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The cross-talk of brassinosteroid signaling and strigolactone signaling during mesocotyl development in rice

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Abstract
The elongation of the mesocotyl is modulated by phytohormones, which have been shown to exert both synergistic and antagonistic influences on this process. However, the complex regulatory networks involved remain to be fully understood. To investigate the interplay between strigolactone (SL) and brassinosteroid (BR) signaling pathways in the regulation of mesocotyl elongation, we analyzed the elongation phenotypes of mutants deficient in SL and BR signaling. Our findings indicate that the SL signaling pathway operates downstream of the BR signaling pathway during mesocotyl elongation. Furthermore, a comprehensive analysis of genome-wide expression profiles in response to BR treatment demonstrated that BR signaling activation inhibits carotenoid biosynthesis, while the inhibition of SL signaling alters the mesocotyl's sensitivity to BR. This suggests a potential convergence of SL and BR pathways in regulating shared target genes during mesocotyl development. The results indicate that both SL and BR signaling pathways can influence mesocotyl elongation through the action of BBX6. These findings will enhance our understanding of the underlying mechanisms of mesocotyl development and provide valuable insights for the breeding elite rice varieties suitable for direct seeding.

Keywords Brassinosteroid · Strigolactone · Mesocotyl · Rice

Introduction

Rice transplanting has traditionally been a prevalent method in rice cultivation; however, rising labor costs have posed significant challenges to the sustainability of this practice. In contrast, direct seeding, which involves the establishment of plants from seeds sown directly in the field, is a more straightforward and cost-effective alternative to transplanting seedlings. Nonetheless, direct seeding is associated with certain risks, including the potential failure of seedlings to emerge from the soil and the susceptibility of mature plants to lodging, both of which can lead to decreased grain yields (Kumar and Ladha 2011). To mitigate these risks, deep sowing is frequently employed to enhance the resilience of plants cultivated through direct seeding (Ohno et al. 2018). The rice mesocotyl, a structure located between the nodes of the coleoptile and the base of the seedling, is critical for seedlings emergence from the soil (Hu et al. 2010). Previous research indicates that the emergence rate of rice seedlings is closely associated with mesocotyl length and the sowing depth (Ohno et al. 2018). When seeds are planted at comparable depths, seedlings possessing shorter mesocotyls generally demonstrate a reduced ability to emerge from the soil, in contrast to those with longer mesocotyls, which enhance this emergence process (Lee et al. 2017). Consequently, rice varieties that are optimized for direct seeding should ideally possess elongated mesocotyls (Lee et al. 2017).

Numerous environmental and developmental factors had an impact on the growth of rice mesocotyls (Zhan et al. 2020). First, temperature is a significant factor influencing mesocotyl elongation; optimal temperatures enhance seed germination and mesocotyl growth, thereby improving seedling emergence and survival rates. Conversely, exposure to extreme heat or cold can inhibit mesocotyl elongation, resulting in suboptimal seedling emergence (Radford

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and Henzell 1990). Second, light plays a crucial role in regulating mesocotyl elongation, with mesocotyls typically elongating in the absence of light and experiencing suppression upon exposure. The inhibitory effects of various light wavelengths on mesocotyl elongation have been observed, with red light exerting a greater inhibitory effect than far-red light (Takano et al. 2009). Additionally, soil moisture levels significantly influence the elongation of rice mesocotyls, with variations in mesocotyl length corresponding to differing water content (Takahashi 1978).

In addition to environmental influences, developmental factors, particularly plant hormones are essential in the modulation of mesocotyl development (Zhan et al. 2020). Key hormones involved in this regulatory process include gibberellin (GA) (Lyu et al. 2024), cytokinins (CTK) (Hu et al. 2014), abscisic acid (ABA), brassinosteroid (BR) (Sun et al. 2018), ethylene (ETH) (Xiong et al. 2017), jasmonic acid (JA) (Xiong et al. 2017), and strigolactones (SLs) (Hu et al. 2010). SLs, a class of terpenoid phytohormones, are known to regulate multiple aspects of plant development, such as branching, germination, and mesocotyl elongation. The biosynthetic pathway of SLs involves several critical enzymatic processes. The enzyme D27 facilitates the conversion of 9-cis-β-carotene from all-trans-β-carotene, which is subsequently transformed into carlactone (CL) by the actions of D17/CCD7 and D10/CCD8 (Alder et al. 2012). CL serves as an intermediate in the in vivo biosynthesis of SLs (Abe et al. 2014). CL is further converted into both canonical and non-canonical SLs by different members of CYP711A subfamily cytochrome P450 oxygenases (Cardoso et al. 2014; Zhang et al. 2014). In the absence of SLs, the protein D53 (DWARF 53) interacts with the transcriptional co-repressor TPR (TOPLESS RELATED PROTEIN), thereby inhibiting the expression of downstream genes associated with SL signaling (Jiang et al. 2013; Zhou et al. 2013). Conversely, when SLs are present, the receptor D14 (DWARF 14) detects the SLs and forms a complex involving D14, D3 (DWARF 3), and D53, which leads to the ubiquitination of D53 by the SCF^{D3}. Subsequently, the ubiquitinated D53 was degraded via the 26 S proteasome pathway, which releases the downstream transcription factor activity to regulate downstream gene expression (Jiang et al. 2013; Zhou et al. 2013). The phenotypes of seedlings of SL mutant grown in the dark has demonstrated that SL inhibits mesocotyl elongation by repressing cell division. Furthermore, the mesocotyls of SL-deficient mutants and SL-insensitive mutants are longer than those of the wild type (Hu et al. 2010).

BRs represent a category of steroid hormones that play a critical role in the regulation of various agronomic traits in rice, including plant height, leaf angle, grain size, tiller number, heading date, senescence, and environmental adaptation (Han et al. 2023). The biosynthetic pathway for BRs involves the conversion of campesterol (CR) to brassinolide (BL) through two distinct pathways (Han et al. 2023). The *D11* encodes a novel cytochrome P450 enzyme that is integral to the BR biosynthesis pathway (Tanabe et al. 2005). BR signaling is mediated by the leucine-rich repeat receptor kinase D61/OsBRI1 (BRASSINOSTEROID INSENSITIVE 1) and its co-receptor BAK1(BRI1 ASSOCIATED KINASE), which activates the BSKs (BRASSINOSTEROID-SIGNALING KINASE). This activation subsequently leads to the inhibition of the GSK2 (Glycogen synthase kinase 3 -like kinase 2) by the BSU1(BRI1 suppressor 1)-like phosphatase, thereby modulating the phosphorylation of BRASSINAZOLE-RESISTANT 1(OsBZR1) and other transcription factors (Han et al. 2023). Within the BR signaling pathway, the phosphorylation of transcription factors such as OsBZR1, REDUCED LEAF ANGLE1 (RLA1) /SMALL ORGAN SIZE 1 (SMOS1), and DWARF AND LOW-TILLERING (DLT)/ SMALL ORGAN SIZE 2 (SMOS2) by OsGSK2 kinase (Fang et al. 2020; Tong et al. 2012), which is essential for regulating BR-dependent gene expression associated with BR responses. Previous investigations on BR biosynthesis and signaling mutants has indicated that BRs promote mesocotyl elongation (Sun et al. 2018).

Understanding of the hormonal regulation of plant growth and the development of rice mesocotyls offers valuable insights for breeding rice varieties with elongated mesocotyls that are more conducive to direct seeding (Zhan et al. 2020). It is noteworthy that an antagonistic relationship exists among various phytohormones in the regulation of mesocotyl elongation. Previous studies have demonstrated that SLs inhibit mesocotyl elongation by modulating cell division, with SL deficient mutants exhibiting longer mesocotyls when treated with synthetic CTKs such as kinetin (Hu et al. 2014). The gene *CYCLOIDEA/PCF 5* (*OsTCP5*) is implicated in the regulation of mesocotyl elongation under the influence of SLs and CKs in dark conditions (Hu et al. 2014). Additionally, ethylene has been shown to suppress the expression of jasmonic acid (JA) biosynthesis genes, such as *GAOYAO 1* (*GY1*), through the transcription factor EIN-3 LIKE 2 (OsEIL2), thereby reducing JA levels and promoting mesocotyl elongation (Xiong et al. 2017). Moreover, Karrikins (KARs), a novel plant growth regulator structurally similar to SLs, has been found to regulate mesocotyl elongation in rice under dark conditions through D14L-mediated pathways. KAR signaling pathway and SL signaling pathway operate in additive and parallel manners, respectively, and both of them require the function of D3 (Zheng et al. 2020). The gene *CYC U2*, which is specifically expressed in the mesocotyl and encodes a U-type cell cycle protein, is phosphorylated by OsGSK2. BRs facilitate mesocotyl elongation by inhibiting the phosphorylation of

CYC U2 by OsGSK2; however, the phosphorylated CYC U2 is recognized and degraded by D3 (Sun et al. 2018).

An examination of the interactions between BR and SL signaling pathways will enhance our understanding of mesocotyl development. In this study, our findings confirmed the antagonistic effects of SLs and BRs on the regulation of mesocotyl elongation. The intactness of SL signal transduction pathway is essential for the BR-mediated promotion of mesocotyl elongation, while BR signaling inhibits the expression of SL biosynthetic genes. Moreover, we revealed that BBX6, a protein containing a B-box, plays a regulatory role in mesocotyl elongation downstream of both SL and BR pathways. Elucidating the crosstalk of BR- and SL- signaling will provide new insight into mesocotyl development. A comprehensive understanding of the molecular regulatory mechanisms that govern SL and BR signaling in the context of mesocotyl development is crucial for the advancement of breeding strategies aimed at producing new rice varieties that are well-suited for dry direct seeding practices.

Results

Blocking SL signaling attenuates BR enhanced mesocotyl elongation in rice

BRs have been shown to positively influence mesocotyl elongation in rice, as evidenced by the observation that both BR-deficient mutant *d11* and BR-insensitive mutant *d61* exhibit shorter mesocotyls (Yamamuro et al. 2000; Hong et al. 2002). Conversely, SLs negatively impact mesocotyl elongation, with the SL-insensitive mutant *d14* displaying a longer mesocotyl compared to wild type (WT) (Hu et al. 2010). In the absence of BR, CYC U2 undergoes phosphorylation by GSK2, which is subsequently recognized by D3 for ubiquitination, resulting in the degradation of CYC U2 via the 26 S proteasome pathway. The activation of BR signaling inhibits the kinase activity of GSK2, thereby repressing the phosphorylation of CYC U2, which leads to the accumulation of CYC U2 and subsequent mesocotyl elongation. These findings suggest that both SL and BR signaling pathways necessitate the function of D3 to modulate mesocotyl development (Sun et al. 2018). To further elucidate the mechanisms underlying the interaction between SL and BR signaling pathways during mesocotyl development, we generated double mutants *d14/d11* and *d14/d61* through the crossing of *d14* with *d11* and *d61*, respectively (Supplemental Fig. 1). Plant height and number of tillers in *d14/d11* and *d14/d61* are similar to those in *d14*. Leaf color in *d14/d11* and *d14/d61* is similar to that of *d11* and *d61* (Supplementary Fig. 1).

To explore the mechanism underlying the cross-talk between SL and BR signaling, we compared the mesocotyl length in WT, *d11*, *d61*, *d14*, *d14/d11* and *d14/d61* with or without BR treatment (Fig. 1A-E). Without BR treatment, *d14/d11* and *d14/d61* had a similar mesocotyl length to *d14* (Fig. 1A, 1 C, and 1E). The mesocotyl length of *d14*, *d14/d11* and *d14/d61* is longer than that of WT, *d11* and *d61*, respectively (Fig. 1A, 1 C, and 1E). These results indicated that the decrease in mesocotyl elongation caused by defects in BR biosynthesis or BR signaling was suppressed when SL signaling-mediated negative regulation of mesocotyl elongation was blocked. Application of 1 μM BR treatment increased the length of mesocotyls in WT, *d11*, *d14*, and *d14/d11*, but not in *d61* and *d14/d61* (Fig. 1B and D, and 1E). However, the length of mesocotyls in *d14* and *d14/d11* was shorter than that in WT and *d11* with BR treatment (Fig. 1B and D, and 1E). These results suggest that when SL signaling was blocked by loss of function of *d14*, the mesocotyl elongation response to BR signaling activation was attenuated.

Arabidopsis *DWARF4* (*AtDWF4*) is a direct target of BZR1, activation of BR signaling resulted in inhibition of *DWARF4* expression, providing a native feedback regulation mechanism of BR signaling (He et al. 2005). DWARF4 and DWARF4L1/D11 in rice are closely related to AtDWF4 (Sakamoto et al. 2006). The exogenous application of BL decrease the expression of *DWF4* and *D11* (Sakamoto et al. 2006). To confirm the specificity of the response to BR treatment, we compared the expression of *DWF4*, *BZR1* and *CYC U2* in WT, *d11*, *d61*, *d14*, *d14/d11* and *d14/d61* with or without BR treatment (Fig. 1F-H). Application of 1 μM BR leads to a decrease in the expression of *DWF4* in WT and *d11*, but not in *d61*. The inhibitory effect of BR treatment on *DWF4* expression was attenuated in *d14* and *d14/d11* compared to WT and *d11*. However, in contrast to in *d61*, the application of 1 μM BR resulted in a slight decrease in the expression of *DWF4* in *d14/d61* (Fig. 1F). In addition, the expression of *BZR1* with or without BR treatment correlates well with the expression of *DWF4* (Fig. 1G). Application of 1 μM BR resulted in a decrease in the expression of *CYC U2* in WT, *d11* and *d14*. However, the inhibition of *CYC U2* expression by BR was attenuated in *d14* compared to WT. On the contrary, application of 1 μM BR led to a increase in the expression of *CYC U2* in *d61*, *d14/d61* and *d14/d11* (Fig. 1H). These results indicate that the crosstalk between SL and BR signaling in mesocotyl development may not be limited to the regulation of *CYCU2* transcription levels. These results suggest that the crosstalk between SL and BR signaling pathway regulates the transcription of downstream genes.

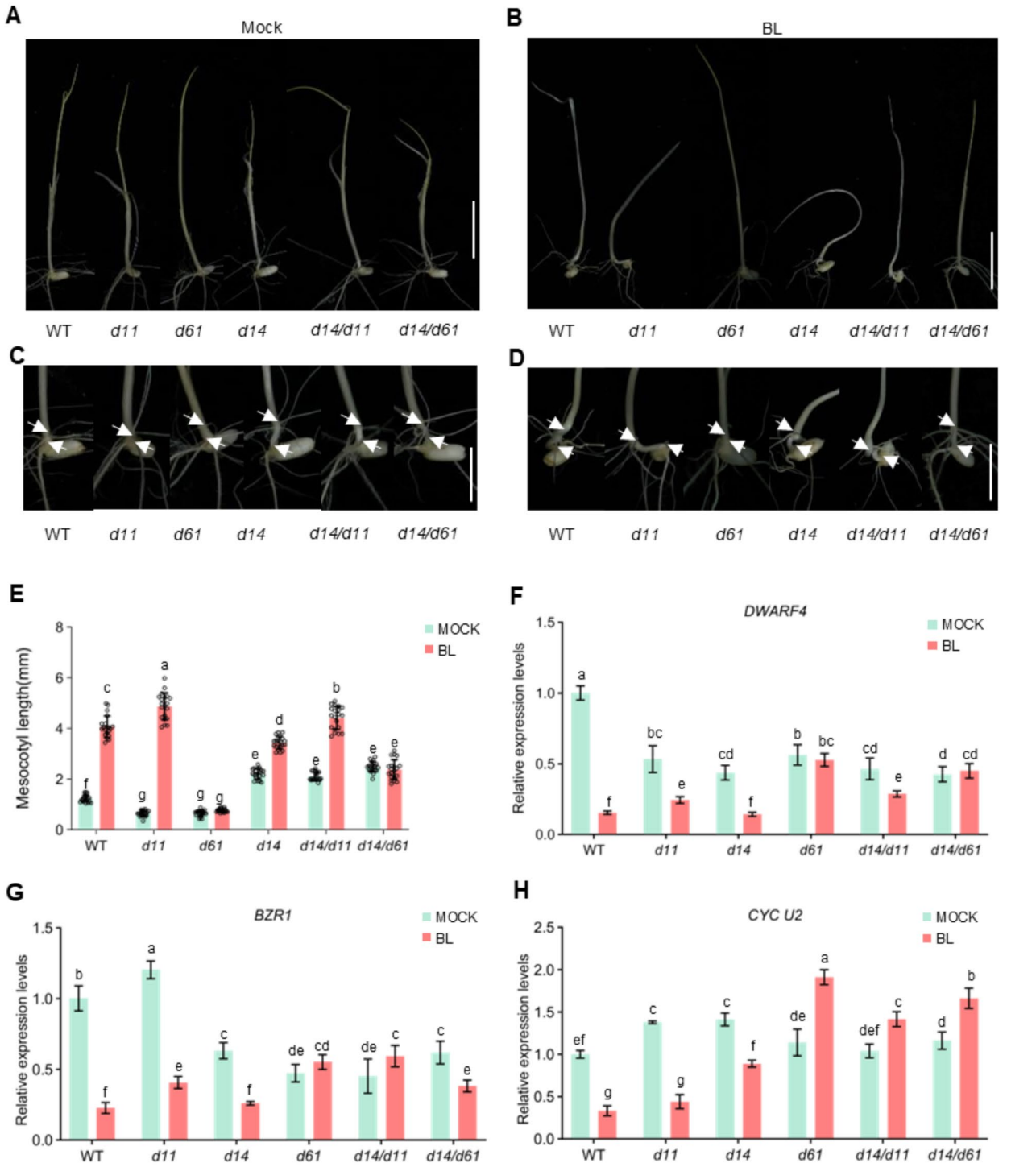

Fig. 1 ***d14*** acts downstream of ***d11*** and ***d61*** during mesocotyl development. **A.** The seedling of WT, *d11, d61, d14, d14/d11* and *d14/d61* without treatment. **B.** The seedling of WT, *d11, d61, d14, d14/d11* and *d14/d61* with 1 μM Brassinolide treatment. **A-B**, scale bars 2cm **C.** The mesocotyl of WT, *d11, d61, d14, d14/d11* and *d14/d61* without treatment. **D.** The mesocotyl of WT, *d11, d61, d14, d14/d11* and *d14/d61* treated with 1 μM Brassinolide treatment. **C-D**，scale bars 1cm. **E.** The mesocotyl length of WT, *d11, d61, d14, d14/d11* and *d14/d61* with and without BR treatment. Data shown are means ± SE. (n = 20). One-way ANOVA, different letters indicate significant differences between samples according to Duncan's test ($P < 0.05$). **F.** The relative expression levels of *BZR1* in WT, *d11, d61, d14, d14/d11* and *d14/d61*. **G.** The relative expression levels of *DWF4* in WT, *d11, d61, d14, d14/d11* and *d14/d61*. **H.** The relative expression levels of *CYC U2* in WT, *d11, d61, d14, d14/d11* and *d14/d61*. **F-H**, Rice ACTIN1 was used as an internal reference gene, and three biological replicates were conducted; data shown are means ± SE. (n = 3). One-way ANOVA, different letters indicate significant differences between samples according to Duncan's test ($P < 0.05$)

Activation of BR signaling lead to inhibition of carotenoid biosynthesis

Since the plant hormone treatment experiments suggested that the SL and BR pathways may converge to regulate common target genes during mesocotyl development. We compared the whole genome expression profile of WT, *d11*, *d61*, *d14*, *d14/d11* and *d14/d61* seedlings grown in the dark for 7 days with or without BR treatment. First, the differentially expressed genes (DEGs) between each sample were identified (Supplemental Fig. 2). There are 1442 and 1870 up-regulated genes in *d11* and *d61* compared to WT, respectively. There are 1458 and 2271 down-regulated genes in *d11* and *d61* compared to WT. It was found that 387 genes are both up-regulated in *d11* and *d61* and 623 genes are both down-regulated in *d11* and *d61* (Fig. 2A and B). Application of 1 μM BR led to 3350 genes up-regulated and 6740 genes down-regulated in WT (Supplemental Fig. 2A). It is known that *d11* is more sensitive while *d61* are less sensitive to BR treatment compared to WT. Consistently, 3595 genes were up-regulated and 5803 genes were down-regulated in *d11* (Fig. 2C and Supplemental Fig. 2A), whereas, 945 genes were up-regulated and 763 genes were down-regulated in *d61* respectively (Fig. 2D and Supplemental Fig. 2A). We then defined the 5542 DEGs between mock and BR-treated plants in both WT and *d11*, but not *d61*, as BR-responsive genes (Fig. 2E F). KEGG analysis showed that the majority of these BR-responsive genes were involved in metabolic pathways and biosynthesis of secondary metabolites (Fig. 2G). Interestingly, the carotenoid biosynthesis pathway was found among the top 20 enriched pathways (Fig. 2G). The heat map showed that the application of BR resulted in the suppression of carotenoid genes in WT, and *d11* but not in *d61* (Fig. 2H). Since the SLs are derived from carotenoids, these results suggested that activation of the BR signaling pathway led to negative regulation of SL biosynthesis.

Blocking of SL signaling altered the sensitivity of mesocotyl to BR

Our analysis revealed that there were 1,516, 2,066, and 2,018 genes that exhibited upregulation in the *d14*, *d14/d11* and *d14/d61*, respectively, when compared to the WT. On the contrast, we identified 1,175, 1,842, and 1,599 genes that were downregulated in the *d14*, *d14/d11* and *d14/d61* respectively, in comparison to WT (Supplemental Fig. 2A). 1250 genes were found to be differentially expressed between *d14* and WT, but not between *d14/d11* and *d14*, nor between *d14/d61* and *d14* (Supplemental Fig. 3A and 3B). The majority of these genes were involved in metabolic pathways and biosynthesis of secondary metabolites (Supplemental Fig. 3C). The carotenoid biosynthesis pathway was also found among the top 20 enriched pathways of these genes (Supplemental Fig. 3C). In addition, 289 genes were found to be differentially expressed between *d14* and WT, between *d14/d11* and *d11*, and between *d14/d61* and *d61* (Supplemental Fig. 3D and 3E). Among of these genes, steroid and carotenoid biosynthesis pathways were found to be among the top 20 enriched pathways (Supplemental Fig. 3F). These results indicated that blockade of SL signaling resulted in altered expression of steroid and carotenoid biosynthesis genes.

To investigate the effect of blocking SL signaling on the expression of BR-responsive genes, we compared the BR-responsive gene set (5452) with the BR-regulated DEGs in *d14*, *d14/d11* and *d14/d61* (Supplemental Fig. 4). 753 BR-responsive genes were found not to be differentially expressed in *d14* (Supplemental Fig. 4A). 1118 BR-responsive genes were found not to be differentially expressed in *d14/d11* (Supplemental Fig. 4B). In addition, 2249 BR-responsive genes were found in *d14/d61* (Supplemental Fig. 4C). We further compared the BR-responsive DEGs in in *d14*, *d14/d11* and *d14/d61*. It showed that 1136 genes can be considered as BR-responsive genes even in SL-signaling blocking conditions (Supplemental Fig. 5A and 5B). KEGG analysis revealed that the carotenoid biosynthesis pathway was also among the top 20 enriched pathways (Supplemental Fig. 5C). The heat map showed that the application of BR resulted in the suppression of these carotenoid genes in WT, *d11*, *d14* and *d14/d11*, but not in *d61* nor *d14/d61* (Supplemental Fig. 5D).

By comparing the expression of BR-responsive genes in different genetic backgrounds, 292 DEGs that relies on the function of D14 were identified (Fig. 3A-D). The heat map showed that blocking of SL signaling altered the sensitivity of the expression of these genes to BR (Fig. 3E). The expression of a subset of genes up-regulated in *d11* is significantly enhanced in *d14/d11*. A known BR signaling component *BRASSINOSTEROID UPREGULATED1* (*BU1*) was found among these genes (Tanaka et al. 2009) (Fig. 3F). In addition, a number of carotenoid biosynthesis pathway genes (Fig. 3G), including ABA biosynthesis genes were also found to be more sensitive to BR treatment in *d14/d11* than in *d11* (Fig. 3H). Taken together, these results revealed that blocking of SL signaling altered the sensitivity to BR during mesocotyl development.

SL and BR antagonized to regulated mesocotyl elongation

To further dissect the mechanism underlying the cross-talk between the SL and BR signaling pathways during mesocotyl development, we generated the double mutant *d17/*

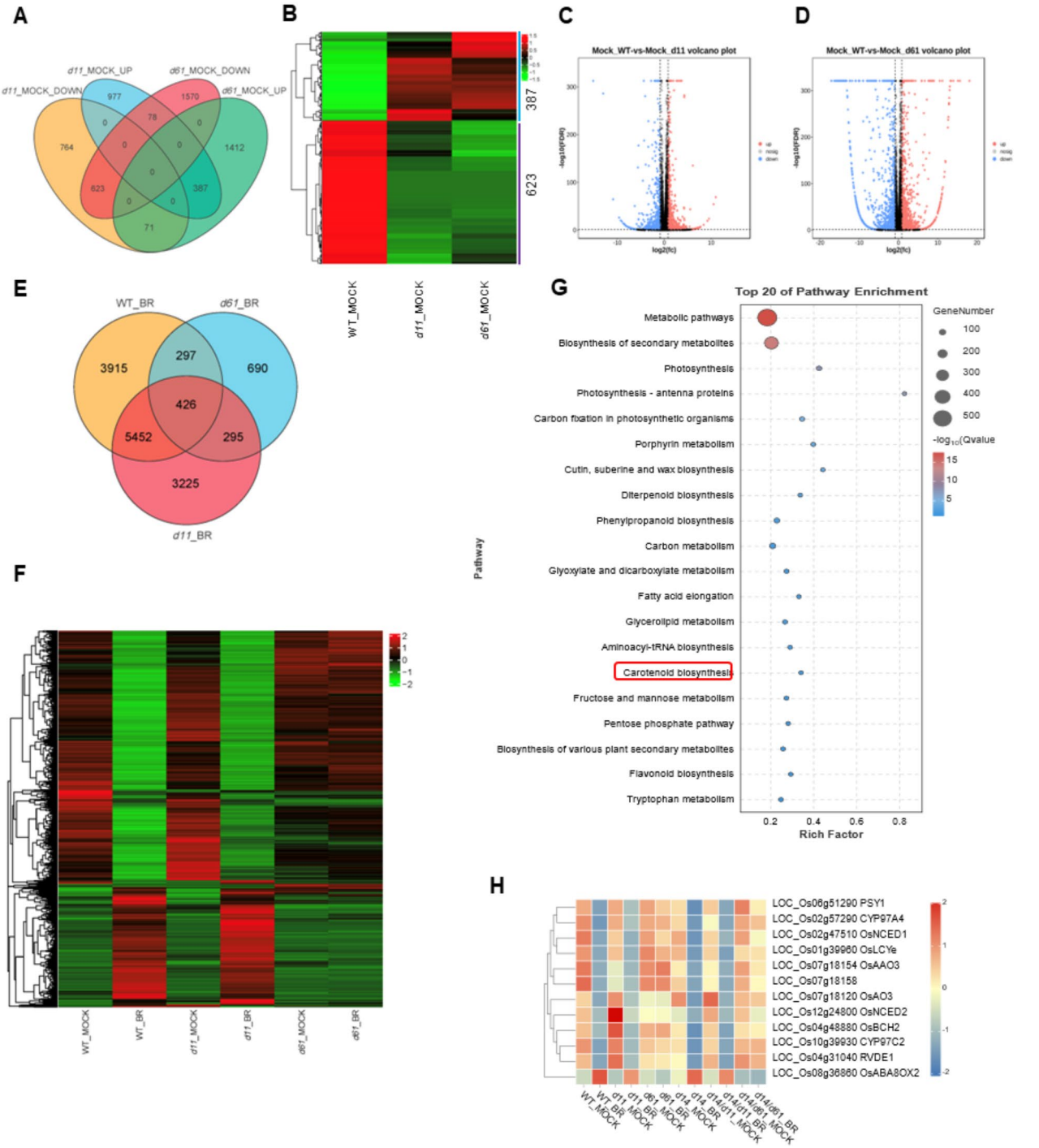

Fig. 2 BR-responsive genes during mesocotyl development. **A.** Venn diagram showing the DEGs in *d11* and *d61* compare to WT. **B.** Heat map showing the DEGs in *d11* and *d61* compared to WT. **C.** Volcano plot showing the DEGs between WT and *d11*. **D.** Volcano plot showing the DEGs between WT and *d61*. **E.** Venn diagram showing the DEGs of between mock and BR treated seedlings of WT, *d11* and *d61*. **F.** Heat map showing the DEGs of between mock treated and BR treated seedlings of WT, *d11* and *d61*. **G.** KEGG of the DEGs of between mock and BR treated seedlings of WT, *d11* and *d61*. **H.** Heat map of carotenoid biosynthesis genes between mock and BR treated seedlings of WT, *d11* and *d61*

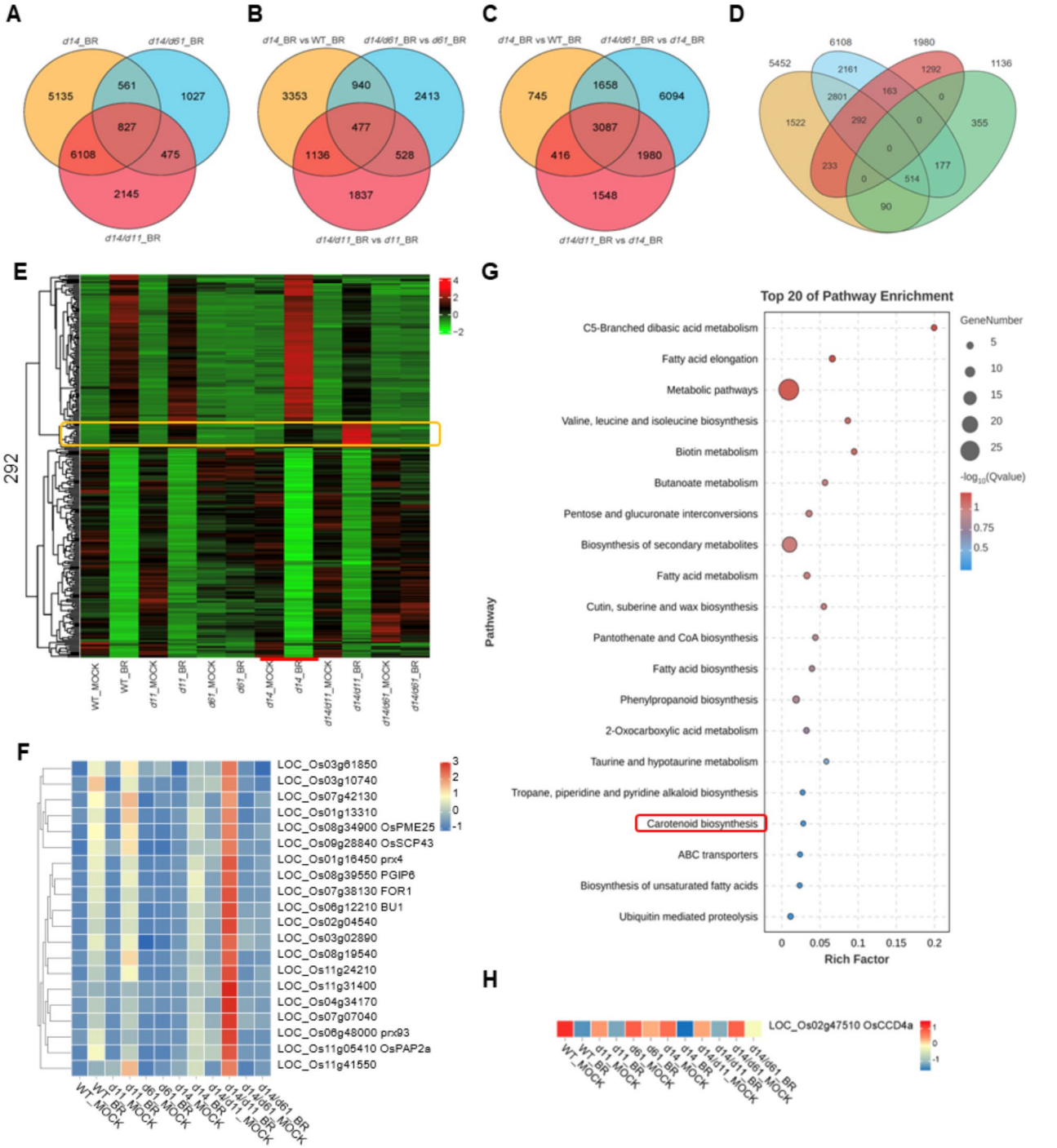

Fig. 3 Blocking of SL signaling altered the sensitivity of mesocotyl to BR. **A**. Veen map showing the DEGs of between mock and BR treated seedlings of *d14, d14/d11* and *d14/d61*. **B**. Veen map showing the DEGs of WT vs *d14, d14/d11* vs *d11* and *d14/d61* vs *d61* without BR treatment. **C**. Veen map showing the DEGs of WT vs *d14, d14/d11* vs *d14* and *d14/d61* vs *d14* with BR treatment. **D.** Veen map showing the DEGs in Fig 2E, Fig 1A, Fig1B and Fig1C. **E**. Heat map show the 292 DEGs between mock and BR treated seedlings. **F**. Heat map showing the DEGs highlighted in Fig3E. **G**. Heat map showing the 292 DEGs between mock and BR treated seedlings. **H.** Heat map of carotenoid biosynthesis genes between mock and BR treated seedlings

d61 by crossing *d17* with *d61*, respectively (Supplemental Fig. 6). Plant height and number of tillers in *d17/d61* are similar to those in *d17*. Leaf color in *d17/d61* is similar to that of *d61* (Supplementary Fig. 6). To determine the role of SL and BR in mesocotyl development, we compared the mesocotyl length of WT, *d11*, *d61*, *d17*, *d17/d61*, *d14*, *d14/d11* and *d14/d61* with mock, 1 μM $GR24^{5DS}$**(a synthetic analogue of SL)**, 1 μM BR and 1 μM $GR24^{5DS}$ plus1 μM BR treatments (Fig. 4 and Supplementary Fig. 7). It showed that the mesocotyl length of *d14/d11*, *d14/d61* and *d17/d61* double mutants without hormone treatment was significantly elongated compared to that of WT, the mesocotyl length of *d17/d61* was similar to that of *d17*, and the mesocotyl length of *d14/d11*, *d14/d61* was similar to that of *d14* (Fig. 4A and B). This indicates that the SL pathway regulates mesocotyl elongation downstream of the BR pathway.

When 1 μM BR was applied, the length of mesocotyls of WT, *d11*, *d14* and *d14/d11* became longer than those without hormone treatment, consistent with BR being able to positively regulate mesocotyl elongation. As expected, the length of mesocotyls of *d61*, *d17/d61* and *d14/d61* treated with 1μM BR is similar to that of those without BR treatment. However, the length of mesocotyls of *d17* with 1μM BR treatment is also similar to that without BR treatment (Fig. 4A and B). These results indicated that the loss of function of *D17* reduces the sensitivity of mesocotyls to BR. With 1μM $GR24^{5DS}$ treatment, the length of WT, *d11* and *d17* mesocotyls was shorter than that without hormone treatment, consistent with the ability of SL to negatively regulate mesocotyl elongation. As expected, the length of mesocotyls in *d14*, *d14/d11* and *d14/d61* treated with 1 μM $GR24^{5DS}$ was similar to that without $GR24^{5DS}$ treatment, respectively (Fig. 4A and B). The length of mesocotyls of *d61* with 1μM $GR24^{5DS}$ treatment is also similar to that without $GR24^{5DS}$ treatment. However, the length of mesocotyls of *d17/d61* with 1μM $GR24^{5DS}$ treatment was shorter than that without $GR24^{5DS}$ treatment (Fig. 4A and B). These results indicated that the loss of function of *D61* reduced the sensitivity of mesocotyls to SL, rather than blocking SL responses. Application of 1 μM BR plus 1 μM $GR24^{5DS}$ made the length of mesocotyls in WT, *d11*, *d14* and *d14/d11* longer than those without hormone treatment (Fig. 4A and E). The length of mesocotyls of *d61*, *d17*, *d17/d61* and *d14/d61* treated with 1 μM BR plus 1 μM $GR24^{5DS}$ is similar to those without BR treatment (Fig. 4A and E). These results indicate that the SL signaling pathway regulates mesocotyl elongation downstream of the BR signaling pathway.

BBX6 negatively regulated mesocotyl elongation

B-box (BBX) proteins represent a category of zinc-finger transcription factors characterized by the presence of a B-box domain (Fan et al. 2012). BBX6 is a member of the BBX family transcription factor, which are a subset of zinc finger proteins characterized by the presence of one or two B-box structural domains. These proteins are integral to various aspects of plant growth and development. Previous studies have established that *BZR1–1D* SUPPRESSOR (BZS1), also referred to as BBX20, functions as a positive regulator of light signaling while simultaneously acting as a negative regulator of BR signaling in Arabidopsis (Fan et al. 2012). The overexpression of BZS1 has been associated with de-etiolation in darkness, a reduction in hypocotyl length under light conditions, diminished sensitivity to BR treatment, and the repression of numerous BR-activated genes (Wei et al. 2016; Lin et al. 2021; Fan et al. 2012). Consequently, BBX20 has been implicated in the regulation of hypocotyl elongation in Arabidopsis, operating downstream of BR signaling pathways (Fan et al. 2012). Notably, while BR signaling leads to a decrease in *BZS1* expression levels. SLs have been shown to enhance *BZS1* expression (Wei et al. 2016; Lin et al. 2021; Fan et al. 2012). Furthermore, transcriptomic analyses have indicated that the expression of *BBX6* is downregulated at *d14* mutant (Zheng et al. 2020). Application of BR can upregulate the expression of *BBX6* in WT and *d11*, but not in *d61*. Therefore, BBX6 may act downstream of the BR signaling pathway during rice mesocotyl development. To determine the role of *BBX6* in rice mesocotyl development, we used CRISPR/Cas9 technology to generate a knock-out mutant of *BBX6* (Fig. 5A). The mutant *bbx6* has an insertion of 2'-Deoxythymidine-5'-triphosphate (dTTP, T) at site 98 after ATG (Fig. 5B). It showed no significant difference in plant morphology between WT and *bbx6* (Fig. 5A). We also generated the *BBX6-GFP* overexpression line, which can be detected by anti-GFP (Fig. 5C). *BBX6-OE* has a decrease in tiller number compared to WT (Fig. 5A), indicated that *BBX6* may involve in the regulation of tiller number in rice.

To determine the function of *BBX6* in regulating rice mesocotyl elongation, we treated WT, *bbx6*, and *BBX6-OE* with mock, 1 μM BR, 1 μM $GR24^{5DS}$, and 1 μM BR plus 1 μM $GR24^{5DS}$ (Fig. 5D F). It was found that the mesocotyl length of *bbx6* mutants without hormone treatment was significantly longer than that of WT, whereas the mesocotyl length of *BBX6-OE* was significantly shorter than that of WT (Fig. 5D F). These results demonstrate that *BBX6* negatively regulates mesocotyl elongation in rice. It was also found that both mesocotyls in *bbx6* and *BBX6-OE* elongated significantly after treatment with the hormone BR (Fig. 5D F). Both *bbx6* and *BBX6-OE* mesocotyls were significantly shortened after further treatment with $GR24^{5DS}$ (Fig. 5D F). The results after treatment with BR plus $GR24^{5DS}$ showed similar results to the BR hormone treatment (Fig. 5D F). These results showed that *BBX6* negatively regulates rice

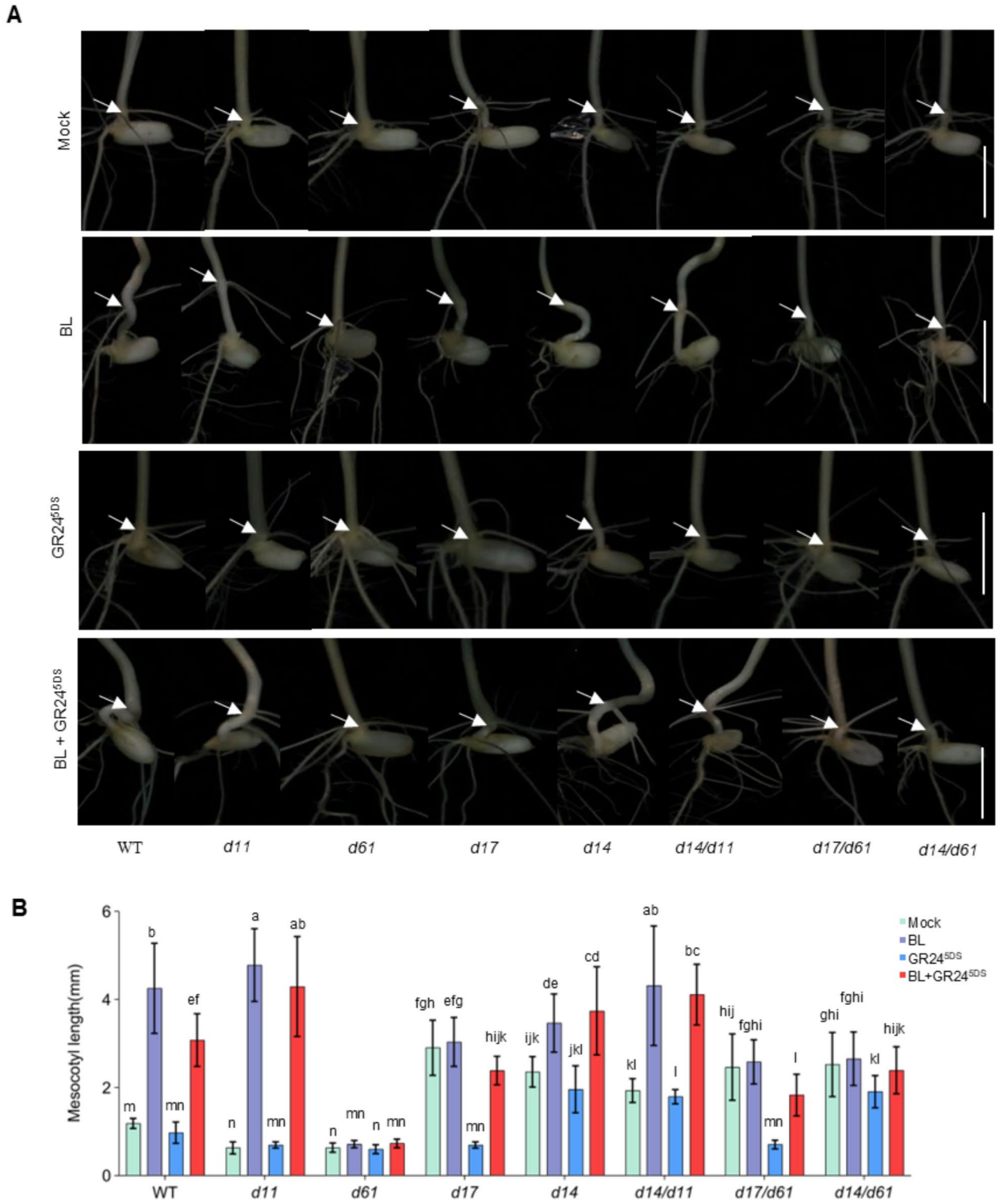

Fig. 4 SL and BR signaling pathways partially antagonized to regulate mesocotyl development. **A.** The mesocotyl of WT, *d11, d61, d17, d14, d14/d11, d17/d61* and *d14/d61* plants treated Mock，1 μM Brassinolide, 1 μM GR245DS and 1 μM Brassinolide plus 1 μM GR245DS. Scale bars, 1cm **B.** The mesocotyl length of WT, *d11, d61, d17, d14, d14/d11, d17/d61* and *d14/d61* plants treated with BR and /or SL. Data shown are means ± SE. (n = 20). One-way ANOVA, different letters indicate significant differences between samples according to Duncan's test ($P < 0.05$)

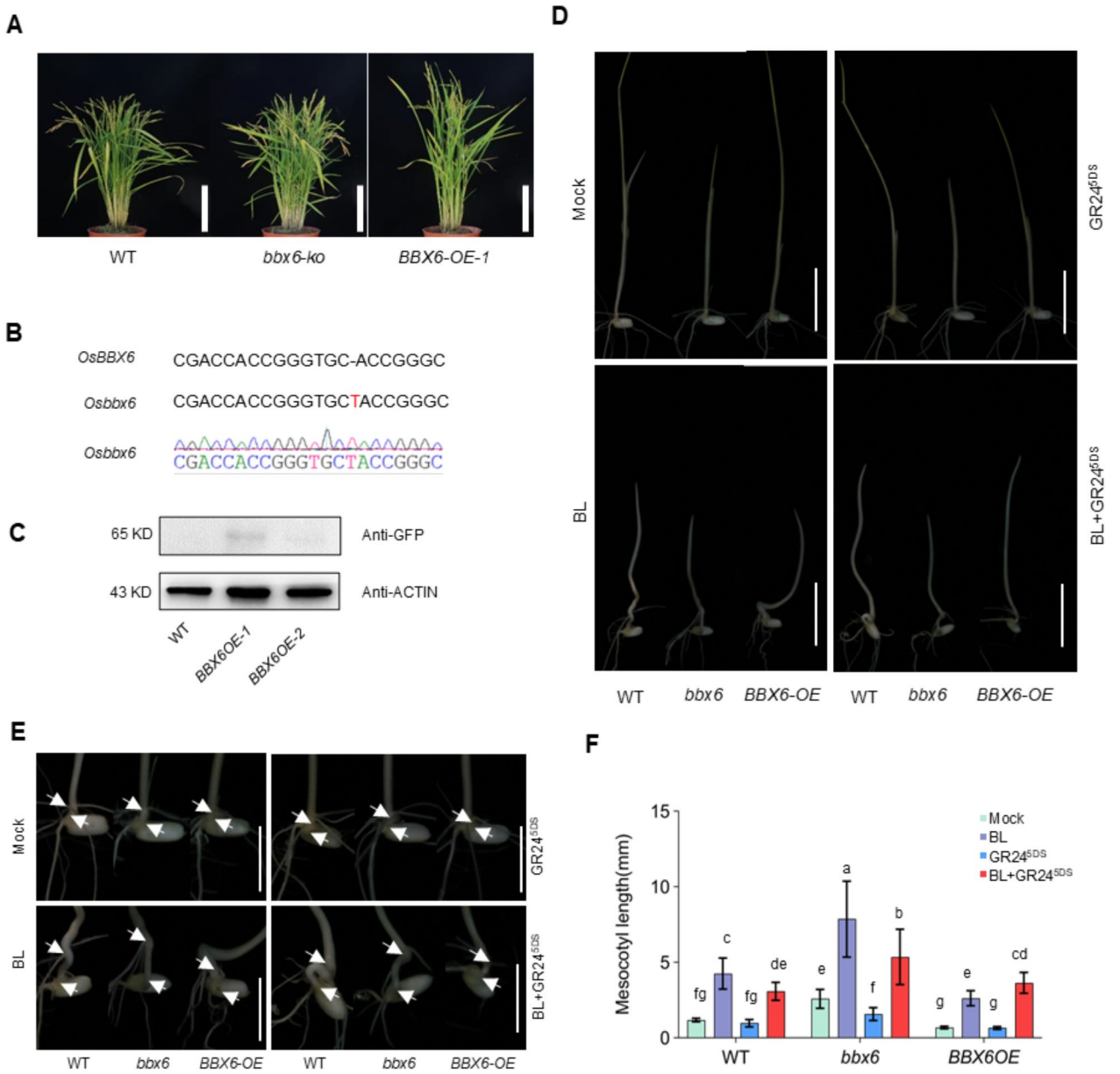

Fig. 5 ***BBX6*** acts downstream of both the BR and SL pathways. **A.** The plant morphology of WT, *bbx6* and *BBX6OE*, Scale bars, 20 cm. **B.** Mutation site of *BBX6* in *bbx6*. **C**. Protein blot of BBX6-GFP in *BBX6-OE* using anti-GFP antibody. **D.** The seedling of WT, *bbx6* and *BBX6OE* with Mock，1 μM Brassinolide, 1 μM GR245DS and 1 μM Brassinolide plus 1 μM GR245DS, Scale bars, 2 cm. **E.** The mesocotyl of in WT, *bbx6* and *BBX6OE* with BR and SL treatment. Scale bars, 1 cm. **F.** The length of mesocotyl in WT, *bbx6* and *BBX6OE* with BR and SL treatment. Data shown are means ± SE. (n = 20). One-way ANOVA, different letters indicate significant differences between samples according to Duncan's test ($P < 0.05$)

mesocotyl elongation. Together, SL and BR signaling pathways jointly regulate rice mesocotyl length through BBX6.

Discussions

SLs are known to influence various processes of plant development, including branching and mesocotyl elongation. Conversely, BRs are steroidal hormones that are pivotal throughout the entirety of plant developmental stages. Research has established that the signaling pathways of SL and BR exert antagonistic effects on rice tillering; specifically, SL inhibits tillering by promoting the degradation of D53 and increasing the expression of *FC1*, while BR signaling facilitates tillering by augmenting the accumulation of the OsBZR1–RLA1–DLT complex and repressing *FC1* expression. This antagonistic regulation of *FC1* by SL and BR signaling pathways allows for a coordinated control of

tillering in rice. The findings of this study further elucidate that SL and BR signaling pathways also antagonistically regulate mesocotyl elongation. Our investigation indicates that *BBX6* is significantly involved in mesocotyl elongation, with its expression modulated by both SL and BR signaling pathways. It is plausible that the antagonistic regulation of *BBX6* expression by SL and BR signaling pathway contributes to the coordinated control of mesocotyl elongation in rice.

Understanding the interactions between SL and BR signaling is essential for future breeding programs aimed at enhancing cereal yield and improving tolerance to environmental stresses. The evidence suggests a close relationship between SL and BR pathways, characterized by substantial overlap in the sensing, signaling, and metabolic processes related to nutrient sensing. This study provides evidence to demonstrate an antagonistic interaction between SL and BR signaling pathways in the context of mesocotyl elongation. This finding suggests that genetic manipulation of SLs or BRs signaling may enhance seedling emergence rates, which is a crucial aspect for developing rice varieties suitable for direct seeding.

It is noteworthy that rice varieties characterized by elongated mesocotyls often demonstrate enhanced drought tolerance. Comparative analysis of whole-genome expression profiles in response to BR treatment revealed that the activation of BR signaling inhibits carotenoid biosynthesis. Notably, the inhibition of carotenoid biosynthesis through BR activation not only modifies SL production but also impacts abscisic acid (ABA) levels, indicating a possible interaction between BR and ABA signaling. Despite the conservation of core components within the ABA and BR signaling pathways in rice, the mechanisms governing the crosstalk between these pathways remain largely unexplored. Therefore, elucidating the interactions between ABA and BR signaling pathways during mesocotyl development is vital for improving rice resilience to abiotic stress. In conclusion, a comprehensive understanding of the mechanisms underlying the crosstalk between BR and SL, as well as between BR and ABA in response to various environmental factors, will facilitate the development of innovative strategies to enhance rice production.

Materials and methods

Plant materials

All mutants and transgenic plants, except those explicitly mentioned, were generated in the genetic background of *Oryza sativa* cv. Nipponbare. *d14*, *d17*, *d11*, *d61* mutants have been described previously. CRISPR/Cas9 genome editing was used to generate the *bbx6* mutant in the Nipponbare genetic background. Double mutants *d17/d61*, *d14/d11* and *d14/d61* were generated by crossing. *pUbi: BBX6-GFP* overexpression lines were obtained by Agrobacterium-mediated genetic transformation.

Mesocotyl length measurements

Prepare 0.7% agar culture medium and sterilize at 121 °C for 20 min. Perform the operation in a laminar flow cabinet and use sterilized forceps to transfer the germinated seeds to the culture medium. Then incubate in a dark rice culture incubator at 30 °C for 7 days. For chemical treatment, 1 µM BR, 1 µM $GR24^{5DS}$ and 1 µM BR plus 1 µM $GR24^{5DS}$ are added to the culture medium for plant hormone treatment, while the same amount of acetone is added as a contrast. A photograph was taken using a ruler to measure the length of the mesocotyl, and then the length of the mesocotyl was measured using the straight or curved measurement function in Image J software.

RNA isolation and RT-PCR analysis

Dark-grown rice seedlings were harvested, snap-frozen in liquid nitrogen and stored at -80°C. Trizol reagent (Invitrogen) was used to extract total RNA from the roots according to the manufacturer's instructions. Total RNA (0.3–0.7 µg) was reverse transcribed into first-strand cDNA using HiScript III RT SuperMix (+ gDNA wiper) (Vazyme). RT-qPCR was performed using the cDNA and gene-specific primers with ChamQ Universal SYBR qPCR Master Mix (Vazyme) on a CFX384 Connect Real-Time System (Bio-Rad) according to the manufacturer's instructions (Supplemental Table 1). Rice ACTIN1 was used as an internal control.

RNA-seq and data analysis

RNA extraction was performed using Trizol reagent according to the manufacturer's instructions. The high-quality RNA obtained was used to construct the mRNA library, which was sequenced on the Illumina Novaseq 6000 platform using 150 bp paired-end reads. Each sample generated 6 Gb of data. Raw data in fastq format were subjected to adapter trimming and quality control using Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic) and FastQC (de Sena Brandine and Smith, 2019) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were aligned to the Oryza sativa reference genome MSU version 7.0, using the gene model annotation file from http://rice.plantbiology.msu.edu/ and Hisat2 from https://daehwankimlab.github.io/hisat2/download/. RNA sequences were quantified at the gene level using featureCounts,

available at https://subread.sourceforge.net/featureCounts.html(Supplemental Table 2). Data were analysed for differential gene expression using OmicShare tools (https://www.omicshare.com/tools), with results presented using Venn diagrams and heat map analysis.

Transgenic protein detection

To identify the overexpression lines of BBX6, extract protein from rice leaf after normal hydroponic treatment for 7 days. Place the leaf in a 2 mL centrifuge tube with steel balls. Quickly freeze the samples using liquid nitrogen, then pulverise the tissue using a grinder. Add an appropriate volume of protein extraction fluid (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% [w/v] SDS and 1× protease inhibitor cocktail) and vortex for 10 s. Place the samples in an ice bath for 5 min and vortex five times. Centrifuge the mixture for 12 min at 12,000 rpm, 4°C. Add 4 X SDS loading buffer to the supernatant. Proceed to the next step of protein immunoblotting by heating at 95 °C for 15 min in a metal bath, anti-GFP (TransGen, HT801-01) at 1: 10,000 was used to detect BBX6-GFP protein levels. Rice internal control ACTIN protein was detected using anti-ACTIN antibody (Abmart, Cat. No. M20009) at a dilution of 1:5000.

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Declarations

Competing interests Authors declare that they have no competing interests.

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