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Molecules and Cells

Overexpression of the Ethylene-Responsive Factor Gene *BrERF4* from *Brassica rapa* Increases Tolerance to Salt and Drought in Arabidopsis Plants

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Ethylene-responsive factors (ERFs), within a subgroup of the AP2/ERF transcription factor family, are involved in diverse plant reactions to biotic or abiotic stresses. Here, we report that overexpression of an ERF gene from *Brassica rapa* ssp. *pekinensis* (*BrERF4*) led to improved tolerance to salt and drought stresses in Arabidopsis. It also significantly affected the growth and development of transgenic plants. We detected that salt-induced expressions of a transcriptional repressor gene, *AtERF4*, and some Ser/Thr protein phosphatase2C genes, *ABI1*, *ABI2* and *AtPP2CA*, were suppressed in *BrERF4*-overexpressing Arabidopsis plants. Furthermore, *BrERF4* was induced by treatment with ethylene or methyljasmonate, but not by abscisic acid or NaCl in *B. rapa*. These results suggest that BrERF4 is activated through a network of different signaling pathways in response to salinity and drought.

INTRODUCTION

Transcription factors play critical roles in changing gene expression in response to environmental stress stimuli, such as salinity, drought and pathogens, or by components of stress-activated signaling pathways, such as jasmonic acid (JA), abscisic acid (ABA), and ethylene (ET) (Ohta et al., 2001; Tran et al., 2007; Yamaguchi-Shinozaki and Shinozaki, 2006). The Arabidopsis genome sequence has revealed 124 genes that encode putative ethylene-responsive factor (ERF) proteins containing an APETALA2 (AP2)/ERF DNA-binding domain of about 60 amino acids (Riechmann et al., 2000). ERF proteins, members of the AP2/ERF family, were first identified in tobacco as proteins that bind to the GCC box, the core sequence of an ethylene-responsive element (Ohme-Takagi and Shinshi, 1995). These proteins are divided into two major subfamilies, the drought-responsive element binding factor (DREB) subfamily (A group) and the ERF subfamily (B group). Each subfamily is further classified into six subgroups, A1 to A6 and B1 to B6, based on conserved domains (Sakuma et al., 2002).

ERF subfamily proteins modulate a variety of stress responses and developmental processes (Alonso and Stepanova, 2004; Yamaguchi-Shinozaki and Shinozaki, 2006). Most identified so far have been shown to function as transcription activators, such as Arabidopsis AtERF1/2/5, tobacco ERF2/4 and Tsi1, and tomato Pti4, in response to biotic and abiotic stresses (Fujimoto et al., 2000; McGrath et al., 2005; Ohta et al., 2000; Park et al., 2001; Zhou et al., 1997). In contrast, some ERF members can act as repressors of transcription including Arabidopsis AtERF3/4 and AtERF7-12, and tobacco ERF3 (Fujimoto et al., 2000; McGrath et al., 2005; Ohta et al., 2001; Song et al., 2005; Yang et al., 2005). These repressors decrease the expression levels of a GCC box-containing reporter gene. The ERF-associated amphiphilic repression (EAR) motif, containing the conserved sequence $^{L}/_{F}DLN^{L}/_{F}(x)P$, is essential for transcriptional repression. Ohta et al. (2001) have shown that mutations within that conserved motif result in abolishment of the repression capacity. Furthermore, the EAR-motif-containing Cys2/His2-type zinc-finger protein Zat7 is directly involved in salt tolerance; a mutation in its EAR motif leads to elimination of salt tolerance (Ciftci-Yilmaz et al., 2007).

EARs play important roles in controlling plant responses to stress. AtERF4 negatively regulates disease resistance and JA-dependent expression of *PDF1.2* (McGrath et al., 2005). In addition, *AtERF4*-overexpressing transgenic plants demonstrate that the protein negatively mediates in response to salt stress, down-regulating expression of ABA-dependent responsive genes in Arabidopsis (Yang et al., 2005). Overexpression of *GmDREB2* or *GmERF4*, which encode the EAR-motif-containing transcription factor from soybean, enhances tolerance to salt and drought by transgenic plants (Chen et al., 2007; Zhang et al., 2010).

Transcriptional alteration of expression of stress-responsive genes is a fundamental aspect when generating transgenic plants that overexpress the transcriptional regulator gene that contributes to stress responses (Lee et al., 2008). In this study, we isolated and characterized a regulatory gene *BrERF4* from Chinese cabbage (*Brassica rapa* ssp. *pekinensis*), an eco-

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nomically important vegetable crop. Overexpression of *BrERF4* led to salt and drought tolerance in transgenic Arabidopsis, which may partially be due to suppression of a transcriptional repressor gene, *AtERF4*, and some Ser/Thr protein phosphatase2C genes, *ABI1*, *ABI2*, and *AtPP2CA*.

MATERIALS AND METHODS

Construction and plant transformation

All Arabidopsis plant materials were in the Columbia background. The cDNA clone KFYP-085F08 (GenBank Accession No. EX112088) from *Brassica* cDNA libraries (http://www.brassica-rapa.org/) using the Lambda-UniZAP XR vector (Stratagene) was amplified to construct *BrERF4*-overexpressing plants. The primer set containing the Gateway partial recombination sequences of attB1 or attB2 was 5′-AAAAAGCAGGC TGACTCACTATAGGGAATTCAC-3′ and 5′-AGAAAGCTGGG TCCTCTAGATGCATGCTCGCGAG-3′. Amplified fragments were re-amplified with the adaptor primers, attB1 (5′-ACAAGTTTG TACAAAAAAGCAGGCT-3′) and attB2 (5′-ACCACTTTGTACA AGAAAGCTGGGT-3′), and the resulting products were inserted into the entry vector pDONR221 by BP recombination according to the manufacturer's instructions (Invitrogen, USA). The recombinant plasmid was used in the LR reaction with destination vector pB2GW7, which contains the *CaMV 35S* promoter (Karimi et al., 2002). This recombinant binary plasmid was introduced into Arabidopsis plants by *Agrobacterium tumefaciens*-mediated transformation (Clough and Bent, 1998). *BrERF4*- overexpressing transgenic plants were placed in soil and verified by spraying them with two applications of 0.1% Basta (Bayer, Korea).

Northern blot analysis

Total RNAs were isolated as described by Kroczek and Siebert (1990). Briefly, 8 μg was separated by electrophoresis on a 1% agarose gel and hybridized as previously reported (Song, 2006). To make gene-specific DNA probes labeled with [α-^{32}P]dATP, PCR amplification was performed using the following primer sets: *BrERF4*, 5′-TGAACTTATCGAAGGTGGTC-3′, 5′-CTCTCCCGATTGAAGTTTTTTTC-3′; *AtERF4*, 5′-TGAGCTT TTAACGGTGTCGT-3′, 5′-TCTCTCTCAGATTTAATCTGTT-3′; *RD22*, 5′-AGTACCAACGTCCAAGTAGG-3′, 5′-GACGTCGCA CAATACTTCTC-3′; *ABI1*, 5′-CCGAGAACGGAGATCTAATG-3′, 5′-AATACTCAGCCGCGGACATC-3′; *ABI2*, 5′-TTCTCCTG CAGTCGCTGTTC-3′, 5′-AATACTCTGCCGCGGACATT-3′; and *AtPP2CA*, 5′-TTGTTGCGGTGTTGTTGGAG-3′, 5′-GTTAAG AGCAACGCCGCATC-3′. Amplification of the RD29A gene was performed with universal primers #1201 and #1212, using EST clone LTI78 as template.

Stress tolerance analyses

For assessing their drought tolerance, wild-type and *BrERF4*-overexpressing transgenic plants were initially grown in soil with normal watering for 2 weeks. Irrigation was withheld for 11 days. To determine the amount of water lost, the aerial portions of 2-week-old plants were separated from their roots, placed on weighing dishes, and air-dried for 5 h on a laboratory bench. To evaluate salt tolerance, 20-day-old plants in soil were watered for 2 h, then exposed to 300 mM NaCl for 7 days. To test the effect of excess salt in growing media, aseptic seeds were placed on a standard MS medium (without NaCl) for 10 days, then transferred to an MS medium containing 300 mM NaCl.

Chemical treatments

Three-week-old, soil-grown *B. rapa* plants were placed in a growth chamber at 25°C, 14-h photoperiod, and 50% relative humidity. They were then sprayed with 50 μM methyljasmonate (MeJA) or ethephon. For ABA or NaCl application, the same type of plants was transferred to soil treated with solutions of 50 μM ABA or 300 mM NaCl. Bacterial culturing and syringe inoculations were performed as previously described (Greenberg et al., 2000). While being infected with the virulent bacterial pathogen *P. syringae* pv. *maculicola* (strain ES4326 DG3), plants were covered with a plastic dome to increase the relative humidity.

Analysis of flowering time

Wild-type and *BrERF4*-overexpressing transgenic plants (> 15 per line) were placed in a growth room at 23°C, under long-day conditions (14 h/10 h day/night). Two indexes were used to describe flowering time: number of days from sowing to bolting, and number of rosette leaves present when inflorescences appeared.

RESULTS

Overexpression of *BrERF4* enhances salt and drought tolerances in Arabidopsis

We performed a functional analysis of randomly selected expressed sequence tag (EST) clones from *Brassica rapa*, which is part of the *Brassica* genome project (http://www.brassica-rapa.org/). Our main focus was on putative transcriptional regulators and their potential use in developing crops tolerant to salt stress. Here, transgenic Arabidopsis plants were generated in which expression of those EST clones was under the control of the *CaMV 35S* promoter. Dozens of each primary transformant, growing in soil, were treated with 300 mM NaCl. Overexpression of KFYP-085F08 (GenBank Accession No. EX112088) resulted in a phenotype with salt tolerance (Fig. 1A). Most plants were still green at 7 days after treatment while others had wilted or died, such as was seen with the wild type (WT). This suggested that the clone is expressed at different intensities within transgenic plants. From the transformants, 15 independent lines were chosen for northern blot analysis. Most lines accumulated high levels of the gene transcripts while some did only very weakly, as was expected (Fig. 1B). Lines 4 and 8 (BrERF4-4 and -8) were selected for further study.

Subsequent DNA sequencing of the plasmid insert revealed that the gene encodes a putative ERF transcription factor closely related to the AtERF proteins. The clone protein shares the highest amino acid sequence homology (83% identity and 89% similarity) with AtERF4 (Fig. 2). We named this gene *Brassica rapa ERF4* (*BrERF4*). Its protein, a homolog of the AtERF family, appears to be associated with an environmental stress response. *BrERF4* has an open reading frame of 636 bp and encodes a polypeptide of 211 amino acids, with a predicted molecular mass of 22.7 kDa.

This involvement in the salt response was further examined by monitoring whether *BrERF4*-overexpressing plants had salt tolerance when grown in soil or on an MS medium. Under such stress, WT plants in soil showed more yellowing and began to die after 7 days of exposure to 300 mM NaCl, in contrast to the transgenics (Fig. 1C). Likewise, after being transferred from a salt-free medium to one containing 300 mM NaCl, most WT plants turned yellow after 4 days while most transgenics still showed green leaves (Fig. 1D).

Transgenic plants also demonstrated enhanced tolerance to drought stress (Fig. 1E). This was evidenced by their rate of water loss during aerial exposure, as measured by the decline in fresh weights when aboveground tissues were detached

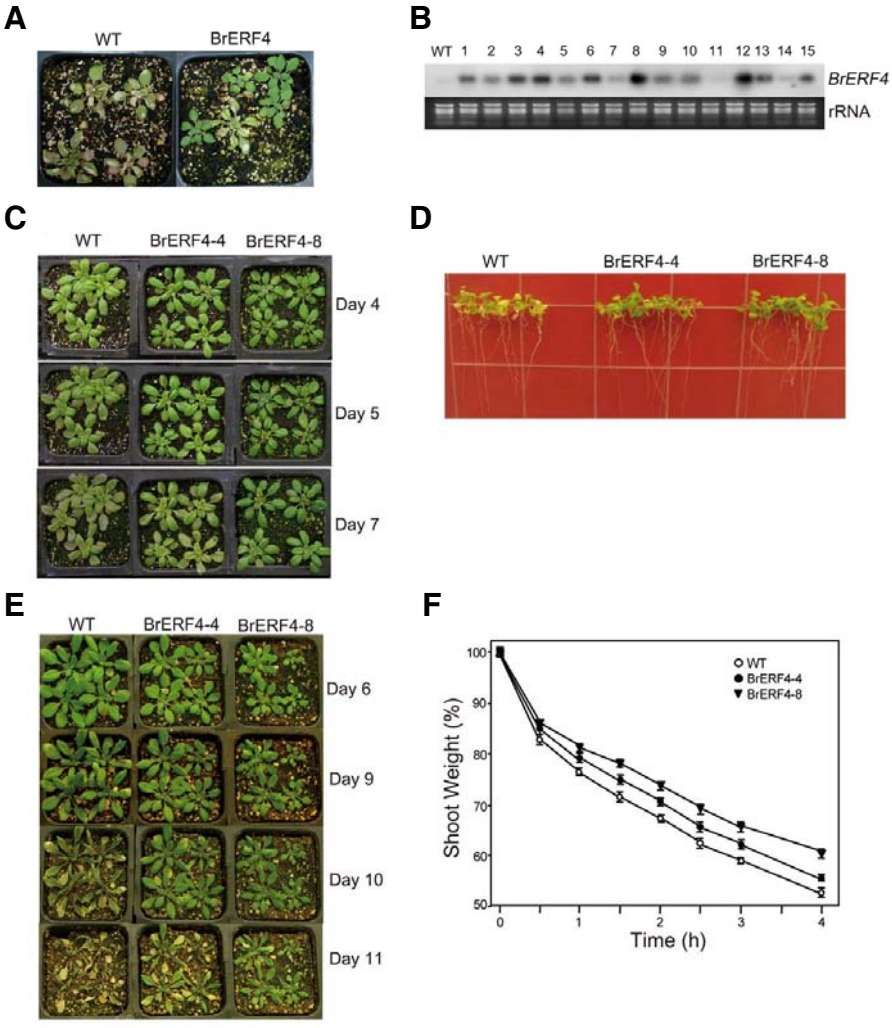

Fig. 1. Response to salt and drought stresses of *BrERF4*-overex-pressing transgenic plants. (A) Conference of salt tolerance. WT and transgenic plants overexpressing *BrERF4* were treated with 300 mM NaCl at 15 days after sowing. Photograph was taken at 7 days after application. (B) *BrERF4*-over expressing transgenic plants. Total RNAs from 3-week-old leaves of untrans-formed control (WT) and 15 independ-ent transgenic lines containing *BrERF4* under control of *CaMV 35S* promoter were extracted and hybridized using *BrERF4*-specific DNA probe. Equal loading of each lane was confirmed by pre-staining gel with ethidium bromide. (C) Response to salt stress. Plants of WT and overexpressing lines BrERF4-4 and BrERF4-8 growing in soil were treated with 300 mM NaCl at 20 days after sowing. Photographs were taken at 4, 5, and 7 days after application. (D) Response to excess salt in MS me-dium. Plants grown for 10 days on salt-free medium were transferred to me-dium containing 300 mM NaCl. Photo-graph was taken 4 days later. (E) Re-sponse to drought stress. WT and transgenic plants were grown in soil with normal irrigation for 2 weeks; water was then withheld for 11 days. (F) Tran-spiration rates. To measure water loss, aerial portions of 2-week-old plants were separated from roots, placed on dishes, and air-dried for 5 h to compare weights before and after.

from the roots (Fig. 1F). For example, after 3 h of dehydration, weights of BrERF4-4 and BrERF4-8 plants were reduced to 65% and 70%, respectively, of their initial values compared with only 60% retention by the WT. We also tested whether the transgenic plants had disease resistance. This hypothesis was based on reports that some ERF proteins function in pathogen resistance and modulate the expression of ET/JA/salicylic acid-dependent genes (Fujimoto et al., 2000; McGrath et al., 2005; Ohme-Takagi and Shinshi, 1995; Yamaguchi-Shinozaki and Shinozaki, 2006). However, our *BrERF4* plants did not manifest any significantly increased resistance to *Pseudomonas syrin-gae* (data not shown). These data indicate that BrERF4 mainly plays an important role in responses to abiotic stresses, such as salt and drought.

Phylogenetic analysis of BrERFs and AtERFs

Multiple alignments showed that the BrERF4 protein has sig-nificant homology with tobacco NtERF3, soybean GmERF4, and Arabidopsis AtERFs, and contains a central 58 amino acid AP2/ERF domain and a conserved EAR motif in the C-terminal region (Fig. 2A). Inspection of its amino acid sequence revealed two putative nuclear localization signal sequences (R24KRP and P38GKKTRV) in the AP2/ERF domain near the N-terminal. Zhuang et al. (2010) have surveyed the *B. rapa* EST database based on the conserved AP2/ERF domain amino acid sequence of Arabidopsis, finding 62 putative AP2/ERF family genes, in-cluding *BrERF4*. Of those 62 genes, 33 are classified into the ERF subfamily, with 10 belonging to subgroup B1. Our phyloge-netic analysis also indicated that BrERF4 is in that subgroup, and is closest to AtERF4 among all Arabidopsis AtERF proteins (Fig. 2B).

Expression patterns of stress-responsive genes in *BrERF4*-overexpressing plants

Northern blot analysis showed that, at the transcriptional level, expressions of the well-known ABA-dependent abiotic stress-inducible genes *RD29A* and *RD22* in transgenic plants were not altered by treatment with 300 mM NaCl (Fig. 3A). Instead, transcript accumulations of the Ser/Thr protein phosphatase2C (PP2C) genes were reduced compared with the WT. Such genes, as well as *ABI1*, *ABI2*, and *AtPP2CA*, have been de-scribed as negative regulators of the ABA signaling pathway (Gosti et al., 1999; Kuhn et al., 2006; Merlot et al., 2001; Yo-shida et al., 2006). This suggests, therefore, that salt tolerance by *BrERF4*-overex-pressing plants is likely conferred by an alternative ABA signal transduction pathway that is mediated by those Ser/Thr PP2C proteins.

Studies of transcription factors have revealed roles for ERF proteins during hormonal stress responses (Singh et al., 2002). Therefore, we investigated the transcript levels of *BrERF4* in 3-

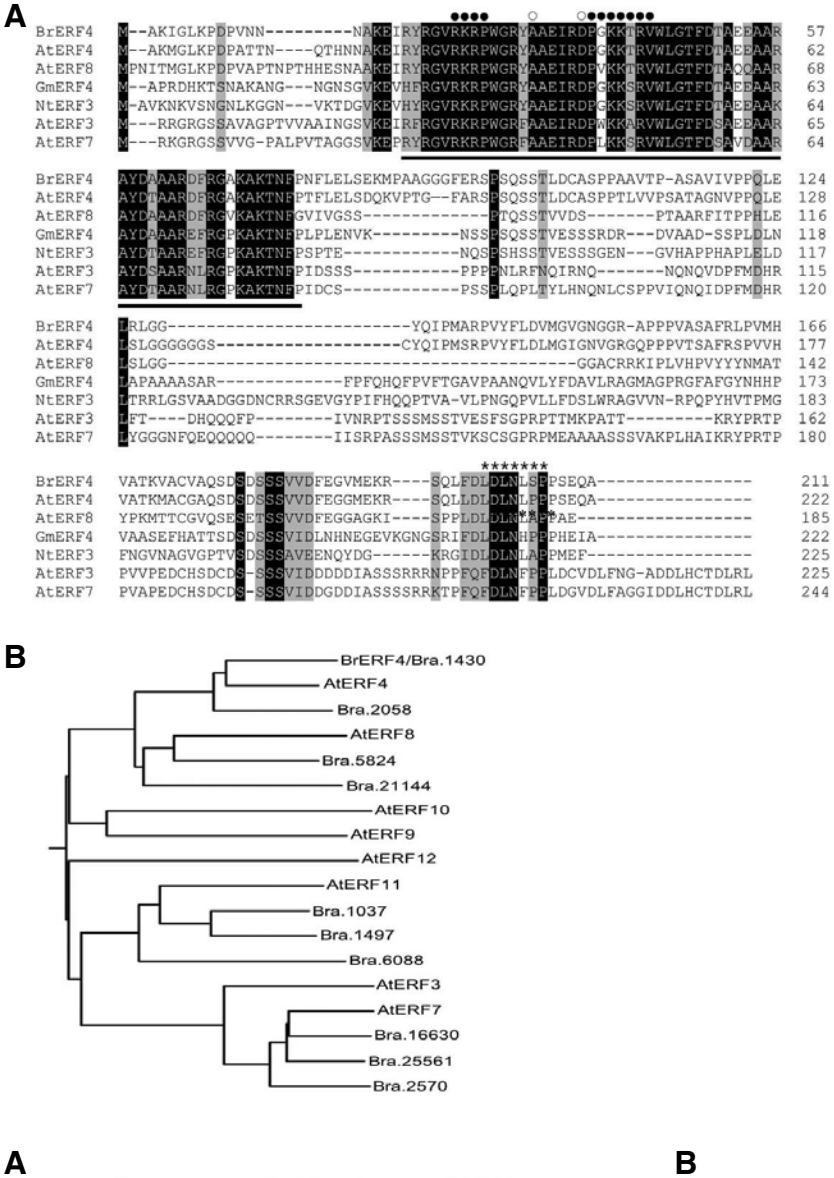

Fig. 2. Analysis of BrERF4 protein and phylogenetic relationship in gene family. (A) Comparison of amino acid sequences for BrERF4 and close homologues Arabidopsis AtERF3/4/7/8, soybean GmERF4, and tobacco NtERF3. Amino acid residues identical or similar within sequences of all 7 proteins are shown in white/black or light gray, respectively. AP2/ERF domain is underlined and EAR motif in C-terminal is shown by asterisks. Nuclear localization signal sequences are indicated by closed circles. Two conserved amino acid residues in AP2/ERF domain (Ala and Asp) are marked by open circles. (B) Phylogenetic analysis of subgroup B1 proteins from *B. rapa* and Arabidopsis. Tree was constructed with ClustalW program (http://clustalw.genome.jp/). Sequences were retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/); GenBank Accession Numbers are: BrERF4, EX112088; Bra.1037, EX133485; Bra.1497, EX138604; Bra.6088, EX062823; Bra.2570, EX135489; Bra.16630, EX094284; Bra.25561, EX128697; Bra.2058, EX092683; Bra.5824, EX131213; Bra.21144, EX099934; AtERF3, AB008105; AtERF4, AB008106; AtERF7, AB032201; AtERF8, AB036884; AtERF9, AB047648; AtERF10, AB047649; AtERF11, AB055882; AtERF12, AB055883; GmERF4, EU681278; and NtERF3, D38124.

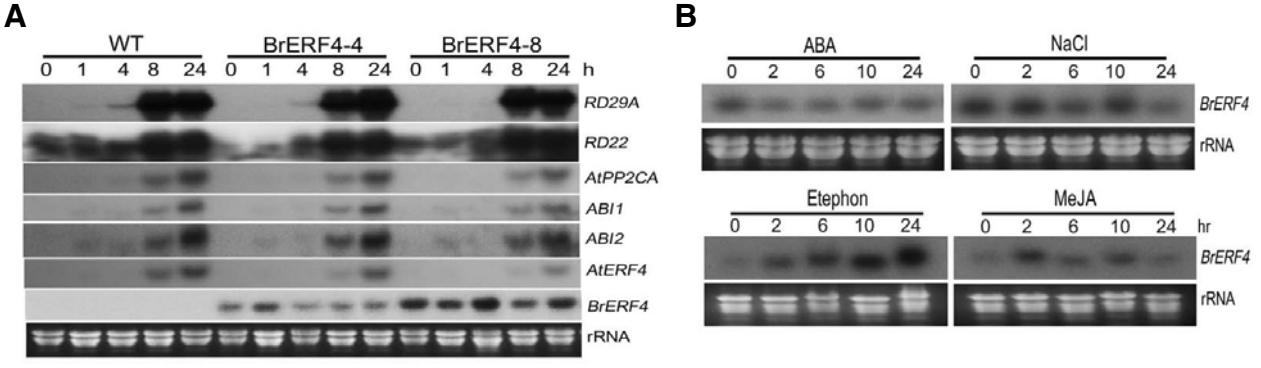

Fig. 3. Northern blot analysis of *BrERF4*. (A) mRNA expression profiles of salt-inducible genes. Three-week-old plants of WT and *BrERF4*-overexpressing transgenic plants were treated with 300 mM NaCl. Leaves were sampled at indicated time points for extracting total RNAs. (B) *BrERF4* expression induced in *B. rapa* by application of ethephon, MeJA, or ABA (50 μM each), or 300 mM NaCl. Total RNAs were analyzed from 3-week-old leaves.

week-old *B. rapa* seedlings under exogenous hormone treatments (Fig. 3B). Expression was significantly increased at 2 h after application of ethephon (an ethylene-releasing agent) or MeJA, but was not induced by ABA or NaCl.

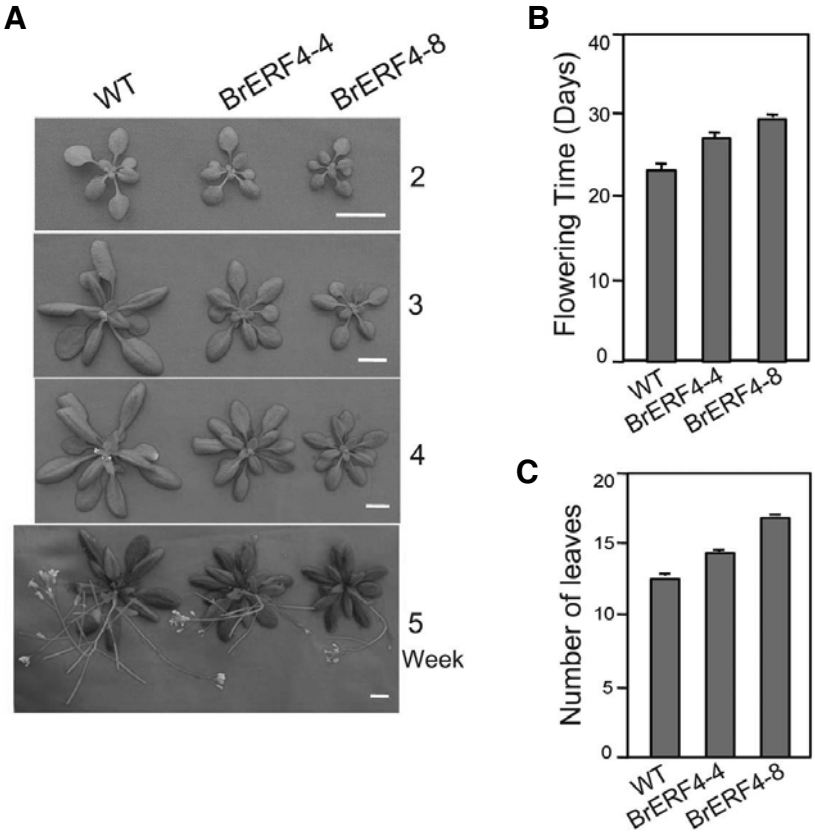

Fig. 4. Growth retardation of transgenic plants. (A) Growth of WT and *BrERF4*-overexpressing plants in soil. Scale bars indicate 1 cm. (B) Flowering times of WT and transgenic plants. (C) Number of rosette leaves when inflorescences appeared.

***BrERF4*-overexpressing plants display a phenotype of growth retardation**

Seeds of *BrERF4* plants germinated uniformly and were of similar size to those from the wild type. However, transgenic plants exhibited retarded vegetative growth that was correlated with the transcript level of the transgene (Fig. 4A). Flowering time also differed between the two genotypes (Fig. 4B). For example, under the same growth conditions, plants of BrERF4-4 and BrERF4-8 began to flower at 27 and 29 days, respectively, after sowing versus 23 days for the wild type. When the number of rosette leaves was used for assigning the time of flowering, transgenic plants had 2 to 4 more leaves than the WT (Fig. 4C). Thus, a delay in flowering also reflected a retardation in the growth and development of *BrERF4* plants.

DISCUSSION

We have now identified *BrERF4* by performing salt tolerance tests with transgenic plants that overexpress genes encoding putative transcription factors. These results enable researchers to develop crop plants with increased tolerance to salinity or drought. Our database analysis of protein sequences demonstrates that BrERF4 is an ERF protein, with an AP2/ERF domain in the N-terminal and an ERF motif in the C-terminal. This suggests that the BrERF4 protein functions like other ERF proteins through an interaction with the GCC box to regulate plant responses to various stresses (see also Alonso and Stepanova, 2004; Yamaguchi-Shinozaki and Shinozaki, 2006). The phylogenetic analysis showed that BrERF4 belongs to subgroup B1 of the AP2/ERF family and is homologous to *Arabidopsis* ERF proteins.

Northern blot analysis revealed that, under high salinity, transcript levels of major abiotic stress-responsive marker genes, such as *RD29A* and *RD22*, are similar between wild-type and transgenic plants, implying that salt tolerance in the latter is not conferred by the proteins encoding these genes. Instead, such enhanced tolerance in *BrERF4*-overexpressing plants may partially be due to suppression of the Ser/Thr PP2C genes, for which proteins negatively regulate the ABA signaling pathway (Gosti et al., 1999; Kuhn et al., 2006; Yoshida et al., 2006). This suggestion is supported by phenotypes of *AtMYB44*-overexpressing transgenic plants that have greater tolerance to salt and drought stresses (Jung et al., 2008). Compared with the WT, those plants exhibit decreased expression of the PP2C genes and transcript levels similar to those of *RD29A* and *RD22*. The role of AtMYB44 as a transcription factor in response to environmental stimuli could be feed-forward regulation of the ABA signaling pathway through repression of those PP2C genes, thereby indicating that *BrERF4*-overexpressing transgenic plants are likely to resemble *AtMYB44* plants in their salinity response.

It is also possible that salt tolerance in our *BrERF4* plants is improved through a reduction in *AtERF4* expression, which represses the *AtERF4*-mediated signaling pathway. This hypothesis is supported by the fact that AtERF4 is a transcriptional repressor that confers hypersensitivity to salt as well as the down-regulation of salt-responsive genes in its transgenic plants (Yang et al., 2005). Its involvement in disease resistance with *AtERF4*-overexpressing transgenic lines and *erf4* mutants also demonstrates that AtERF4 negatively regulates such resistance and the expression of defense genes (McGrath et al., 2005). These data imply that *BrERF4* overexpression could regulate suppression or inhibition of *AtERF4* expression to confer salt tolerance within *BrERF4* transgenic plants. Never-

theless, *BrERF4* overexpression here did not increase resistance to *Pseudomonas syringae*. Therefore, whether such transgenics have any resistance to a necrotrophic pathogen, such as *Fusarium oxysporum*, as has been tested with *AtERF4* transgenic plants, remains to be investigated (McGrath et al., 2005).

In plants, genes from the AP2/ERF family, including subgroup B1, are induced via exogenous application of various extracellular stimuli, including ET, ABA, JA, or NaCl (Ohta et al., 2001; Tran et al., 2007; Yamaguchi-Shinozaki and Shinozaki, 2006). We demonstrated that expression of *BrERF4* in *B. rapa* is responsive to ET and MeJA but not to ABA and NaCl. This suggests that *BrERF4* can be regulated at either the transcriptional or post-transcriptional level in response to certain stimuli. Accumulating evidence indicates that plant responses to biotic and abiotic stresses are regulated through a network of signaling pathways. Differential expression of AtERF genes in response to some stimuli, such as ABA, drought, and NaCl, shows that their proteins probably function by activating or repressing the expression of GCC box-containing stress-responsive genes, either dependent or independent of the ethylene signal (Fujimoto et al., 2000). Therefore, our observation is not unusual that ABA and NaCl did not induce *BrERF4* in *B. rapa*. From this we infer that the role of BrERF4 in responses to salt and drought by those plants could result from cross-talk between different pathways for activating multiple stress responses, and that various stress pathways can be linked by certain transcriptional regulators, e.g., Tsi1 acting in ethylene-signaling and salt stress-signaling pathways (Park et al., 2001). Expression of the *Tsi1* gene, encoding a tobacco ERF protein, also is induced by ET or JA, but not by ABA, and its overexpression leads to resistance toward pathogen attack and salt stress in tobacco. However, the BrERF4 protein might be regulated post-transcriptionally to activate the responses to environmental stimuli. Therefore, a particular stress factor, such as high salinity, or some component of the stress-signaling pathway, such ABA, might act by converting the BrERF4 protein to an active form that is capable of activating GCC box-mediated transcription.

Our study showed that overexpression of *BrERF4* resulted in growth retardation and delayed flowering. Similar phenomena have been observed with transgenic plants under the control of constitutive *CaMV 35S* promoters to over-express transcriptional regulator genes, including rice *OsERF1*, soybean *GmDREB3*, and *AtMYB44*, *DREB1A*, *DREB1B*, and *DREB1C* in Arabidopsis (Chen et al., 2009; Gilmour et al., 2004; Hu et al., 2008; Jung et al., 2008; Kasuga et al., 1999; Liu et al., 1998). To minimize those negative effects, the stress-inducible *RD29A* promoter can be introduced to control expression. For instance, transgenic Arabidopsis, tobacco, rice, wheat, and potato that overexpress *DREB1A* show normal growth as well as improved tolerance to drought and salt stresses (Behnam et al., 2006; Dubouzet et al., 2003; Kasuga et al., 2004; Pellegrineschi et al., 2004; Yamaguchi-Shinozaki and Shinozaki, 1993). Therefore, all of these data indicate that employment of the *RD29A* promoter in *RD29A:BrERF4* transgenic crops, including *B. rapa*, would minimize growth retardation but maintain enhanced salt and drought tolerances.

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