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Ectopic expression of *Expansin3* or *Expansinβ1* causes enhanced hormone and salt stress sensitivity in Arabidopsis

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Abstract   Expansins are cell wall loosening proteins that appear to permit the microfibril matrix network to slide in growing plant cell walls, thereby enabling the wall to expand. To scrutinize possible impacts on plant growth and development when expansins are over-expressed, we characterized phenotypic alterations of the transgenic plants that constitutively expressed *AtEXP3* or *AtEXP-β1* under control of *35S-CaMV* promoter. Our results suggest that both *AtEXP3-OX* and *AtXPβ1-OX* are very sensitive to salt stress. However, the mechanisms underlying their enhanced salt sensitivity appear to be different.

Keywords   *Arabidopsis thaliana* · *COR* genes · Expansin · Ethylene · *IAA* genes · Salt stress

Introduction

The plant cell wall is a heterogeneous polymeric structure containing cellulose microfibrils, hemicellulose, pectin, lignin, and proteins. Entanglement and covalent bonds between these polymers confer structural rigidity to the cell wall. However, cross-linking of this polymeric network loosens during growth. Cell expansion is an essential component of many plant morphogenetic processes such as cell enlargement, fruit softening, pollen tube, and root hair growth, and abscission. Hence, cell expansion is tightly controlled in a highly specific manner (see Cosgrove 1997). Expansin proteins participate in the cell wall loosening process. Although they do not hydrolyze cellulose, expansins can weaken pure cellulose paper via the disruption of hydrogen bonding between cellulose microfibrils or between cellulose and other cell wall polysaccharides (McQueen-Mason et al. 1994, 1995). Thus, various types of expansins play key roles in cell expansion during growth and environmental adaptation (Rose et al. 1997; Catalá et al. 2000; Hiwasa et al. 2003), and different expansin genes have been isolated from rice, cucumber, tomato, cotton, and *Arabidopsis* (Li et al. 2002). The expansin family accounts for 32 and 53 genes in *Arabidopsis* and rice, respectively (Sampedro and Cosgrove 2005). Furthermore, the members of two families, namely the α- and β expansins, exhibit organ-, tissue-, hormone-, and cellular-specific expression patterns (Lee and Kende 2001; Reidy et al. 2001; Kende et al. 2004; Wu et al. 2001; Shin et al. 2005; Vreeburg et al. 2005).

However, since expansins are involved in the cell expansion process, their over-expression may cause harmful defects in growth and development, because

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they are known to be regulated by environmental stimuli (Brummell et al. 1999; Hisawa et al. 2003). Thus, we used transgenic Arabidopsis that constitutively expressed *AtEXP3* or *AtEXP-β1* under control of *35S-CaMV* promoter, to investigate potential impacts of expansin over-expression on stress.

Materials and methods

Plant materials and growth condition

Arabidopsis thaliana plants of the Colombia ecotype were used. Transgenic seedlings that over-expressed *A. thaliana EXP3* (AT2G37640) or *EXP-β1* (AT2G-20750), (*AtEXP3-OX* and *AtXPβ1-OX*, respectively) were generated. All germination and growth experiments were performed in growth chambers at 23°C with photocycles of 16 h light and 8 h dark, at a relative humidity of 70%. Seeds were surface-sterilized and sown in normal Murashige and Skoog (MS) agar (1.2%), pH 7.0, supplemented with 2% (w/v) sucrose. For phenotypic observations, 5-day old seedlings grown on normal MS agar were transferred to media containing hormones (0.1, 1 or 10 µM of absisic acid, ABA; 1-aminocyclopropane-1-carboxylic acid, ACC; or indole-3-acetic acid, IAA) or salt (0, 250 or 300 mM NaCl) under various pH conditions. Phenotypic analyses were performed 1 week later. Twenty seedlings were included in each analysis.

Gene amplification and transformation of *Arabidopsis*

Full-length cDNAs (RIKEN) encoding *Arabidopsis EXP3* and *EXPB1* were amplified by PCR and cloned into *pBI121*, which contains the *cauliflower mosaic virus* (*CaMV*) *35S* promoter. To obtain transgenic plants, *Agrobacterium*-mediated transformations were performed according to Lee et al. (2002). Homozygous T$_4$ transgenic plants were used for all experiments.

Expansin activity assays

Cell wall proteins were extracted and the expansin activity assayed using a protocol described by McQueen-Mason et al. (1995) with some modifications. For extraction of cell wall protein, tissues were ground into a fine powder in liquid N$_2$ and 1 g (fresh wt) was added to 4 ml homogenization buffer [25 mM HEPES (pH 6.8), 1% polyvinylpyrolidone (40,000 MW), 1% Triton X-100, 3 mM sodium metabisulfite, 2 mM EDTA, and 2 mM dithiothreitol]. The homogenate was collected on a 50 µm nylon mesh and excess liquid removed by squeezing. Wall fragments were washed three times in the same volume of the homogenization buffer lacking Triton X-100. Wall fragments were retained on the nylon mesh and then re-suspended at 2 ml g^{-1} original fresh wt in 1 M NaCl, 25 mM HEPES (pH 6.8), 3 mM sodium metabisulfite, 2 mM EDTA, and 2 mM dithiothreitol, and left to extract for 1 h at room temperature. The salt extract was filtered through nylon mesh, its volume measured and proteins were precipitated by the gradual addition of 0.39 g solid ammonium sulfate ml^{-1}. After 10 min on ice, the protein precipitate was recovered by centrifugation (10,000 *g*) for 10 min at 4°C. The supernatant was removed and the pellets stored at −20°C. Immediately prior to expansin assays, precipitates were re-suspended in 1 ml 50 mM sodium acetate (pH 4.5), and desalted in the same solution, using a 5 ml Sephadex G25 column. Expansin assays were performed in a Universal Testing Machine (UTM) using a Whatman No. 113 filter paper strip (2 × 5 cm). Briefly, 2 mm wide strips of composite were placed between the clamps of the UTM and bathed in 1 ml protein solution (1 mg cell wall proteins ml^{-1}). Strips extended by applying a 5 g weight to the lower clamp for 10 min and the maximum force and tensile strength were measured. Expansin activity was calculated using the equation $\sigma_{max} = F_{max}/A$; where A = cross sectional area and F_{max} = maximum load (kg). The crosshead speed was 0.5 mm min^{-1}.

Peroxidase activity assay and ROS (H$_2$O$_2$) staining

Peroxidase activity (guaiacol assay) was measured following the method of Putter (1974). Seedlings were homogenized in liquid N$_2$ and macerated in 0.1 M Tris/HCl buffer, containing 4 ml 8.75% (w/v) polyvinylpyrrolidone (MW 40,000), 2 ml 0.1 M KCl, and 0.1 ml 0.28% Triton X-100, in 35.1 ml. The homogenate was centrifuged at 4,000 g for 30 min and the supernatant was filtered using 0.45 µm membrane filter. Peroxidase activity was determined using guaiacol at 470 nm at room temperature.

Detection of H_2O_2 was performed with 7-day old seedlings according to the method described by Thordal-Christensen et al. (1997) with some modification. The seedlings were washed in 50 mM potassium phosphate buffer (PBT) for several times and incubated for 10 min in solution with 0.3 mg DAB (3,3-diamionbenzidine) in 1 ml PBT. For color reaction, 1 μl 30% H_2O_2 was added to each tube. Then, each seedling was rinsed with PBT twice for 5 min. For dehydration of sample, 100% methanol was used.

Northern blot analysis

Total RNA was isolated from 2-week old seedlings of wild-type and expansin-overexpressing plants (*AtEXP3-OX* and *AtEXPB1-OX*) using a protocol described by Lee et al. (2002). Prior to isolation of RNA, seedlings were grown on 0.6% agar containing MS salts and supplemented with 2% (w/v) sucrose. The seedlings were then exposed for 3 or 6 h to NaCl (300 mM), IAA (10 μM) or ACC (10 μM). For northern blot analysis, total RNA (20 μg per lane) was separated by on 1.5% (w/v) agarose gels containing formaldehyde, and then transferred to a nylon membrane (Amersham Biosciences, Piscataway, NJ, USA). The probes for *COR15a* (*AT2G42540*), KIN1 (*AT5G15960*), *AtEXP3* and *AtEXPB1* were prepared by RT-PCR and radio-labeled using a random primer kit (Amersham Biosciences, Piscataway, NJ, USA). Membranes were pre-hybridized at 58°C for 1 h, and then hybridized against the ^{32}P-labelled probes at 65°C overnight. Membranes were washed twice for 20 min in 1 × SSC, 0.1% SDS and once in 0.1 × SSC, 0.1% SDS for 20 min at 42°C. Membranes were exposed to X-ray film at −70°C.

Statistical analysis

Data are expressed as means ± standard error (SE). Comparisons were made between groups with Student's *t*-tests.

Results

Growth phenotype of *EXP3-OX* or *EXPβ1-OX* plants

To determine the possible impact of expansin over-expression on plant growth responses, we investigated two typical expansin genes, *Arabidopsis* α- and β-expansin (*AtEXP3* and *AtEXPβ1*, respectively). *A. thaliana* was transformed with full-length cDNAs of *AtEXP3* and *AtEXPβ1*, under the control of *35S-CaMV* promoter and we obtained 20 individual transgenic lines expressing for each. Under normal growth conditions, many of the transgenic lines exhibited hyper-expression of these expansin genes (Fig. 1a). For further demonstration, one line was selected for each overexpressed gene, because other lines also exhibited similar responses.

Since expansin is involved in the plant cell elongation process, we examined the growth phenotypes of the transgene-over-expressing lines *AtEXP3-OX* and *AtEXPβ1-OX* under normal growth conditions first. Under light or dark conditions at normal temperature, no significant difference could be detected between wild type, *AtEXP3-OX* or *AtEXPβ1-OX* seedlings. However, when seeds of *AtEXP3-OX* or *AtEXPβ1-OX* were allowed to germinate in soil, they showed significant differences in growth performance (Fig. 1b). The size of the *AtEXP3-OX* leaves exhibit approximately larger than those of the wild type seedlings by 1.5-fold. Interestingly, the petiole lengths of *AtEXPβ1-OX* plants were significantly longer than those of wild type or *AtEXP3-OX* (Fig. 1d).

AtEXP3 and *AtEXPβ1* encode proteins with similar levels of expansin activity

In order to determine whether *AtEXP3* or *AtEXP-β1* encodes a protein with greater expansin activity when expressed in *Arabidopsis*, we partially purified these proteins and performed three replicate experiments to measure their activities using a constant extensometer. Protein samples from *AtEXP3-OX* and *AtEXPβ1-OX* seedlings were found to possess similar levels of exapnsin activity (Fig. 2). During a 30 s incubation period, no difference was detected between *AtEXP3* or *AtEXP-β1* proteins. However, following a longer incubation (5 min), the over-expressed proteins cause a nearly 50% reduction in tensile strength and increase in elongation relative to wild-type. These results clearly demonstrate that the transgenic expansin genes *AtEXP3* and *AtEXP-β1* encode functional proteins, although there is no discernible difference in their expansin activity.

Fig. 1 Overexpression of α- and β-expansin in A. thaliana caused alterations in growth phenotypes. (**a**) Northern blot analysis of expansin mRNA levels in wild-type (Ct) and transgenic lines overexpressing AtEXP3 (left panel) and AtEXPβ1 (right panel). Two-week-old Arabidopsis seedlings were grown on standard MS agar prior to RNA isolation and Northern blot analysis. Total RNA (ca. 20 μg) was used as a loading control. (**b**) Phenotype of wild-type (Col-0), AtEXP3-OX and AtEXPβ1-OX grown in soil for 3 weeks. Petiole lengths (**c**) and leaf diameter (**d**) of wild-type (Col-0), AtEXP3-OX and AtEXPβ1-OX grown in soil for 3 weeks. Values are means of three replicates ($N = 20$ seedlings/assay); error bars denote SE (*$P < 0.01$ in Student's t-test)
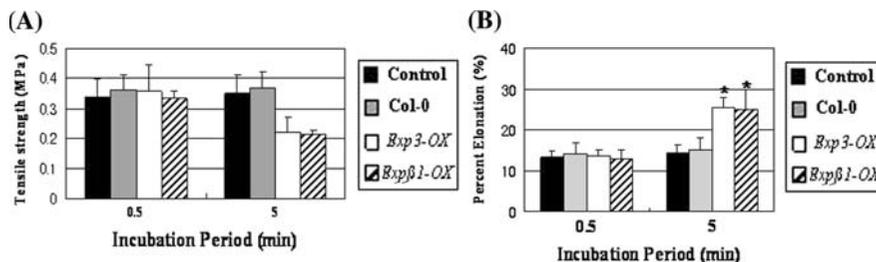

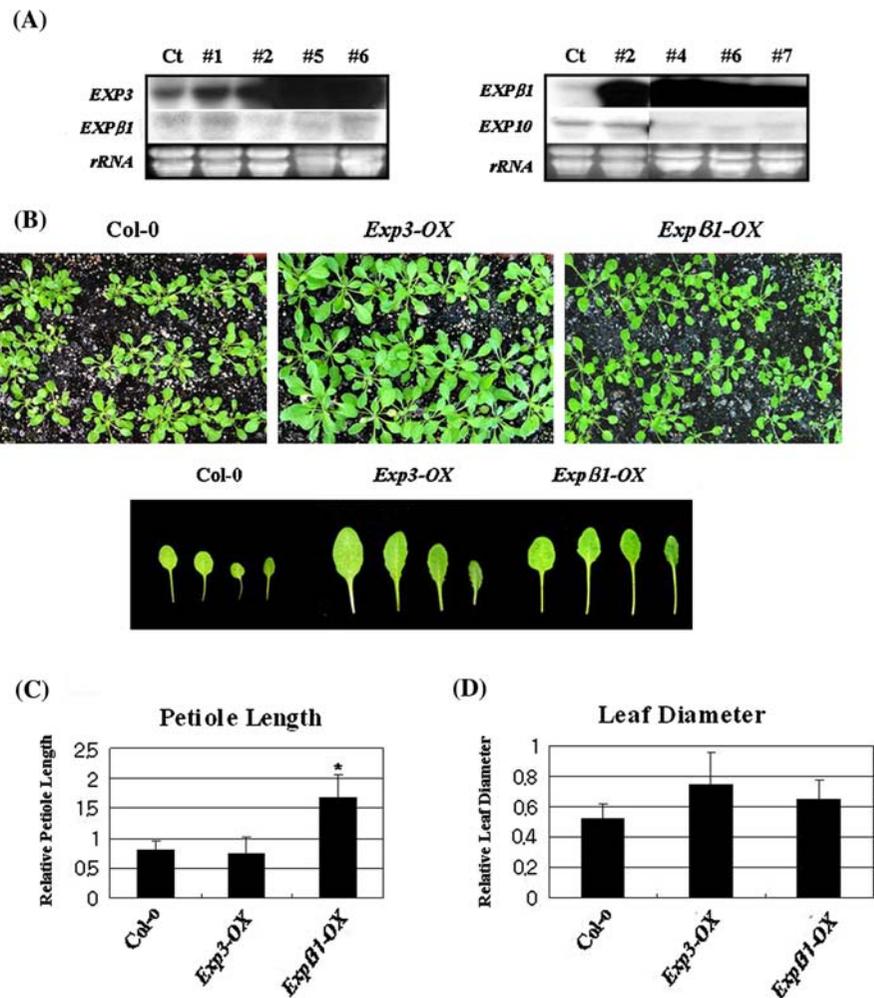**Fig. 2** Measurement of expansin activity in wild-type, AtEXP3-OX and AtEXPβ1-OX seedlings. Cell wall proteins were extracted from 5 g (fresh wt) of two-week-old wild-type, AtEXP3-OX and AtEXPβ1-OX seedlings. Samples were assayed for expansin activity using Whatman filter paper strips (No. 113). (**a**): Measurement of tensile strength using filter paper treated with expansin proteins from wild-type, AtEXP3-OX and AtEXPβ1-OX seedlings. The UTM was used to determine the tensile strength of filter paper strips treated with expansin proteins in 50 mM sodium acetate buffer (pH 4.8). Tensile strength was calculated with the equation $\sigma_{max} = F_{max}/A$ where A = cross sectional area; F_{max} = maximum load (kg). Crosshead speed was 0.5 mm per min. (**b**): Percentage elongation was measured as elongation (% or strain %) = $\Delta L/L \times 100$, where L = specimen gauge length and ΔL = maximum extension (*$P < 0.01$ in Student's t-test)

EXP3-OX or *EXPβ1-OX* plants in response
to abiotic stresses

In order to examine stress tolerance of these trans-
genic plants, wild-type, *AtEXP3-OX* and *AtEXPβ1-
OX* seeds were germinated in normal MS media and
5-day old seedlings transferred to media supple-
mented with NaCl. Both *AtEXP3-OX* and *AtEXPβ1-
OX* plants exhibited increased sensitivity to salt stress
(Fig. 3a and b). Most of the *AtEXP3-OX* and
AtEXPβ1-OX seedlings were dead within 1 week of
transfer to the salt medium (Fig. 3a). However, when
these seedlings were transferred to the media con-
taining ABA (0.1, 1, 10 μM) or mannitol (100, 200,
300 mM), they did not display any distinct differ-
ences to wild type (data not shown). Since both
AtEXPβ1-OX and *AtEXP3-OX* seedlings exhibit a
lethal phenotype in response to salt stress, we
investigated this sensitivity further. Initially, we
hypothesized that *AtEXP3-OX* or *AtEXPβ1-OX* seed-
lings might generate more reactive oxygen species
(ROS) when exposed to high salt stress. Although
AtEXP3-OX seedlings produced more ROS, even
under normal growth conditions, we did not detect
any increase in ROS levels in *AtEXPβ1-OX* seedlings
under either normal or salt stress conditions (Fig. 3c).
Next, we investigated whether or not the altered salt
stress response of *AtEXP3-OX* and *AtEXPβ1-OX*

seedlings was as a result of defects in the regulation
of genes associated with abiotic stress signaling. We
performed a northern analysis using probes for the
stress-responsive genes, *COR15a* and *KIN1*. The
seedlings were grown for 2 weeks on normal media
prior to exposure to 300 mM NaCl for 3 or 6 h.
AtEXPβ1-OX seedlings exhibited different *KIN1* and
Cor15a expression patterns than either wild-type or
AtEXP3-OX (Fig. 4a). Following salt treatment, both
stress responsive genes were expressed at high levels
in the wild type and *AtEXP3-OX* plants, but *KIN1*
expression was reduced significantly in *AtEXPβ1-OX*
seedlings. As shown in the Fig. 4b, enhanced perox-
idase activity was observed in both the *AtEXP3-OX*
and *AtEXPβ1-OX* seedlings in normal condition.

EXP3-OX or *EXPβ1-OX* plants in response
to hormone

Since expansin genes are responsive to auxin (IAA)
and ethylene (Catalá et al. 2000; Vreeburg et al.
2005; Hiwasa et al. 2003), we grew wild-type (col-0)
seedlings on standard MS agar for 2 weeks and then
transferred them to 3 MM filter paper saturated with
IAA or ACC as the precursor of the ethylene for 3 or
6 h. *AtEXP3* was induced by IAA only and *AtEXPβ1*
was induced by ACC only in wild type seedlings
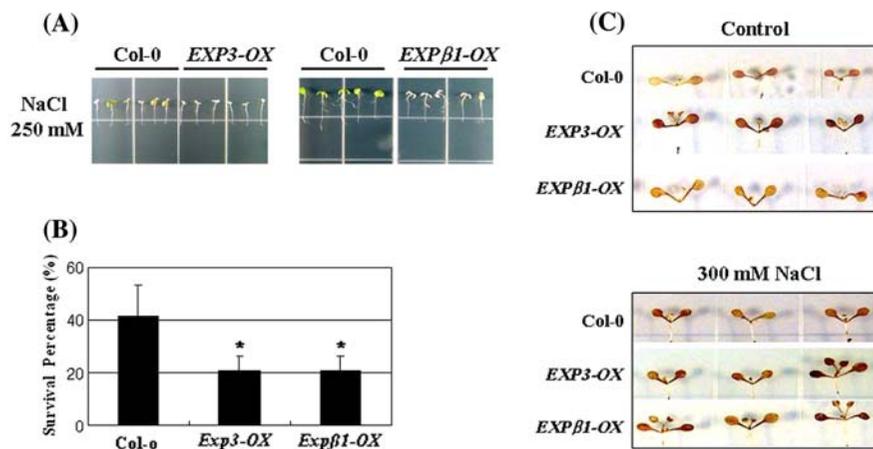(Fig. 5a). However, *AtEXPβ1* was not induced by

Fig. 3 Salt sensitivity of *AtEXP3-OX* and *AtEXPβ1-OX*
plants. (**a**) Phenotypic alterations of *AtEXP3-OX* and
AtEXPβ1-OX in response to salt stress. Five-day-old seedlings
of wild-type, *AtEXP3-OX* and *AtEXPβ1-OX* were transferred
to salt media and allowed to grow for 1 week. (**b**) Survival
rates of *AtEXP3-OX* and *AtEXPβ1-OX* seedlings in response to
salt stress. Values are means of three replicates ($N = 20$

seedlings/assay); error bars denote SE (*$P < 0.01$ in Student's
t-test). (**c**) ROS levels under control and salt stress conditions.
Each plant was grown in 1.2 % MS media for one week. Wild-
type (Col-0) and *AtEXP-OX* plants were transferred to stress
media containing 300 mM NaCl and incubated for 3 h. ROS
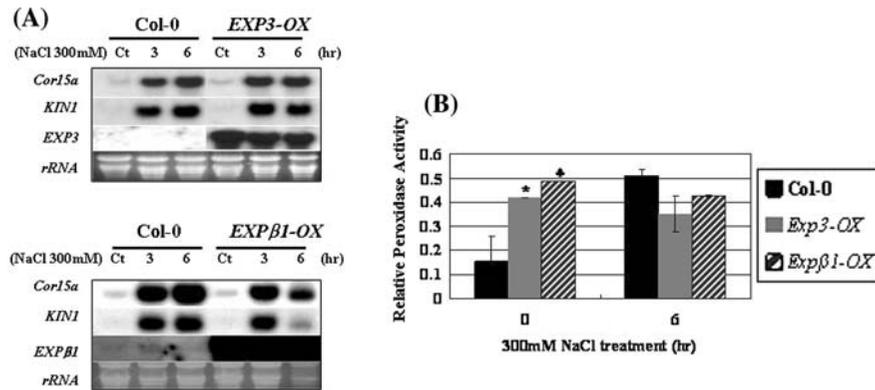staining was performed using DAB (3,3′-diaminobenzidine)

Fig. 4 The levels of the stress-inducible genes and peroxidase activities in *AtEXP3-OX* and *AtEXPβ1-OX* plants. (**a**) Transcription of *AtEXP3* (top panel) or *AtEXPβ1* (lower panel) was compared with the stress-inducible genes *Cor15a* and *KIN1*, under salt stress in *AtEXP3-OX* or *AtEXPβ1-OX* plants. Two-week-old wild-type (Col-0), *AtEXP3-OX* and *AtEXPβ1-OX* seedlings grown on standard MS media were transferred to media containing 300 mM NaCl for 3 and 6 h. Ct, untreated control. *Cor15a* and *KIN1* encode environmental stress responsive genes. Each gene was detected using ^{32}P-labeled probes and then visualized by autoradiography. Total RNA was used as a loading control. (**b**) Peroxidase activities under control and salt stress conditions. Each plant was grown in 1.2% MS media for 3 weeks. Wild-type (Col-0) and *AtEXP-OX* plants were transferred to stress media containing 300 mM NaCl and incubated for 3 or 6 h. Values are means of three replicates; error bars denote SE (*$P < 0.01$ in Student's *t*-test)
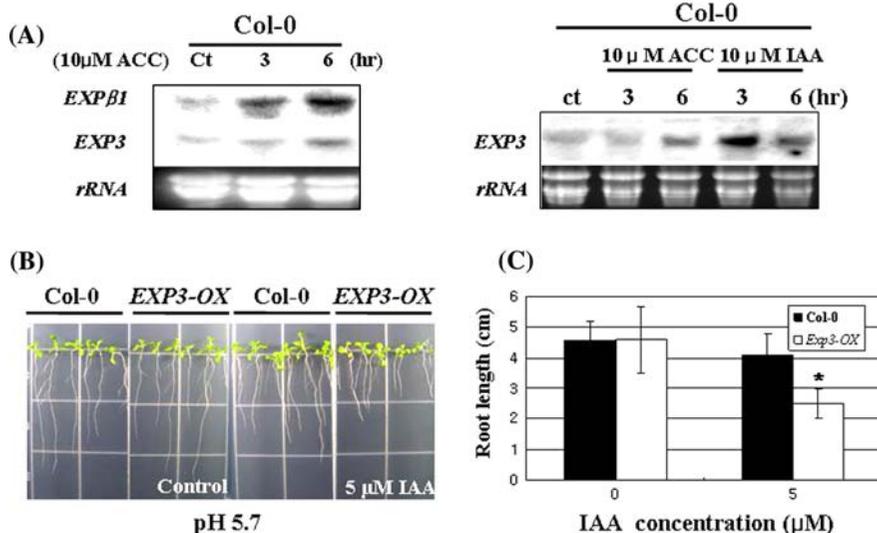

Fig. 5 Auxin and ethylene sensitivity of *AtEXP3* and *AtEXPβ1*. (**a**) Northern blot analysis of wild-type expansin genes induced by IAA (indole-3-acetic acid) and ACC (1-aminocyclopropane-1-carboxylic acid). The two-week-old wild-type (Col-0) seedlings were incubated in media containing ACC or IAA for 3 or 6 h. Each lane contains approximately 20 µg total RNA. Ct, untreated control. Total RNA was used as a loading control. (**b**) Growth performance of wild-type (Col-0) and *AtEXP3-OX* in response to IAA (left panel) or wild-type (Col-0), *AtEXP3-OX* and *AtEXPβ1-OX* to pH 5.0 (right panel). After 3 days of stratification at 4°C, seeds were germinated on standard MS agar supplemented with 2% sucrose and transfer to the test media after 5 days and allowed to grow for 10 more days. Twenty seedlings were included in every analysis. (C) Root lengths of auxin-treated 10-day old seedlings. Values are means of three replicates ($N = 20$ seedlings/assay); error bars denote SE (*$P < 0.05$ in Student's *t*-test)

IAA in wild type seedlings (data not shown). We then examined whether or not these transgenic plants exhibited different responses to phyto-hormones or various pH and found distinct phenotypic changes to some treatments. For example, the *AtEXP3-OX* seedlings were sensitive to auxin and *AtEXPβ1-OX*
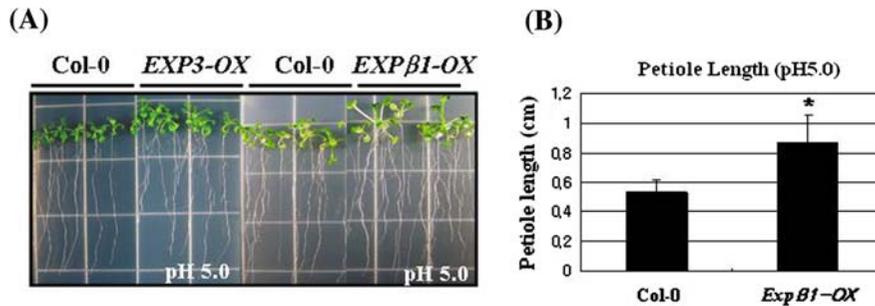
Fig. 6 pH sensitivity of *AtEXPβ1-OX* plants. (**a**) Phenotypic alterations of *AtEXP3-OX* and *AtEXPβ1-OX* in response to pH 5.0. Five-day-old seedlings of wild-type, *AtEXP3-OX* and *AtEXPβ1-OX* were transferred to MS media (pH 5.0) and allowed to grow for 1 week. (**b**) Root lengths of auxin-treated 10-day old seedlings. Values are means of three replicates $N = 20$ seedlings/assay); error bars denote SE (*$P < 0.01$ in Student's *t*-test)

seedlings exhibited elongated petiole growth in MS medium adjusted to pH 5.0 (Figs. 5 and 6).

Discussion

Currently, there is substantial interest worldwide in developing ethanol as biofuel and thus, ethanol production has increased (Ragauskas et al. 2006; Cardona and Sanchez 2007; Service 2007). One strategy for producing cheap biofuel has focused on the conversion of lignocellulosic biomass to ethanol. This conversion employs various steps including saccharification and fermentation, whereby enzymatic hydrolysis of cellulose into glucose is followed by fermentation to ethanol. The great challenge to this process centers upon the removal of lignin from lignocellulosic materials in order to obtain pure cellulose. Expansins could be used to address this problem because they loosen the cross-linking of the cell wall polymeric network. Thus, co-treatment of samples with both expansins and cellulases may lead to substantial improvement in ethanol production. Many trials have concentrated on producing functional expansin proteins in plant systems. However, since expansins are involved in the cell expansion process, their over-expression may cause harmful defects in growth and development. Thus, we used transgenic Arabidopsis that constitutively expressed *AtEXP3* or *AtEXP-β1* under control of *35S-CaMV* promoter, to investigate potential impacts of expansin over-expression on stress. In this study, to scrutinize possible impacts on plant growth and development when these genes are over-expressed, we characterized phenotypic alterations of these transgenic plants in responses to various stimuli.

As reported in previous studies, both *EXP3* and *EXPβ1* genes appear to be involved in cell expansion because transgenic plants with both genes exhibit increased cell elongation (Fig. 1). Since large-scale production of expansin proteins can be achieved in genetically modified plants, the growth performance of field-grown isolates will be of great importance. Both *AtEXP3-OX* and *AtXPβ1-OX* exhibited enhanced growth, next we determined whether *AtEXP3* or *AtEXP-β1* encodes a protein with greater expansin activity when expressed in *Arabidopsis* (Fig. 2). These results clearly indicate that *AtEXP3* and *AtXPβ1* encode functional expansin proteins.

Our results (Fig. 3) suggest that both *AtEXP3-OX* and *AtXPβ1-OX* are very sensitive to salt stress to similar levels. However, the mechanisms underlying their enhanced salt sensitivity appear to be different. For example, although ROS levels are elevated in the *AtEXP3-OX* seedlings, their expression of stress-responsive genes is unaltered (Figs. 3c and 4a). In contrast, *AtEXPβ1-OX* seedlings exhibited slightly reduced expression of the stress-responsive gene (Fig. 4a), but no significant difference in ROS levels compared to wild-type plants. The measurement of peroxidase activity indicates that both *AtEXP3-OX* and *AtXPβ1-OX* plants are under stressful conditions at normal growth environment, and may be vulnerable to abiotic stresses (Fig. 4b).

Differences in cellular responses of *AtEXP3-OX* and *AtXPβ1-OX* plants to salt stress may be originated from their differentiated roles, because we observed that these two expansins exhibited somewhat different

responses to the various growth conditions tested. This suggestion is supported by the findings that wild-type *AtEXP3* or *AtEXP-β1* genes exhibit distinct responses to different hormones (Fig. 5). For example, *AtEXP3-OX* seedlings showed increased sensitivity to auxin, whereas this phenomenon was not observed in *AtEXPβ1-OX*. Although *AtEXP3* and *AtEXPβ1* were induced by ethylene, both *AtEXP3-OX* and *AtXPβ1-OX* did not show any significant phenotypic alterations to this hormone (data not shown). We also found that *AtEXPβ1-OX* seedlings produced elongated petioles when grown in the media adjusted to pH 5.0 and soil (Fig. 5 and Fig. 1), but that the *AtEXP3-OX* did not. These results suggest that the individual expansin proteins may perform distinct cellular functions, leading to the varied sensitivity of the transgenic plants to environmental stimuli.

Since both expansin over-expressing plants exhibited increased levels of expansin activity in conjunction with sensitivity to salt stress, their potential for commercial production will require further investigation. However, it appears that the *AtEXP3* would be a better choice because its transgenic plants grow much better when compared to the wild type and *AtEXPβ1-OX* plants. Alternatively, it is possible that transgenic plants expressing other expansins may not demonstrate similar salt stress sensitivity.

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