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Arabidopsis Brassinosteroid Mutants *det2-1* and *bin2-1* Display Altered Salt Tolerance

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Abstract Exogenous brassinosteroid (BR) has been reported to improve plant resistance to abiotic stress, but little is known about the role of endogenous BR in plant stress responses. In this study we investigated the involvement of endogenous BR in salt stress response using BR mutants *det2-1* and *bin2-1* of *Arabidopsis*. Seed germination and seedling growth of *det2-1* and *bin2-1* were more sensitive to salt stress than that of Columbia wild type (WT). The transcript levels of salt- and ABA-induced genes *COR78* and *P5CS1* were less induced in *det2-1* than in WT under 200 mM NaCl. In addition, the basal proline level and, to a lesser extent, the proline level induced by 200 mM NaCl or 50 μM ABA in both *det2-1* and *bin2-1* was enhanced, resulting in decreased proline accumulation. On the other hand, exogenous 24-epibrassinolide (EBR) could enhance proline accumulation, promote root elongation of WT, and partially rescue the growth of *det2-1* under salt stress. These results suggested that endogenous BR is positively involved in the plant response to salt stress in *Arabidopsis*.

Keywords Brassinosteroid · *Bin2-1* · *Det2-1* · Mutant · Proline · Salt stress

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Introduction

Phytohormones such as abscisic acid (ABA), ethylene, and jasmonic acid have been reported to be involved in plant responses to abiotic stress. Brassinosteroids (BRs) are a class of plant polyhydroxysteroids that are structurally similar to animal and insect steroid hormones and control a broad range of plant developmental processes (Clouse and Sasse 1998). Several BR signaling components have been identified and characterized using BR-deficient or BR-insensitive mutants. The membrane-localized receptors BRI1 and BAK1 transfer the BR signal into the cytoplasm to inhibit BIN2, a glycogen synthase kinase-3/SHAGGY-like kinase. BIN2 negatively regulates downstream signaling by phosphorylating two transcription factors BES1 and BZR1 and inhibiting their binding to BR target promoters (Wang and He 2004; Li and Jin 2006). *Arabidopsis DET2* encodes a BR biosynthetic enzyme that shares significant sequence identity with the mammalian steroid 5α-reductase. The defects in root and leaf development of the mutant *det2-1* could be rescued by the application of brassinolide (Li and others 1996).

Application of exogenous BR has been reported to improve plant resistance to many environmental stresses such as salinity stress (Anuradha and Rao 2001, 2003), drought stress (Kagale and others 2007), thermal stress (Dhaubhadel and others 2002; Singh and Shono 2005; Ogweno and others 2008), and heavy-metal stress (Hayat and others 2007). However, the function of endogenous BR under stress is not clear. Recently, a rice knockout mutant of a salt-responsive *OsGSK1*, an ortholog of *Arabidopsis BIN2*, was reported to be hypersensitive to BR and more tolerant to abiotic stress (Koh and others 2007).

From this study we provide preliminary evidence that endogenous BR is involved in salt stress response by demonstrating that the *Arabidopsis* BR-deficient mutant

det2-1 and BR-insensitive mutant *bin2-1* are hypersensitive to salinity stress during seed germination and/or the seedling growth stage. Moreover, the hypersensitivity correlated with the inhibited induction of stress-related genes *COR78* and *P5CS1* and proline accumulation under 200-mM-NaCl stress. Addition of 24-epibrassinolide (EBR) could improve the NaCl-induced proline accumulation and alleviate the inhibition of root elongation in wild type (WT). Our data suggested that BR plays an important role in the *Arabidopsis* response to environmental stresses.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia was used as WT control. BR-deficient mutant *det2-1* and BR-insensitive mutant *bin2-1* were obtained from Dr. Zhiyong Wang and Dr. Jun Zhao. WT and *det2-1* seeds were surface-sterilized in 10% sodium hypochlorite and rinsed five times in sterile water. The seeds were then sown on half-strength MS (1/2 MS) agar medium with 1% sucrose for normal germination, or on the same basic medium supplemented with different concentrations of NaCl and ABA for stress treatment. After being stored at 4°C for 72 h, seeds were germinated in a growth chamber at 22°C under 16 h of light (120 $\mu Em^{-2}\ s^{-1}$), E represents Einstein.

Growth Measurement

The salt sensitivity of mutant and wild-type seedlings was analyzed by transferring seedlings grown on 1/2 MS medium (1% sucrose) for 4 days (*det2-1*) or 7 days (*bin2-1*) onto NaCl-containing medium with or without different concentrations of EBR. The seedlings were allowed to grow horizontally for the desired period of time. The seedlings were then carefully pulled out from the medium for photographing, determination of fresh weight (dead seedlings were counted as zero), and root length measurement. The inhibition of fresh weight or root elongation of WT and mutants was calculated as follows: [(control seedlings − treated seedlings) × 100] ÷ control seedlings.

Salt and ABA Treatment

Sterilized seeds of mutant and WT were germinated on nylon mesh placed on 1/2 MS medium. The mesh with 10-day-old seedlings was lifted and incubated in liquid 1/2 MS medium containing 200 mM NaCl or 50 μM ABA, with or without preincubation for 3 days in medium with EBR. The seeds were harvested at the desired time points and frozen in liquid nitrogen for proline content measurement and RNA extraction.

Proline Content Determination

Proline content of the seedlings was measured according to the method of Bates and others (1973). Twenty to twenty-five seedlings were pooled in each replica for proline determination. Folds of proline accumulation were calculated as follows: proline content of treated sample ÷ proline content of control.

Isolation of RNA and Semiquantitative RT-PCR

Total RNA was extracted from 10-day-old seedlings as described by Hua and others (2001). Twenty to twenty-five seedlings were pooled for total RNA extraction. First-strand cDNA was synthesized by Superscript II following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). PCR, using the first-strand cDNA as a template, was performed using the following primers: *COR78*F: 5′-TGG AACAGAGGATGTAACGAG-3′ and *COR78*R: 5′-CGGA GTCAACTTCTCATCAAC-3′ to amplify a 1084-bp *COR78* (At5g52310) fragment. *RD22*F: 5′-ATGGCGATT CGGCTTCCTCTG-3′ and *RD22*R: 5′-CTAGTAGCTGA ACCACACAAC-3′ were used for full-length *RD22* (At5g25610) cDNA. The primers for *P5CS1* (At2g39800) were *P5CS1*F: 5′-AGTCT-ATGCTTGATTTGAGGGT-3′ and *P5CS1*R: 5′-AAGCTCATTAAGCACAGCATTC-3′. A 380-bp fragment of *ACTIN7* (At5g09180), an internal control, was amplified by *ACTIN7*F: 5′-TGCACAAGTCA-TAACCATCGG-3′ and *ACTIN7*R: 5′-TGTGAACAATCG ATGGACCTGAC-3′. PCR amplification of all four genes was performed by an initial denaturation at 94°C for 4 min followed by 25 cycles of denaturing at 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 60 s, and a final extension at 72°C for 7 min. Quantification of the amplified signals was performed by photographing the gel and analyzing it using the software Band Leader (Version 3.0, Magnitec Ltd.)

Results

Hypersensitivity of *det2-1* to Salt Stress During Germination

To understand whether endogenous BR is involved in the salt response during seed germination, the seeds of *det2-1* and WT were germinated on salt-containing medium. The germination rates were counted every day for 7 days. The germination rates of WT were 100% under all NaCl concentrations tested within 7 days. On the other hand, the

germination rate of *det2-1* was close to 100% only under control conditions and was only 84 and 75% under 50 and 100 mM NaCl, respectively (Fig. 1a). Under 200 mM NaCl, less than 45% of *det2-1* seeds germinated whereas WT seeds reached 100% (data not shown). Not only the germination rate but also the speed of germination was reduced in *det2-1* under salt stress. Under 0 mM NaCl, WT seeds germinated within the first day, whereas only 24% of *det2-1* seeds germinated. Under salt stress, the germination of *det2-1* was further delayed compared with that of WT (Fig. 1a).

It is well established that phytohormone ABA could inhibit seed germination and is involved in a plant's response to salt stress. Because germination of *det2-1* was hypersensitive to salt stress, its sensitivity to ABA treatment was also checked. The germination rate of *det2-1* was reduced more than that of WT after 0.1-μM-ABA treatment (Fig. 1b), which is in agreement with a previous report (Steber and McCourt 2001) that the germination of *det2-1* was hypersensitive to ABA. These results indicated that endogenous BR has a positive effect on seed germination under salt stress or ABA treatment.

Altered Salt Stress Sensitivity of *det2-1* During Seedling Growth

To characterize the salt sensitivity of *det2-1* at the seedling growth stage, 7-day-old seedlings were grown on 1/2 MS medium with 50, 100, and 150 mM NaCl for 3 days. On the control medium, *det2-1* seedlings showed darker green leaves and shorter roots than WT. Under 100 mM NaCl, leaves of *det2-1* seedlings turned pale rapidly, whereas the leaves of WT were still partially green (Fig. 2a, panels a and b).

We further examined the root length and fresh weight of *det2-1*. As shown in Fig. 2b, the root elongation of *det2-1* seedlings was inhibited more severely than that of WT at all concentrations of NaCl. The inhibition of *det2-1* root elongation was 86.7% under 100 mM NaCl, twice as much as that of WT. The fresh weight of *det2-1* was inhibited more than that of WT under 50 and 100 mM NaCl (Fig. 2b). The inhibition of fresh weight of *det2-1* was 100% because all the *det2-1* seedlings were dead under 150 mM NaCl. These results indicate that *det2-1* is hypersensitive to salt stress during the seedling stage.

To investigate whether the salt sensitivity of *det2-1* is due to its lack of wild-type level of BR, different concentrations of EBR were added back to the salt-containing medium. As shown in Fig. 2a (panels c and d), the bleaching of the leaves of *det2-1* was slowed greatly by 10 nM of EBR, suggesting that addition of BR can partially rescue the growth of *det2-1* under salt stress.

Altered Salt Stress-Induced Gene Expression and Proline Accumulation of *det2-1*

To gain some insight into the possible molecular mechanism underlying the salt hypersensitivity of *det2-1*, the expression in *det2-1* of some marker genes known to be induced by salt stress and ABA was compared with that in WT. *COR78* and *RD22* were reported to be induced by abiotic stress such as salinity, desiccation, cold, and osmotic stress (Zhu 2002; Yamaguchi-Shinozaki and Shinozaki 2006). As shown in Fig. 3a and b, during salt stress the induction of *COR78* transcription in *det2-1* was significantly decreased compared with that in WT, especially at 6 h after treatment, but the salt-induced *RD22* expression was maintained in both genotypes.

The expression of *P5CS1*, which encodes the key enzyme of proline biosynthesis, was also examined. The induction of *P5CS1* in *det2-1* after a 4-h treatment with 200 mM NaCl was greatly inhibited compared with that in WT (Fig. 3a, b). We have further examined the proline content of *det2-1* under NaCl treatment and found that the proline level under the stress condition was not significantly different from that of WT. However, the folds of proline accumulation in *det2-1* were less than in WT due to a higher basal proline content in *det2-1* (Fig. 3c). Our

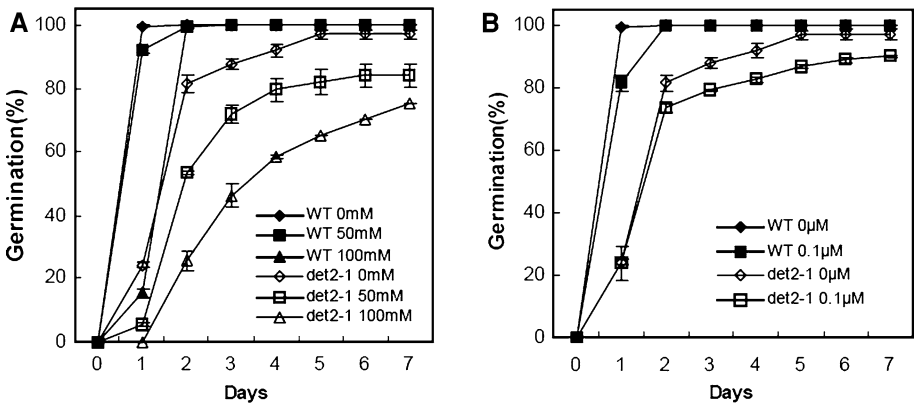

Fig. 1 Effects of salt stress (**a**) or ABA treatment (**b**) on the germination rate of *det2-1*. Data are the mean ± standard deviation of three independent measurements of 300 seeds

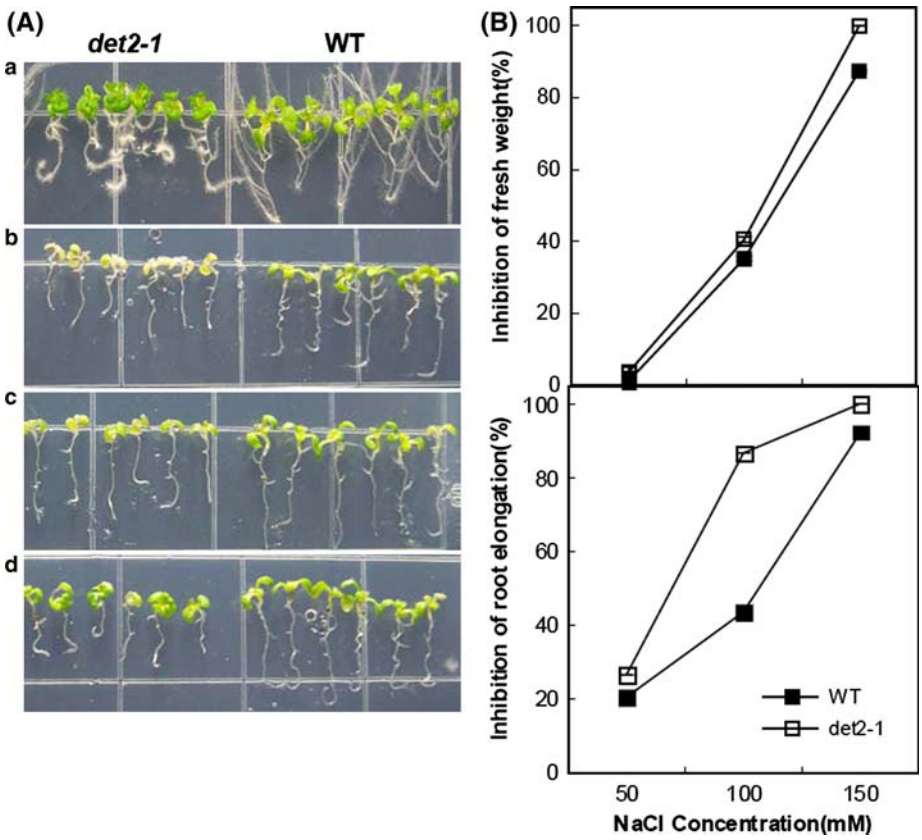

Fig. 2 Effects of salt stress on *det2-1* seedling growth. **a** Effects of EBR on 100-mM-NaCl-stressed *det2-1* and WT seedlings. Seedlings grown on normal medium for 7 days were transferred onto NaCl-containing medium and grown for another 3 days; (a) CK, (b) 100-mM-NaCl treatment, (c) cotreatment with 100 mM NaCl and 1 nM EBR, (d) cotreatment with 100 mM NaCl and 10 nM EBR. **b** Relative inhibition of fresh weight and root length of *det2-1* (mean ± SD, $n = 4$) under salt stress. CK: seedlings grown on normal 1/2 MS without NaCl

results seem to suggest that endogenous BR may be involved in the salt response by modulating the expression of some stress-inducible genes and flux through the proline biosynthetic pathway.

We also tested whether addition of BR could rescue proline accumulation in *det2-1*. As demonstrated in Fig. 3c, the basal level of proline in *det2-1* was reduced to a level similar to that in WT, which resulted in enhanced proline accumulation.

Hypersensitivity to Salt Stress and Proline Accumulation of *bin2-1* Seedling

To obtain more evidence about the involvement of BR in a plant's salt stress responses, we tested the salt sensitivity of BR-insensitive mutant *bin2-1* at the seedling stage. Because the homozygous *bin2-1* mutant is sterile, we used the seeds derived from the heterozygous *bin2-1*, which would segregate out plants corresponding to homozygous *bin2-1*, heterozygous *bin2-1*, and WT. Seven days after germination the seedlings of heterozygous *bin2-1* phenotype were selected and transferred to 1/2 MS medium containing different NaCl concentrations. Almost all *bin2-1* seedlings were bleached at 150 and 200 mM NaCl (Fig. 4a), and root growth was more severely inhibited than in WT at each NaCl concentration tested. The survival rate (Fig. 4b), relative root elongation (Fig. 4c), and fresh weight (Fig. 4d) all showed that heterozygous *bin2-1*seedlings are more sensitive to salt stress than WT. Similar to the results in *det2-1*, 200-mM-NaCl-induced *P5CS1* upregulation (Fig. 4e, f) and proline accumulation (Fig. 4g) were also inhibited in *bin2-1* seedlings.

Exogenous BR Enhanced the Seedlings Growth and Proline Accumulation Under Salt Stress

The above results indicated that the WT level of endogenous BR and BR signaling are both necessary for normal seedling growth during salt stress. Thus, we further tested whether exogenous applied EBR could improve the growth of WT seedlings under salt stress. Four-day-old wild-type seedlings were grown on medium containing 75 mM NaCl, with or without 0.1 μM EBR for 7 days. The result showed that addition of 0.1 μM EBR in the growth medium under 75 mM NaCl could significantly improve the root elongation of the seedlings, but no improvement was observed at higher BR concentrations (Fig. 5a).

The effect of BR on salt-induced proline accumulation was also analyzed. Ten-day-old wild-type seedlings were first pretreated with 0.1, 0.3, or 0.5 μM of EBR for 3 days before being incubated under 200 mM NaCl. Proline

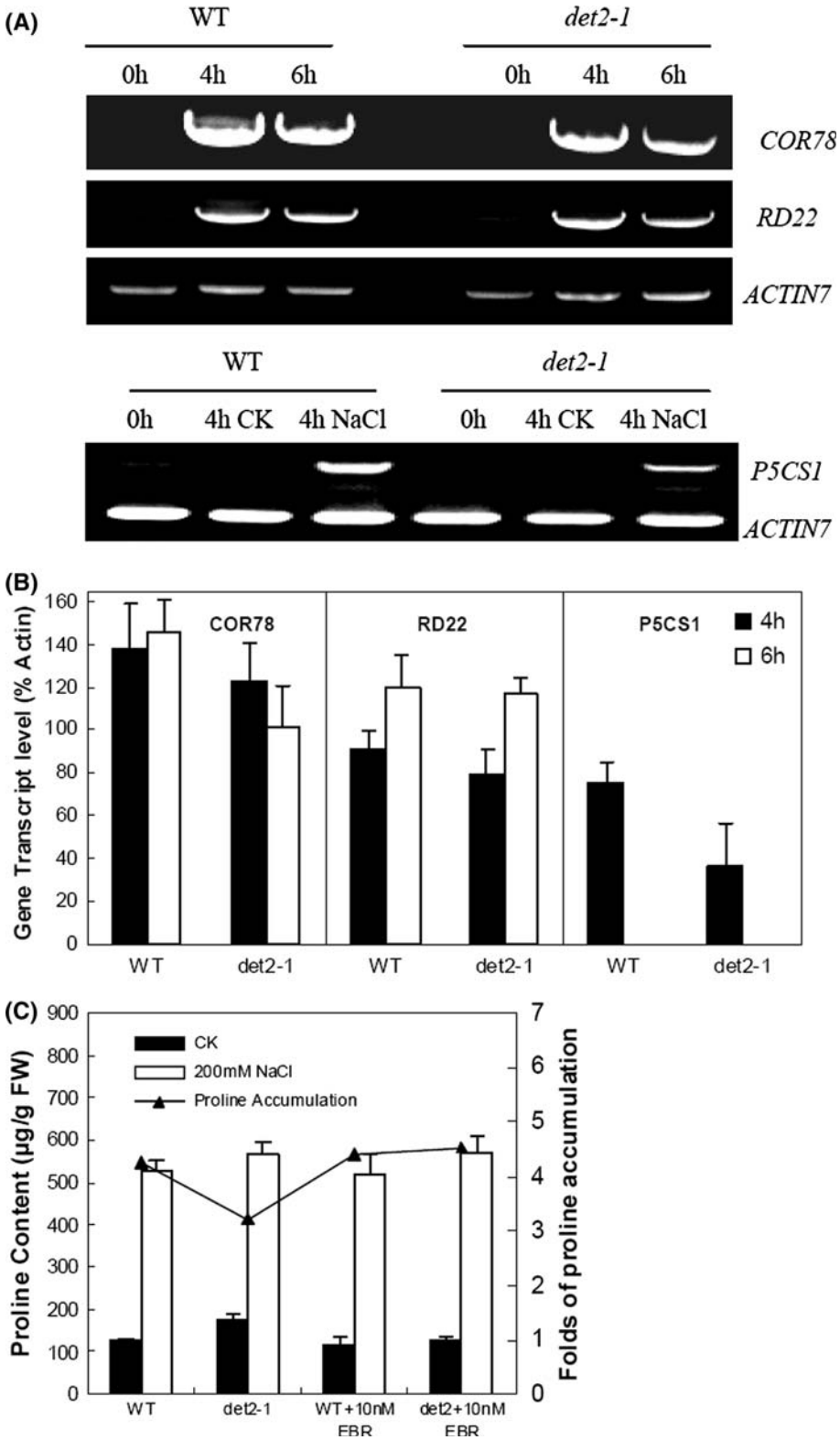

Fig. 3 Salt-induced gene expression and proline accumulation in *det2-1*. **a** RT-PCR analysis of *COR78*, *RD22*, and *P5CS1* in *det2-1* and WT under 200-mM-NaCl stress for 0, 4, and 6 h. *ACTIN7* was used as an internal control. **b** Quantitative results of RT-PCR for transcript level (mean ± SD, $n = 3$ for *COR78* and *RD22*; $n = 4$ for *P5CS1*). **c** Content and folds of proline accumulation of WT and *det2-1* (mean ± SD, $n = 5$) under 200-mM-NaCl treatment for 24 h with or without 10 nM EBR. CK: seedlings grown on normal 1/2 MS medium

accumulation induced by salt treatment was enhanced by EBR in a dose-dependent manner (Fig. 5b). These results showed that exogenous BR could enhance salt tolerance and salt-induced proline accumulation.

Discussion

Most plant growth hormones are first discovered as substances that stimulate plant development; however, some,

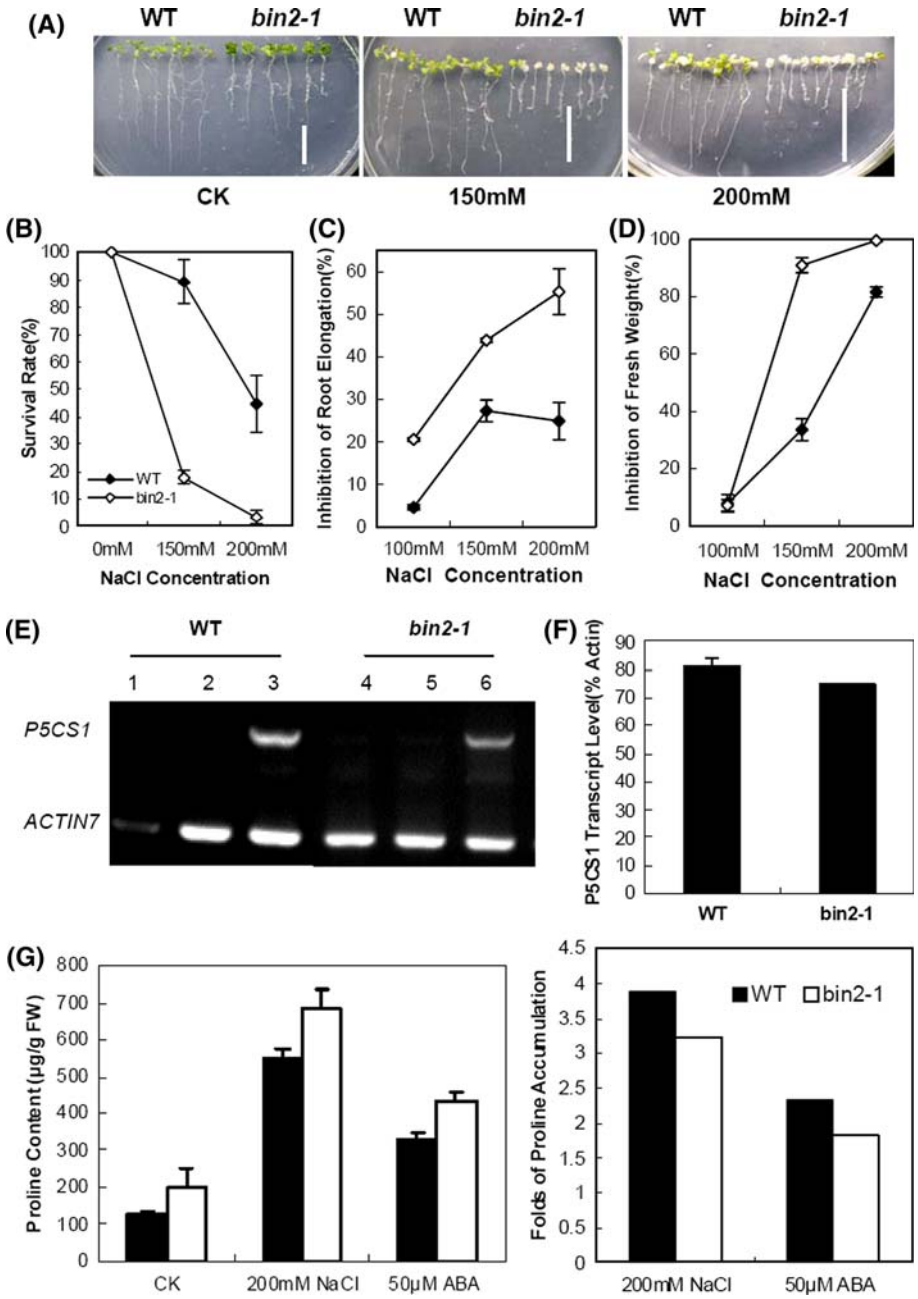

Fig. 4 Altered sensitivity to salt stress and proline accumulation of *bin2-1* seedlings. **a** Growth of *bin2-1* seedlings on medium with different NaCl concentrations. Seedlings grown on normal medium for 7 days were transferred onto NaCl-containing medium and grown for another 3 days. *Scale bars*: 2 cm. Survival rate (**b**), relative inhibition of root length (**c**), and fresh weight (**d**) of *bin2-1* (mean ± SD, $n = 4$) under different concentrations of NaCl. **e** RT-PCR analysis of *P5CS1* transcript level in WT and *bin2-1* at 0 h (lanes 1, 4), 4 h control (*lanes* 2, 5), and 200-mM-NaCl treatment for 4 h (*lanes* 3, 6), *ACTIN7* was used as an internal control. **f** Quantitative results of RT-PCR for *P5CS1* transcript level (mean ± SD, $n = 4$). **g** Content and folds of proline accumulation in WT and *bin2-1* (mean ± SD, $n = 5$) treated with 200 mM NaCl or 50 μM ABA. CK: seedlings grown on 1/2 MS medium

such as abscisic acid (Shinozaki and others 2003; Zhang and others 2006; Lin and others 2007), ethylene (Mizoguchi and others 1997; Wang and others 2007), and jasmonic acid (Takahashi and others 2007; Walia and others 2007), are later found to also modulate the plant response to environmental stimuli. Previous reports on BR's function in plant stress tolerance were based mostly on its exogenous application. The involvement of endogenous BR in stress response can best be investigated using mutants deficient in endogenous BR production or BR signaling. To this end, BR mutants *det2-1* and *bin2-1* were used to study the function of endogenous BR in salt tolerance. Examination of seed germination rates and measurements of relative root elongation and relative fresh weight of the seedlings demonstrated that *det2-1* was hypersensitive to salinity at both germination and the early growth stage, and this growth defect can be partially rescued by the exogenous addition of 10 nM EBR. In addition, the seedling growth of the BR-insensitive mutant *bin2-1* also showed hypersensitivity to salt stress, suggesting that endogenous BR and BR signaling are important for salt tolerance of *Arabidopsis*.

It has been widely reported that there might be an antagonistic interaction between BR and ABA signaling

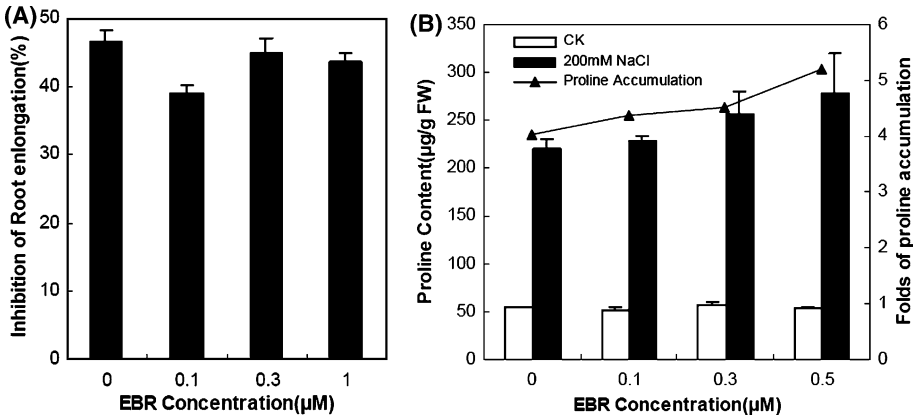

Fig. 5 Effect of EBR on seedling root elongation and proline accumulation under salt stress. **a** Effect of EBR on root length of salt-stressed (75 mM) seedlings (mean ± SD, $n = 4$); root length was measured at 7 days after stress. **b** Effect of EBR pretreatment on proline accumulation of WT under 200-mM-NaCl stress (mean ± SD, $n = 5$). Seedlings were preincubated with different concentrations of EBR for 3 days before addition of 200 mM NaCl; CK: seedlings pretreated with 1/2 MS medium with 70% ethanol added (solvent of EBR) for 3 days

pathways. For example, expression of three closely related BR-responsive bHLH transcription factors, BEE1, BEE2, and BEE3, was inhibited by ABA, and overexpressing BEE1 could reduce ABA sensitivity in *Arabidopsis* (Friedrichsen and others 2002). *bin2-1* displayed a hypersensitivity to ABA in root elongation profiles (Li and others 2001). Recently, it has been reported that ABA could inhibit BR signaling by modulating the phosphorylation status of BES1 and BR-responsive gene expression (Zhang and others 2009).

Altered sensitivity of *det2-1* seedlings to salt stress was manifested not only in germination and seedling growth, but also in the expression of genes known to be related to salt response. Salt induction of *COR78* and *P5CS1* expression is decreased in *det2-1*, suggesting that BR might promote salt tolerance by upregulating a subset of salt-responsive genes. In a genome-wide search for BR-regulated genes, *COR78* is among the few stress-related genes that are responsive to BR, whose expression in the BR-deficient mutant *dwf1-6* was found to be slightly decreased compared to that in WT, but downregulated by BR treatment (Müssig and others 2001). Therefore, it is not clear whether BR directly regulates *COR78* expression. In our data, the expression of *COR78* was undetectable under control conditions in both the wild type and *det2-1*. However, its salt-induced expression was reduced. We reason from these data that BR may not directly regulate the promoter of *COR78* and *P5CS1* (which is not on the list of BR-regulated genes in the above-cited article); rather, BR may interact with a salt signaling component to affect the salt-induced expression of *COR78* and *P5CS1*. Another possibility is that BR may regulate *COR78* and *P5CS1* promoters only under stress conditions. The E-box consensus sequence (CANNTG), present in many BR-induced promoters (Nemhauser and others 2004), was found in the promoter of *P5CS1* (at −86 and −128 bp) and *COR78* (at −297 bp).

Proline accumulation appears to play a protective role in plants under salt and drought stresses (Savouré and others 1995) and is regulated by signal molecules such as calcium (Knight and others 1997), PLC (Parre and others 2007), and PLD (Thiery and others 2004), which are integrated in ABA-dependent and ABA-independent pathways (Savouré and others 1997; Strizhov and others 1997).

Our results showed that the induction of the *P5CS1* transcript level in *det2-1* and *bin2-1* was attenuated compared with that in WT after treatment with 200 mM NaCl (Figs. 3a, 4e). However, the absolute proline level during stress was not significantly changed or even slightly increased. The decreased proline accumulation is mainly due to the enhanced basal proline level (Figs. 3c, 4g). It is possible that here the *P5CS1* expression level is not the sole determinant for the proline level. It seemed that expression of the proline dehydrogenase gene was also decreased in *det2-1* compared with that in WT (data not shown). The role of proline accumulation in protecting the stressed plant is a matter of debate. It is not clear whether the role of proline accumulation is achieved by the proline molecule itself or by the proline biosynthetic process, which consumes large amounts of $NADPH^+$ reported to accumulate during many kinds of environmental stresses (Hare and Cress 1997).

On the other hand, exogenous EBR could increase proline accumulation of WT under 200 mM NaCl (Fig. 5b), supporting a previous report that exogenous EBR could enhance proline accumulation induced by 20% PEG treatment in *Sorghum* (Vardhini and Rao 2003). The proline accumulations of both *bin2-1* (Fig. 4g) and *det2-1*

(data not shown) under 50-μM-ABA treatment were less than that of WT, raising the possibility that decreased proline accumulation in *det2-1* and *bin2-1* under 200 mM NaCl may result from the antagonistic effect of BR on the ABA-dependent pathway. Taken together, these results suggest that the role of BR in the salt tolerance of *Arabidopsis* may be accomplished partially by regulating proline accumulation; however, more work needs to be done to elucidate the precise role of BR in stress-induced proline accumulation.

In contrast to our results, Abraham and others (2003) have shown that the induction of *P5CS1* transcription after NaCl treatment for 24 h was inhibited by EBR in WT and enhanced in *det2-1*. The apparent discrepancy may be due to different growth conditions, especially different light regimes. In our experiment, *Arabidopsis* seedlings were grown under a 16-h light/8-h dark cycle, whereas the seedlings of Abraham and others were grown under an 8-h light/16-h dark cycle. The activity of BR could be affected by light (Neff and others 1999; Turk and others 2005). We have also tried BR treatment under short-day conditions; preliminary results showed that BR could suppress proline accumulation. Different lengths of treatment may also affect the induction of gene expression because *P5CS1* has been shown to be mostly induced at the onset of stress, reaching a peak at about 4–8 h after 1% NaCl stress (Savouré and others 1995). All our results on *P5CS1* expression shown here were obtained with 4-h NaCl treatment.

In conclusion, we have presented evidence that endogenous BR and BR signaling are important for the response of *Arabidopsis* to salt stress. BR could enhance salt tolerance by affecting the expression of some stress-related genes and proline accumulation. Our results, therefore, have provided more insights into the role of BR as a plant growth hormone not only in plant development but also in adaptation to environmental stresses.

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