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# Comprehensive analysis of downstream transcriptomic features in the competitive relationships between BEH3 and other BES/BZR transcription factors

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Members of a plant-specific BES/BZR transcription factor (TF) family including BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1) regulate various developmental processes and environmental responses. Recently, we reported that BES1/BZR1 Homolog 3 (BEH3) exhibited a competitive effect toward other BES/BZR TFs. In this study, we analyzed transcriptome profiles in *BEH3*-overexpressing plants and compared them with those of BES1 and BZR1 double gain-of-function mutants. We identified 46 differentially expressed genes (DEGs), which were downregulated in the gain-of-function mutants of BES1 and BZR1 but upregulated upon *BEH3* overexpression. In these DEGs, putative BES1 and BZR1 direct-targeted genes were highly enriched. In addition, these DEGs contained not only known brassinosteroid biosynthetic enzymes, but also some NAC TFs, which negatively regulate brassinosteroid-inactivating enzymes. Moreover, the iron sensor and the iron-deficient response-related bHLH TFs were also included. Taken together, our findings indicate that a competitive relationship between BEH3 and other BES/BZR TFs exists in various BES/BZR binding target genes.

**Key words:** transcription factor, BES1, BZR1, brassinosteroid, vascular development

In *Arabidopsis thaliana*, BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1) are well-known key transcription factors (TFs) in the brassinosteroid (BR) signaling pathway, which regulates various plant developmental processes and stress responses (Kono and Yin, 2020; Nolan et al., 2020). In the BR response, BES1 and BZR1 regulate both activation and repression of gene expression, depending on the target gene (Sun et al., 2010; Yu et al., 2011). When BZR1 and BES1 act as an activator, they are considered to cooperate with other TFs, such as the bHLH TFs PIF4 and BIM1, to positively regulate BR-induced genes (Nolan et al., 2020). On the other hand, BZR1 represses gene expression by a specific DNA-recognizing mechanism without any other partner TF (Nolan et al., 2020; Nosaki et al., 2022). The Arabidopsis genome encodes six BES/BZR homologs that are thought to have redundant functions in BR signaling (Nolan et al., 2020). In a previous paper, we revealed that one of the BES/BZR homologs, BES1/BZR1 Homolog 3 (BEH3), has functions opposite to those of other BES/BZR TFs in the regulation of vascular stem cells (Furuya et al., 2021). A BES1 gain-of-function mutant, *bes1-D*, displays accelerated vascular stem cell differentiation and consequently exhibits fewer (pro)cambial cell layers during secondary vascular development (Kondo et al., 2014; Saito et al., 2018), whereas stable transgenic lines of β-estradiol-inducible *BEH3*-overexpressing plants show an increase in the number of (pro)cambium layers (Furuya et al., 2021).

To investigate comprehensive changes in the transcriptomic profile following *BEH3* overexpression, we performed RNA-seq analysis using eight-day-old seedlings of the β-estradiol-inducible *BEH3* overexpression line *pER8:BEH3-CFP #3-4* treated with 10 μM β-estradiol (ind.BEH3 Est.) or DMSO (ind.BEH3 Mock)

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for 9 h. Comparative analysis between ind.BEH3 Est. and ind.BEH3 Mock (ind.BEH3 Est. vs. Mock) revealed 552 upregulated and 293 downregulated differentially expressed genes (DEGs) (the false discovery rate (FDR) was defined as less than 0.05) (Fig. 1A). For comparison, we also analyzed the transcriptome of 9-day-old seedlings of the constitutively active double mutant *bes1-D bzr1-D* and Columbia-0 (Col-0; wild-type) (*bes1-D bzr1-D* vs. Col-0). Consequently, 808 upregulated and 564 downregulated DEGs were identified (Fig. 1B). The well-known BES1- and BZR1-upregulated direct target genes *SMALL AUXIN UPREGULATED 15 (SAUR15)* and *INDOLE-3-ACETIC ACID INDUCIBLE 19* (*IAA19*) were found in the upregulated DEGs, whereas the well-known downregulated direct target genes *DWARF4* (*DWF4*) and *BRASSINOSTEROID-6-OXIDASE 2* (*BR6ox2*) were included in the downregulated DEGs. To investigate the competitive effect of BEH3 toward BES1 and BZR1, we compared the DEGs from *bes1-D bzr1-D* vs. Col-0 and ind.BEH3 Est. vs. Mock and focused on oppositely regulated genes between these comparisons (Fig. 1C, 1D). Among the 564 downregulated genes in *bes1-D bzr1-D*, 46 were found in upregulated DEGs from ind.BEH3 Est. vs. Mock (*bes1-D bzr1-D* down ind.BEH3 up). However, among the 808 upregulated genes in *bes1-D bzr1-D*, only 15 were included in the downregulated genes of ind.BEH3 (Fig. 1C, 1D). These results indicate that the opposite effects of *BEH3* overexpression mainly occur in the *bes1-D bzr1-D* downregulated DEGs.

Based on chromatin immunoprecipitation microarray (ChIP-chip) experiments, 3,410 putative BZR1 target genes and 1,609 putative BES1 target genes were identified (Sun et al., 2010; Yu et al., 2011). Both BZR1 and BES1 target genes were enriched in the 46 DEGs (*bes1-D bzr1-D* down/ind.BEH3 up) more than in the other groups of DEGs (Fig. 1E). We then analyzed the promoter regions of these 46 DEGs using Analysis of Motif Enrichment (AME) software and the ArabidopsisDAPv1 database, which is based on DNA affinity purification sequencing (DAP-seq) experiments (McLeay and Bailey, 2010; O'Malley et al., 2016). We found 23 putative loci of BES/BZR homolog-associated binding motifs in the promoter region of 19 genes in the 46 DEGs (*bes1-D bzr1-D* down/ind.BEH3 up) (Fig. 1F). Recently, it was reported that BZR1 preferentially binds to 10-bp elements of DNA fragments containing a YR (Y: pyrimidine; R: purine) base-pair step, such as CA, TG and CG, flanking the 3′ or 5′ side of the G-box or NN-BRRE-core to allow tight DNA recognition for the repression of downregulated BR-responsive genes (Nosaki et al., 2022). These features were observed in the identified motifs (13 of 23 loci; Fig. 1F). These data suggest that BEH3 oppositely functions via binding competition to BES1 and BZR1 on these motifs.

*DWF4* and *BR6ox2*, which were listed in the 46 DEGs (*bes1-D bzr1-D* down/ind.BEH3 up), encode BR-biosynthetic enzymes (Wei and Li, 2020). The repression of these genes by BES1 and BZR1 is known as the negative feedback regulation of BR signaling (Kono and Yin, 2020; Nolan et al., 2020). Thus, BEH3 may inhibit the negative feedback regulation of BR signaling through competition. To extend the characterization of the 46 DEGs, we used the plant gene co-expression database ATTED-II v11 (Obayashi et al., 2022) and conducted gene ontology analysis using STRING v11.5 (Szklarczyk et al., 2021) and identified a total of six gene modules (Fig. 1G). Module-1 contained BR biosynthetic genes, such as *DWF4*, *CPD* and *BR6ox2* (Wei and Li, 2020). Module-6 contained three ATAF subgroup members of NAC TFs: ATAF1 (ANAC002), ATAF2 (ANAC081) and ANAC102 (Christianson et al., 2010). It is known that these ATAFs, together with the core circadian clock regulator CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), transcriptionally suppress the genes that encode two cytochrome P450 enzymes (*PHYB ACTIVATION TAGGED SUPPRESSOR 1* (*BAS1*) and *SUPPRESSOR OF PHYB-4 7* (*SOB7*)) that function in the inactivation of BRs (Peng et al., 2015; Peng and Neff, 2021). In addition, the expression of these *ATAF* genes is suppressed by BR and white light (Peng and Neff, 2021). Taken together, these results suggest that *BEH3* overexpression induces transient accumulation of active BRs by upregulating BR biosynthetic enzymes and downregulating BR inactivation enzymes, followed by negative feedback loops consisting of both module-1 and module-6 to control BR levels. Indeed, the expression levels of *DWF4*, *CPD*, *BR6ox2* and *BAS1* were not very different among constitutive *BEH3*-overexpressing plants, *beh3* mutants and wild-type plants under normal culture conditions (Van Nguyen et al., 2021). However, tissue- and timing-specific regulation of active BR levels by BEH3 may contribute to developmental and stress response processes. Module-5 is the largest module and is associated with the GO term "Iron ion homeostasis (GO:0055072)". This module contained the Fe sensor BRUTUS (BTS) and the Ib bHLH subgroup proteins bHLH100 and bHLH101, which are key regulators of the Fe-deficient response (Wang et al., 2013; Liang, 2022). Negative effects of BRs on iron uptake and translocation in cucumber and rice have been reported, but the molecular mechanism of this process has not yet been elucidated (Wang et al., 2012, 2015). Our findings suggest that BEH3 and other BES/BZRs also have opposing functions in iron homeostasis.

In summary, our previous reports and this study suggest that BEH3 inhibits the transcriptional repressor activities of other BES/BZR TFs in the transcriptional regulation of various BES1/BZR1 downregulated target genes, including those related to BR homeostasis and stress responses. Further tissue- or response-specific analyses of BEH3 and other BES/BZR TFs will help to

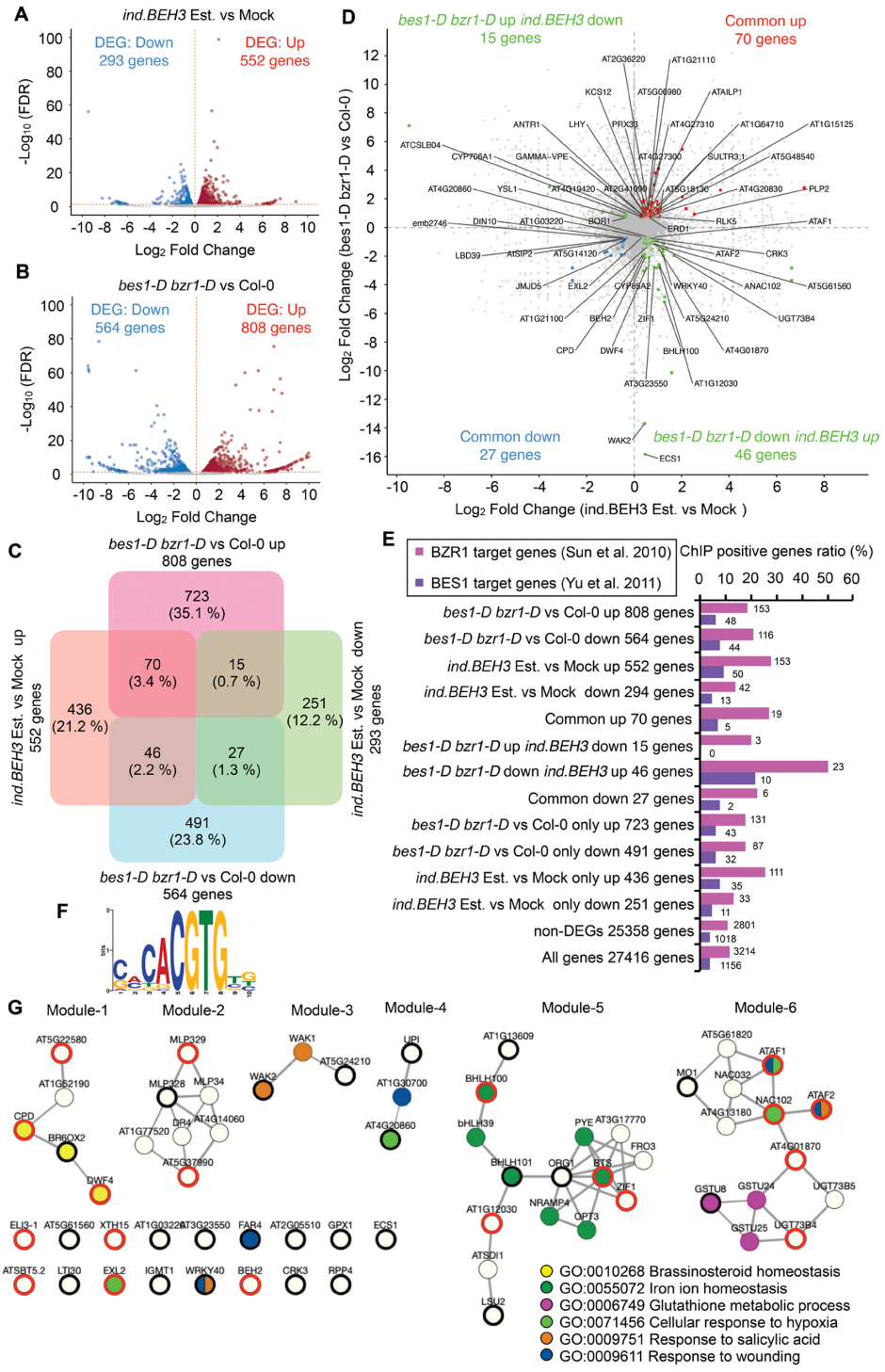


Fig. 1. Comparative transcriptomic analysis of *BEH3*-overexpressing plants and BES1 and BZR1 gain-of-function mutants. (A) Volcano plot of differentially expressed genes (DEGs) of *pER8:BEH3-CFP #3-4* treated with 10 μM β-estradiol (ind.BEH3 Est.) compared with that treated with DMSO (ind.BEH3 Mock). Red and blue dots indicate significantly upregulated and downregulated DEGs (FDR < 0.05), respectively. (B) Volcano plot of DEGs of *bes1-D bzr1-D* compared with Columbia-0 (Col-0). Red and blue dots indicate significantly upregulated and downregulated DEGs (FDR < 0.05), respectively. (C) Venn diagrams for DEGs in both comparisons (ind.BEH3 Est. vs. Mock and *bes1-D bzr1-D* vs. Col-0). (D) Correlation plot for DEGs in both comparisons (ind.BEH3 Est. vs. Mock and *bes1-D bzr1-D* vs. Col-0). Colored dots indicate significant DEGs (FDR < 0.05) in both comparisons. (E) The percentage of BZR1 target genes (Sun et al., 2010) and BES1 target genes (Yu et al., 2011) identified using ChIP-chip experiments relative to the total number of DEGs in each group correlated with (C). The number on the right side of each bar indicates the number of BZR1 or BES1 target genes in DEGs in each group. (F) The sequence logo of BES/BZR homolog-associated binding motifs in the promoter region of the DEGs (*bes1-D bzr1-D* down ind.BEH3 up). (G) Gene co-expression network analysis of the DEGs (*bes1-D bzr1-D* down ind.BEH3 up) using ATTED-II. Bold circles indicate the DEGs. Red bold circles indicate genes with BES/BZR homolog-associated binding motifs.

uncover the mechanisms of fine-tuned regulation in each developmental and environmental context.

## DATA AVAILABILITY

All data are available in the manuscript. All sequence reads were deposited in the DDBJ and are available through the Sequence Read Archive (SRA) under the accession number DRA015623.

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