

RhEXPA4, a rose expansin gene, modulates leaf growth and confers drought and salt tolerance to *Arabidopsis*

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Abstract Drought and high salinity are major environmental conditions limiting plant growth and development. Expansin is a cell-wall-loosening protein known to disrupt hydrogen bonds between xyloglucan and cellulose microfibrils. The expression of expansin increases in plants under various abiotic stresses, and plays an important role in adaptation to these stresses. We aimed to investigate the role of the *RhEXPA4*, a rose expansin gene, in response to abiotic stresses through its overexpression analysis in *Arabidopsis*. In transgenic *Arabidopsis* harboring the *PRO_{RhEXPA4}::GUS* construct, *RhEXPA4* promoter activity was induced by abscisic acid (ABA), drought and salt, particularly in zones of active growth. Transgenic lines with higher *RhEXPA4* level developed compact phenotypes with shorter stems, curly leaves and compact inflorescences, while the lines with relatively lower *RhEXPA4* expression showed normal phenotypes, similar to the wild type (WT). The germination percentage of transgenic *Arabidopsis* seeds was higher than that of WT seeds under salt stress and ABA treatments. Transgenic plants showed enhanced tolerance to drought and salt stresses: they displayed higher survival rates after drought, and exhibited more lateral roots and higher content of leaf chlorophyll *a* under salt stress. Moreover, high-level *RhEXPA4* overexpressors have multiple modifications in leaf blade

epidermal structure, such as smaller, compact cells, fewer stomata and midvein vascular patterning in leaves, which provides them with more tolerance to abiotic stresses compared to mild overexpressors and the WT. Collectively, our results suggest that *RhEXPA4*, a cell-wall-loosening protein, confers tolerance to abiotic stresses through modifying cell expansion and plant development in *Arabidopsis*.

Keywords Abiotic stress · Abscisic acid · *Arabidopsis* · Rose *RhEXPA4*

Abbreviations

ABA	Abscisic acid
GUS	β-Glucuronidase
MDA	Malondialdehyde
MS	Murashige and Skoog
RT-PCR	Reverse transcription polymerase chain reaction
RWC	Relative water content
SEM	Scanning electron microscopy
WT	Wild type

Introduction

Plants are subjected to environmental stresses, such as drought and salt, over their whole life span, which results in an increased level of the stress hormone abscisic acid (ABA) and consequent retardation of plant growth and development (Kasuga et al. 1999; Wang et al. 2003). Plants have developed a series of complex mechanisms in order to acclimatize to such adverse environmental stresses. These

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inducible adaptation processes have evolved throughout the plant kingdom, and are essential for survival. Stress signal transduction leads to various physiological and metabolic responses, including stress responsive gene expression. Cell-wall modification is also a vital aspect of plant acclimatization to environmental stresses. Cell-wall architecture changes mediated by various cell-wall-modifying proteins, including expansins, assist a plant in adjusting to environmental changes by regulating growth (Sasidharan et al. 2011).

Expansins are cell-wall-loosening proteins, and include α -expansin, β -expansin, and expansin-like A and expansin-like B subfamilies (Sampedro and Cosgrove 2005). Expansins are generally considered to act through a non-enzymatic mechanism, with expansins specifically responsible for disrupting non-covalent binding and dissociating a polysaccharide complex that links microfibrils together (McQueen-Mason and Cosgrove 1994, 1995). Various expansin proteins have been identified in different species, and are involved in a variety of developmental processes, resulting in cell-wall modification (Cosgrove 2000). For instance, they have been found to play an essential role in root growth (Soltys et al. 2012), internode elongation (Choi et al. 2003), leaf development (Pien et al. 2001; Sloan et al. 2009) and floral development (Zenoni et al. 2004, 2011). In ornamental plants, α - and β -expansins have a role in opening and senescence of *Mirabilis jalapa* flowers (Gookin et al. 2003). Two expansin genes, *DcEXPA1* and *DcEXPA2*, are associated with petal growth and development during carnation flower opening (Harada et al. 2011). In roses, *RhEXPA1*, one of three α -expansin genes (*RhEXPA1–RhEXPA3*) is mainly involved in the petal expansion (Takahashi et al. 2007; Yamada et al. 2009).

There is increased evidence for the essential role of expansins in different response mechanisms of various plant species to environmental stresses. The expression level of expansin proteins in different plant species is up-regulated by heat shock, shade stress, anaerobic conditions and pathogen infection (Colmer et al. 2004; Xu et al. 2007; Fudali et al. 2008; Sasidharan et al. 2008; Zhao et al. 2012). Under drought stress, expansins are also strongly regulated at the transcriptional level in maize (Wu and Cosgrove 2000), wheat (Xing et al. 2009) and in the resurrection plant *Cratogeomys plantagineum* (Jones and McQueen-Mason 2004). This upregulation was considered as a preliminary step for drought acclimation through adjusting cell-wall extensibility (Harb et al. 2010). The overexpression of a wheat expansin gene, *TaEXPB23*, which increases the flexibility of cell walls, improves drought tolerance in tobacco (Li et al. 2011). Recently, expansins have also been reported to be involved in the response to salt stress. β -expansin transcript abundance was induced in *Sorghum bicolor* under saline conditions (Buchanan et al. 2005). β -expansin protein levels were

higher in a salt-resistant maize cultivar than the levels in a sensitive cultivar (Geilfus et al. 2010, 2011). ABA acts as a signaling component in mediating plant adaptive responses to drought and salt stresses. ABA has been considered not to change the expression of expansin genes (Wu et al. 2001; Vreeburg et al. 2005), but the recent evidence showed that ABA enhanced wheat coleoptile growth mainly by increasing expansin expression during osmotic stress (Zhao et al. 2012). Thus, it remains unclear how expansin improves plant adaptive response to ABA-mediated drought or salt stress.

In our previous work, we observed drought tolerance of *RhEXPA4*-overexpressing *Arabidopsis* with normal phenotypes (Dai et al. 2012). We noticed that high-level *RhEXPA4* overexpression resulted in abnormal phenotypes of transgenic plants. In this study, we further characterized the *RhEXPA4* function by investigating the activity of the *RhEXPA4* promoter and its possible roles in conferring abiotic stress tolerances to transgenic *Arabidopsis*. Observations showed that high-level *RhEXPA4*-overexpressed *Arabidopsis* plants displayed a characteristic phenotype, with smaller rosette size, shorter leaf petioles, and curly leaves. Under drought, high salt stress and ABA treatment, transgenic plants displayed enhanced tolerance during vegetative developmental stages, both in soil and culture medium.

Materials and methods

Plant material and growth conditions

Sterilized *Arabidopsis thaliana* L. (Columbia ecotype) seeds (obtained from the Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH, USA) were sown on Murashige and Skoog (MS) medium containing 1 % sucrose. After stratification for 3 days at 4 °C, the plates were transferred to a growth room at 22 °C, 16/8 h light/dark photoperiod and a light intensity of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For phenotypic analysis of young plants, 8-day-old seedlings were transplanted into soil (rich soil:vermiculite, 1:1, w/w) and grown to maturity. All the transgenic plants were generated with floral-dip procedures using *Agrobacterium tumefaciens* strain GV3101 (Clough and Bent 1998). Green kanamycin-resistant *Arabidopsis* seedlings were selected, planted in soil and grown in a greenhouse. Seeds of T2 homozygotes were collected at maturity and stored at room temperature.

Semi-quantitative RT-PCR

To determine the expression levels of *RhEXPA4* transgenic *Arabidopsis* plants, we extracted total RNA from leaves

collected from 4-week-old transgenic plants (T2 generation), and wild-type (WT) plants, using the Trizol agent (Invitrogen, Carlsbad, CA, USA). cDNAs were synthesized from 1 µg total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). The relative expression levels of *RhEXPA4* were analyzed using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). The PCR was performed with 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The following gene-specific primers were used: *RhEXPA4-F* (5'-ATGA AAATGGCTTCTGCTGTGTT-3') and *RhEXPA4-R* (5'-TAGCGGAACTGGGCACCA-3'); while *Actin-F* (5'-CA TCCTCCGCTTGACCTTGC-3') and *Actin-R* (5'-TGG ACCTGCCATCATACTCG-3') were also used to amplify the *Actin* gene as an internal control.

Detection of expansin activity

Extraction of cell-wall proteins and detection of expansin activity were carried out according to the methods reported by McQueen-Mason et al. (1992) with some modifications. For extraction of cell wall protein, tissues were ground into a fine powder in liquid N₂ and was added to homogenization buffer [20 mM sodium acetate, pH 4.5, 2 mM disodium ethylenediaminetetra acetate (EDTA)]. Wall material was collected on a nylon screen (70 µm mesh) and suspended for 12 h on ice in extraction buffer (1 M NaCl, 25 mM Hepes, pH 7.0, 3 mM sodium metabisulfite, 2 mM EDTA, 5 mM dithiothreitol). The proteins in supernatant were slowly precipitated with 0.39 g of ammonium sulfate per milliliter for 1 h. Precipitated proteins were pelleted by centrifugation (12,000g, 15 min, 4 °C) and subsequently re-suspended in sodium acetate (50 mM, pH 4.5). The protein concentration was adjusted to 1 mg ml⁻¹. Expansin activity was assayed by measuring the increase in extension rate of wheat coleoptiles after addition of the extract. All activity detection was replicated three times.

Construction of plant expression vectors and *Arabidopsis* transformation

For the *ProRhEXPA4::GUS* fusion construct, a 1,613 bp DNA sequence upstream of the ATG of *RhEXPA4* gene was amplified by PCR and inserted in front of the β-glucuronidase (*GUS*) reporter gene using pBI121 vector.

Histochemical staining and quantitative assay of GUS activity

Plants expressing the *ProRhEXPA4::GUS* construct were treated with distilled water for 3 h, 75 mM NaCl for 2 h, dehydration for 30 min, or 50 µM ABA for 3 h, then the samples were subjected to histochemical GUS staining and

quantitative assay as described by Jefferson (1987). All experiments were performed independently three times.

Drought, salt stresses and ABA treatments of transgenic *Arabidopsis*

The growth conditions of T3 transgenic and control *Arabidopsis* plants were the same as described above. For germination analysis, ca. 100 seeds from each line (WT, vector, L4, L13, L15, L22) were sown on MS medium containing 0, 0.2, 0.8 µM ABA or 100 mM NaCl, respectively. The germination percentage was determined at the indicated time. To measure root growth, the *Arabidopsis* seeds were germinated on MS agar for 7 days, and the seedlings were transferred to fresh medium containing 0, 20, 60 µM ABA or 100, 150 mM NaCl. The plates were positioned vertically on shelves to assist the evaluation of root growth rates. For the drought–recovery experiments, 8-day-old seedlings were transplanted into a 7-cm pot filled with 100 g substrate and grown for 14 days. The plants were well watered, and then held in dry conditions for an additional 16 days. The plants were then rewatered regularly as a recovery process. The survival rates were recorded after a 15-day recovery period. All experiments were performed independently three times.

Measurements of relative water content, malondialdehyde content, relative electrolyte leakage and chlorophyll content

Relative water content (RWC) was measured as follows: the shoot of a stressed plant was detached from its root, immediately weighed (fresh weight, FW), then the samples were immediately hydrated by floating on deionized water to full turgidity for 3–4 h (45–50 % relative humidity, 25 °C). After hydration, the samples were removed from the water and, after any surface moisture had been dried quickly and lightly with filter/tissue paper, were immediately weighed to obtain fully turgid weight (TW). Samples were then oven-dried to a constant dry weight (DW) at 80 °C for 24 h. RWC was evaluated according to the formula: $RWC (\%) = (FW - DW)/(TW - DW) \times 100$.

Malondialdehyde (MDA) content and relative electrolyte leakages were determined according to the methods reported by Zhang et al. (2012). Chlorophyll content was measured according to Arnon's (1949) method using methanolic extracts from leaves.

Microscopic analysis

Rosette leaves of 6-week-old WT, *RhEXPA4*-transgenic lines (L4 and L22) were examined by scanning electron microscopy (SEM). Samples of small leaves were taken and

immediately placed into a fixative mixture of 2.5 % glutaraldehyde with phosphate buffer (pH 7.2) for 3 days, as described by Lü et al. (2010) with slight modification. The samples were dehydrated in a graded ethanol series. Explants were dried by CO₂ critical point drying and coated with gold. The adaxial and abaxial epidermal surfaces were examined using a Hitachi SEM (HITACHI S-3400N, Japan) and photographed.

Light microscopy analysis of the leaf blade and stem anatomical structure was performed as described previously (Myers et al. 2011). Explants were fixed initially in formalin/acetic acid/alcohol (1:1:18, by volume). Paraffin embedding was used to prepare permanent tissue sections. Microtome sections (thickness, 10 µm) were stained with Safranin and Fast Green and further examined under a Nikon (AFX-IIA) photomicroscope.

Statistical analysis

All measurements were replicated as mentioned in each section. Statistical analysis was conducted using the procedures of SPSS statistics 17.0. Statistical significance was tested using Duncan's test at 0.05 probability levels.

Results

Analysis of structure and activity of *RhEXPA4* promoter

We isolated the 1,613 bp promoter region of the *RhEXPA4* gene (JN903506) and evaluated its putative *cis*-acting regulatory elements using the PLACE program (Higo et al. 1999). A number of *cis*-elements in the promoter of *RhEXPA4* were observed and annotated subsequently. The *cis*-elements identified include salt-, dehydration- and ABA-responsive and tissue-specific elements (Fig. 1a; Supplementary Fig. S1). Among these *cis*-elements, five G T-1 *cis*-elements, located at positions –151/–146, –442/–437, –630/–625, –1222/–1217 and –1540/–1535, play a pivotal role in salt-induced *SCaM-4* gene expression (Park et al. 2004), and one ABRE-related sequence motif (MACGYGB), located at position –268/–262, was found to be essential for ABA response (Kaplan et al. 2006). For the dehydration response, nine MYC (CANNTG) and four MYB motifs (WAA CCA), sited at positions –173/–168, –271/–266, –326/–321, –360/–355, –378/–373, –602/–597, –864/–859, –887/–882, –923/–918, –1338/–1333, –1390/–1385, –1476/–1471 and –1519/–1514, respectively, were previously identified in *Arabidopsis* (Abe et al. 2003; Chakravarthy et al. 2003). We also found four putative GATA boxes situated at –397/–394, –467/–464, –873/–870 and –1008/–1005 locations, respectively (Fig. 1a; Supplementary Fig. S1); these

were considered to be involved in light regulation and tissue-specific expression (Reyes et al. 2004).

Our previous results indicated that *RhEXPA4* is involved in dehydration tolerance in rose petals (Dai et al. 2012). To assess the activity of the *RhEXPA4* promoter during plant development and abiotic stress, we investigated the spatial and temporal expression patterns conferred by the *RhEXPA4* promoter region. In this regard, we have obtained 17 independent transgenic *Arabidopsis* homozygous lines harboring the *ProRhEXPA4::GUS* cassette. GUS activity was detected in the transgenic *Arabidopsis* throughout its life cycle using histochemical staining (Fig. 1b). GUS activity was clearly detectable 5 days after germination (Fig. 1b-1). The expression was also observed in different vegetative tissues, including hypocotyls, stems and leaves until the third week of germination (Fig. 1b-2). Cotyledons, as well as young and mature leaves, were strongly stained in all the analyzed lines, with a continuous decline of GUS expression, while the leaves were maturing (Fig. 1b-1, 2, 5). Roots exhibited strong staining in the lateral root primordia (Fig. 1b-4). Moreover, GUS activity was also detected in reproductive organs, such as silique ends (Fig. 1b-3) and floral organs (Fig. 1b-6). The results indicate that the *RhEXPA4* gene is expressed almost ubiquitously in various plant organs, especially in periods of organ development.

In examining if the *RhEXPA4* promoter is inducible by ABA, dehydration or NaCl, we observed GUS expression in transgenic plants subjected to ABA, dehydration or NaCl. *RhEXPA4* promoter activity was strongly induced by ABA, dehydration and salt in comparison to untreated *ProRhEXPA4::GUS* plants (Fig. 1c). In a word, the *RhEXPA4* promoter could be recognized in *Arabidopsis* plants, and its expression is strongly induced by ABA, dehydration and salt treatment.

High-level overexpression of *RhEXPA4* in *Arabidopsis* resulted in plant morphological and reproductive abnormalities

To examine the function of *RhEXPA4*, we obtained transgenic *Arabidopsis* lines harboring *35S::RhEXPA4*. The expression of *RhEXPA4* genes in all transgenic lines were monitored by semi-quantitative RT-PCR. As depicted in Fig. 2a, the expression level of *RhEXPA4* can generally be categorized into three distinct groups: transgenic lines L15, L16 and L22 showed stronger expression, and are defined as high-level overexpressors; L4 and L13 are mild overexpressors; and L5 and L9 as low expressors. Transgenic lines L4, L13, L15 and L22 were further chosen for subsequent analysis based on the *RhEXPA4* expression level. Expansin activities of cell-wall protein extracts from growing leaves of selected lines were measured. Extracts from growing leaves of WT and control plants showed a

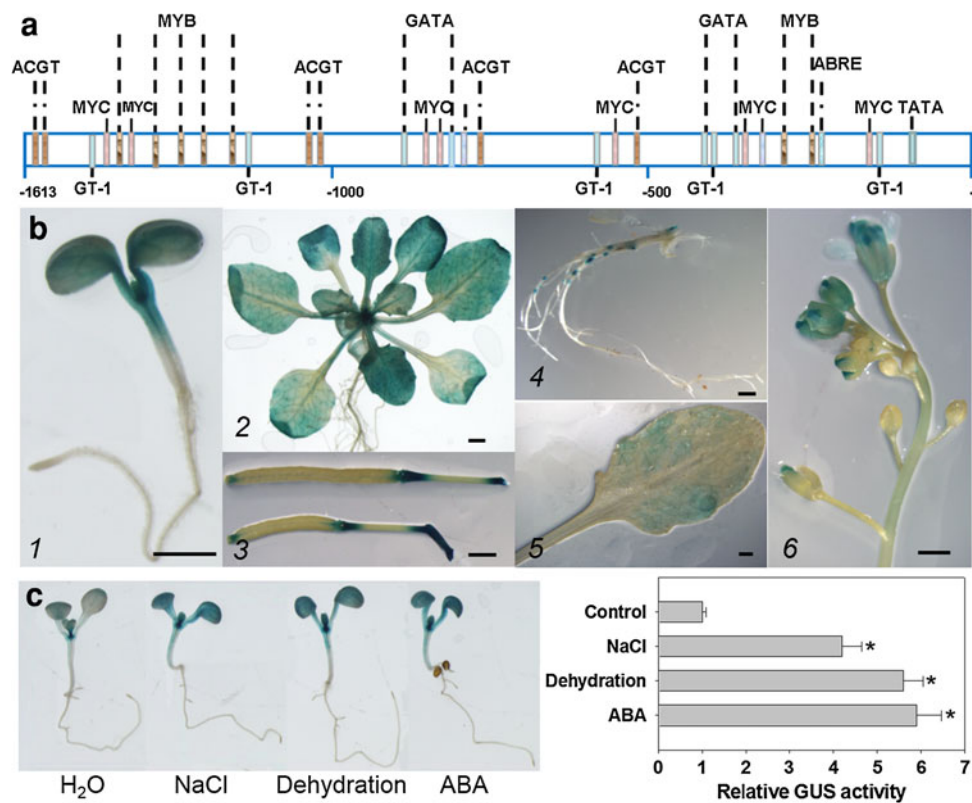


Fig. 1 The activity of *RhEXPA4* promoter. **a** Pictorial representation of *RhEXPA4* promoter region with potential *cis*-elements binding sites. **b** Histochemical localization of GUS expression driven by the *RhEXPA4* promoter in transgenic *Arabidopsis*. 1 5-day-old *Arabidopsis* seedling, 2 shoot from 3-week-old plants, 3 mature siliques, 4 root from 3-week-old plant, 5 rosette leaf from 4-week-old plant, 6 flowers, bar 1 mm. **c** Histochemical analysis of *Pro_{RhEXPA4}::GUS*

transgenic *Arabidopsis* under NaCl, dehydration and ABA treatments. 6-day-old plants were treated with distilled water for 3 h, 75 mM NaCl for 2 h, dehydration treatment for 30 min or 50 μM ABA for 3 h before being subjected to histochemical analysis. The relative GUS activity is shown in the *right hand panel*. Data are the mean of eight individual plants (mean ± SD, *n* = 8). Asterisk indicate significant difference from the control at *P* < 0.05

basal level of expansin activity. However, expansin activities were obviously higher in all transgenic plants than in the WT and control plants, consistent with the *RhEXPA4* expression (Fig. 2d).

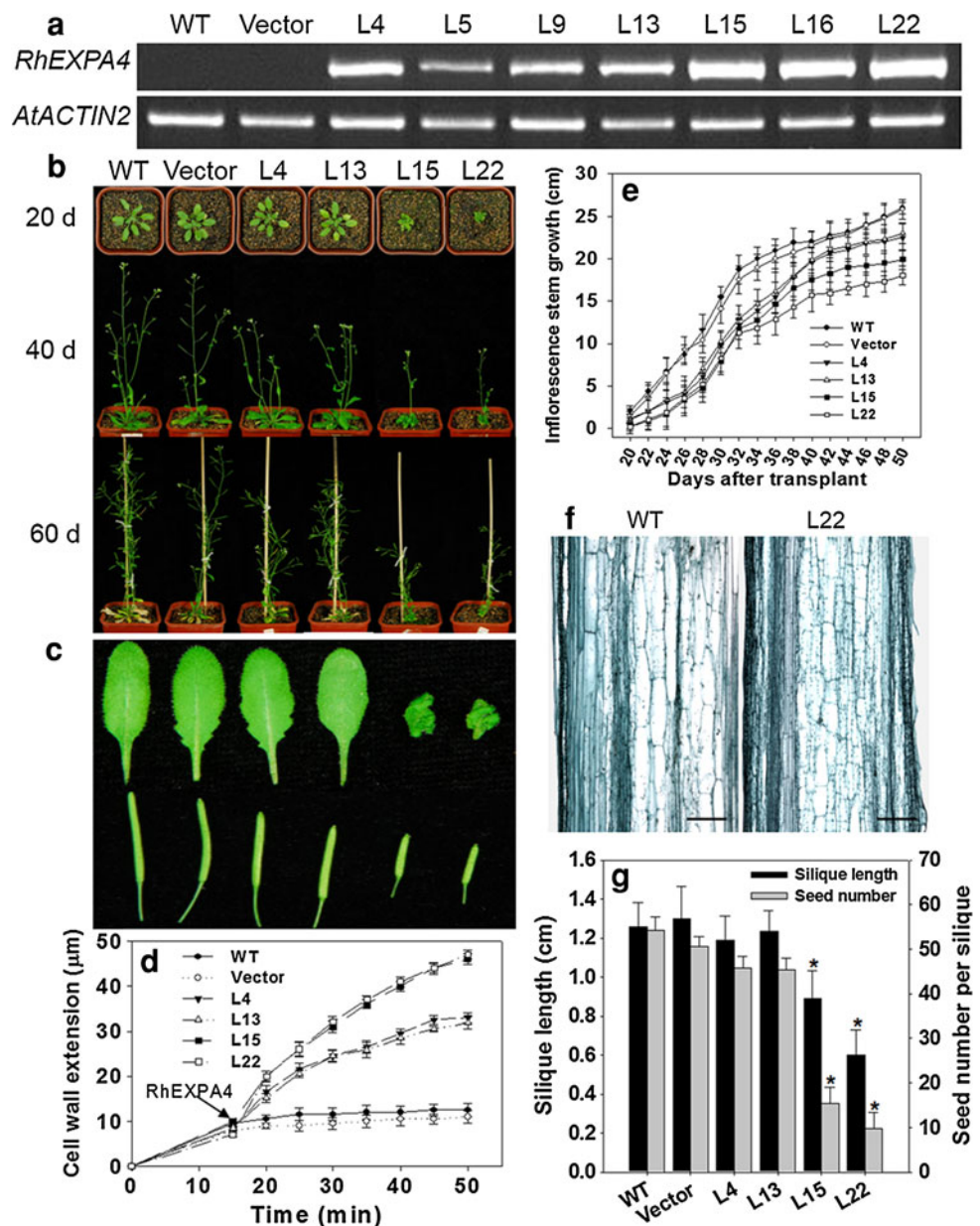
A diverse range of phenotypic characteristics was observed among different transgenic lines due to variation in expression levels of *RhEXPA4*. The mild overexpressors, L4 and L13, did not exhibited obvious changes in leaf shape under normal growth condition when compared with WT and vector plants, whereas high-level overexpressors showed compact plant phenotypes with smaller rosette size, shorter leaf petioles and curly leaves (Fig. 2b, c). The number of rosette leaves and seedling age when flower buds appeared was similar among WT and transgenic plants (data not shown). The height of transgenic plants showed ca. 60–80 % reduction as compared to the WT (Fig. 2b), with a slower stem elongation rate than in control plants (Fig. 2e). The difference in stem and internode length between WT and transgenic plants was probably due to slower longitudinal enlargement of cells (Fig. 2f). *RhEXPA4* overexpression at high levels in planta affected

fertility, resulting in a reduced number of inflorescences and flowers. High-level *RhEXPA4* expression in *Arabidopsis* causes approximately 80 % loss in seed production (Fig. 2c, g). This low productivity was also observed with some other functional proteins when they were overexpressed under the control of the 35S promoter (Xing et al. 2007; Lin et al. 2009).

RhEXPA4 overexpression confers enhanced drought, salt tolerance and ABA insensitivity in transgenic *Arabidopsis*

In the *RhEXPA4* promoter region, there is a CBF/ABRE binding *cis*-element (CACGCGC) and several GT-1 boxes (GAAAAA) (Fig. 1a; Supplementary Fig. S1), suggesting that *RhEXPA4* might be involved in ABA pathway and salt stress tolerance. First, we evaluated the effect of *RhEXPA4* on seed germination in transgenic *Arabidopsis* under ABA and high salt. More than 95 % germination rate was recorded for seeds sown on control MS medium. When exposed to 0.8 μM ABA and 100 mM NaCl for 7 days, the

Fig. 2 Morphological phenotypes of *35S::RhEXPA4* overexpressing plants. **a** *RhEXPA4* expression analysis in *Arabidopsis* transgenic lines using RT-PCR. **b** *RhEXPA4* overexpressors at different stages under normal growth conditions. The pictures were taken 20, 40 and 60 days after sowing. **c** Representative rosette leaf and silique. **d** Expansin activities of different transgenic lines and WT. *Arrows* indicate when the sodium acetate buffer (50 mM, pH 4.5) was switched to the extract. **e** Inflorescence elongation (mean \pm SE, $n = 8$ –12). **f** *Longitudinal sections* of inflorescence stems for WT and L22. *bar* 100 μ m. **g** Silique length and seed numbers. The fourth and fifth siliques of each plant were measured with a ruler (mean \pm SE, $n = 20$). Seeds for each silique were counted ($n = 20$). *Asterisk* indicate significant difference from the WT at $P < 0.05$



germination rate of WT and vector seeds decreased by more than 98 %, and the germination rate of transgenic seeds was significantly higher than that of the WT and vector seeds under these treatments (Fig. 3).

Because primary root growth is an important indicator of plant tolerance to stresses, we also observed primary root growth of transgenic seedlings under ABA and high-salt treatments. On control MS medium, primary root growth was similar for L4, L13, WT and the vector plants, but roots in the high-level overexpressors, L15 and L22, were ca. 30 % shorter than that of the WT and vector plants (Fig. 4). Exogenous ABA or NaCl treatments inhibited primary root growth of 7-day-old *Arabidopsis* seedlings, and the relative primary root length of the control and mild

overexpressors, L4 and L13, were ca. 70 and 60 % under 20 μ M ABA and 100 mM NaCl, respectively (Fig. 4). However, high-level overexpressors L15 and L22 still had more than 90 % relative primary root growth under ABA and NaCl treatment. The relative primary root growth of high-level overexpressors was still significantly higher than other lines when subjected to more severe stress conditions (Fig. 4). We also compared the lateral root growth and leaf chlorophyll content between transgenic and control plants under salt stress. The number of lateral roots per centimeter of primary root in transgenic plants was significantly higher than that of the control at 100 and 150 mM NaCl (Fig. 4b). In addition, transgenic plants showed significantly higher chlorophyll content in contrast to the control

Fig. 3 Seed germination rate of *RhEXPA4* overexpressors under ABA and NaCl stresses. Transgenic lines L4, L13, L15, L22, WT and vector plants grew on MS medium supplemented with 0, 0.2, 0.8 mM ABA or 100 mM NaCl, respectively, for 15 days after sowing. Three independent experiments were performed. 100 seeds were sown in each plate for each experiment

	ABA (μM)			NaCl (mM)	seed germination greening ratio (%)			
	0	0.2	0.8	100	0	0.2	0.8	100 mM
WT					99.1% (107/108)	83.3% (90/108)	0.9% (1/108)	0% (0/108)
Vector					98.1% (106/108)	80.6% (87/108)	0 (0/108)	0 (0/108)
L4					100% (108/108)	90.7% (98/108)	5.6% (6/108)	1.9% (2/108)
L13					98.1% (106/108)	91.7% (99/108)	4.6% (5/108)	0.9% (1/108)
L15					99.1% (107/108)	96.3% (104/108)	17.6% (19/108)	16.7% (18/108)
L22					99.1% (107/108)	97.2% (105/108)	66.7% (72/108)	63% (68/108)

under both normal growth condition and salt stress (Fig. 4b).

Increasing evidence has indicated that transgenic plants overexpressing expansin genes show improved water stress tolerance (Li et al. 2011; Han et al. 2012). Our preliminary results also showed that mild overexpressors L4 and L13 had increased the water stress tolerance in *Arabidopsis* (Dai et al. 2012). Here, we further compared the effect of drought stress on survival rate of *RhEXPA4* overexpressors, especially high-level overexpressors. A 2-week-old transgenic and WT plants were subjected to drought, which generally results in reduced plant growth rate. After 16 days of drought, leaves of WT plants became wilted, whereas transgenic plants, especially high-level overexpressors, displayed only slight wilting (Fig. 5). To examine the survival rates of transgenic plants under severe water deficit condition, the plants with severe visible damage were rewatered after 21 days drought. After a 15-day recovery period, almost all high-level overexpressor plants survived, while ca. 56.8 and 29.3 %, respectively, of L4 and L13 (mild overexpressors) survived, as compared to ca. 10 % in control plants (Fig. 5). In addition, leaves of WT plants showed visible damage, while transgenic plants remained healthy. The improved drought tolerance of the *RhEXPA4* transgenic plants was also correlated with changes in the RWC, MDA contents and electrolyte leakage levels of these plants. As shown in Fig. 5, when the plants were exposed to drought stress, the RWC was

significantly higher in the *RhEXPA4* transgenic plants than in the control plants, whereas the MDA contents and electrolyte leakage levels were lower in the *RhEXPA4* transgenic plants than in the control plants. These results indicate that *RhEXPA4* overexpression could confer salt and drought stress tolerance and decrease ABA sensitivity in *Arabidopsis*; in particular, high-level overexpressors showed more tolerance during earlier seed germination and later developmental stages.

Microscopic analysis of high-level overexpressors displays multiple modifications in blade epidermal structure

To investigate the potential mechanism of *RhEXPA4* conferring tolerance of abiotic stresses in transgenic *Arabidopsis*, we examined the leaf blade epidermal structure of high-level overexpressors by SEM. When compared with adaxial and abaxial leaf epidermal cells of the WT (Fig. 6a-1, 2) and mild overexpressors (Fig. 6a-3, 4), the leaf epidermal cells in high-level overexpressors (Fig. 6a-5, 6) appear to be smaller and compact. The number of pavement cells per $250 \times 195 \mu\text{m}$ leaf area at the abaxial epidermis of the high-level overexpressor blades was about 35 % more than in the WT (Fig. 6b). The smaller size of these pavement cells may suggest a major defect in cell expansion in high-level *RhEXPA4* overexpressors. Under SEM, we also noticed that *RhEXPA4* altered stomatal

Fig. 4 ABA sensitivity and salt tolerance of *RhEXPA4*-overexpressing seedlings. **a** Root growth of *RhEXPA4*-overexpressors under ABA stresses, bar 1 cm. The relative root length is shown in the right hand panel. **b** Plants of *RhEXPA4* overexpressors under salt stress. 7-day-old seedlings grown on MS medium were transferred to MS medium containing 0, 20, 60 mM ABA or 100, 150 mM NaCl. The relative root length, lateral roots density and contents of chlorophyll *a* are shown in right hand panel. The photos and measurements were taken 7 days after transfer. Asterisk significant difference from the WT at $P < 0.05$

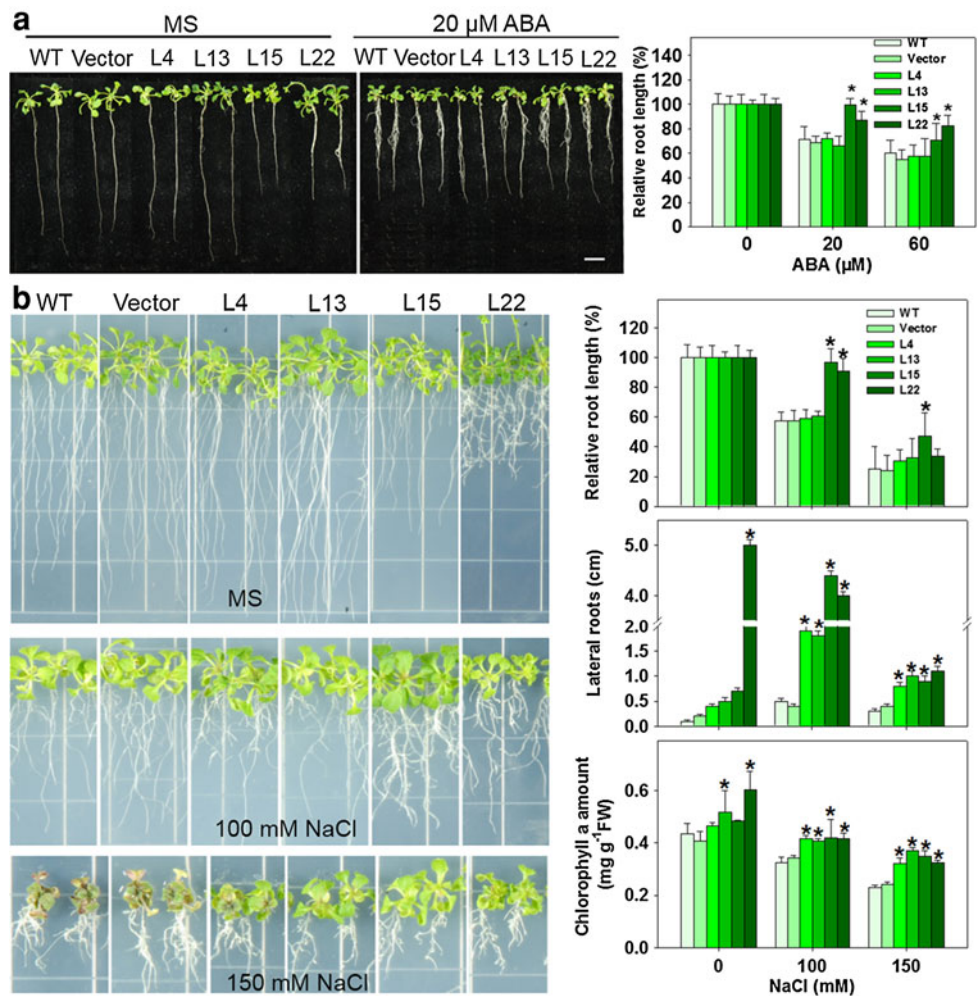
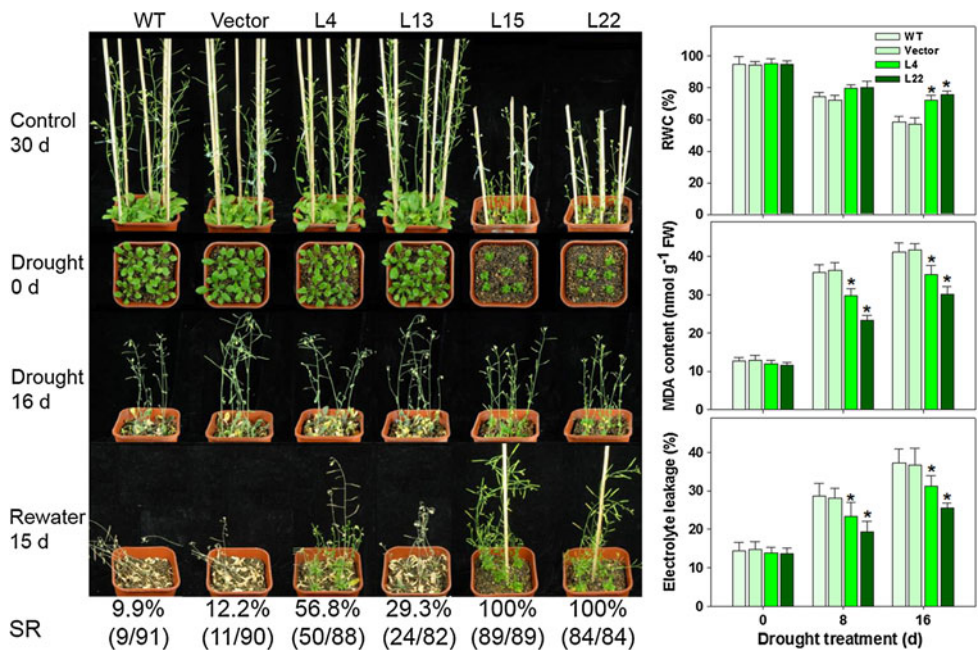


Fig. 5 Tolerance of *RhEXPA4*-overexpressing *Arabidopsis* to drought. T3 homozygous transformants were used in this experiment. Control 30 days, 30-day-old plants growing under normal watering condition; drought 0 day, 14-day-old well-watered plants; drought 16 days, 16 days after withholding water; rewater 15 days, 15 days after rewatering; SR, survival rate. The RWC, MDA content and relative electrolyte leakage are shown in the right hand panel. The data represent the means of three replicates (mean ± SD, $n = 3$). Asterisk significant difference from the WT at $P < 0.05$



development in *Arabidopsis*, resulting in a lower stomatal density. Stomatal precursor cells, such as meristemoids or guard mother cells, were detected in leaves of overexpressed *RhEXPA4* plants, but were almost negligible in control plants, indicating that stomatal cell development may be delayed in transgenic plants (Fig. 6c). These results imply that *RhEXPA4* overexpression affects the enlargement of pavement cells and decreases stomatal density with less leaf transpiration.

Transverse sections of the leaf blade showed that the mesophyll tissue was not well differentiated in high-level overexpressors, especially on the adaxial side. In contrast, palisade mesophyll cells (adaxial side) are uniformly cylindrical, whereas spongy mesophyll cells (abaxial side) are irregular in the WT (Fig. 6d-1). In L22, although cells on either side are not identical, the contrast between palisade and spongy mesophyll cells is inconspicuous (Fig. 6d-2). Transverse sections through the midvein showed that the xylem and phloem cells maintained their relative adaxial and abaxial positions, respectively, but failed to differentiate further (Fig. 6d-3, 4).

Taken together, these results suggested that high-level *RhEXPA4* overexpression in transgenic plants had resulted in multiple modifications in blade epidermal structure including smaller, compact cells, fewer stomata and midvein vascular patterning in leaves, thus contributing to higher abiotic stress tolerance in high-level *RhEXPA4* overexpressors.

Discussion

Expansins are cell-wall proteins that induce cell-wall extension in vitro and cell expansion in vivo by disrupting hydrogen bonds between cellulose microfibrils and xyloglucan (matrix polymers), thus enhancing the plasticity of the cell wall (Sampedro and Cosgrove 2005). There is increasing evidence showing that expansins are involved in stress responses in plants. Expansins are strongly regulated by water stress (Jones and McQueen-Mason 2004; Xing et al. 2009), heat tolerance, shade stress, low oxygen, pathogens, high salinity and ABA also up-regulate the expression of expansins in plants (Colmer et al. 2004; Xu et al. 2007; Fudali et al. 2008; Sasidharan et al. 2008; Geilfus et al. 2010; Zhao et al. 2012). Here, we further characterize the roles of a dehydration-induced expansin gene, *RhEXPA4*, in transgenic *Arabidopsis*.

Expansin activity is generally linked with cell-wall loosening in growing cells (Lee et al. 2001). Localized expression of expansins is normally associated with meristems and growth zones of stem and root (Sampedro and Cosgrove 2005). In this study, histochemical staining of seedlings revealed that *RhEXPA4* was localized within

cotyledons, leaves, roots, silique stems and flowers during seedling development (Fig. 1b). Abiotic stresses and ABA treatment can enhance the expression of the *GUS* reporter gene (Fig. 1c). These results suggest that *RhEXPA4* is expressed in most tissues in *Arabidopsis*, and the *RhEXPA4* gene is responsive to ABA, salt and dehydration.

Transgenic plants overexpressing stress-induced genes exhibit improved tolerance to abiotic stresses, but occasionally show some adverse phenotypic features, such as severe growth retardation and/or problems in seed development (Magome et al. 2008; Kodaira et al. 2011). In our results, the phenotypes of overexpressors depend on expression level of *RhEXPA4* (Fig. 2). *RhEXPA4* overexpression decreased the rate of cell enlargement contributing to shorter stems and siliques and smaller or curly leaves (Fig. 2b, c). Furthermore, high-level *RhEXPA4* overexpressors show more strongly curled leaves and compact plant architecture, and produce fewer seeds compared with mild overexpressors and the WT (Fig. 2b, c, g). However, the timing of plant developmental processes of *RhEXPA4* overexpressors showed no obvious changes. These results indicate that *RhEXPA4* may mainly affect cell enlargement, but not cell differentiation. Silencing of expansin genes is associated with a strong inhibition of growth, whereas overexpression results in faster or abnormal growth (Cho and Cosgrove 2000; Sampedro and Cosgrove 2005). The phenotypes of high-level overexpressors were similar to *AtEXP10* antisense transgenic plants with malformed leaves and small rosette size (Cho and Cosgrove 2000). As excessive ectopic expression of a gene may silence the endogenous expression of its homologous genes (Napoli et al. 1990; Vaucheret et al. 1998), we also wondered if *RhEXPA4* silenced the homologous *Arabidopsis* genes *AtEXP1*, *AtEXP10* and *AtEXP15* according to the phylogenetic analysis of our previous study (Dai et al. 2012). RT-PCR analysis showed that the expression level of these three genes did not display any obvious differences in the high-level overexpressor, L22, as compared to control plants (data not shown). These results indicate that abnormal leaves of *RhEXPA4* overexpressors may result from its action on cell-wall rheology, as reported previously (Cho and Cosgrove 2000), rather than homologous gene silencing in *Arabidopsis*.

An effective strategy for plant drought acclimatization or adaptation is to reduce transpirational water loss. Stomatal closure or lower stomatal density is among the earliest responses to drought stress (Chaves et al. 2003). In this study, *RhEXPA4* overexpression conferred stronger tolerance in the transgenic *Arabidopsis* with higher survival rate, especially in high-level overexpressors under drought conditions (Fig. 5). The enhanced drought tolerance of *35S::RhEXPA4* plants was partially a result of decreased stomatal density. Plants respond to water stress

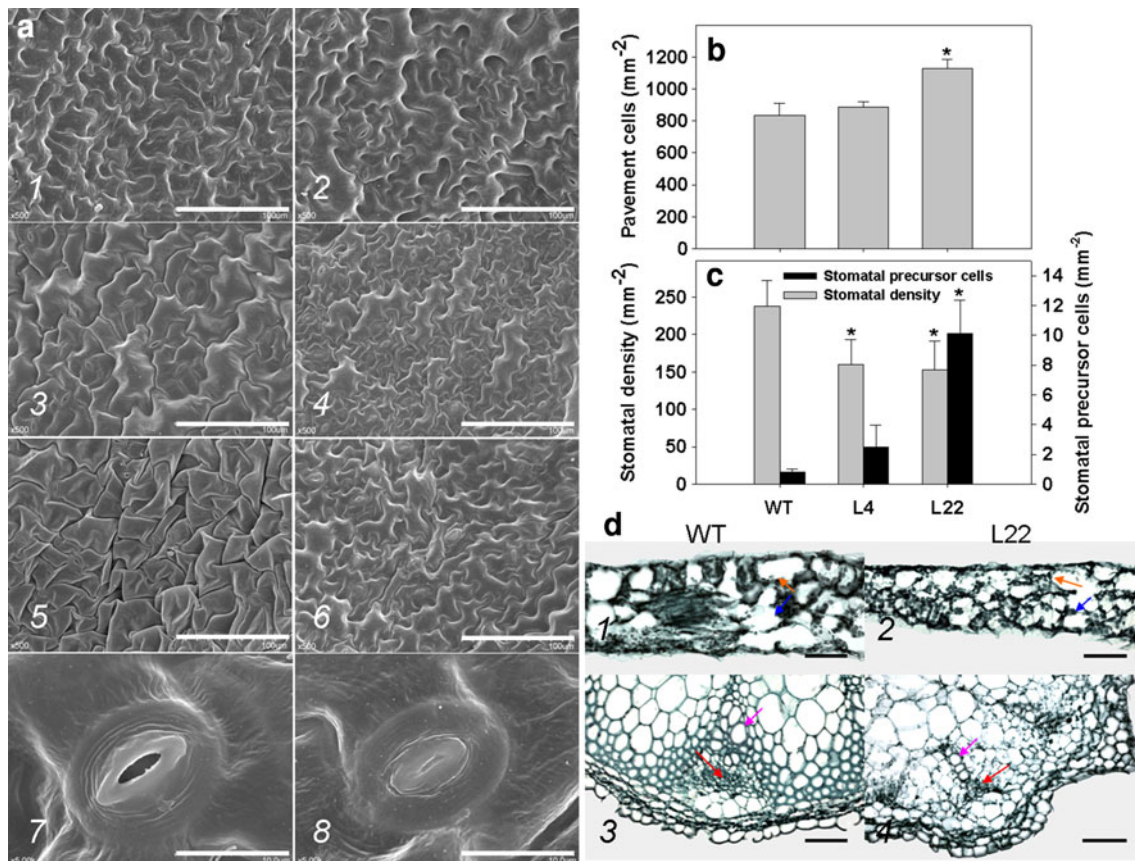


Fig. 6 Effect of *RhEXPA4* overexpression on *Arabidopsis* leaf blade morphological structure. **a** Representative images of leaf adaxial and abaxial epidermal layers from 6-week-old WT (1, 2), L4 (3, 4), L22 (5, 6), bar 100 μm ; functional stoma (7) and stomatal precursor cell (8), bar 10 μm . **b** Pavement cells analyzed in the leaf abaxial epidermal layers of WT, L4 and L22. **c** Stomatal density and the number of stomatal precursor cells. Data are the mean of eight

individual plants (mean \pm SD, $n = 8$). Asterisk significant difference from the WT at $P < 0.05$. **d** Transverse section through WT and L22 leaf blades (1, 2) showing palisade mesophyll (orange arrow) and spongy mesophyll (blue arrow) cells; and inflorescence stems (3, 4) showing xylem (pink arrow) and phloem (red arrow) vessels, bar 50 μm

by decreasing cell enlargement, resulting in the reduction of growth. The overexpression of a wheat expansin protein gene, *TaEXPB23*, improves drought tolerance in tobacco. The transgenic plants showed improved tissue integrity during water stress, suggesting that expansins increase the flexibility and extensibility of the cell wall (Li et al. 2011). The data presented here show that high-level *RhEXPA4* overexpressors displayed smaller rosette size with compact epidermal cells (Figs. 2c, 6a), indicating that *RhEXPA4* improves drought tolerance by modulating leaf growth.

Generally, plants developed salt tolerance by increasing their ability for osmotic adjustment to maintain growth, Na^+ exclusion and compartmentalization of Na^+ and Cl^- at the cellular and intracellular level to avoid toxicity (Munns and Tester 2008). Salinity may affect water uptake, turgor generation and/or cell-wall properties, and therefore restrict cell expansion and root development (Taleisnik et al. 2009). The overexpression of expansins could increase the cell-wall flexibility to avoid stress-caused damages through folding the cell walls (Wu et al. 2001; Li

et al. 2011). In our study, the germination rate of transgenic seeds was significantly higher than that of the WT and vector seeds under salt stress (Fig. 3), indicating that *RhEXPA4* was involved in salt stress tolerance. We also found that *RhEXPA4* transgenic plants had longer primary roots and more lateral roots under salt stress (Fig. 4b). This phenomenon is consistent with the recent findings that *TaEXPB23* may contribute to the maintenance of root elongation under salt stress (Han et al. 2012). These results indicate that *RhEXPA4* may confer this capacity to remodel cell wall composition and to maintain cell wall flexibility in roots under NaCl stress, contributing improved root architecture and salt tolerance in *RhEXPA4* overexpressors.

The plant hormone ABA plays a major role in plant responses to drought and salt stress, which both result in water stress (Zhang et al. 2006). Expression of water-stress-inducible genes is mainly governed by ABA-independent and ABA-dependent pathways (Yamaguchi-Shinozaki and Shinozaki 2005). ABA-responsive element

(ABRE) and dehydration-responsive element/C-Repeat (DRE/CRT) are *cis*-acting elements that function in ABA-dependent and ABA-independent gene expression, respectively, in response to abiotic stress (Yamaguchi-Shinozaki and Shinozaki 1994, 2005). In our study, the promoter of *RhEXPA4* contains one ABRE-related *cis*-element (Fig. 1a; Supplementary Fig. S1). Furthermore, ABA induced the expression of the *GUS* gene in *Pro^{RhEXPA4}::GUS Arabidopsis* (Fig. 1c), suggesting that *RhEXPA4* expression might be involved in an ABA-dependent pathway. However, *RhEXPA4* overexpression in *Arabidopsis* decreased ABA sensitivity with significantly higher seed germination rates (Fig. 3) and more root growth under ABA treatments (Fig. 4a). These results may be due to the modifications in leaf epidermal cells of *RhEXPA4* transgenic plants. *RhEXPA4* overexpression altered stomatal development, resulting in fewer functional stomata and more stomatal precursor cells (Fig. 6c), and thus decreased the sensitivity to exogenous ABA (Figs. 3, 4a).

There are several explanations concerning the mechanism whereby expansin improves stress tolerance in plants. Expansin enhanced plant adaptation to environmental stress through the regulation of cell growth and extension in a pH-dependent manner (Cosgrove et al. 2002). High levels of expansin proteins may acidify the cell wall and promote the growth rate of the stem (Vreeburg et al. 2005), and cell-wall modifying proteins may alter plant organ structures to adapt to various environmental changes by regulating cell-wall growth and development (Sasidharan et al. 2011). Here, our results showed that *RhEXPA4* transgenic *Arabidopsis* displayed a higher tolerance to salt and drought stresses and decreased sensitivity to exogenous ABA (Figs. 3, 4, 5). More interestingly, high-level *RhEXPA4* overexpression results in compact plant architecture with higher tolerance to abiotic stresses compared with mild overexpressors. The analysis by SEM revealed that high-level overexpressors exhibit multiple modifications in leaf blade epidermal structure with compact pavement cells and lower stomatal density (Fig. 6a). The overexpression of expansin may disrupt the elaborate microtubule arrays, cellulose deposition and cell-wall thickening that are required for the development of stomatal guard cells and their adjacent cells during stomatal morphogenesis (Nadeau and Sack 2002; Sampedro and Cosgrove 2005). Our results demonstrated that *RhEXPA4* perhaps influenced stomatal development and the arrangement of pavement cells through affecting cell-wall structures (Fig. 6), and therefore contributed to the higher tolerance ability of high-level *RhEXPA4* overexpressors to abiotic stresses.

In summary, our results demonstrated that *RhEXPA4* overexpression enhanced tolerance to salt and drought

stresses and decreased ABA sensitivity in *Arabidopsis*. High-level *RhEXPA4* overexpressors showed compact plant phenotypes with leaf structure modifications, which contribute to its higher abiotic stress tolerance.

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