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Antagonistic Regulation of Arabidopsis Growth by Brassinosteroids and Abiotic Stresses

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To withstand ever-changing environmental stresses, plants are equipped with phytohormone-mediated stress resistance mechanisms. Salt stress triggers abscisic acid (ABA) signaling, which enhances stress tolerance at the expense of growth. ABA is thought to inhibit the action of growth-promoting hormones, including brassinosteroids (BRs). However, the regulatory mechanisms that coordinate ABA and BR activity remain to be discovered. We noticed that ABA-treated seedlings exhibited small, round leaves and short roots, a phenotype that is characteristic of the BR signaling mutant, *brassinosteroid insensitive1-9* (*bri1-9*). To identify genes that are antagonistically regulated by ABA and BRs, we examined published Arabidopsis microarray data sets. Of the list of genes identified, those upregulated by ABA but downregulated by BRs were enriched with a BRRE motif in their promoter sequences. After validating the microarray data using quantitative RT-PCR, we focused on *RD26*, which is induced by salt stress. Histochemical analysis of transgenic Arabidopsis plants expressing *RD26pro:GUS* revealed that the induction of GUS expression after NaCl treatment was suppressed by co-treatment with BRs, but enhanced by co-treatment with propiconazole, a BR biosynthetic inhibitor. Similarly, treatment with bikinin, an inhibitor of BIN2 kinase, not only inhibited *RD26* expression, but also reduced the survival rate of the plant following exposure to salt stress. Our results suggest that ABA and BRs act antagonistically on their target genes at or after the BIN2 step in BR signaling pathways, and suggest a mechanism by which plants fine-tune their growth, particularly when stress responses and growth compete for resources.

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INTRODUCTION

Due to their sessile nature, plants have developed strategies to cope with abiotic challenges and biotic stresses (Chung et al., 2012; Kim et al., 2014; Maharjan and Choe, 2011). Plants exposed to abiotic stresses display severe growth retardation and reduced productivity. Growth is regulated by plant hormones, which modify endogenous programs in response to exogenous signals. However, the hormone-dependent mechanisms by which growth is inhibited under stress conditions are not fully understood. The molecular mechanisms that impart tolerance to water stress can be divided into abscisic acid (ABA)-dependent and ABA-independent pathways (Shinozaki and Yamaguchi-Shinozaki, 2007). ABA plays vital roles in adaptation to environmental changes, seed dormancy, and the regulation of stomatal closure (Grill and Himmelbach, 1998; Lee and Luan, 2012).

Under stressed conditions, plants rapidly produce ABA, which stimulates the resistance mechanism. In the ABA-dependent pathway, ABA binds to soluble receptors of the PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCARs) family, which induces the *de novo* synthesis of ABA through 9-cis epoxycarotenoid dioxygenase (NCED) as part of a positive feedback mechanism (Ma et al., 2009; Park et al., 2009). Once ABA activates SnRK2s, SnRK2s phosphorylate and thereby activate the transcription of ABA-dependent transcription factors, such as ABA-responsive element binding factors (*ABFs*/*AREBs*) (Furihata et al., 2006; Uno et al., 2000). ABFs/AREBs then bind to the ABA response element (ABRE) in the promoter region of their target genes (Mundy et al., 1990). These conserved elements contain PyACGTGGC (Busk and Pages, 1997; Guiltinan et al., 1990; Yamaguchi-Shinozaki et al., 1990) and ACGTG[G/T]C (Hobo et al., 1999) residues. ABA regulates approximately 10% of the genes in Arabidopsis, and many of these genes are also regulated by other hormones (Nemhauser et al., 2006).

In addition to ABFs/AREBs, other ABA-induced transcription factors are known to participate in the ABA response and stress tolerance. For instance, the NAC (NAM, ATAF1/2 and CUC2) transcription factor family, which largely consists of NO APICAL MERISTEM (NAM) and *Arabidopsis thaliana* transcription activation factor (ATAF1/2) transcription factors, is known to function in the stress response (Aida et al., 1997). ATAF1/ANAC002 directly regulates ABA biosynthesis through the transcriptional activation of *NCED3* (Jensen et al., 2013; Tran et al., 2004).

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RD26 is a member of the ATAF family and is reported to function in ABA-dependent stress-response pathways (Fujita et al., 2004; Ooka et al., 2003).

Brassinosteroids (BRs) are a class of plant steroidal hormones (Chung and Choe, 2013). Like mammalian steroid hormones such as estrogen, ecdysone, and progesterone, BRs play key roles in plant development, regulating processes such as cell elongation, vascular system differentiation, senescence, and stress responses (Choe, 2006; Clouse and Sasse, 1998). BRs and other phytohormones have numerous target genes in common, and complex crosstalk mechanisms exist among these hormone signal transduction pathways (Chung et al., 2011; Nemhauser et al., 2006). Brassinolide (BL), the most active form of BRs, binds to an island domain in the extracellular domain of *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*) (Kinoshita et al., 2005; Sun et al., 2013). In the absence of BL, BKI1 binds to the cytoplasmic kinase domain of BRI1 and thereby inhibits its phosphorylation function (Wang et al., 2008). However, when BL binds to BRI1, BKI1 is released from BRI1 and BRI1 forms a heterodimer with BRI1-ASSOCIATED-KINASE 1 (BAK1) (Russinova et al., 2004; Wang and Chory, 2006). Then, BR-SIGNALING KINASE 1 (BSK1) is released from the cytoplasmic domain of BRI1 and transduces the signal into the nucleus via a protein phosphatase, *bri1 SUPPRESSOR1* (*BSU1*) (Kim et al., 2009; Tang et al., 2008). BSU1 inactivates the BR-INSENSITIVE 2 (BIN2) kinase that acts as a negative regulator in BR signaling pathways (Choe et al., 2002; Li and Nam, 2002; Li et al., 2001). BIN2 negatively regulates BR-specific transcription factors, including BZR1 and BES1 by phosphorylation (Kim et al., 2009), and is degraded in response to BL (Peng et al., 2008). BZR1 binds to the BR response element (BRRE) motif (CGTG[T/C][G/A]) (He et al., 2005) to both activate BR response genes and inhibit BR biosynthetic genes, including *DWF4* (Choe et al., 2001; Kim et al., 2006; 2013). Weak alleles of *bri1* include *bri1-5* (Noguchi et al., 1999) and *bri1-9*, and exhibits a semi-dwarf phenotype (Jin et al., 2007).

In contrast to the ABA pathway, the majority of signaling components have been elucidated in the BR signaling cascade (Vriet et al., 2013). Given the importance of ABA and BRs, much research has focused on identifying the mechanism of crosstalk between BRs and ABA. It has been demonstrated that ABA and BRs antagonistically regulate each other during seed germination and root growth inhibition (Steber and McCourt, 2001; Zhang et al., 2009)

In this study, we sought to understand the mechanisms by which BRs and ABA interact. First, we selected putative marker genes that are antagonistically regulated by BRs and ABA from a microarray database deposited in AtGenExpress (Nemhauser et al., 2006). Secondly, we found that chemical inhibition of BIN2 decreased the plant's tolerance to salt stress, suggesting that BIN2 is involved in ABA-mediated salt tolerance processes. Based on our findings, we propose a model in which transcription factors that bind to common target genes and are specific to either BRs or ABA are antagonistically regulated by each other to bring about ABA-dependent stress responses.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis plants of Columbia ecotype were grown in 0.5× Murashige and Skoog (MS) medium containing 0.5% sucrose and 0.8% plant agar. Plants or seedlings were kept in a growth room at 22°C with a 16 h light/8 h dark cycle. To measure the survival rate in salt media, seeds were germinated and grown in MS agar medium and transferred to medium containing the indicated concentrations of hormone or chemical. After 1 day, seedlings were transferred again to medium containing NaCl. *epi*-Brassinolide (BL) was dissolved in DMSO, and ABA stock was prepared in ethyl alcohol (EtOH). The controls contained the same volume of DMSO or EtOH as chemicals added to the treated aliquots, and considered a mock treatment.

Quantitative RT-PCR

RNA extraction and cDNA synthesis were conducted according to a previous report (Chung et al., 2012). Quantitative RT-PCR analysis was performed using SYBR-mix (KAPA Biosystems). *UBQ10* was used as an internal control. Primer sets used for PCR are listed in Supplementary Table S2.

GUS histochemical assay and quantification of GUS activity

Five-day-old *RD26pro:GUS* transgenic seedlings were first treated with the indicated concentrations of hormone or chemical. Following pre-incubation, each seedling was transferred to NaCl-containing agar medium supplemented with the same concentration of hormone or chemical. After 3 days, seedlings were transferred to GUS buffer (1 mM 5-bromo-4-chloro-3-indoyl-β-d-GlcUA, 100 mM sodium phosphate (pH 7), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 10 mM EDTA, and 0.1% (v/v) Triton X-100) and incubated for 2 h. Serially-diluted EtOH was used to clear the chloroplasts and to reduce background staining. Micrographs were taken using a stereomicroscope (Olympus). To quantify the *in vivo* GUS activity of *RD26pro:GUS* in each treatment, 0.5 cm of the root tip of treated seedlings was excised and transferred to 96-well plates pre-filled with a substrate solution (Blazquez et al., 1998). Seedlings in the substrate solution were incubated for 1 h at 37°C and the reaction was stopped by the addition of 100 μl of cold 0.2 M Na_2CO_3 solution. The fluorescence intensity was measured using a fluorescence spectrophotometer (Varian, USA) with an excitation wavelength of 360 nm and an emission wavelength of 465 nm. The values of 12 seedlings were averaged and plotted with their standard error. The standard curve was calculated using known concentrations of 4-methylumbelliferol solution.

Motif prediction by MEME

MEME (http://meme.sdsc.edu) was used to search for motifs conserved in the promoter region 1000 bp upstream of the start codons of genes antagonistically regulated by ABA and BR (Bailey and Elkan, 1995). A MEME search revealed that the optimum width for motifs was 6 to 9 bp.

RESULTS

Morphological similarity between ABA-treated seedlings and the BR-deficient dwarf mutant

We observed that treatment of Arabidopsis seedlings with ABA often resulted in phenotypes that resembled those of BR-deficient dwarf mutants. The small, curled leaves of ABA-treated Col wild-type plants were similar to those of mock-treated *bri1-9* plants (Figs. 1A and 1B), which bear a loss-of-function mutation in the BR receptor (Jin et al., 2007). Previously, we also showed that BR mutants are more sensitive to ABA treatment (Choe et al., 2002). These findings suggest that ABA and BRs interact to regulate Arabidopsis growth.

Because ABA treatment mimicked the BR mutant phenotype, we reasoned that *bri1-9* would be more sensitive to ABA than the wild type. To test this, we performed a dose-response assay in which we examined the key characteristics of seedling development, including root growth, germination, and post-

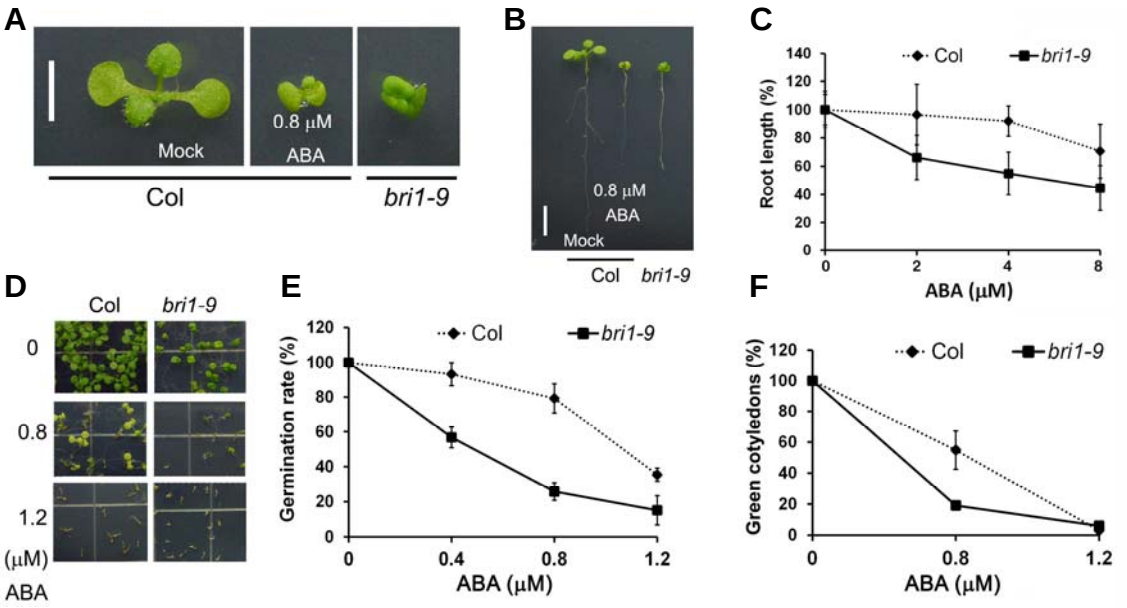

Fig. 1. Response of wild-type and *bri1-9* seedlings to ABA treatment. (A) Top view of Arabidopsis wild-type seedlings treated with mock or ABA, and *bri1-9* treated with mock only. Scale bar = 0.5 cm. (B) Whole seedling morphology of 10-day-old Col and *bri1-9* plants. From left, mock-treated wild type, ABA-treated wild type, and *bri1-9*. Scale bar = 1 cm. (C) Statistical representation of the dose-dependent effect of ABA on root growth. Seedlings were germinated on MS medium before being transferred to medium containing 0, 2, 4, or 8 μM ABA for a further 5 days of growth. The growth of *bri1-9* roots was retarded to a greater extent by ABA treatment than was that of wild-type roots. Values show root length relative to that of an untreated wild-type control. (D) Germination of Col and *bri1-9* on ABA-supplemented media. Photographs were taken ten days after sowing. Germination is suppressed to a greater extent in the *bri1-9* plates. (E) Statistical analysis of germination rates in response to different ABA doses. Seeds with visible radicles were scored as successful germination events. Average values are represented with standard deviations of three independent biological replicates. Again, *bri1-9* was more sensitive than the wild type to ABA treatment. (F) Establishment of green cotyledons in the presence of ABA. Seedlings with green cotyledons were scored. Error bars in (C, E, and F) are standard deviations (n = 3).

germination growth, under various concentrations of ABA. The root growth rate of wild-type plants was reduced in response to ABA treatment in a dose-dependent manner, and the degree of reduction was much greater in *bri1-9* (Fig. 1C). In addition, we scored the percentage of seeds with emerged radicles 3 days after sowing on mock, 0.4, 0.8, and 1.2 μM ABA medium (Fig. 1D). On 0.8 μM ABA, 80% of the Col seeds germinated, whereas only 25% of *bri1-9* seeds did, suggesting that *bri1-9* is more sensitive to exogenous ABA treatment than is the wild type (Fig. 1E). Of the germinated seeds, only 55% and 19% of the cotyledons of wild-type and *bri1-9* seedlings remained green, respectively, at 0.8 μM ABA (Fig. 1F). Thus, the growth of *bri1-9* plants was more sensitive to ABA than the wild type, suggesting that ABA controls BR signaling pathways to achieve optimal growth and development during the ABA-mediated stress response.

BRs and ABA antagonistically regulate overlapping sets of genes

To identify the genes that are oppositely regulated by ABA and BRs, we analyzed a set of publicly available microarray data that were reported by Nemhauser et al. (2006). They reported that 383 genes upregulated by BRs, 268 were downregulated by BRs, 1440 were upregulated by ABA, and 1476 were downregulated by ABA (Nemhauser et al., 2006). Because BRs and ABA have a tendency to reverse the effects on Arabidopsis growth (Fig. 1), we focused on genes that respond oppositely to BRs and ABA. We determined the union (62) of genes downregulated by BRs and upregulated by ABA, and the union (50) upregulated by BRs and downregulated by ABA (Fig. 2A). These genes were chosen for further investigation.

Because BZR1 can function either as an inducer or repressor of BR-responsive genes (He et al., 2005), we determined the number of BR-response elements (BRREs, CGTG[TC][GA], (He et al., 2005)) in the promoter DNA sequences of these genes (Fig. 2B). Whereas 25% of genes in the whole genome contained the BRRE motif, 48% of genes upregulated by ABA and downregulated by BR contained this motif (Fig. 2B). Since the BRRE was not enriched in the group of genes downregulated by ABA and upregulated by BR, we propose that genes that are upregulated by ABA and downregulated by BR are likely targeted by BZR1 through direct binding to BRRE in the promoter sequences.

We next determined the frequency of the BRRE in genes reported to be regulated by other hormones or a biosynthetic precursor of hormone, such as 1-aminocyclopropane-1-carboxylic acid (ACC), indole-3-acetic acid (IAA), methyl jasmonate (MeJA), gibberellins (GA), and cytokinins (CK). Whereas the percentage of genes possessing the BRRE is enriched in the ABA-upregulated group (36.4%) and BR-downregulated group (35.2%) compared to the whole genome control (25%), the percentage was similar for other hormone-response genes (Table 1), except for those upregulated by MeJA, suggesting that BRs negatively regulate JA signaling, as previously noted (Kim et al., 2013).

Of the genes upregulated by ABA and downregulated by BRs,

Table 1. Proportion of genes containing the BRRE

	ABA		BRs		ACC		IAA		GA		MeJa		CK	
Up or Down	U	D	U	D	U	D	U	D	U	D	U	D	U	D
No. genes	1440	1476	268	383	167	365	430	355	40	82	806	701	332	163
BRRE-containing gene	524	356	59	135	45	87	123	78	11	26	272	181	81	46
BRRE/Total	36.4	24.1	22.0	35.2	26.9	23.8	28.6	22.0	27.5	31.7	33.7	25.8	24.4	28.2

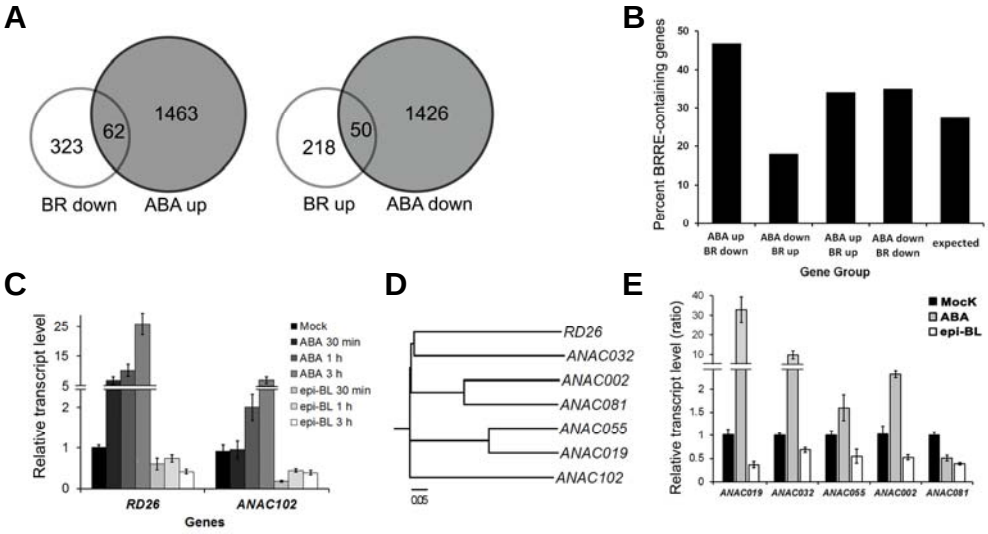

Fig. 2. Identification of genes co-regulated by BRs and ABA in publicly available microarray data. (A) Venn diagram of ABA- and BR-responsive genes. Genes that are antagonistically regulated by these hormones were selected from previously-reported data sets (Nemhauser et al., 2006). Left, Venn diagram of genes downregulated by BRs but upregulated by ABA; right, genes upregulated by BRs but downregulated by ABA. Fisher's exact test was conducted to determine the *p*-value of overlapping genes ($P < 0.09$ for genes upregulated by ABA and downregulated by BR; $P < 0.0051$ for genes downregulated by ABA and upregulated by BR). (B) Enrichment of the BR-response element (BRRE) in the -1000 bp promoter sequences of 62 and 50 genes co-regulated by BRs and ABA, respectively. The percentage of genes that harbor at least one BRRE (CGTG[T/C][G/A]) was determined by searching the Arabidopsis genome sequence database. (C) Time-course analysis of *RD26* and *ANAC102* transcript levels in seven-day-old wild-type seedlings treated with 1 μM *epi*-BL or 50 μM ABA in liquid MS media. Results were normalized using *UBQ10*, and converted to values relative to a mock-treated control. Data are mean ± standard deviation of three technical replicates. (D) *RD26* belongs to the *NAC* transcription factor family, which is composed of at least 94 members (Christianson et al., 2010). Of the entire phylogenetic tree of this gene family, a clade containing *RD26* is shown. The length of the bar indicates the number of changes in amino acid residues per unit length of the horizontal branches. (E) qRT-PCR analysis of the expression of *ANAC* genes clustered with *RD26* in (D). *UBQ10* levels were used as loading controls. Besides *ANAC081*, all tested *ANAC* genes responded similarly to *RD26;* i.e., they were upregulated in response to ABA, and downregulated after exposure to *epi*-BL.

seven encoded *ABF* transcription factors (Supplementary Table S1). qRT-PCR analysis showed that *RD26* and *ANAC102* transcription was induced and repressed by ABA and *epi*-BL, respectively (Fig. 2C). *RD26*, *ANAC102*, and *ANAC092* encode *NAC* transcription factors, which contain a *NAC* domain (Fig. 2D). Moreover, *RD26* and *ANAC102* are closely clustered in the phylogenetic tree, belonging to the *ATAF* subfamily of *NAC* domain proteins (Fujita et al., 2004). We extended our analysis to other genes in the *ATAF* subfamily, and found that these genes are also regulated by BRs (Fig. 2E). We measured the transcript level of each gene in response to a 3-h treatment with ABA or BRs, and found that all the genes except for *ANAC081* were downregulated by BRs (Fig. 2E). Although *ANAC081* did not respond to *epi*-BL, it was reported to be upregulated by NaCl and drought stress (Fujita et al., 2004). Previously, chromatin-immunoprecipitation microarray (ChIP-Chip) experiments revealed that *ANAC102*, *ANAC032*, *ANAC019*, *ANAC081*, *ANAC055*, and *RD26* are the direct targets of BZR1 (Sun et al., 2010), suggesting that BZR1 directly regulates the expression of these genes. Considering that this subfamily of *NACs* is regulated by various environmental stimuli in addition to ABA, it is likely that BRs regulate this group of genes to balance growth and stress pathways.

Overexpression of *RD26* results in a weak BR-deficient mutant phenotype

Similar to BR mutants, *35Spro:RD26* overexpression lines were previously reported to have round leaf blades and short petioles (Fujita et al., 2004). We thus were given the DNA of *35Spro:RD26* and generated the transgenic lines to re-examine the responses after hormone and stress treatments. We confirmed that *RD26* overexpression lines exhibited a semi-dwarf

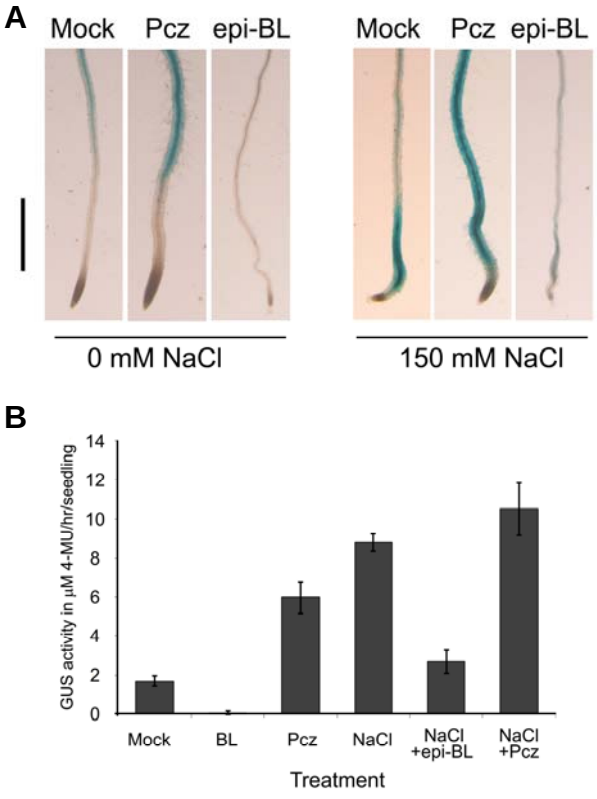

Fig. 3. BR-mediated regulation of *RD26*. (A) GUS histochemical staining pattern of *RD26pro:GUS* seedlings. Four-day-old *RD26pro:GUS* seedlings were transferred to media containing *epi*-BL or Pcz and further incubated for two days (left panel), or transferred to media containing 150 mM NaCl plus the indicated chemicals and incubated for two more days. Scale bar = 0.5 mm. (B) Quantitative assay of GUS activity using the bottom 5 mm of tissue from root tips. Error bars represent ± SE (N > 12).

phenotype, with a reduction in leaf size and inflorescence height (Supplementary Fig. S1A). The phenotype was more pronounced in lines with high levels of *35Spro:RD26* expression, suggesting that these phenotypes are attributable to *RD26* (Supplementary Figs. S1B and S1C). A contrasting phenotype was observed when *RD26* activity was suppressed by an inhibitory SRDX epitope fused to the carboxy-terminus of RD26. Such plants had elongated petioles and downward-curled leaves, which are characteristics of constitutive BR mutants (Chung et al., 2012; Fujita et al., 2004; Kim et al., 2013). These findings suggest that the genetic pathways controlling the stress response and growth are tightly coupled by the actions of BRs and ABA.

Interestingly, previous reports showed that overexpression of other transcription factors involved in the response to abiotic stresses, including *ABF3* and *ABF4*, also resulted in a retarded growth phenotype (Kang et al., 2002). Furthermore, overexpression of the *CBF3* transcription factor, which is essential for cold stress tolerance, resulted in a semi-dwarf phenotype (Sakuma et al., 2006). Taken together, it is likely that stress tolerance mechanisms, which protect the plant from abiotic stresses, limit energy-requiring BR-dependent growth.

Transcriptional induction of *RD26* in salt stress conditions is inhibited by BRs and stimulated by propiconazole treatment

It was reported that BR mutants are hypersensitive to ABA. Moreover, BR-overproducing and constitutive BR signaling mutants tend to be less tolerant to stresses (Chung et al., 2012; Kim et al., 2013), suggesting that BR negatively regulates stress responses. Since *RD26* responds not only to ABA but also to ABA-dependent water stresses (Fujita et al., 2004), we chose *RD26* as a marker gene that is antagonistically regulated by BR and ABA during ABA-dependent stresses. To investigate if *RD26* transcription is inhibited by BRs when the plant is subjected to stress, we examined *RD26* expression in a transgenic Arabidopsis plant expressing *RD26pro:GUS*. In general, *GUS* staining was confined to the roots. In mock conditions, the 6-day-old seedlings displayed weak GUS activity in the root region above the elongation zone (Fig. 3A). However, when seedlings were treated with the BR biosynthetic inhibitor propiconazole (Pcz) (Hartwig et al., 2012) for 3 days, GUS expression became markedly stronger. In contrast, GUS expression was barely detectable in the slender roots of seedlings treated with *epi*-BL (Fig. 3A).

Previously, it was reported that *RD26pro:GUS* expression was rapidly induced in all tissues by salt stresses (Fujita et al., 2004). When 6-day-old *RD26pro:GUS* seedlings were incubated in 150 mM NaCl for 2 days, GUS staining was strongly detected, especially in the root tip region (Fig. 3A). To determine the effects of Pcz and BL, seedlings were pre-treated with Pcz or BL for 2 days before being transferred to NaCl media supplemented with Pcz and *epi*-BL, respectively. Pretreatment with Pcz synergistically induced the expression of *RD26pro:GUS* in transgenic seedlings subjected to 150 mM NaCl, indicating that Pcz accelerates the induction of *RD26* expression in response to stress. However, *epi*-BL pre-treated samples displayed little staining in the roots, with only marginal induction by NaCl.

To quantify the expression of *RD26pro:GUS*, root tips (~5 mm) were excised and GUS activity was measured. Consistently, GUS activity was noticeably increased in samples treated with Pcz, whereas almost no signal was detected in *epi*-BL-treated samples (Fig. 3B). Moreover, treatment with 200 mM NaCl caused physical damage to plants than treatment with 150 mM NaCl, as tissues disintegrated when the osmotic balance was disrupted. However, the phenotype of roots exposed to 200 mM NaCl alone for 2 days was different from that of samples pre-treated with *epi*-BL or Pcz and then exposed to 200 mM NaCl for 2 days (Supplementary Fig. S2). The roots of Pcz-treated seedlings were harder and thicker than those treated with *epi*-BL, which were weak and fragile. Taken together, BRs repress *RD26* expression, both in ambient and stress conditions, while Pcz enhances *RD26* expression.

BIN2 increases salt stress tolerance

We have demonstrated that BRs repress the expression of the ABA-responsive genes to ameliorate ABA-dependent stress tolerance. To test which component in the BR signaling pathway is the focal point of ABA and BR crosstalk in the salt stress tolerance mechanism, we first examined the salt stress tolerance of wild-type seedlings treated with the BIN2 inhibitors, Lithium (Klein and Melton, 1996; Stambolic et al., 1996) and bikinin (De Rybel et al., 2009). Lithium inhibits BIN2 activity by competing with Mg^{2+} (Klein and Melton, 1996; Stambolic et al., 1996). In presence of Lithium (LiCl), the effects of NaCl was greatly enhanced such that seedlings turned yellow and died (Fig. 4A). More quantitatively, the survival rate of seedlings treated with 3 mM LiCl was 42% that of control seedlings treat-

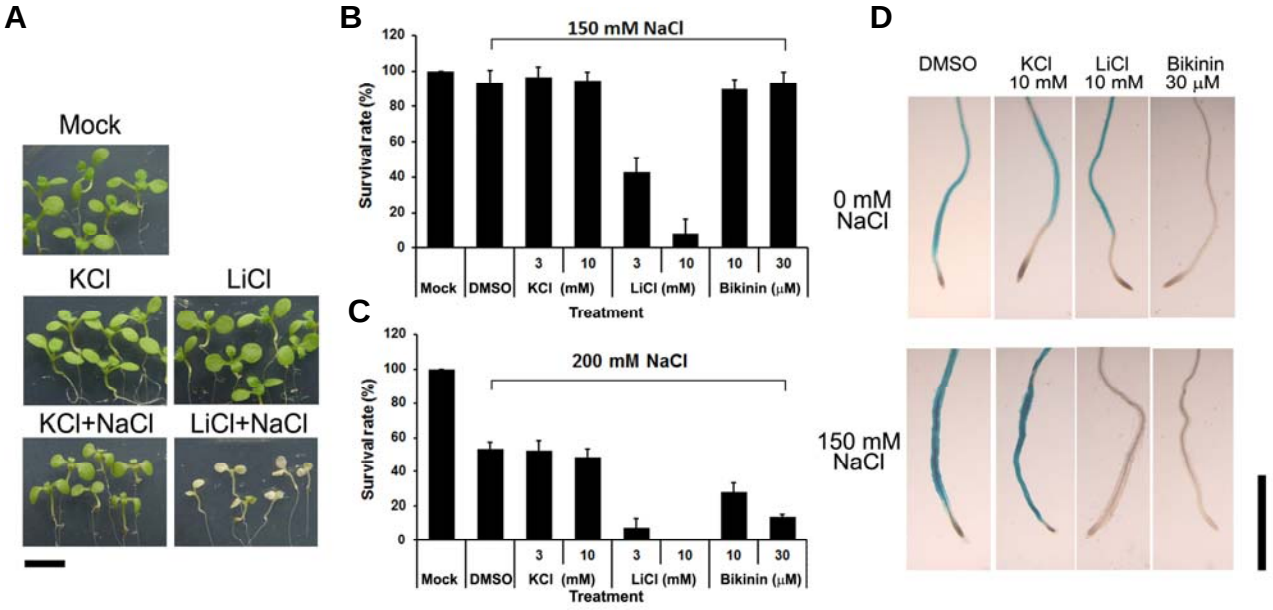

Fig. 4. Reduction in salt stress tolerance upon inhibition of BIN2. (A) Morphology of seedlings treated with mock or various salts. Seedlings were treated separately and arranged in a new plate to obtain a collective image of responses. First row, mock-treated control. Second row, treatment with KCl or LiCl alone. Third row, NaCl treatment in combination with KCl or LiCl. Scale bar = 5 mm. (B, C) Survival rate of Arabidopsis seedlings grown in 150 mM (B) or 200 mM NaCl media (C) supplemented with salts or bikinin (a BIN2 inhibitor). Survival rate was measured as the ratio of the number of green vs. pale yellow seedlings. Error bar = SD (n = 3). (D) GUS staining pattern in the roots of *RD26pro:GUS* seedlings. Scale bar = 1 mm.

ed with 3 mM KCl (Figs. 4A and 4B). We obtained the similar results when repeating this experiment using bikinin. Induction of GUS activity in the *RD26pro:GUS* line by 150 mM NaCl was also inhibited by LiCl and bikinin (Fig. 4D and Supplementary Fig. S3), implying that the low survival rate of plants treated with salt and LiCl was mainly due to the inhibition of ABA-response genes by BRs, especially by the pathway regulated by the BIN2 kinase. Taken together, ABA suppresses the BR pathway, which acts via BIN2, to impart salt stress tolerance.

Conserved motifs in the co-regulated genes

To further understand how the genes are co-regulated by BRs and ABA, we compared the promoter sequences of the gene identified and displayed in Fig. 2. Firstly, we found that the motif sequence conserved among the ABA-upregulated and BR-downregulated genes was clearly an ABRE (motif 1, Fig. 5A). Motif 1 is similar to the BRRE; therefore, it is tempting to suggest that BZR1 and ABF/AREB transcription factors may competitively regulate their target genes by binding to the same response elements.

Secondly, in the case of the BR-up and ABA-downregulated genes, a GAGA motif was enriched (Fig. 5B). Previously, it was reported that BASIC PENTACYSTEINE (BPC) proteins bind to this GAGA motif (Meister et al., 2004; Monfared et al., 2011; Sing et al., 2009). It is likely that BPC may function in the antagonistic regulation of ABA and BR. However, we cannot rule out the possibility that conserved sequences can be bound by currently unidentified transcription factors (Rozhon et al., 2010; Yan et al., 2009).

DISCUSSION

BR-deficient mutants are known to be hypersensitive to ABA (Steber and McCourt, 2001; Zhang et al., 2009), suggesting that ABA can efficiently induce ABA responses when growth is minimized. Therefore, we identified the genes antagonistically regulated by ABA and BRs, including *RD26* and the *ANAC* genes, which were previously shown to function in various stress responses (Christianson et al., 2010). In addition, we provided evidence that BIN2 is necessary to maintain the balance between ABA and BR signaling under salt stress conditions.

Growth inhibition mechanism under stress conditions

Since it is not fully understood how ABA inhibits growth, we studied the mechanisms controlled by ABA and the growth-promoting BRs. Plants grown in medium supplemented with ABA or NaCl displayed a growth retardation phenotype similar to that of BR-deficient mutants (Fig. 1). One explanation for this is that BR downregulates the genes that may not be directly required for stress-tolerance processes. In support of this, the expression of *RD26* and other genes encoding stress-related *NAC* transcription factors was reduced by treatment with *epi*-BL. Likewise, *elongated-D (elg-D)*, a gain-of-function allele of *BAK1* with an enhanced growth phenotype, exhibited reduced photosynthetic efficiency upon salt stress, possibly due to the reduced expression of genes involved in stress responses (Chung et al., 2012). Conversely, a recent microarray analysis revealed that many of the cold stress-responsive genes, including *WRKY* and *CBFs*, are upregulated in *bri1-9*, suggesting that BR signaling antagonistically regulates stress-response genes (Kim et al., 2010).

Salt-treated roots tend to break easily and this effect is severe when BR is added. In contrast, when Pcz is added, roots remain thick and relatively intact compared to those subjected to salt treatment alone (Supplementary Fig. S2). In Arabidopsis,

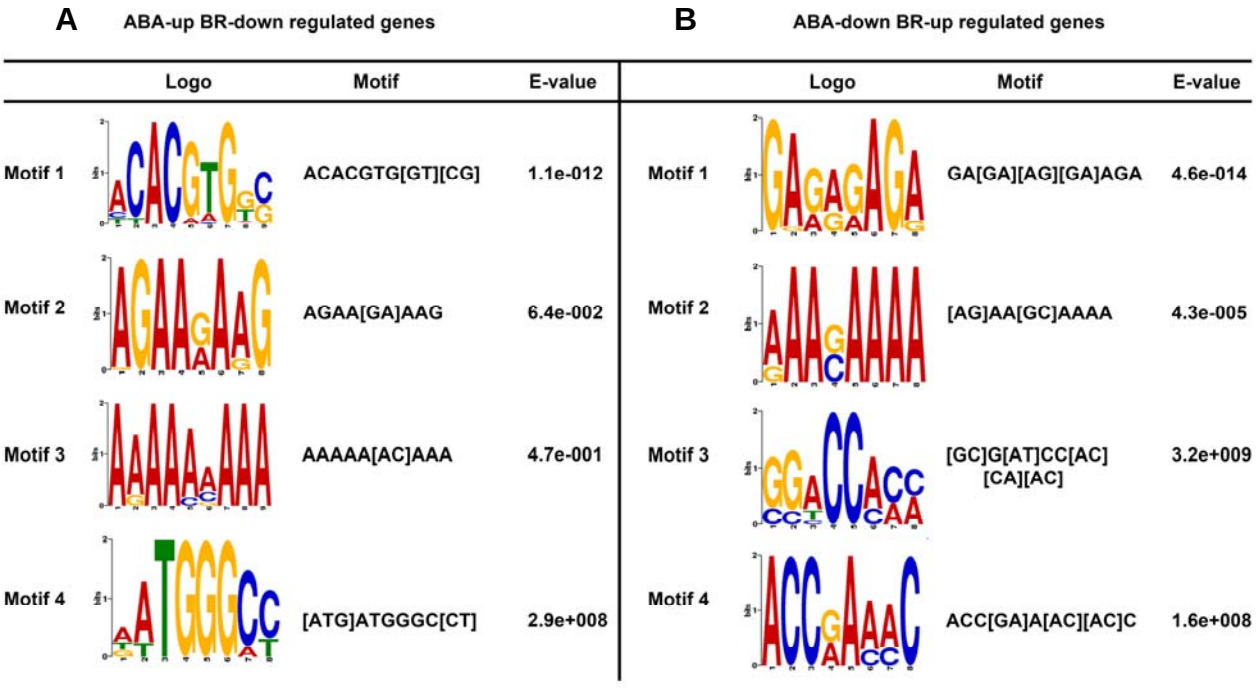

Fig. 5. Consensus sequence elements in the promoter of genes co-regulated by both ABA and BRs. The top fourmost over-represented motifs found in the -1000 bp proximal promoter of genes are shown. Genes that are (A) upregulated by ABA and downregulated by BR and (B) downregulated by ABA and upregulated by BR. Sequence logos for (A) and (B) were generated using the MEME analysis tool. The total information content of each logo is given in bits; the height of each nucleotide in the logo represents the positional probability of that nucleotide multiplied by the information content of the logo. E-value means the possibility of finding the motifs merely by chance.

cell wall loosening is temporarily required for BR-mediated cell expansion, and this is catalyzed by *XYLOGLUCAN ENDOHYDROLASES* (*XEH*) (Fanutti et al., 1993). Previously, it was demonstrated that the salt-responsive transcription factor *OsMPS*, which downregulates the expression of cell-wall biosynthetic genes and *EXPANSIN* (*EXP*) genes, is repressed by growth hormones such as BRs and auxin, suggesting that *OsMPS* functions in the crosstalk that balances adaptive growth with survival under salt stress (Schmidt et al., 2013). Pcz treatment might have corrected the altered root shape in plants exposed to salt stress by effective disruption of the BR signaling (Supplementary Fig. S2).

It was interesting to observe that *RD26* overexpression resulted in semi-dwarfism similar to that observed in BR mutants (Supplementary Fig. S1). This morphological similarity suggests that *RD26* antagonistically regulates the expression of growth-promoting genes regulated by BRs. Indeed, transgenic lines overexpressing *RD26* or *ABF3* and *ABF4* consistently displayed a semi-dwarf phenotype, but enhanced drought tolerance (Kang et al., 2002). Taken together, stress-responsive transcription factors appear to limit energy expenditure on growth to enhance stress tolerance, possibly by downregulating BR-responsive genes.

BR and ABA signaling pathways converge at BIN2

Seedlings in which BIN2 activity was inhibited by either LiCl or bikinin displayed a decreased survival rate when subjected to a salt stress of 200 mM NaCl (Fig. 4). GUS activity was absent in *RD26pro:GUS* plants treated with LiCl and bikinin. These results suggest that BIN2 controls the expression of genes involved in salt stress tolerance, including *RD26*. BIN2 is a protein kinase that phosphorylates BR-specific transcription factors, such as BZR1 and BES1. It is possible that BIN2 regulates stress-associated genes, especially those that are upregulated by ABA and downregulated by BR, through controlling the activities of BZR1 and BES1. Additionally, overexpression of *AtGSK1*, the closest homolog of *BIN2*, results in enhanced salt stress tolerance in Arabidopsis, suggesting that BIN2 may function in the salt stress response (Piao et al., 2001). Based on these findings, we propose a model that illustrates the interaction between BRs and ABA in controlling growth and stress tolerance (Supplementary Fig. S4).

We predict that salt stress controls a step below BIN2 in the BR signaling pathway, because NaCl treatment in combination with BIN2 inhibition altered the expression of *RD26* and rendered the seedlings less tolerant than those treated with salt alone. Future research should examine how NaCl signals modify the activity of BIN2. The underlying mechanism may function directly or indirectly through another signaling component that has yet to be discovered.

In conclusion, we sought to determine why stressed plants display stunted growth. We analyzed the relationship between BRs, which promote growth, and ABA, which is involved in the response to environmental stress. Based on the phenotypic similarities between ABA-treated seedlings and *bri1-9*, we searched for and identified genes that are oppositely regulated by ABA and BRs in previously reported microarray data sets. Of these genes, *RD26,* which belongs to the *ANAC* family, was chosen as representative and shown to be specifically induced by ABA, but suppressed by BRs. We provided evidence that BIN2 plays a key role in balancing the growth and the stress responses during periods of stress. Thus, our results suggest

that ABA and BRs may regulate a set of target genes to fine-tune Arabidopsis growth during the stress response.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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