Arabidopsis zinc finger homeodomain transcription factor BRASSINOSTEROID-RELATED HOMEOBOX 2 acts as a positive regulator of brassinosteroid response

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Abstract The brassinosteroid (BR) phytohormone is an important regulator of plant growth. To identify novel transcription factors that regulate BR responses, we screened chimeric repressor gene silencing technology (CRES-T) plants, in which transcription factors were converted into chimeric repressors by the fusion of SRDX plant-specific repression domain, with brassinazole (Brz), an inhibitor of BR biosynthesis. We identified that a line that expressed the chimeric repressor for zinc finger homeobox transcription factor, BRASSINOSTEROID-RELATED-HOMEOBOX-2 (BHB2-sx), exhibited Brz-hypersensitive phenotype with shorter hypocotyl under dark, dwarf and round and dark green leaves similar to BR-deficient phenotype. Similar to BHB2-sx plants, bbh2 knockout mutant also exhibited Brz hypersensitive phenotype. In contrast, ectopic expression of BHB2 (BHB2-ox) showed hypocotyl elongation phenotype (BR excessive), showing decrease to Brz sensitivity. The expression of the DWF4 and CPD BR biosynthesis genes was repressed in BHB2-sx plants, whereas it was enhanced in BHB2-ox plants. The BR deficient-like phenotype of BHB2-sx plant was partially restored by treatment with brassinolide (BL), indicating that the BR deficient phenotype of BHB2-sx plant may be due to suppression of BR biosynthesis. Our results indicate that BHB2 is a positive regulator of BR response may be due to the promotion of BR biosynthesis genes.

Key words: brassinosteroid (BR), chimeric repressor gene silencing technology, plant hormone, transcriptional regulation.

The brassinosteroid (BR) steroid phytohormones play important roles in various processes of plant growth and development in response to diverse environmental stimuli (Nolan et al. 2020). The BR signaling cascade has been intensely studied. Activation of BR1 SUPPRESSOR 1 (BSU1) by phosphorylation inactivates BRASSINOSTEROID INSENSITIVE2 (BIN2) glycerol synthase kinase 3-like kinase, which allows the nuclear transfer of the BRZ-INSENSITIVE-LONG 1 (BIL1)/BRASSINAZOLE-RESISTANT 1 (BZR1) and BR1-EMS SUPPRESSOR 1 (BES1/BZR2) transcription factors that regulate the expression of BR-responsive genes (Chen et al. 2017; He et al. 2002; Kim et al. 2011; Li et al. 2009; Mora-García et al. 2004; Oh et al. 2012; Yu et al. 2010). Deficiencies in BR biosynthesis have been reported to cause drastic growth defects in plants (Clouse et al. 1996; Fujioka et al. 1997; Hong et al. 2003). DEETIOLATED2 (DET2) and DWARF4 (DWF4) BR biosynthesis genes are known to play crucial roles in BR biosynthesis (Chory et al. 1991; Fujioka et al. 1997; Fujiyama et al. 2019; Schuler 1996; Werck-Reichhart and Feyereisen 2000). Brassinazole (Brz), a BR biosynthesis inhibitor, binds to and inactivates DWF4 (Asami et al. 2000, 2001). Moreover, Brz has been used to identify BR-related genes mainly by screening mutants that exhibit responses to Brz that are different from wild type; mostly based on the altered hypocotyl length under dark growth conditions (Shimada et al. 2015; Yamagami et al. 2017).

Maintaining BR homeostasis in each tissue is critical for regulating plant growth. Antagonistic regulation has been demonstrated between BR responses, such as the suppression of BR biosynthetic genes in response to...
BHB2 positively regulates BR response

the excess level of endogenous BR (Mathur et al. 1998; Tanaka et al. 2005). The expression of BR biosynthesis genes, DWF4 and CPD, is repressed by Brz (Mathur et al. 1998; Tanaka et al. 2005). BZR1/BIL1 represses the expression of DWF4 and CPD by binding to their promoter regions (Sun et al. 2010). BR homeostasis is regulated through BR negative feedback pathway. Although intensive studies of the inhibition of BR biosynthesis have been achieved, little is known the positively regulation of BR biosynthesis.

Only a small number of transcription factors that act as key regulators of BR responses have been identified among BR-related genes. This could be probably because of the high redundancy of plant transcription factors, due to which single knockout mutants often fail to exhibit an informative phenotype. Thus, we employed Brz, BR biosynthesis inhibitor, and chimeric repressor gene silencing technology (CRES-T), wherein a transcription factor is converted into a chimeric repressor by the fusion of a SRDX plant-specific repression domain, resulting in the dominant repression of target genes to overcome genetic redundancy (Hiratsu et al. 2003; Oshima et al. 2011).

We screened Arabidopsis plants expressing a chimeric repressor driven by the CaMV 35S promoter (CRES-T lines). The chimeric repressor was constructed by fusing a transcription factor with the plant-specific repression domain derived from SUPERMAN (SUPERMAN REPRESSION DOMAIN ver.X; SRDX: LDLDLELRLGFA; Hiratsu et al. 2003). We screened approximately 20,000 Arabidopsis CRES-T lines covering ca. 1,700 independent chimeric repressors to isolate plants that exhibited differential sensitivity to Brz as compared to that of wild type, using the hypocotyl length under dark conditions as an index.

Figure 1. BHB2-sx plants are brassinazole (Brz) insensitive and exhibit brassinosteroid (BR) excessive-like phenotype. (A) Hypocotyls of wild-type (WT; left) and BHB2-sx-#11 (right), treated with (right panel) and without (left panel) 3 µM Brz, grown under continuous dark for 7 days. Scale bar = 2.0 mm. (B) Measurement of the length of hypocotyl of WT and 2 lines of BHB2-sx (#1 and #11) treated with (right panel) and without (left panel) 3 µM Brz, grown under continuous dark for 7 days. Error bars represent means ± s.d. (n=21); * p < 0.01 using Student's t-test. (C) Phenotypes of BHB2-sx-#11 and WT plants. BHB2-sx plants exhibited slender, dwarf, early flowering, and epinastic leaves, common in BR-excessive mutants. Scale bar = 1.0 cm. (D) Schematic representation of the BHB2 gene and edited position of bhb2-1 by CRISPR/Cas9. (E) Hypocotyls of WT and bhb2-1 treated with (right panel) and without (left panel) 3 µM Brz, grown under continuous dark for 7 days. Scale bar = 2.0 mm. (F) Measurement of the length of hypocotyl of WT and bhb2-1 treated with (right panel) and without (left panel) 3 µM Brz, grown under continuous dark for 7 days. Error bars represent means ± s.d. (n=18); * p < 0.01 using Student's t-test. (G) Expression analysis of DWF4 (left) and CPD (right) BR biosynthesis genes in BHB2-sx (#1 and #11), grown on medium for 10 days by qRT-PCR. Error bars represent means ± s.d. (n=3); * p < 0.01 using Student's t-test.
under a 16/8h light and dark cycle. Transgenic plants expressing each of the chimeric repressors were grown on MS medium containing 3 µM Brz. After growth for 7 days in the dark, seedlings showed longer or shorter hypocotyls than WT when picked up and their transgenes were identified by genomic PCR followed by DNA sequencing.

We isolated a CRES-T line for AT1G14440, in which the hypocotyl length was significantly shorter than that of the wild type under dark conditions, both in the presence and absence of Brz (Figure 1A, B). We found that AT1G14440 belongs to zinc finger homeodomain (ZF-HD) transcription factor family and encodes homeobox protein 31 (Tan and Irish 2006) and designated as BRASSINOSTEROID-RELATED HOMEobox 2 (BHB2).

We noticed that rosette plants that express BHB2 chimeric repressor (Pro35S:BHB2-SRDX; hereafter referred to as BHB2-sx) exhibited a typical BR-deficient phenotype, including dwarf, dark green, and round leaves (Figure 1C). We prepared a knockout line of bhh2-1 by using the pTTK352 CRISPR/Cas9 vector (Tsutsui and Higashiyama 2017; Supplementary Figure S1A, B, C). We noticed that the hypocotyl length of bhh2-1 under dark conditions was significantly shorter than wild type similar as in BHB2-sx plants, both in the presence and absence of Brz (Figure 1D, E, F).

In contrast to BHB2-sx plants, we found that the ectopically expressing BHB2 transgenic Arabidopsis (Pro35S:BHB2, hereafter referred to as BHB2-ox) exhibited a BR-excessive phenotype, that is, a longer hypocotyl than the wild type, both in the presence and absence of Brz (Figure 2A, B). In addition, the expression of DWF4 and CPD BR biosynthesis genes was upregulated in BHB2-ox (Figure 2C). The ectopic expression of BHB2 in BHB2-ox plants was confirmed by RT-PCR. These results indicated that BHB2 acts as a positive regulator of BR responses.

We observed that both wild type and det2-1 BR biosynthesis mutants were characterized by elongated hypocotyls whereas such responses to BL were absent or minimal in bri1-5 BR signaling mutants, as previously reported (Noguchi et al. 1999) (Figure 3A). We found that the hypocotyl elongation was induced by BL dose-dependently in wild type and det2-1 plants (Figure 3A, B). BHB2-sx plants were characterized by elongated hypocotyls by BL dose-dependently similar as in wild-type or det2-1 plants, indicating that the BR-deficient-like phenotype of BHB2-sx plants may be due to deficient of endogenous BR.

In BR receptor mutant bri1-5, the expression of BR biosynthesis enzyme genes DWF4 and CPD was significantly enhanced than wild type in the absence of BR by feedback regulatory mechanism in BR signaling, but it was partially suppressed in the presence of BL, because of the diminishing of the BR feedback regulation by the deficient of BR signaling (Figure 3C). In contrast, although the expression of DWF4 and CPD in BR biosynthesis mutant det2-1 was significantly enhanced in the absence of BL similar as in bri1-5 mutant, it was more severely suppressed in det2-1 in the presence of BL than BR treated bri1-5 (Figure 3C). This is because det2-1 mutant possesses higher sensitivity to BR than wild-type and bri1-5 due to BR deficient condition. The level of expression of BR biosynthesis genes, DWF4 and CPD, decreased 0.5 times in BHB2-sx when compared with that in wild type in the absence of BL (Figure 1G). In the presence of 100 nM BL, the ratio decrease of the expression level of DWF4 and CPD due to feedback regulation was 0.5 times in wild type when compared with none-treated, while that was 0.7 times in BHB2-sx (Figure 3C). This results indicate that the feedback regulation of BR biosynthesis genes was enhanced in BHB2-sx than wild type similar to det2-1 and bri1-5.

The dwarf hypocotyl as BR deficient phenotype (Figures 1A, B, 3A, B) and repressed BR biosynthetic gene expression (Figures 1G, 3C) in BHB2-sx plants would be due to the part of deficiency of BR synthesis. These results indicated that BHB2 might have function to enhance the expressions of BR biosynthesis genes directly. In other words, BHB2 acts as a positive regulator of BR response.
probably through the promotion of BR biosynthesis.

The BR phytohormone regulates various plant physiological aspects, including agricultural traits. Alteration of endogenous levels of BR results in drastic morphological changes in plants, indicating that maintenance of BR homeostasis is important for normal plant growth. Plant growth is promoted by BR with crosstalk between another phytohormones. In this study, we identified a novel BR positive regulator, BHB2, using the chimeric repressor method and the Brz, BR inhibitor. BHB2 belongs to ZF-HD transcription factor family containing a homeodomain (HD) and a zinc finger motif binding to DNA (Tan and Irish 2006). The dwarf phenotype of BHB2-sx plants is likely attributed to BR deficiency because the expression of DWF4 and CPD was significantly repressed in BHB2-sx plants. In summary, our study highlighted BHB2 to be an important transcription factor that regulates plant growth by enhancing BR responses probably promoting BR biosynthesis. In the future, we intend to elucidate the mechanism that positively regulates BR biosynthesis through BHB2.

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References

Figure 3. BHB2-sx showed BR responsivity by BL treatment. (A) 13-days-old seedlings of WT, BHB2-sx (#1 and #11), bri1-5 and det2-1 mutants grown on medium with 0, 1, 10 and 100 nM BL. Scale bar=5.0 mm. (B) Measurement of the elongation rate of 13-days-old hypocotyls of WT, bri1-5, det2-1 and BHB2-sx (#1 and #11) treated with 0, 1, 10, 100 nM BL. Error bars represent means±s.d. (n=14); *p<0.01 using Student’s t-test. (C) The expression analysis of BR biosynthesis genes, DWF4 (upper) and CPD (lower), in 13-days-old seedlings of WT, bri1-5, det2-1 and BHB2-sx (#1 and #11) plants treated with 0, 1, 10, 100 nM BL by qRT-PCR. Error bars represent means±s.d. (n=3); *p<0.05 and **p<0.01 using Student’s t-test.


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