in WL while specifically promoting *DWF4* and *PHYB ACTIVATION TAGGED SUPPRESSOR 1 (CYP2B1/BAS1)* expression in WL supplemented with far-red (WL + FR), a treatment that simulates shade. In addition, BBX21 represses BR signaling genes, such as *PACLOBUTRAZOL RESISTANCE1 (PRE1)*, *PRE3* and *ARABIDOPSIS MYB-LIKE 2 (MYBL2)*, and auxin-related and expansin genes, such as *INDOLE-3-ACETIC ACID INDUCIBLE 1 (IAA1)*, *IAA4* and *EXPANSIN 11* in short-term shade. By a genetic approach, we found that BBX21 acts genetically upstream of BRASSINAZOLE-RESISTANT 1 (BZR1) for the promotion of *DWF4* and *BAS1* gene expression in shade. We propose that BBX21 integrates the BR homeostasis and shade-light signaling, allowing the fine-tuning of hypocotyl elongation in Arabidopsis.**

**Keywords:** Arabidopsis • BBX21 • Brassinosteroids (BRs) • BZR1 • Shade light

Brassinosteroids (BRs), a group of polyhydroxylated plant steroid hormones, are involved in many aspects of plant growth and development in a dosage-dependent manner (Lin et al. 2021). Maintenance of BR homeostasis is therefore critical for optimal functions of BRs. Considering that BRs cannot undergo long-distance transport, their biosynthesis and catabolism are two critical antagonistic processes to maintain BR homeostasis in plants (Symons and Reid 2004, Zhao and Li 2012). BR biosynthesis involves parallel and highly networked pathways leading to the production of $C_{27}$, $C_{28}$ and $C_{29}$ steroids based on the total number of carbons (Fujioka and Yokota 2003). $C_{28}$ are the most abundant and ubiquitous BRs in plants that involve the early C-22 oxidation pathway and the late C-6 oxidation pathway (Nomura et al. 2001, Fujioka et al. 2002). In the early steps of BR biosynthesis, five C-22 hydroxylation reactions are catalyzed by cytochrome P450 monooxygenases. CYTOCHROME P450 90B1 (DWF4/CYP90B1) is mainly responsible for these reactions acting as a rate-limiting step between the sterols and C-6-oxidation intermediates in Arabidopsis (Choe et al. 1998, Fujita et al. 2006, Fujiyama et al. 2019). BRASSINOSTEROID-6-OXIDASES BR6OX1 (CYP85A1) and BR6OX2 (CYP85A2) are two central C-6 oxidation enzymes that catalyze several end-metabolic reactions of BR intermediates, in which C-6 position of 6-deoxocastasterone, 6-deoxotyphasterol (6-deoxoTY), 3-dehydro-6-deoxoteasterone



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(6-deoxo-3-DT) and 6-deoxoteasteasterone are oxidized. At the end of $C_{28}$ BR biosynthesis pathway, castasterone (CS) is converted into brassinolide (BL) by the enzymatic activity of BR6OX2 (Kim et al. 2005, Nomura et al. 2005). Furthermore, PHYB ACTIVATION TAGGED SUPPRESSOR 1 (CYP2B1/BAS1) encodes a cytochrome P450 monooxygenase implicated in BR catabolism as well as photomorphogenesis (Neff et al. 1999, Turk et al. 2003).

The BR signaling pathway involves the perception of BL, the most active BR compound, by the receptor BR-INSENSITIVE 1 (BRI1) and the co-receptor BRI1-ASSOCIATED RECEPTOR KINASE (BAK1), which ultimately inactivate BR-INSENSITIVE 2 (BIN2), triggering the accumulation of unphosphorylated BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI-EMS-SUPPRESSOR 1 (BES1). The unphosphorylated forms of both transcription factors bind to the gene targets and promote cell elongation (Li et al. 1996, Wang et al. 2002, Yin et al. 2002). BES1 and BZR1 not only regulate the expression of thousands of genes involved in plant growth but are also responsible for feedback inhibition by directly binding to the promoters of multiple BR biosynthesis genes, repressing their expression (He et al. 2005, Sun et al. 2010, Yu et al. 2011). Furthermore, BZR1 interacts with PHYTOCHROME-INTERACTING FACTOR 4 (PIF4), one of its target gene products, forming heterodimers that bind to the promoters of common target genes (Bai et al. 2012, Oh et al. 2012). The *pif1 pif3 pif4 pif5* quadruple mutant displays reduced hypocotyl elongation in darkness and suppresses BZR1-mediated hypocotyl elongation, indicating that both BZR1 and the PIFs are required to promote cell elongation (Oh et al. 2012). A recent study demonstrated that BES1 can also inhibit the expression of BR biosynthesis genes during the day, and elevated levels of PIF4 compete for BES1, resulting in de-repressed BR biosynthesis at dawn (Martínez et al. 2018). In addition, PIF4 and PIF5 directly bind to the promoters of *DWF4* and *CYP85A2*/*BR6OX2* to enhance their expression, resulting in elevated levels of BRs (Park et al. 2003, Wei et al. 2017).

B-box-containing zinc finger transcription factors (BBX) mediate transcriptional regulation and protein–protein interactions in plant growth and development, often integrating environmental information with hormone signaling network (Gangappa and Botto 2014, Song et al. 2020b, Yadav et al. 2020). Some BBX transcription factors are involved in the BR signaling of seedling photomorphogenesis. For example, BBX32, a repressor of seedling photomorphogenesis, interacts with BZR1 and PIF3 to promote BR-mediated cotyledon closure in response to light (Ravindran et al. 2021). Very recently, it has also been demonstrated that BBX28 and BBX29 suppress seedling photomorphogenesis through the physical interaction with BR-ENHANCED EXPRESSION 1 (BEE1), BEE2 and BEE3, which ultimately increase the levels of BZR1 to promote cell elongation (Cao et al. 2022). In contrast, BBX20/BZS1, a positive regulator of seedling photomorphogenesis, is repressed by BZR1 transcription factor (Fan et al. 2012). In addition, BBX21, a close homolog of BBX20, physically interacts with ELONGATED HYPOCOTYL 5 (HY5) to promote together the transcription of several genes during seedling photomorphogenesis (Datta et al. 2006, Xu et al. 2016, 2018, Bursch et al. 2020). The simultaneous overexpression of both transcription factors results in the additive inhibition of seedling hypocotyl elongation, suggesting that BBX21 can also act independently or regulate post-transcriptionally HY5 to promote photomorphogenesis (Job et al. 2018). Interestingly, it has been demonstrated that BBX21 can regulate HY5 at the post-transcriptional level by interfering with HY5 binding on the *ABI5* promoter to fine-tune ABA signaling (Xu et al. 2014). In addition, the stability of BBX21 is regulated by CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) through the 26S proteosome machinery in darkness (Xu et al. 2018).

Shade avoidance syndrome (SAS) is a strategy to compete for light between neighboring plants by elongation of stems and petioles (Ballaré and Pierik 2017). When plants begin to be shaded by neighbors, a burst of molecular signals are triggered to reallocate resources, promoting plant elongation to compete for photosynthesis light. In the canopies, the transmitted light is especially depleted in red (R) and blue (B) wavelengths due to the absorption by chlorophylls, and the photosynthetically active radiation (PAR) is lowered according to plant density. The reduction of R/FR (red/far-red) ratios and B photons inactivate phytochrome B (phyB) and cryptochrome 1 (cry1), respectively, enhancing the activity of PIF transcription factors that promote the shade-induced transcriptome and activate COP1 and SUPPRESSOR of phyA-105 ubiquitin E3 ligase complex that mediates the stability of SAS-induced proteins (Casal 2013). SAS signaling pathways together with the activity of phytohormones ultimately optimize the resource allocation patterns and the plant developmental configuration under the shade (Alabadí and Blázquez 2009). The hypocotyl elongation response in the SAS, once the de-etiolation requirements have been fulfilled, is under the tight control of several BBXs (Crocco et al. 2010, Wang et al. 2013). For example, BBX24 promotes the SAS through the physical interaction with DELLAs, which enhances PIF4 activity to induce the expression of cell-elongation genes (Crocco et al. 2015). In addition, BBX24 and JASMONATE-INSENSITIVE 3 regulate DELLA activity, coregulating GA and jasmonic acid (JA) signaling pathways downstream phyB to induce the hypocotyl elongation in the SAS (Saura-Sánchez et al. 2023). In opposition, BBX21 can inhibit the hypocotyl growth, downregulating the expression of auxins, BRs and ethylene genes downstream COP1 in the SAS (Crocco et al. 2010, 2011, Gangappa et al. 2013). However, the BBX21-dependent mechanisms and the hormonal pathway underlying the repression of cell elongation remain unknown. Here we combined genetic, metabolic, physiological and gene-expression experiments to understand the BBX21-dependent mechanisms that suppress the hypocotyl elongation responses in Arabidopsis.

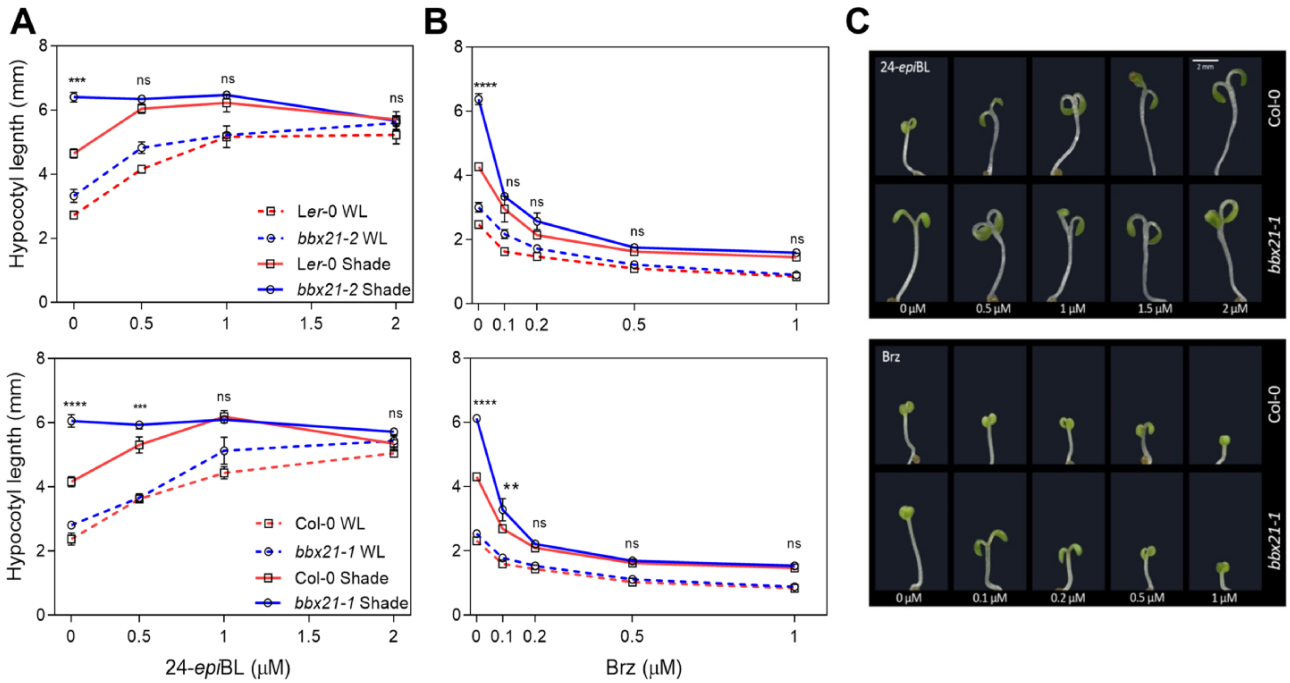


Fig. 1 Col-0 and L*er* mimic the hypocotyl phenotype of the *bbx21-1* and *bbx21-2* mutant with the addition of exogenous 24-*epi*BL under shade. A. 24-*epi*BL and B. Brz dose-response curves of *bbx21-1* and *bbx21-2* mutants and their wild-type Col-0 and L*er*-0, respectively, under WL and shade. C. Representative photographs of Col-0 and *bbx21-1* seedlings grown at different doses of 24-*epi*BL and Brz under shade for 5 d. In A and B, each point indicates mean ± SE ($n = 3$). Two-way ANOVA followed by Tukey's test for comparisons between means. Asterisks indicates significant differences between genotypes under shade at the same 24-*epi*BL or Brz doses (****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, ns: no significant difference).

## Results

### BBX21 represses hypocotyl elongation by reducing 24-epiBL sensitivity under shade

In a previous work, we demonstrated that BBX21 inhibits the hypocotyl growth of Arabidopsis seedlings under simulated shade in part through COP1 signaling, but additional mechanisms are also on place (Crocco et al. 2010). Given the involvement of several hormones in the promotion of the hypocotyl elongation—particularly under shade, we examined the effects of synthetic inhibitors of auxin transport [*N*-1-naphthylphthalamic acid (NPA)], GAs [paclobutrazol (PAC)] and BRs [brassinazole (Brz)] biosynthesis on the hypocotyl elongation of L*er*-0 and *bbx21-2* seedlings under white light (WL) and shade. The hypocotyl length was short and similar among genotypes under WL (**Supplementary Fig. S1**). We found that *bbx21-2* mutant had longer hypocotyls than L*er*-0 when seedlings were grown in a media with PAC and NPA under shade. In contrast, the Brz inhibitor (1 μM) inhibited the hypocotyl elongation of *bbx21-2* seedlings to reach the levels of L*er*-0 under shade (**Supplementary Fig. S1A**). The *bbx21-2* mutant mimics the wild-type (WT) phenotype under simulated shade only with the Brz inhibitor, suggesting that BRs may mediate the BBX21-dependent hypocotyl growth.

To better characterize the involvement of BRs in the BBX21-dependent hypocotyl elongation, we compared the phenotypes of Col-0, L*er*-0 and two independent null mutant lines of BBX21 (i.e. *bbx21-1* and *bbx21-2*, respectively) with different doses of 24-*epi*brassinolide (24-*epi*BL) and Brz under WL and shade (**Fig. 1**). The addition of BR (24-*epi*BL) increased the hypocotyl elongation independently of the genotype under WL (**Fig. 1A** and **Supplementary Fig. S2**). However, the effects of the 24-*epi*BL were different between genotypes under shade. In wild-type genotypes, the hypocotyl elongation increased and saturated between 0.5 or 1 μmol of 24-*epi*BL, while the *bbx21-1* and *bbx21-2* mutants showed constitutively taller hypocotyls independently of the 24-*epi*BL doses (**Fig. 1A, C**). In contrast, low doses of Brz (0.1 μmol) suppressed the hypocotyl elongation of both *bbx21* mutants with respect to the wild-type genotypes under shade (**Fig. 1B, C**). The 24-*epi*BL dose-response curve of *35S:myc-BBX21* seedlings grown in WL showed a lower response to BR, suggesting that BBX21 is involved in this hormonal signaling pathway (**Supplementary Fig. S3**). We confirmed these results by comparing the hypocotyl phenotype in Col-0, *35S:YFP-BBX21* and *35S:myc-BBX21*-overexpressing seedlings grown in agar under WL or shade or in an agar media supplemented with 24-*epi*BL (1 μmol) or with BZR (0.5 μM) under shade. The hypocotyl length was similar between genotypes under WL, while the overexpression of BBX21 significantly reduced the hypocotyl elongation under shade. Moreover, the addition of 24-*epi*BL increased the hypocotyl length of wild-type seedlings under shade but not in

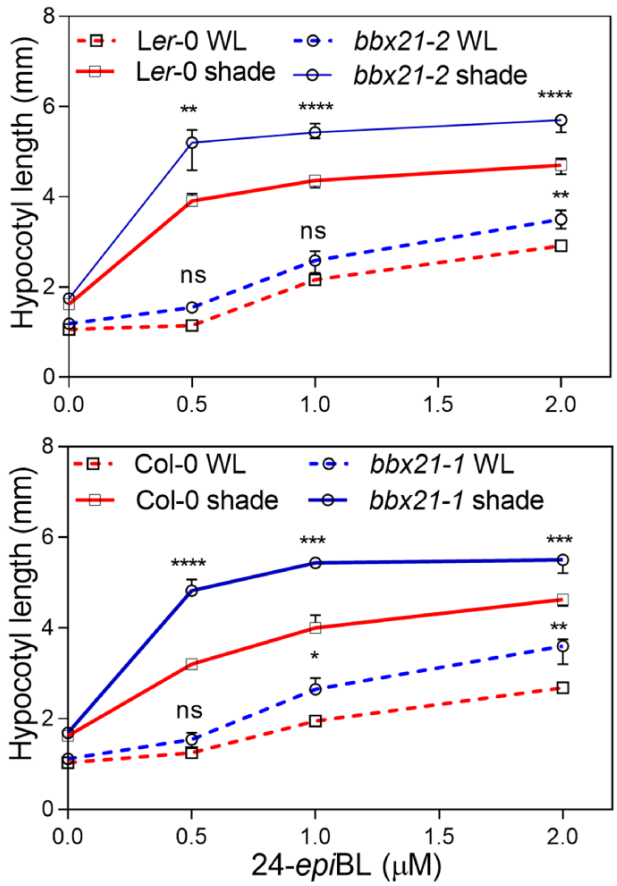


Fig. 2 Hypocotyl growth sensitivity response to 24-*epi*BL under WL or shade. Seedlings of L*er*-0, Col-0, *bbx21-1* and *bbx21-2* were grown with 24-*epi*BL in presence of 0.5 μM Brz under WL or shade for 5 d. Each point indicates mean ± SE ($n = 3$). Two-way ANOVA followed by Tukey's test for comparisons between means. Asterisks indicate significant differences between genotypes in WL or shade at the same doses (****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, ns: nosignificant difference).

the BBX21-overexpressing lines, while the addition of Bzr inhibited the growth independently of the genotype (**Supplementary Fig. S4**). These results agree with the inhibitory function of BBX21 in the BR signaling under shade. We designed an additional experiment to evaluate the BR sensitivity response in the hypocotyl growth at different doses of 24-*epi*BL with the addition of 0.5 μmol Brz to inhibit the endogenous synthesis of BRs. The *bbx21-1* and *bbx21-2* seedlings showed a higher sensitivity response to 24-*epi*BL than wild-type genotypes principally at very low doses and shade (**Fig. 2**). Taken together, these results strongly suggest that BBX21 inhibits the hypocotyl growth by affecting the BR biosynthesis and/or signaling pathways.

## BBX21 inhibits the synthesis of intermediate and active BRs under WL and shade

Next, we investigated whether BBX21 regulates the formation of intermediates and end products of the BR biosynthetic pathway. We quantified the content of 13 BRs, including 11 intermediates and 2 active BRs, BL and its less active analogue 28-homobrassinolide (28-homoBL), in Col-0 and *bbx21-1* seedlings grown under WL and shade for 5 d. The levels of 10 of 13 BRs were significantly higher in the *bbx21-1* mutant compared with Col-0 under WL and/or shade, indicating that BBX21 inhibits their synthesis (**Fig. 3**). The active BL was around 3-fold higher in the *bbx21-1* than in Col-0 under WL, and the 28-homoBL was 5-fold higher in the mutant with respect to WT in both light conditions, with stronger effects under shade ($P = 0.0002$ in *bbx21-1* between WL and shade, **Fig. 3C**). Four intermediates belonging to the most common $C_{28}$-BR biosynthesis pathway, including teasterone (TE), typhasterol (TY) and CS, and two intermediates belonging to $C_{27}$ and $C_{29}$ BR biosynthesis pathways, such as 28-norcastasterone (28-norCS) and 28-homocastasterone (28-homoCS), respectively, were significantly higher in the *bbx21-1* mutant than Col-0 under both light conditions (**Fig. 3B**). Furthermore, two important sterols, campesterol (CR) and campestanol (CN), were produced at higher levels in the *bbx21-1* mutant under WL and shade, respectively (**Fig. 3A**). The levels of 6-oxocampestanol intermediate were not affected by BBX21, and two intermediates of BR biosynthesis, 6-deoxo-3-DT and 6-deoxoTY, were down-regulated in the *bbx21* mutant under WL and shade, respectively (**Fig. 3**). Altogether, these results suggest that BBX21 down-regulates the de novo biosynthesis of BRs under WL and shade.

## BBX21 regulates BR metabolism and signaling gene expression

BR homeostasis is maintained in part by transcriptional feedback regulation loops that control the expression of key metabolic enzymes (Bajguz et al. 2020). To have a better understanding of the BBX21 control on the biosynthesis of BRs, we evaluated the expression of *DWF4*, a rate-limiting enzyme between the sterols and C-6-oxidation intermediates, *BR6OX1* and *BR6OX2*, two central C-6 oxidation enzymes that catalyze several end-metabolic steps of BR intermediates (**Supplementary Fig. S5**), and *BAS1*, which encodes a cytochrome P450 monooxygenase implicated in the catabolism of BL and CS (**Supplementary Fig. S6**). The expression of the three BR anabolic genes was up-regulated under WL in the *bbx21-1* mutant compared with Col-0, suggesting that BBX21 inhibits their expression (**Fig. 4A**). The expression of *DWF4* and *BAS1* was significantly lower in the *bbx21-1* than WT, while *BR6OX1* and *BR6OX2* expression was similar between both genotypes in WL + 1 h-FR, a treatment that simulates shade (**Fig. 4A**). In addition, the expression of *BR6OX2* and *BAS1* was promoted in the BBX21-overexpressing line in WL + 1 h-FR (**Fig. 4B**). The higher expression of both *BR6OXs* correlates with the reduced levels of 6-deoxyTY and 6-deoxo-3-DT in *bbx21-1* seedlings (**Fig. 3B**), suggesting that BBX21 inactivates the early C-6 oxidation pathway and consequently can reduce the synthesis of BL and/or increase the BR catabolism under shade. Furthermore, the down-regulated and up-regulated expression of *BAS1* in *bbx21-1* mutant and 35S:*myc*-BBX21-overexpressing line, respectively, support the idea that BBX21 reduces endogenous BR accumulation in plants cultivated under shade.

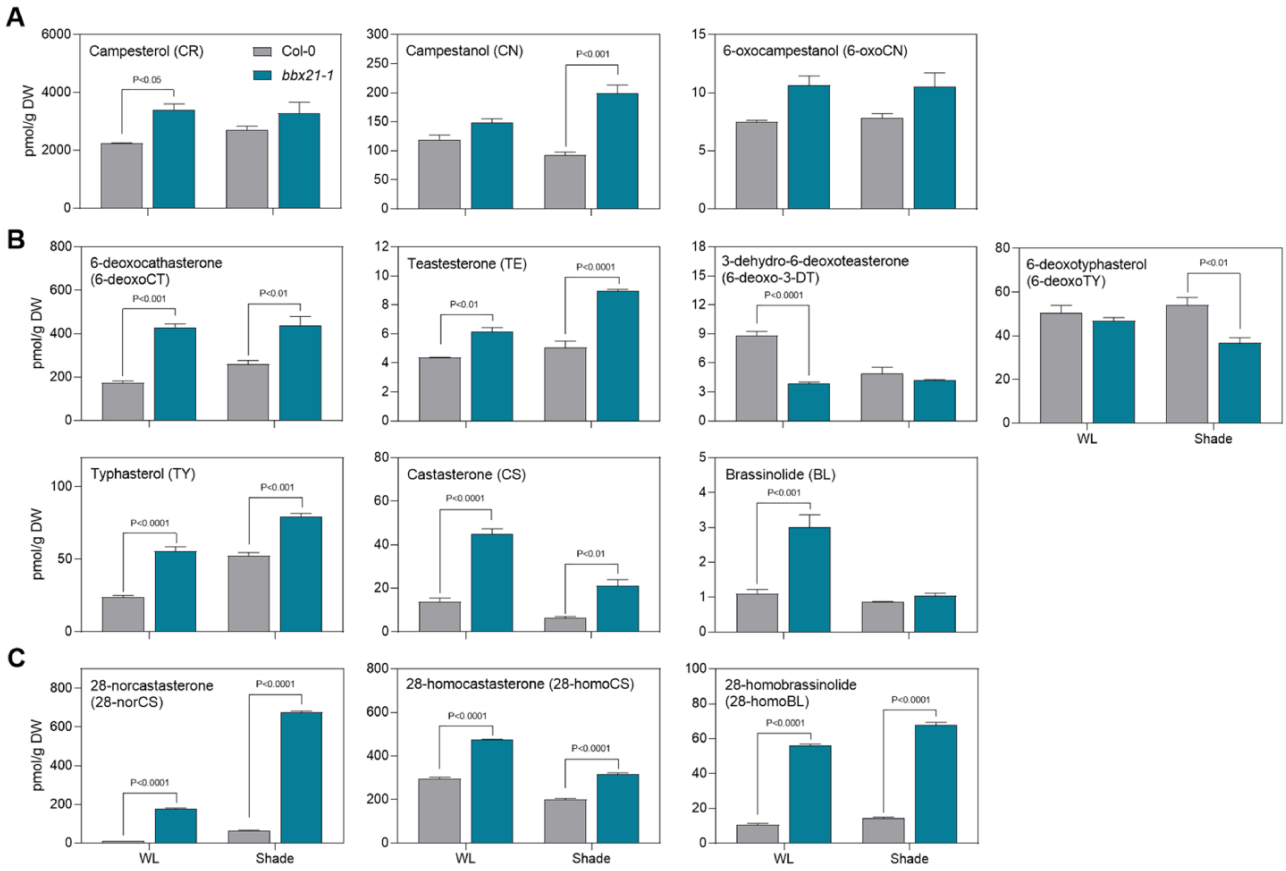


Fig. 3 BBX21 inhibits the biosynthesis of intermediate and active brassinosteroids under WL and shade. Precursor and active content of BRs in Col-0 and *bbx21-1* seedlings grown under WL or shade for 5 d. A. Sterol intermediates of BRs. B. $C_{28}$-related intermediates and active BRs. C. $C_{27}$-related and $C_{29}$-related intermediates and active BRs. Each bar indicates mean ± SE ($n = 3$). Two-way ANOVA followed by Tukey's test for comparisons between means. Significant differences between genotypes in each light condition are indicated.

However, these observations also suggest that BBX21 may be involved in other mechanisms associated with BR homeostasis for the growth regulation under shade. Then, we reasoned that BBX21 transcription factor can regulate the gene expression of BR signaling. Then, we measured the expression of genes regulated by BR such as *BRASINAZOLE-RESISTANT (BZR1)*, *PACLOBUTRAZOL-RESISTANCE 5 (PRE5)* and *ARGOS-LIKE (ARL)* in WT, *bbx21-1* and *35S:myc-BBX21* seedlings exposed to WL or WL + 1 h-FR. We found that the three transcripts were up-regulated in the *bbx21-1* mutant (**Fig. 5A**), but this negative regulation was not confirmed in BBX21-overexpressing seedlings grown in WL (**Fig. 5B**). Furthermore, *PRE5* and *ARL* were up-regulated in the *bbx21-1* mutant, and PRE5 was also down-regulated in the *35S:myc-BBX21*-overexpressing line when seedlings were exposed to WL + 1 h-FR (**Fig. 5**). These results suggest that BBX21 can inhibit some BR-regulated genes under shade.

Previous work on the BBX21 function under shade suggested that this transcription factor is part of the 'gas-and-brake' mechanism that is activated immediately upon shade treatment and operates as negative regulator of the positive regulators involved in the promotion of growth under shade (Crocco et al. 2010). In order to have a better understanding of the mechanisms in which BBX21 is involved, we designed a new experiment to perform RT-qPCR analysis of selected genes in a short-term and a long-term shade conditions. We cultivated WT and *bbx21-1* de-etiolated seedlings for 5 d in WL and then, in the last photoperiod, we exposed them to WL, to WL supplemented with 1 h-FR (WL + 1 h-FR) or to WL supplemented with 5 h-FR (WL + 5 h-FR) to study the effects of short- and long-term shade on gene expression. We measured the gene expression of BR- and growth-related genes such as *MYB-LIKE2 (MYBL2)*, *ILI1 BINDING BHLH 1 (IBH1)*, *PRE1*, *PRE3*, *PIF4*, *INDOLE-3-ACETIC ACID INDUCIBLE 1 (IAA1)*, *IAA4* and *EXPANSIN 11 (EXP11)*. The *MYBL2*, *PRE1*, *PRE3*, *PIF4*, *IAA4* and *EXP11* gene expression was significantly lower in *bbx21-1* compared with WT in WL + 1 h-FR but not in longer shade (**Fig. 6**), suggesting that BBX21 can promote the gene expression in short-term shade.

## BBX21, BZR1 and PIF4 signaling co-regulate gene expression under shade

To have a better understanding of the BBX21 function under shade, we revisited three previous transcriptome profilings to

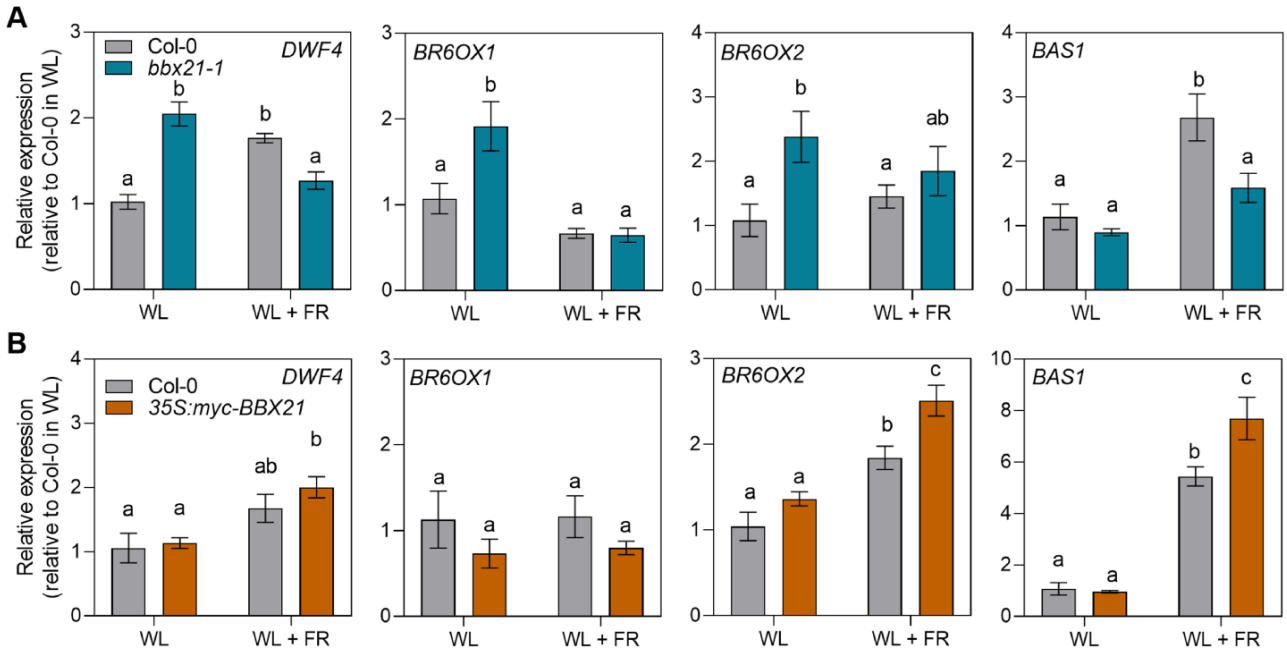


Fig. 4 BBX21 regulates the expression of BRs biosynthesis genes under WL and WL + FR. Relative gene expression of *DWF4*, *BAS1*, *BR6OX1* and *BR6OX2* in 5-d-old Col-0 and *bbx21-1* (A) and Col-0 and *35S:myc-BBX21* (B) seedlings grown under WL or WL supplemented with 1 h-FR at the end of the photoperiod. Each bar indicates mean ± SE ($n = 3$). Two-way ANOVA followed by Tukey's test for comparisons between means. Different letters indicate significant differences in the expression of each gene.

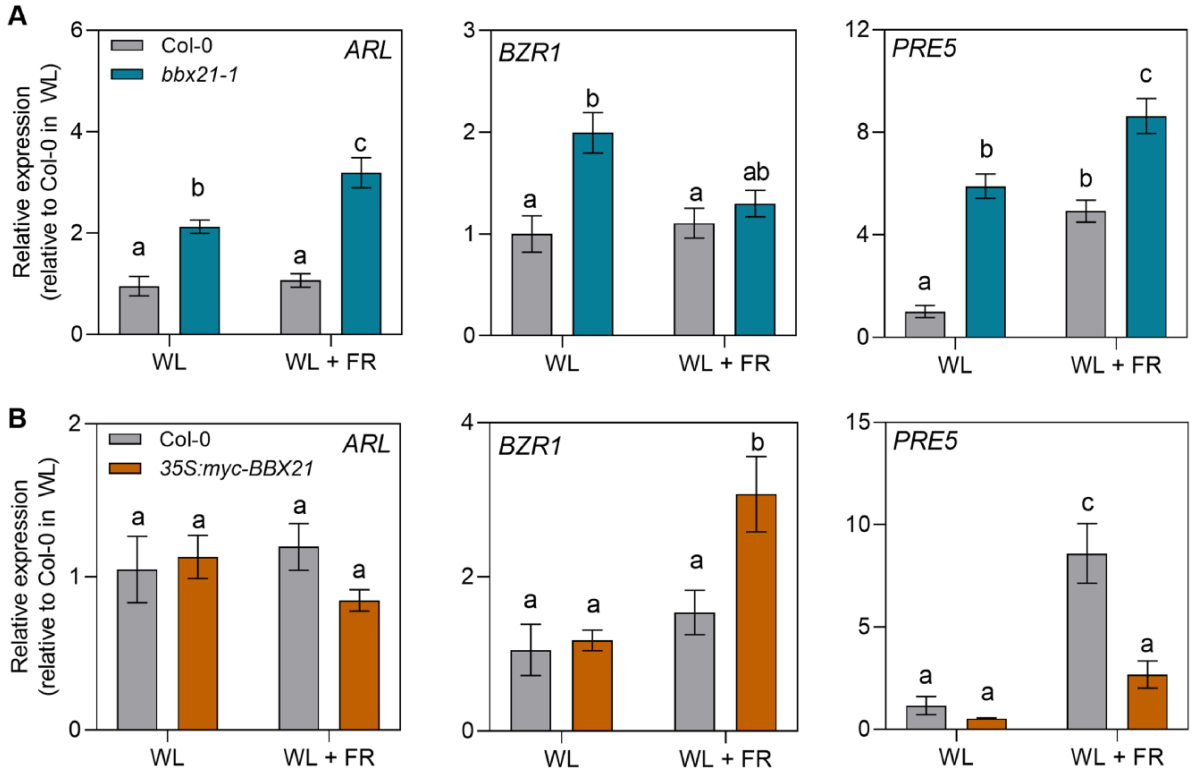


Fig. 5 BBX21 regulates the expression of BR signaling genes under WL and WL + FR. Relative gene expression of *BZR1*, *PRE5* and *ARL* in 5-d-old Col-0 and *bbx21-1* (A) and Col-0 and *35S:myc-BBX21* (B) seedlings grown under WL or WL supplemented with 1 h-FR at the end of the photoperiod. Each bar indicates mean ± SE ($n = 3$). Two-way ANOVA followed by Tukey's test for comparisons between means. Different letters indicate significant differences in the expression of each gene.

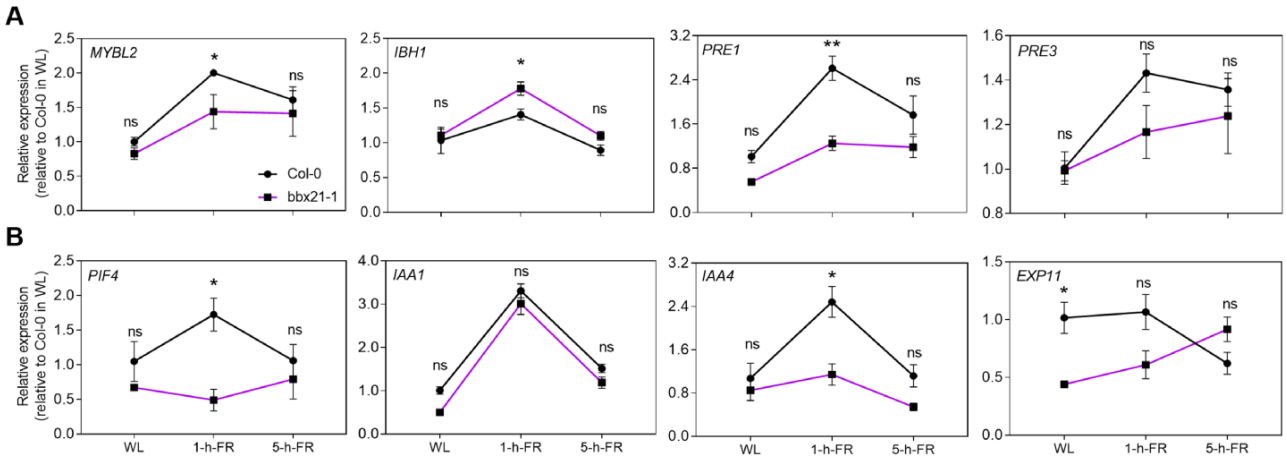


Fig. 6 Relative gene expression in 5-d-old Col-0 and *bbx21-1* mutant seedlings grown under WL or WL supplemented with 1 h-FR and 5 h-FR at the end of the photoperiod of A. genes co-regulated by BBX21 and BZR1 and B. genes co-regulated by BBX21 and PIF4. Each point indicates mean ± SE (n = 3). The black line is Col-0 and the purple line is the *bbx21-1* mutant. Asterisks indicate significant differences between genotypes in the same light condition by *t* student test (***$P < 0.001$, **$P < 0.01$*$P < 0.05$, ns: no significant difference).

identify co-regulated genes between BBX21, BZR1 and PIF4 transcription factors. We compared the 576 BBX21-regulated genes whose expression is significantly affected by simulated shade with 3412 BZR1-regulated genes and 1538 PIF4-binding target genes that promote hypocotyl elongation (Bai et al. 2012, Oh et al. 2012). We found 128 BBX21-regulated genes co-regulated by PIF4 and BZR1 (**Supplementary Table S1**). By DNA-Chip analysis (dChip), we classified those co-regulated genes in five clusters (**Fig. 7A** and **Supplementary Fig. S7**). The cluster (a) includes 26 genes co-regulated by BBX21 and BZR1-binding targets with BR regulation such as *ARABIDOPSIS PIN-FORMED 7*, *LEUCINE-RICH REPEAT/EXTENSIN 3* and *MYBL2*. The cluster (b) includes 19 genes co-regulated by BBX21, BZR1-binding targets with BR regulation and PIF4-binding targets such as *IBH1*, *OBF BINDING PROTEIN 4* and a bZIP transcription factor, *ZIP61*. The cluster (c) is represented by 28 genes co-regulated by BBX21 and BZR1-binding targets without BR regulation including *JAR1*, *CRF2*, *ILR1* and *CY707A1*. The cluster (d) is represented by 14 genes co-regulated by BBX21, BZR1-binding targets without BR regulation and PIF4-binding targets, including RPT2 and NCED4. Finally, the cluster (e) includes 41 genes co-regulated by BBX21 and PIF4-binding targets such as *PIF4*, *IAA1* and *GAST1 PROTEIN HOMOLOG 4*, among others (**Supplementary Fig. S7** and **Supplementary Table S1**). We found a significant enrichment of genes co-regulated by BBX21 and BZR1-binding targets with BR regulation [$P = 0.004$, representation factor (RF) = 1.5] and with PIF4-binding targets ($P = 6.47e^{-05}$, RF = 1.6). Then, we evaluated the expression of *MYBL2* (cluster a), *IBH1* (cluster b) and two key genes promoting cell elongation through BR signaling, *PRE1* and *PRE3*, in Col-0 and *bbx21-1* seedlings grown under WL and simulated shade for 5 d. The gene expression of *MYBL2*, *PRE1* and *PRE3* was strongly induced by simulated shade in the *bbx21-1* mutant, suggesting that BBX21 inhibits their expression (**Fig. 7B**). We also evaluated the expression of genes of PIF4 signaling belonging to the cluster (e), including *PIF4* and *IAA1* and two additional genes related with the cell growth (*IAA4* and *EXP11*). The results indicate that BBX21 strongly inhibits the gene expression of *IAA1*, *IAA4* and *EXP11* but not *PIF4* under simulated shade (**Fig. 7C**). Taken together, these results suggest that BBX21 represses the expression of genes related to BR and PIF4 signaling under simulated shade.

## BBX21 interacts genetically with BZR1 to control hypocotyl length under shade

The previous results demonstrated that BBX21 inhibits the hypocotyl elongation, in part, by controlling the expression of genes downstream BZR1 and PIF4 under shade (**Fig. 7**). Furthermore, BZR1 interacts with PIFs to regulate the expression of cell elongation-related genes (Oh et al. 2012), and PIF4 is a central transcription factor together with PIF5 and PIF7 in the SAS (Lorrain et al. 2008, Leivar et al. 2012, Li et al. 2012). Thus, we hypothesized that BBX21 would interact genetically with BZR1 and PIF4 to inhibit hypocotyl elongation under shade. We crossed *bbx21-1* with *bzr1-1D* to select *bbx21-1 bzr1-1D* double mutant for phenotyping in our light conditions. Seedlings of *bzr1-1D*, *bbx21-1* and *bbx21-1 bzr1-1D* showed longer hypocotyls than Col-0 under shade but not under WL, suggesting that BBX21 and BZR act redundantly in the same genetic pathway for the control of hypocotyl elongation under shade (**Fig. 8A**). Then, we measured gene expression of *DWF4*, *BR6OX1*, *BR6OX2* and *BAS1* in WT, *bbx21-1*, *bzr1-1D* and *bbx21-1 bzr1-1D* under WL or WL supplemented with 1 h-FR (WL + FR). We found that BBX21 induces the expression of *DWF4* and *BAS1* in short-term shade and that BZR1 acts downstream of BBX21 (**Fig. 8B**). The *DWF4* and *BAS1* expression were down-regulated not only in the *bbx21-1* mutant but also more intensely in the *bbx21-1 bzr1-1D* mutant, suggesting that an additional mechanism can be regulating their expression in simulated shade (**Fig. 8B**).

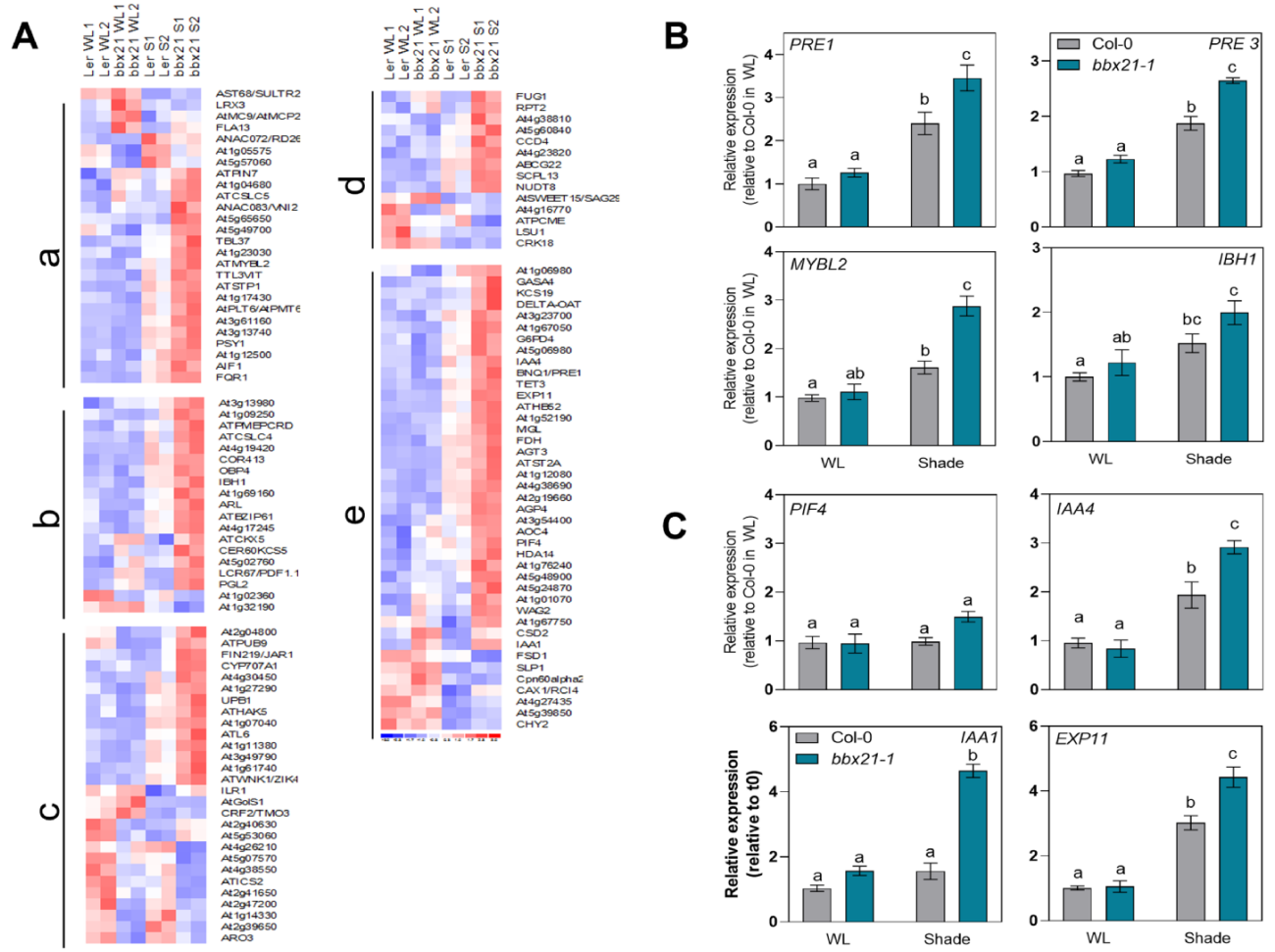


Fig. 7 Coregulation of BBX21-regulated genes under shade and BZR- and PIF4-binding genes. A. dCHIP analysis of genes regulated by BBX21 under shade that are also regulated by BZR1 (with and without BR regulation; Bai et al. 2012) and/or PIF4 (Oh et al. 2012). The 128 co-regulated genes were classified into five clusters (a = 26, b = 19, c = 28, d = 14 and e = 41 genes). B and C. Relative expression of genes co-regulated by BBX21 and BZR1 (B) and by BBX21 and PIF4 (C). Seedlings were exposed to WL or shade for 5 days in the same growth chamber. Each bar indicates mean ± SE ($n = 3$). Two-way ANOVA followed by Tukey's test for comparisons between means. Different letters indicate significant differences in the expression of each gene.

It is well known that BR deficiency can cause a drastic dwarf phenotype in the plant hypocotyl in the dark (Szekeres et al. 1996, Wang et al. 2002). To exclude the possibility that a BBX21-dependent phenotype in BR signaling is induced in darkness, we designed a physiological experiment with WT, *bzr1-1D*, *bbx21-1* and *bbx21-1 bzr1-1* double mutant seedlings grown in agar or agar + 0.5 μM Brz in total dark for 5 d (**Supplementary Fig. S8A**). Previously, we had found that 0.5 μM was the lower concentration of Brz that induced a saturated inhibition response in darkness (**Supplementary Fig. S8B**). All genotypes showed similar hypocotyl length in darkness; the addition of Brz inhibitor reduced significantly the growth of Col-0 and *bbx21-1* seedlings but did not have effect on the mutants carrying the dominant allele of *bzr1-1D*, neither in the single mutant nor in the *bzr1-1D bbx21-1* double mutant. These results suggest that BBX21 activity on BR signaling is independent of the dark because no differences in hypocotyl growth response were detected between WT and *bbx21-1* seedlings.

## Discussion

Here, we demonstrated that BBX21 represses the hypocotyl elongation response by affecting the BR homeostasis. We found that BBX21 down-regulates the biosynthesis of several intermediates and that two active BRs (BL and 28-homoBL) are drastically elevated in *bbx21-1* compared with Col-0 seedlings grown under WL and simulated shade, suggesting that BBX21 represses hypocotyl elongation by affecting the synthesis of active BRs. Moreover, here we showed that BBX21 activity on BR signaling is independent of the dark because no differences in hypocotyl growth response were detected between Col-0 and L*er*-0 and *bbx21-1* and *bbx21-2* seedlings, respectively (**Supplementary Fig. S8**). BBX21 also represses the synthesis of intermediates

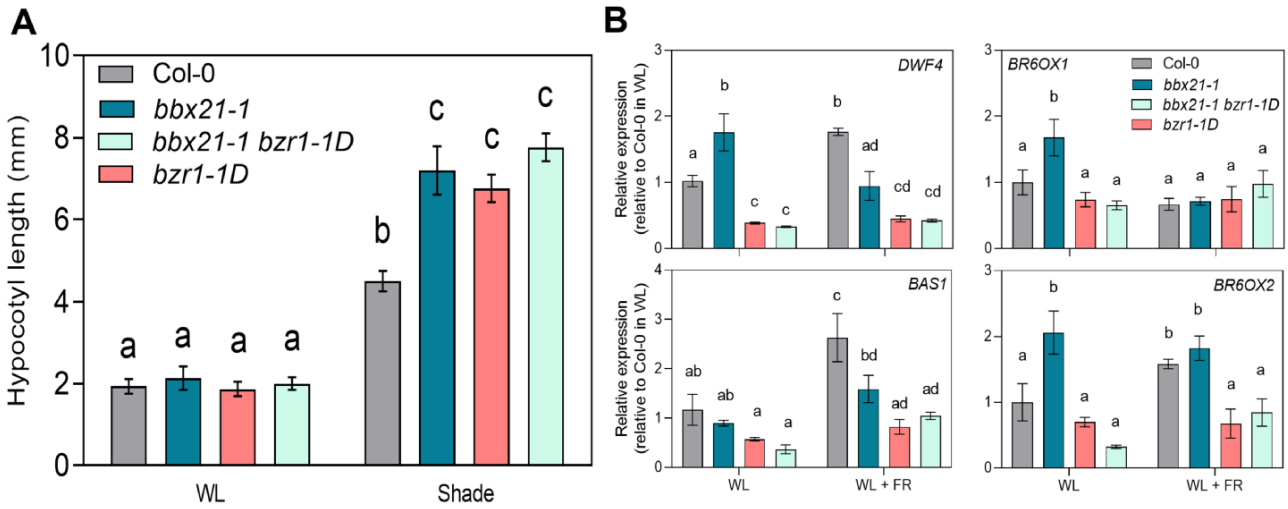


Fig. 8 BBX21 interacts genetically with BZR1 under shade. A. Hypocotyl length of Col-0, *bbx21-1*, *bzr1-1D* and *bbx21 bzr1-1D* seedlings grown in WL or shade for 5 d. B. Relative gene expression of *DWF4*, *BR6OX1*, *BR6OX2* and *BAS1* in Col-0, *bbx21-1*, *bzr1-1D* and *bbx21 bzr1-1D* seedlings grown under WL or WL supplemented with 1 h-FR at the end of the photoperiod. Each bar indicates mean ± SE ($n = 3$). Two-way ANOVA followed by Tukey's test for comparisons between means.

involved in sterol biosynthesis pathway (CR and CN), the C-6 oxidation pathway (TE, TY, CS, 6-deoxoCT, 6-deoxoTY and 6-deoxo-3-DT) and the $C_{27}$ and $C_{29}$ BR pathways (28-norCS and 28-homoCS, respectively). In addition, BBX21 suppresses the transcription of two critical genes that encode for enzymes involved in the biosynthesis of BRs, *BR6OX1* and *BR6OX2*, under WL (**Fig. 4** and **Supplementary Fig. S5**). These observations agree with previous reports showing light-dependency in the BR biosynthesis enzymes. It has been documented that *BR6OX2* expression is controlled by light and circadian clock (Nomoto et al. 2013). Moreover, *BR6OX2* expression is impaired in *hy5* and *phyA phyB* mutants, suggesting that its expression is induced by HY5 and phytochromes during seedling photomorphogenesis (Hamasaki et al. 2020). Furthermore, the expression pattern of *DWF4*, *BR6OX1* and *BR6OX2* is organ-specific, being particularly higher in the hook of Arabidopsis seedlings in response to light (Hamasaki et al. 2020). The transcriptional induction of BR biosynthesis enzymes by light is also common in other species. In seedlings of rice, it has been documented that 6 h of blue light and WL, but not far-red light, up-regulates the expression of *CYP85A1/OSDWARF*, encoding the homolog of *BR6OX1* of Arabidopsis, and increases endogenous BR levels, especially TY and CS (Asahina et al. 2014). The down-regulation of *BAS1* expression in *bbx21-1* mutant under WL + FR and the opposite response in the 35S:*myc*-BBX21 overexpression line suggest that BBX21 could be a point of control for the integration of shade light signals and the reduction of the level of active BRs. These results are consistent with the early study, showing that BAS1 and its homolog SOB7 act redundantly to promote seedling photomorphogenesis by reducing endogenous CS and active BRs (Turk et al. 2005).

The BR profile of seedlings grown under shade is also dependent on the activity of BBX21 (**Fig. 3**). However, there are some significant qualitative and quantitative differences in the BR profile of seedlings grown under WL and simulated shade. For example, 28-homoCS, CS and BL were more strongly inhibited by BBX21 under WL, while 28-norCS and CN biosynthesis was more intensely suppressed under shade (**Fig. 3**). In addition, there are differences in the gene expression of critical BR biosynthetic enzymes between WL and simulated shade. We found null effects of BBX21 on the regulation of *BR6OX1* and *BR6OX2* gene expression, but positive effects of BBX21 on the expression of *DWF4* when seedlings were grown in WL + FR (**Fig. 4**). In Arabidopsis, two active C-6 oxidation pathways have been documented. The 'early' C-6 oxidation pathway predominates when hydroxylation takes place after the oxidation of C-6 by BR6OXs enzymes, and the 'late' C-6 oxidation pathway occurs when hydroxylation metabolic steps occur before the oxidation of C-6 (Fujioka et al. 2002). The higher levels of TE and TY intermediates in *bbx21-1* mutant seedlings grown under shade suggest that the 'early' C-6 oxidation pathway is more relevant (**Fig. 3**, WL vs. shade in *bbx21-1*, $P = 0.0004$ and 0.0006, respectively). Interestingly, the BR profile under shade shares some similitudes with the BRs measured in etiolated seedlings. It has been suggested that the 'early' C-6 oxidation pathway is predominant during the skotomorphogenesis, while the 'late' C-6 oxidation pathway is active in the promotion of photomorphogenesis (Noguchi et al. 2000).

Regarding the previous observations on the biosynthetic pathways of active BR mediated by light, we reasoned that additional BBX21-dependent mechanisms would be involved in the control of hypocotyls under shade. The higher BR sensitivity of *bbx21-1* and *bbx21-2* mutant seedlings suggests that BBX21 may negatively modulate the BR signaling pathway (**Fig. 2**). Here we collected some evidence supporting this hypothesis. First, we found a significant enrichment of BBX21-dependent genes

differentially expressed under shade that are co-regulated by BZR1 and PIF4 (**Fig. 7A**, **Supplementary Fig. S7**). Second, we demonstrated that BBX21 down-regulates the expression of BR- and PIF4-signaling genes under shade (**Fig. 6**). Third, we found that BBX21 and BZR1 act redundantly in the same genetic pathway controlling the hypocotyl growth under shade (**Fig. 8**). It has been reported that BZR1 can interact with PIF4, one of its target gene products, to form heterodimers binding to promoters of their common targets (Bai et al. 2012, Oh et al. 2012). We hypothesize that BBX21 can inhibit the BZR1-PIF4 transcriptional module repressing the gene expression of BR signaling genes. We cannot discard that phyA and BBX21 may also act in the same pathway to inhibit BR signaling under shade. Previous reports documented that BBX21 promotes seedling photomorphogenesis in continuous FR mediated by phyA signaling (Datta et al. 2007) and also that the phyA negatively regulates the hypocotyl growth under strong shade by repressing the BR pathway (Martínez-García et al. 2014, Yang et al. 2018, Song et al. 2020a). In fact, the phyA can reduce COP1 nuclear speckle, which may lead to a reduction on the levels of PIF4 proteins that suppress the expression of BR genes (Song et al. 2020a). More studies are required to connect the function of BBX21 in the phyA-dependent signaling under shade.

In opposition to BBX21, there are other BBXs that enhance the BR signaling pathway during seedling de-etiolation. For example, BBX32, a repressor of seedling photomorphogenesis, interacts physically with BZR1 and PIF3 to mediate cotyledon closure (Ravindran et al. 2021). Furthermore, two other members of the same structural group V, BBX28 and BBX29, also suppress seedling photomorphogenesis by the physical interaction with BEE1, BEE2 and BEE3 that increase the levels of BZR1 to promote cell elongation. BBX20, a close homolog of BBX21 included in the structure group IV, partially suppresses the *cop1* phenotype in the dark and its expression is inhibited by BZR1 (Fan et al. 2012). A distinctive feature among BBXs belonging to the structural groups IV and V is the presence of two or one B-Box motifs, respectively, together with Val-Pro (VP) motifs (Crocco and Botto 2013, Job et al. 2018). Future studies may focus on the interplay between BR signaling and the function of the B-Box domains and VP motifs that may confer contrasting functions as negative and positive regulators of BR responses.

The findings of this work suggest that BBX21 can modulate the biosynthesis and signaling of BRs to finely integrate endogenous and environmental signals for the control of the hypocotyl growth by light. BBX21 is also active in other responses across the life cycle. Previous reports demonstrated that BBX21 has critical functions in different physiological developmental stages in some crop species explored. In the peel of pear fruits, PpBBX21 protein directly interacts with PpBBX18 and PpHY5, inhibiting anthocyanin biosynthesis in transient overexpression experiments (Bai et al. 2019). In potato, the overexpression of BBX21 reduces the photoinhibition effects on photosynthesis by increasing the synthesis of photoprotector pigments (Crocco et al. 2018). In addition, BBX21-overexpressing potato plants increase tuber yield by enhancing mesophyll conductance for photosynthesis and ABA tolerance under moderated water restrictions (Gómez-Ocampo et al. 2021). The function of BBX21 in BR-mediated responses in other physiological responses, such as the increase of tuber size in potato plants, may be explored in the future in conjunction with the effects of abiotic stresses to generate superior genotypes with enhanced resilience to harsh conditions in the context of climate change.

## Materials and Methods

### Plant material

Mutants and transgenic lines used in this study have been described previously: *bbx21-1* (Crocco et al. 2010), *35S:YFP-BBX21*, *35S:myc-BBX21* ((Xu et al. 2016) and *bzr1-1D* (Wang et al. 2002) in Col-0 background and *bbx21-2* in L*er*-0 background (Crocco et al. 2010). *pif4-101* was obtained from the Nottingham Resource Center. The double mutant *bbx21-1 bzr1-1D* was generated by crossing *bbx21-1* with *bzr1-1D* line. Homozygous progenies were selected by PCR using primers listed in **Supplementary Table S2**. The *bzr1-1D* mutation was selected by MspI/HpaII enzyme restriction reaction.

### Growth conditions and light treatments

Seeds were sown in clear plastic boxes on 0.8% agar/water and incubated in darkness at 4 °C to reduce dormancy and homogenize germination. After 4 days, imbibed seeds were exposed to a 2 h-WL pulse and incubated in darkness for 24 h at 22 °C to induce germination. Then, the boxes with seedlings were transferred for 2 days to a photoperiod of 10 h WL + 14 h darkness at 22 °C for the full de-etiolation seedling. Three-day-old seedlings were exposed to WL or shade for 5 days in the same growth chamber. The WL treatment consisted of 100 μmol $m^{-2}$ $s^{-1}$ of PAR (between 400 and 700 nm) provided by a mixture of fluorescent and halogen lamps (R/FR = 1.2). The shade treatment was achieved by using a green acetate filter (LEE filters 138, 121 or 089; Paolini 2031; La Casa del Acetato, Buenos Aires, Argentina) on the top of the boxes with seedlings, reducing the PAR to 20 μmol $m^{-2}$ $s^{-1}$ and the R/FR ratio to 0.2. The WL + FR treatment that simulates shade consisted to grow seedlings under WL for 5 d and then supplementing with a 1 h pulse of far-red light (i.e. FR = 28 μmol $m^{-2}$ $s^{-1}$) given laterally at the end of the photoperiod following the methodology described previously (Fernández-Milmanda et al. 2020). The simulated shade treatment used for the validation by RT-qPCR of the expression of genes selected from the comparative transcriptomic analysis was the same as indicated in (Crocco et al. 2010). For all experiments, three replicates per treatment (i.e. three individual acrylic boxes with medium in which the seedlings were grown) were analyzed. Spectral photon fluences were estimated with a Li-Cor (Li-188B, LiCor Corp., Lincoln, NE, USA), and PAR and R/FR were measured using a SpectroSense2 attached to a SKR-1850SS2 light sensor (Skye Instruments Ltd., Powys, UK).

### Experiments with synthetic hormones and inhibitors

Seeds were sown in agar/water medium supplemented with a BR synthetic hormone (24-*epi*BL, E1641 Sigma-Aldrich®, St Louis, MO) or BR inhibitor (Brassinazole, Brz, SML1406, Sigma-Aldrich®, St Louis, MO). For the Brz–dose curves, seeds were first sown on a wet filter paper for germination and full de-etiolation. At the third day, the filter paper was transferred to the agar plastic boxes containing different doses of Brz before the beginning of the WL or shade treatments. On the fifth day of light treatments, seedlings were photographed to measure the hypocotyl length using ImageJ software (Schneider et al. 2012).

## BRs quantification

Extraction and quantification of inactive and active endogenous BR were performed as described previously (Tarkowská et al. 2016, Oklestkova et al. 2017). Three replicates of approximately 5 mg dry weight of Arabidopsis seedlings were extracted in ice-cold 60% acetonitrile, and 25 pmol of deuterium-labeled internal standards of BRs was added to each sample (OlChemIm Ltd., Olomouc, Czech Republic). After 12 hours, the samples were centrifuged and supernatants were purified using 50 mg Discovery™ DPA-6S cartridges (Supelco®, Bellefonte, PA, USA). After evaporation to dryness, samples were reconstructed in 40 μL of methanol and analyzed by liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) using an ACQUITY UPLC® I-Class System (Waters, Milford, MA, USA) with the use of triple quadrupole mass spectrometer Xevo™ TQ-S MS (Waters MS Technologies, Manchester, UK). The UHPLC-MS/MS analysis is described in Oklestkova et al. (2017) and Tarkowská et al. (2016).

## RT-qPCR experiments

For qPCR experiments, 100 mg of fresh seedlings were harvested and frozen immediately in liquid nitrogen. Total RNA was extracted using a Spectrum™ plant total RNA kit (Sigma-Aldrich, St Louis, MO). Crude RNA preparations were treated with 1.5 units of RNase-free DNase I (http://www.promega.com). cDNA was synthesized from 2 μg of DNA-free RNA template using an oligo(dT) primer and M-MLV reverse transcriptase (http://www.promega.com). RT-qPCR analyses were performed on an optical 96-well plate using LightCycler 480 SYBR Green I Master mix (Roche, https://www.roche.com/) and a LightCycler 480 II real-time PCR system (https://www.roche.com/). The thermal cycle used was 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Specific primer pairs for each gene were designed using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in **Supplementary Table S2**.

## Transcriptomic analysis in silico

The transcriptomes of *bzr1-1D* (Gene Expression Omnibus database accession number GSE35408; Bai et al. 2012), *pif4* (GEO accession number GSE37160; Oh et al. 2012) and *bbx21-1* in shade (Crocco et al. 2010) were compared to find genes coregulated by BZR1, PIF4 and BBX21. Venn diagram was obtained using Bioinformatics and Evolutionary Genomics (https://bioinformatics.psb.ugent.be/webtools/Venn/). Coregulated genes were hierarchically clustered using DNA-Chip Analyzer (Li and Wong 2003). Among these genes, *PRE1*, *PRE3*, *MYBL2*, *IBH1*, *PIF4*, *IAA1*, *IAA4* and *EXP11* were selected to validate the gene expression by RT-qPCR in Col-0 and *bbx21-1* seedlings grown in WL or long-term shade for 5 days. The harvest was done at the end of the photoperiod. Statistical significance of the overlap between two groups of genes was calculated as the RF, which is the number of overlapping genes divided by the expected number of overlapping genes drawn from two independent groups (http://nemates.org/MA/progs/overlap_stats.html).

## Supplementary Data

Supplementary data are available at *PCP* online.

## Data Availability

The accession numbers of the genes used in this study are as follows: AT1G75540 (BBX21), AT5G38970 (BR6OX1), AT3G30180 (BR6OX2), AT1G75080 (BZR1), AT2G44080 (ARL), AT3G28857 (PRE5), AT3G50660 (DWF4), AT2G26710 (BAS1), AT1G20190 (EXP11), AT4G14560 (IAA1), AT5G43700 (IAA4), AT2G43060 (IBH1), AT1G71030 (MYBL2), AT2G43010 (PIF4), AT5G39860 (PRE1) and AT5G39860 (PRE3). The data that support the findings of the study are available from the corresponding author upon request.

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## Author Contributions

C.D.C. and J.F.B. conceived the project and designed most of the experiments. C.D.C., G.G.-O., and J.C. performed and analyzed the experiments. S.M.-G. genotyped the *bzr1-1D bbx21* mutant. J.O. and D.T. contributed with the measurement of BR hormones. J.L.P.-P. and M.A.B. contributed with reagents. J.F.B. supervised the experiments, analyzed data and wrote the paper with the contribution of all the authors. All the authors read the manuscript and approved this submission.

## Disclosures

The authors have no conflicts of interest to declare.

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