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SHORT COMMUNICATION

Transcriptional network regulation of the brassinosteroid signaling pathway by the BES1–TPL–HDA19 co-repressor complex

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Abstract

Main conclusion **The brassinosteroid-related BES1 and BZR1 transcription factors dynamically modulate downstream gene networks via the TPL–HDA19 co-repressor complex in BR-signaling pathways in *Arabidopsis thaliana.***

Abstract Brassinosteroids (BRs) are plant steroid hormones that are essential for diverse growth and developmental processes across the whole life cycle of plants. In *Arabidopsis thaliana*, the BR-related transcription factors BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1) regulate a range of global gene expression in response to BR and several external signaling cues; however, the molecular mechanisms by which they mediate the reprogramming of downstream transcription remain unclear. We here report that formation of a protein complex between BES1 and BZR1 and Histone Deacetylase 19 (HDA19) via the conserved ERF-associated amphiphilic repression (EAR) motif proved essential for regulation of BR-signaling-related gene expression. Defects in BR-related functions of BES1 and BZR1 proteins containing a mutated EAR motif were completely rescued by artificial fusion with EAR-repression domain (SRDX), TOPLESS (TPL), or HDA19 proteins. RNA-sequencing analysis of *Arabidopsis* plants over-expressing *bes1-DmEAR* or *bes1-DmEAR-HDA19* revealed an essential role for HDA19 activity in regulation of BES1/BZR1-mediated BR signaling. In addition to BR-related gene expression, the BES1–HDA19 transcription factor complex was important for abiotic stress-related drought stress tolerance and organ boundary formation. These results suggested that integrating activation of BR-signaling pathways with the formation of the protein complex containing BES1/BZR1 and TPL–HDA19 via the EAR motif was important in fine-tuning BR-related gene networks in plants.

Keywords BES1 · Brassinosteroids · Histone deacetylase · Plant hormone · RNA-seq · Stress response · TOPLESS

Abbreviations

BR	Brassinosteroid
EAR	ERF-associated amphiphilic repression
TPL	TOPLESS
HDA19	Histone deacetylase 19
BES1	BRI1-EMS-SUPPRESSOR 1
BZR1	BRASSINAZOLE-RESISTANT 1
DEG	Differentially expressed genes
GO	Gene ontology

Introduction

Brassinosteroids (BRs) are plant growth hormones that play critical regulatory roles in diverse developmental processes through tight connections with environmental and other signaling cues. BR-signaling pathways are initiated by the perception of biologically active BRs by a complex anchored to the plasma membrane consisting of the co-receptors BRASSINOSTEROID INSENSITIVE 1 (BRI1) and BRI1-ASSOCIATED KINASE 1 (BAK1) (Kim and Wang 2010).

Hyemin Kim and Donghwan Shim contributed equally to this work as the first authors.

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The signaling cues are then transmitted to two BR-signaling-related transcription factors, BES1 and BZR1, by the nuclear accumulation of dephosphorylated forms (Yin et al. 2002, 2005; Ryu et al. 2007). The activated transcription factors modulate an extensive range of transcriptional reprogramming to ensure proper responses to BR. The transcriptional changes in the nucleus rely on the ability of BES1 and BZR1 to form a variety of protein complexes with several different transcription factors, including bHLHs, Myb, and histone-modification-related factors, thus modulating transcriptional repression or activation (Li et al. 2018). The ERF-associated amphiphilic repression (EAR) motif in the C-terminal region of BES1 and BZR1 has been shown recently to play a critical role in direct interactions of these proteins with the co-repressor TOPLESS (TPL)–HISTONE DEACETYLASE 19 (HDA19) complex (Oh et al. 2014; Ryu et al. 2014).

BR signaling is also important in regulating cellular proliferation in the shoot and root meristem. In the shoot apical meristem (SAM), BRs specifically modulate a group of small, rarely dividing cells called organ boundaries that separate the developing organ from the meristem (Fletcher 2002; Reddy et al. 2004). A transcription factor involved in BR signaling, BZR1, directly represses expression of the *CUP-SHAPED COTYLEDON 1*, *2*, and *3* (CUC1-3) genes, which determine the identity of the organ boundary, leading to the separation of organ boundaries (Bell et al. 2012; Gendron et al. 2012). In addition, BRs regulate cell division in the quiescent center (QC) through a cell-autonomous pathway that is mediated by the R2R3 MYB transcription factor BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER (BRAVO), independently of QC cell division promoted by auxin and ethylene signaling (González-García et al. 2011; Lee et al. 2015). The co-repressor TPL acts in BR-regulated gene expression in root and shoot meristems in a manner crucial for organ boundary initiation and maintenance, and for preserving low QC cell division rates (Espinosa-Ruiz et al. 2017). Formation of the BES1/BZR1–TPL–HDA19 complex, therefore, is likely to be important in several BR responses. Despite these insights, molecular and genetic evidence for the essential role of the EAR motifs present in BES1 and BZR1 in initiating histone deacetylase activity, thus modulating expression of the BR-related global gene network, remains limited.

We found that formation of the BES1/BZR1–TPL–HDA19 protein complex, mediated by the evolutionarily conserved EAR motif, was essential for a broad range of BR responses in plants. Defects in BR-related functions of BES1 and BZR1 proteins containing a mutated EAR motif (mEAR) were completely rescued by artificial fusion with either an EAR motif-based artificial transcriptional repression domain (SRDX; LDLDLELRLGFA), TPL, or HDA19 proteins. Physiological and global transcriptomic analysis of plants over-expressing *bes1-DmEAR* or *bes1-DmEAR-HDA19* suggested that HDA19 activity had an essential role in modulating BES1/BZR1-mediated BR signaling. HDA19 activity acting through BES1 was also important for drought stress tolerance and cell proliferation during initiation of the meristem-related organ boundary. EAR motif-associated recruitment of TPL–HDA19 thus appears to be required for regulation of BES1/BZR1-mediated BR signaling and fine-tuning of BR-related gene networks in plants.

Materials and methods

Plant materials, transgenic plants, and growth conditions

Arabidopsis thaliana ecotype Col-0 was used as wild-type controls and genetic backgrounds of all transgenic lines. *Arabidopsis* seeds were germinated in media (pH 5.7) containing 1/2 × Gamborg B5 salts (Duchefa, Haarlem, Netherlands), 1% sucrose and 0.8% phytoagar under long-day conditions (16-h light/8-h dark cycles) at 20–22 °C. The same materials of *35S-bes1-D*(*mEAR*) and *35S-bzr1-1D*(*mEAR*) transgenic plants reported previously (Ryu et al. 2014) were used. To generate the artificial fusion of SRDX, TPL and HDA19 proteins to *bes1-DmEAR* and *bzr1-1DmEAR*, the cDNA fragments of them were subcloned into *pCB302ES* containing the *35S-bes1-DmEAR* and *35S-bzr1-1DmEAR* and FLAG tag sequences. All transgenes were transformed by *Agrobacterium*-mediated floral dipping methods. The transgene expression was verified by immunoblotting with an anti-FLAG antibody (Sigma).

RNA-seq and real-time qRT-PCR analysis

To determine the expression levels of transcripts, total RNA was isolated from 7-day-old seedlings using a Total RNA extraction kit (Intron Biotechnology, Seoul, Korea) according to the manufacturer's instructions. An RNA integrity number > 8 was selected and used for library preparation. The each paired-end cDNA library was conducted according to the TruSeq RNA Sample Preparation Guide (Illumina). To perform real-time qRT-PCR, double-strand cDNA was synthesized from 1 µg RNA with a first-strand synthesis KIT (Enzynomics, Daejeon, Korea) with oligo (dT) primers. Quantitative real-time PCR was performed with 1 µl of cDNA and gene-specific primer sets according to the instructions provided for a Quant Studio 3 (Applied Biosystems) instrument using SYBR Green real-time PCR Master Mix (Applied Biosystems, USA). The data analysis and fold change calculation was done with the delta Ct method provided for the Quant Studio 3 software. The sequences of all

gene-specific primers used in this study are listed in Suppl. Table S1.

Physiological analysis

For hypocotyl elongation assays, T3 homozygous transgenic plant seeds of Col-0, *35S-bes1-D, 35S-bzr1-1D, 35S-bes1-D(mEAR)-SRDX/TPL/HDA19* and *35S-bzr1-1D(mEAR)-SRDX/TPL/HDA19* were plated on half-strength Gamborg B5 medium containing 1% sucrose and 1 μM BRZ (Sigma) for 1 day in 16-h light/8-h dark condition and then further incubated for 4 days in the dark condition. To evaluate the drought stress tolerance of *35S-bes1-DmEAR-HDA19* transgenic line, normal watering was withheld from 2-week-old pot-grown Col-0 and *35S-bes1-DmEAR-HDA19* transgenic plants for 12 days, and then re-watered for 3 days. The restored plants were monitored.

Gene ontology (GO) enrichment analysis

AgriGO (http://bioinfo.cau.edu.cn/agriGO/) is used for GO enrichment analysis which is the functional hierarchical enrichment of DEGs (references). The heatmaps of differentially expressed genes related to GO terms were generated using matrix2png interface (https://matrix2png.msl.ubc.ca/bin/matrix2png.cgi).

Accession number

Nine raw data of each RNA-seq samples were deposited with the Accession Number SRP144745 in the NCBI Short Read Archive database.

Results and discussion

We previously identified a functional EAR motif (LxLxL) in the primary amino acid sequence of BES1 from *A. thaliana* (Ryu et al. 2014). This motif is essential for recruiting the TPL–HDA19 complex, a Groucho/Tup1-like transcriptional corepressor (Causier et al. 2011). To confirm the evolutionary conservation of the EAR motif in other plants, we aligned the primary amino acid sequences of the orthologous BES1 and BZR1 proteins from legumes (*Medicago truncatula*), tomato (*Solanum lycopersicum*), and rice (*Oryza sativa*; Suppl. Fig. S1). The EAR motif sequences were well conserved across the different species, as were previously characterized functional regions, including phosphorylation domains, 14-3-3 binding domains, and PEST domains, suggesting the likely involvement of the EAR motif in transcriptional regulation of BR-signaling pathways by BES1 and BZR1.

Next, we explored whether formation of the TPL-HDA19 co-repressor complex with BES1/BZR1, mediated by the EAR motif, was involved in regulating BR signaling. We compared brassinazole (BRZ)-resistant hypocotyl elongation phenotypes in Col-0 plants and artificial chimeric *SRDX*, *TPL*, and *HDA19*-tagged *bes1-DmEAR* (Ryu et al. 2014) or *bzr1-1DmEAR* overexpression lines (Fig. 1a). As previously reported (Wang et al. 2002; Yin et al. 2002; Ryu et al. 2014), overexpression of *bes1-D*, *bzr1-1D,* and artificial chimeric *SRDX*, *TPL*, and *HDA19*-tagged *bes1-DmEAR* resulted in gain-of-function mutants that showed not only BRZ-resistant hypocotyl elongation but also repression of the BR biosynthetic genes *CPD* and *DWARF 4* (*DWF4*) (Fig. 1a, b). Similar to the *bes1-DmEAR* mutant, the BR-defective responses of *bzr1-1DmEAR* were completely rescued by tagging with

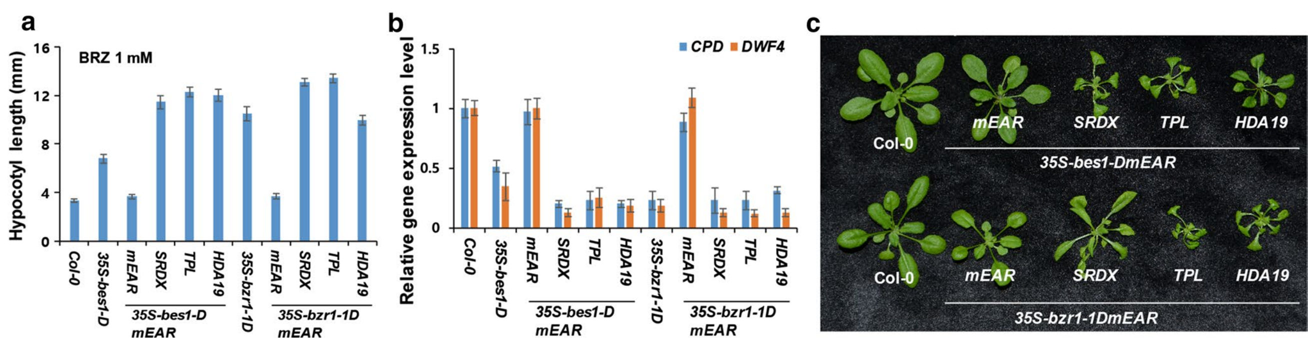

Fig. 1 EAR motif-mediated BES1/BZR1–TPL–HDA19 corepressor complex formation is essential for BR signaling. **a** BR-related hypocotyl elongation phenotype of wild-type (Col-0), *bes1-D*(*mEAR*), *bzr1-1D*(*mEAR*) and artificial chimeric SRDX, TPL and HDA19 tagged *bes1-DmEAR* or *bzr1-1DmEAR* overexpression lines on the BRZ-containing media. The transgenic plants were grown on 1 μM BRZ in the dark for 5 days. The hypocotyl length was measured (mean ± SE, $n=10$). **b** Relative fold change of BR-responsive gene expressions in Col-0 and indicated transgene overexpression lines was determined by a real-time qRT-PCR (mean ± SE, $n=3$). **c** The 3-week-old Col-0 and indicated transgenic lines were shown. The *bes1-DmEAR* or *bzr1-1DmEAR* overexpression lines shows similar phenotype with Col-0, but artificial chimeric SRDX, TPL and HDA19 tagged *bes1-DmEAR* or *bzr1-1DmEAR* overexpression lines shows strong BR-related phenotypes

an artificial SRDX sequence consisting of repeated EAR motifs (Fig. 1). Mutation of the EAR motif (mEAR) completely abolished the effects of over-expressing *bes1-D* and *bzr1-1D*, indicating that this motif was essential for BES1 and BZR1 functions. In addition, plants over-expressing artificial chimeric *TPL* and *HDA19* fused with *bzr1-1DmEAR* showed hyper-sensitive BR-related responses, including BRZ-resistant long hypocotyls, curled rosette leaves, and lower expression of *CPD* and *DWF4* (Fig. 1). HDA19 recruited by the EAR motif, therefore, appeared to have an essential role in BR-signaling pathways modulated by transcriptional regulation of BES1/BZR1.

EAR motif-mediated integration of either TPL or HDA19 was essential for BES1/BZR1-mediated BR-signaling pathways (Fig. 1). We performed RNA-sequencing (RNA-Seq) analysis to confirm whether TPL and its integrated HDA19 activity were required for transcriptional regulation of BR-signaling-related transcription factors. Total RNA extracted from Col-0, *bes1-DmEAR*, and *bes1-DmEAR-HDA19* transgenic plants was used in a comparative transcriptomic analysis. For a statistical evaluation of samples used in the comparative analyses, high-quality reads were mapped onto the annotated *Arabidopsis thaliana* transcriptome (TAIR10), and expression abundance was calculated using RSEM 1.3.0 software. Differentially expressed genes (DEGs) were identified using EdgeR software and distributions of DEGs between Col-0, *bes1-DmEAR*, and *bes1-DmEAR-HDA19* transgenic plants were visualized using MA plots (Fig. 2a). In total, 379 up-regulated and 157 down-regulated DEGs were highly enriched in *bes1-DmEAR-HDA19* transgenic plants compared to Col-0. Col-0 and *bes1-DmEAR* plants clustered together in pairwise comparisons of DEGs with similar gene expression patterns, but *bes1-DmEAR-HDA19* transgenic plants were grouped separately (Fig. 2b); these groupings resembled the phenotypic differences and BR responses observed in transgenic plants (Fig. 1). For comparative transcriptomic analyses, high-quality reads were mapped to the assembled transcriptome, and expression abundance was calculated. In total, 695 genes were differentially expressed (using a cutoff greater than twofold change with a *P* value below 0.01) in all pairwise comparisons (Suppl. Fig. S2). Among

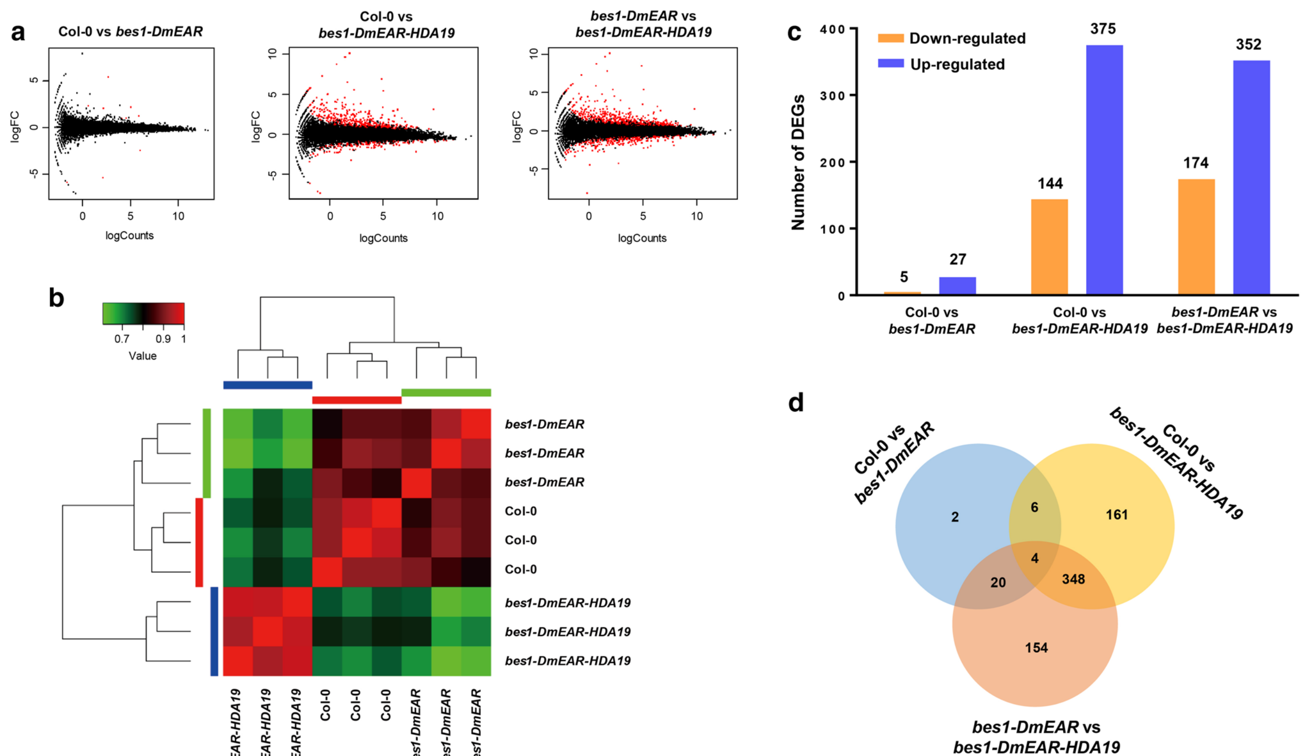

Fig. 2 HDA19 is required for the BES1-mediated downstream gene regulation network. **a** MA plots for the comparison of expression of transcripts among Col-0, *bes1DmEAR* and *bes1DmEAR-HDA19*, reciprocally. **b** Clustered heatmap showing the Pearson correlation matrix for pairwise sample comparisons. The color key was adjusted on the basis of the minimal and maximal values for optimal visual differentiation of the differences, and a dendrogram illustrates the relationship distance between the samples (three biological replicates). **c** The number of up- and down-regulated genes among differentially expressed genes in transgenic lines in RNA-seq. **d** Venn diagram shows differentially expressed genes within each transgenic lines

the DEGs, 27 and 5 were significantly up- and down-regulated in *bes1-DmEAR* overexpression plants compared with those of a wild-type Col-0 control, respectively. In *bes1-DmEAR-HDA19* transgenic plants, 519 transcripts were differentially expressed compared to Col-0, and 375 and 144 showed up or down-regulated expression (Fig. 2c). Also, we compared the transcriptomes between *bes1-DmEAR* and *bes1-DmEAR-HDA19* transgenic plants and identified 526 transcripts as reliable DEGs. Among them, 352 were up-regulated and 174 were down-regulated in the *bes1-DmEAR-HDA19* transgenic plants compared with those of *bes1-DmEAR DmEAR* mutants (Fig. 2c). We confirmed common- or specific- expressed genes among the each compared DEGs (Fig. 2d and ESM_1). Venn diagram showed that Col-0 and *bes1-DmEAR* overexpression showed a few common expressed genes because their expression patterns and plant phenotypes were very similar (Figs. 1, 2b). Whereas, *bes1-DmEAR-HDA19* transgenic plants vs. other plant samples showed a lot of common or specific expressed genes (Fig. 2d). These results indicate that HDA19 protein was required for the proper regulation of the BES1 transcriptional network in BR-signaling pathways.

To confirm the biological relevance of BR signaling and HDA19 activity, we compared DEGs in plants expressing *bes1-DmEAR-HDA19* with previously characterized BR-responsive genes. As expected, expression patterns of BR-responsive genes correlated with DEGs in plants expressing *bes1-DmEAR-HDA19* (Suppl. Tables S2 and S3). Most notably, expression of BR-responsive biosynthetic genes, including *DWF3, 4* and *5*, *DEETIOLATED 2* (*DET2*), and *CYP85A2 (BR6ox2)*, was greatly reduced in BR-activated plants expressing *bes1-DmEAR-HDA19* (Suppl. Fig. S3). All these results suggested that the transcriptional networks of the BR-signaling pathway were required for HDA19 activity induced via the EAR motif of the BR-related transcription factors BES1 and BZR1.

We performed gene ontogeny (GO) enrichment analysis for the annotated genes to characterize the functions of the DEGs. In total, 24 GO categories related to biological processes were enriched in the group of DEGs with P values < 0.01 (Suppl. Table S4). As with the BR-related physiological responses, many categories related to (a) biotic stress responses, plant hormone stimulus, cell wall, and development were significantly enriched in the DEGs (Suppl. Table S4). To determine the relationship between the BR-related physiological responses and the gene expression pattern, we confirmed the expression patterns of selected DEGs from each category. Gene set enrichment analysis (GESA) and hierarchical GO trees indicated that DEGs were significantly over-represented in the jasmonic acid (JA), salicylic acid (SA), ABA, and auxin pathways, which are related to defense, abiotic stress, and development (Suppl. Fig. S4). In particular, expression of *REGULATORY COMPONENT OF ABA RECEPTOR* (*RCAR9*), *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), *PATHOGENESIS RESPONSIVE* (*PR*) genes, the defensin-like (*DEFL*) gene family, *Jasmonate-ZIM-domain protein 7 (JAZ7)* and developmental-related genes was significantly altered (Suppl. Tables S5-7) (Mondragón-Palomino et al. 2017). These results correlated with recent studies of the positive roles of BR in multiple stress tolerance responses and diverse developmental processes.

Finally, we evaluated the GO terms identified in DEGs that were related to development. BR is known to play important roles in various developmental processes of plants (Clouse et al. 1996; Kauschmann et al. 1996; Clouse and Sasse 1998). Recent studies have identified key roles for BR-signaling pathways in stem cell homeostasis and organ boundary formation by modulating expression of related genes (González-García et al. 2011; Heyman et al. 2013; Vilarrasa-Blasi et al. 2014). Data from our genome-wide transcriptomic analysis confirmed differential expression patterns of genes such as *WUSCHEL* (*WUS*)*, FLOWERING LOCUS C* (*FLC*), and *RETINOBLASTOMA-RELATED1* (*RBR1*) that are related to cell differentiation and development (Suppl. Table S8).

To validate the representative GO enrichment and RNA-Seq analyses, we performed real-time quantitative RT-PCR analyses of several stress, development, and BR-responsive genes selected from the DEGs (Fig. 3a). Expression patterns of the selected genes, which included the *JAI1*, *WRKY33*, and *MYB96* transcription factors, *ARCK1*, *CBF1*, *CUCs*, *CPD*, and *DWF4*, correlated with the RNA-Seq data ($R^2 = 0.0.8432$; Fig. 3a, Suppl. Fig. S5). We consistently confirmed the drought stress tolerance and abnormal organ boundary phenotypes of BR-activated plants expressing *bes1-DmEAR-HDA19* (Fig. 3b, c). HDA19 activity, therefore, appeared to play an essential role in BR-signaling pathways through formation of the BES1/BZR1-mediated transcriptional network.

The EAR motif is important for direct interactions between the C-terminal regions of BES1 and BZR1 and the Groucho/TLE-like transcriptional co-repressor TPL/TPL-related (TPR; Oh et al. 2014; Ryu et al. 2014). TPL and its paralogous TPRs are required for transcriptional reprogramming of diverse developmental processes and hormonal signaling pathways (Liu and Karmarkar 2008; Causier et al. 2011). This study provided molecular and physiological evidence indicating that the TPL-HDA19 co-repressor complex functioned as an essential regulator of BES1/BZRR1-mediated BR-signaling pathways. Our complementation of mutated EAR motifs by artificial fusion with HDA19 instead of SRDX or TPL suggested that HDA19 activity played a critical role in the transcriptional network regulating BR-signaling pathways. HDA19 activity was sufficient for complete rescue of the BR-defective responses found in *bes1-DmEAR* and *bzr1-1DmEAR* plants, such as hypocotyl

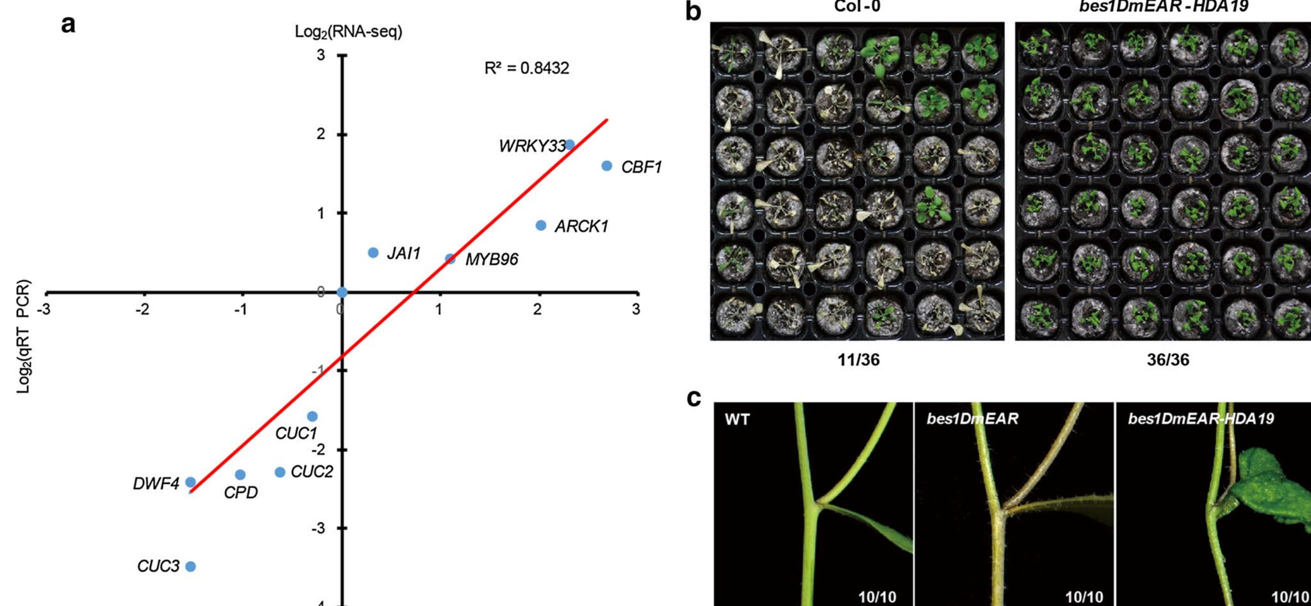

Fig. 3 BR-signaling pathways are integrated into stress and developmental regulatory network by BES1–TPL–HDA19 co-repressor complex. **a** The expression patterns of selected DEGs were validated by real-time qRT-PCR. RNA-seq values (*y* axis) and qRT-PCR values (*x* axis) were compared using log2 fold change in the correlation analysis. **b** Drought stress response of WT and *bes1DmEAR-HDA19* overexpression plants. 14-day-old indicated plants were subjected to drought by water withholding for 12 d, and then re-watered for 3 d. WT (left panel) and *bes1DmEAR-HDA19* overexpression plants (right panel). The numbers in the bottom of subpanels represent the frequency of survived plants. **c** Overexpression of *bes1DmEAR-HDA19* plants but not that of *bes1-DmEAR* was resulted in abnormal stem bending and organ fusion of the cauline leaf and axillary branch to the main stem. The numbers in the bottom of subpanels represent the frequency of the observed phenotype

elongation, curled rosette leaves, lower expression of BR biosynthetic genes, stress tolerance, and organ boundary formation (Figs. 1, 3). These results were correlated with broad spectrum of crosstalk mechanisms of BR signaling with diverse developmental and stress-signaling pathways (Ryu and Cho 2015; Ha et al. 2016). Also, many recent studies have revealed the positive effects of BR-signaling pathways on abiotic stress tolerance (Ryu and Cho 2015; Ha et al. 2016; Nolan et al. 2017; Lee et al. 2018). However, a recent study showed antagonistic interaction of RD26 and BES1 for drought stress tolerance and growth regulation (Ye et al. 2017), but our RNA-seq data supported the *RD26* expression was highly enriched in the *bes1-DmEAR-HDA19* over-expressing plants (Suppl. Table S5). The up-regulation of *RD26* was correlated with drought stress tolerance phenotype of the *bes1-DmEAR-HDA19* over-expressing plants (Fig. 3). These suggest that complicated feedback loops between BR-mediated growth and stress tolerance signals might be involved in plant growth and development. The EAR motif is present in several transcription factors and mediates transcriptional repression through interactions with TPL and homologous TPL-related (TPR) proteins (Kagale and Rozwadowski 2011). In addition, HDA19, a histone deacetylase (HDAC), has a critical role in epigenetic gene suppression of various biological processes by inducing heterochromatin (Liu et al. 2014). Recent studies suggest that HDACs may activate transcription through direct deacetylation of TATA-BINDING PROTEIN–ASSOCIATED FACTOR 9 or by activating transcription elongation machinery (Nusinzon and Horvath 2005; Greer et al. 2015; Jian et al. 2017). Although not all the roles of HDAC have been identified fully, its function as an activator is likely to be crucial in fine-tuning gene regulation. Our RNA-Seq analysis of plants over-expressing *bes1-DmEAR-HDA19* consistently identified many up-regulated DEGs (Fig. 2). These data suggest the possibility that other mechanisms modulate HDA19-mediated regulation of BES1/BZR1 transcriptional activity.

In conclusion, a genome-wide transcriptomic analysis of plants over-expressing *bes1-DmEAR* or *bes1-DmEAR-HDA19* suggested an essential role for HDA19 activity in the regulation of BES1-mediated BR signaling, and its further involvement in abiotic stress, defense, and developmental pathways. Resolving the complex transcriptional regulatory network generated by the TPL-HDA19 co-repressor complex will enable understanding of the molecular mechanisms associated with plant growth and development.

Author contribution statement HK, DS, SM, JL, WB, and HR performed the experiments. HK, DS, HC, KK and HR

designed the experiments and analyzed the data. HK, DS and HR wrote the manuscript.

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