

Cloning and characterization of an expansin gene *AbEXP* from *Achyranthes bidentata*

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Received: 28 March 2017 / Accepted: 15 August 2017
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Abstract *Achyranthes bidentata*, a traditional perennial medicine herb in China, contains a rich source of important pharmaceutically active phytoecdysteroids including 20-hydroxyecdysone (20E). The growth of *A. bidentata* plants and 20E production are suppressed under abiotic stress condition. Expansins are plant cell wall-loosening proteins to play important roles in different environment stresses. In this study, we tried to clone an expansin gene (designed as *AbEXP*) from *A. bidentata* and characterize its transcriptional response to abiotic stresses (salt stress and nutrient deficiency). The cDNA of *AbEXP* contained 1584 nucleotides with a complete opening reading frame of 786 nucleotides to encode a peptide of 261 amino acids. The expression of the gene was detected in stems, roots or leaves, and was relatively abundant in stems. The expression levels of *AbEXP* under the salinity stress (25 mM NaCl) were increased 51.7-folds in roots but decreased in stems significantly. P and K deficiency enhanced the expression of *AbEXP* in roots and stems, but down-regulated the levels in leaves compared with control conditions. Under N deficiency *AbEXP* expression levels in roots and leaves were down-regulated by 9.95-fold and 4.58-fold, respectively. The expression level in roots was 43.9-fold of the control

level under Fe deficiency while no significant changes of the expression were detected in stems and leaves. Although the growth of *A. bidentata* seedlings was inhibited under both the salinity stress and nutrient deficiency, 20E content in roots was enhanced significantly under N and P deficiency. These results demonstrate that *AbEXP* could be involved in the responses to abiotic stresses and manipulation of the gene expression of *AbEXP* could promote growth and 20E production of *A. bidentata*.

Keywords *Achyranthes bidentata* · Expansin · 20-Hydroxyecdysone · Salinity stress · Nutrient deficiency

Introduction

The dried roots of *Achyranthes bidentata* Blume (Amaranthaceae family), a well-known traditional Chinese medicine (Niuxi), have been widely used to nourish the liver and kidneys, strengthen bones and muscles, invigorate circulation (Tang and Eisenbrand 1992). Pharmacological and phytochemical investigations have revealed that phytoecdysones and triterpenoid saponins are the main bioactive compounds of *A. bidentata* (Meng and Li 2000). 20-Hydroxyecdysone (20E) is one of the major phytoecdysones in air-dried *A. bidentata* roots (Li et al. 2007), which displayed a wide variety of biomedical activities including growth-promoting, wound-healing, nephroprotection and antidepressant effect (Lafont and Dinan 2003; Dinan and Lafont 2006). The commercial supply of 20E for agricultural and therapeutic uses is primarily obtained by extraction of plant material, such as *Ajuga turkestanica*, *Cyanotis arachnoidea* and *A. bidentata* (Thiem et al. 2016). Air-dried leaves, stems and roots from *A. bidentata* have been reported to contain 20E at 0.19–0.46 mg/g (Li et al. 2007). A growing demand for

Electronic supplementary material The online version of this article (doi:10.1007/s10725-017-0313-0) contains supplementary material, which is available to authorized users.

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20E in pharmaceutical industrial has resulted in a transition from wild collection to cultivation of *A. bidentata*. The seed germination, growth and development characteristics of different breeding types of *A. bidentata* have been investigated (Zhang et al. 2010). Gnanaraj et al. (2012) developed the micropropagation in vitro of *A. bidentata* using nodal explants. During cultivation, the growth of *A. bidentata* is severely affected by various abiotic stresses. Duan et al. (2014) reported that seed germination of *A. bidentata* was totally inhibited by NaCl at 250 mM. The germination rate decreased by 48.84% at chilling temperature (4 °C for 6 h), while the root growth was inhibited by 7.33–50.43%. The retardation of root growth and lateral root development was caused by NaCl at 62.5–125 mM (Ding et al. 2010). On the other hand, the production of active components from medicinal plants is sensitive to various environmental factors. The biosynthesis of most secondary metabolites in plants is part of the defense responses of plants to pathogen infection and environmental stresses (Wang and Wu 2013). Therefore, enhancement of the tolerance-related traits would have a significant impact on plant growth and production of bioactive metabolites in *A. bidentata*.

Plant growth under stress is apparently a conflict between stiffening and loosening cell walls (Tenhaken 2015). Expansins are cell wall proteins to promote plant cell wall extension, encoded by a gene superfamily including α -expansin (EXPA), β -expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB) (Cosgrove 2000). Expansin genes are often transcriptionally up-regulated by abiotic stress conditions. Wu and Cosgrove (2001) reported that water deficits enhanced expression of expansin genes (*Exp1*, *Exp5*, *ExpB2*, and *ExpB8*) in maize roots. Buchanan et al. (2005) reported a salinity-induced increase in β -expansin transcript abundance for *Sorghum bicolor*. The overexpression of a cotton expansin gene *GhEXPA1* in *Arabidopsis* led to a substantial increase in seed size (Xu et al. 2013a). Transgenic tobacco plants overexpressing expansin gene *PpEXPI* showed enhanced tolerance to heat stress at 42 °C (Xu et al. 2013b). The genetic manipulation of expansin could become a hopeful approach in enhancing resistance of plants to abiotic stresses.

In our previous work, a gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), a key enzyme involved in triterpene acids biosynthesis, was cloned and characterized in *A. bidentata* (Wang et al. 2017). Li et al. (2016) conducted transcriptome analysis of *A. bidentata* to identify putative genes involved in biosynthesis and transportation of 20E and oleanolic acid. However, growth related genes including expansins have not been cloned and reported in *A. bidentata*. Thus, as a follow-up to our previous works (Wang et al. 2013, 2017) on the molecular characterization of functional genes and biotechnological production of bioactive metabolites from this plant, we tried to clone

and characterize a full-length cDNA encoding an expansin from *A. bidentata*, which was designated as *AbEXP* (GenBank Accession No. KY705376). The expression pattern of *AbEXP* in roots, stems and leaves was also studied. The transcriptional response of *AbEXP* and 20E production in *A. bidentata* roots were also investigated under abiotic stresses (salt stress and nutrient deficiency). This is the first report about the isolation and characterization of a novel expansin gene in *A. bidentata*.

Materials and methods

Plant materials

Achyranthes bidentata seeds were collected in June 2010 from the suburb of Nanjing, China with its voucher specimen (SCU-100812) identified by Prof. C.Y. Liu and were deposited in the Herbarium of Soochow University. *A. bidentata* seeds were sterilized with 0.1% HgCl₂ for 60 s, washed three times with sterile distilled water and germinated on hormone-free Murashige and Skoog (MS) (Murashige and Skoog 1962) medium containing 3% sucrose (w/v) at pH 5.8. The culture was maintained at 25 °C under a 14 h photoperiod with white fluorescent light at 3000 lux 4-week-old *A. bidentata* seedlings were used for gene cloning.

Salt stress and nutrient deficiency treatments

Salinity stress

In order to analysis the expression changes of *AbEXP* under salt stress, *A. bidentata* seedlings were treated according to the method described by Abbasi et al. (2015) with slight modification. 10-day-old seedlings were selected and transplanted into 6-L containers covered with a foamed plastic plate containing evenly spaced holes. The composition of the basic nutrient media was (g/L): (NH₄)₂SO₄ 48.2, MgSO₄ 65.9, K₂SO₄ 15.9, KNO₃ 18.5, Ca(NO₃)₂ 59.9, KH₂PO₄ 24.8, Fe citrate 5, MnCl₂·4H₂O 0.9, ZnSO₄·7H₂O 0.11, CuSO₄·5H₂O 0.04, H₂BO₃ 2.9, H₂MoO₄ 0.01 and the pH was 6.0 ± 0.5. The pH of each container was adjusted into 6.0 ± 0.5 daily using HCl or NaOH. The nutrient solution in the pots was continuously aerated using aquatic pumps and solution was changed every 5 days. Seedlings were treated with salt stress by adding 25 mM NaCl into the basic nutrient solution. The experiment was laid out as a completely random block design with three replications (five plants per replicate). *A. bidentata* seedlings grown in basic nutrient solution were used as control. Roots, stems and leaves were collected after 1 month and were used for RNA isolation and gene expression analysis.

Nutrient deficiency

In order to analyse the expression changes of *AbEXP* under nutrient deficiency stresses, *A. bidentata* seedlings were treated as described by Li et al. (2014) with slight modification. 15-day-old seedlings with uniform growth were selected and then treated under low nitrogen (–N), low phosphorus (–P), low potassium (–K) and low iron (–Fe) conditions for 15 days. The full-strength nutrient solution contained 250 mM KH_2PO_4 , 3000 mM KNO_3 , 2000 mM $\text{Ca}(\text{NO}_3)_2$, 250 mM MgSO_4 , 25 mM MgCl_2 , 12.5 mM H_3BO_3 , 1 mM MnSO_4 , 1 mM ZnSO_4 , 0.25 mM CuSO_4 , 0.25 mM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ and 25 mM Fe-Na-EDTA. For –N treatment, KNO_3 and $\text{Ca}(\text{NO}_3)_2$ were replaced by 750 μM K_2SO_4 and 1200 μM CaCl_2 , respectively. KH_2PO_4 was replaced by 125 μM K_2SO_4 for –P treatment. For –K treatment, KNO_3 and KH_2PO_4 were replaced by 1500 μM $\text{Ca}(\text{NO}_3)_2$ and 250 μM NaH_2PO_4 , respectively. Fe-Na-EDTA was fully withdrawn for –Fe treatment. The experiment was laid out as a completely random block design with three replications (five plants per replicate). The nutrient solution in the pots was continuously aerated using aquatic pumps and solution was changed every 5 days. *A. bidentata* seedlings grown in the full-strength nutrient solution were used as control. Roots, stems and leaves were collected after 15 days and were used for RNA isolation and gene expression analysis.

Sequencing of full-length cDNA of *AbEXP*

Total RNA was extracted from *A. bidentata* using RNeasy Pure Plant Kit (Qiagen Biotech, Beijing, China) following the manufacturer's instructions. cDNA was amplified from total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, San Jose, USA) according to the manufacturer's instructions. All PCR products were cloned into pGEM (Promega, Southampton, UK) vector and transformed into *Escherichia coli* Top 10 for sequencing. A pair of primers EXPF and EXPR were designed and synthesized according to the conserved regions of previously reported expansins of other plant species, such as *Arabidopsis thaliana* (Shcherban et al. 1995), *Oryza sativa* (Cho and Kende 1997) and *Zea Mays* (Wu et al. 2001). Firstly, conventional PCR was performed to clone the conserved sequence of *A. bidentata*. The 25 μL reaction mixture contained 10 ng/L cDNA, 1 μL 10 mM dNTPs, 0.5 μL 10 mM EXPF and EXPR primers, 2.5 μL 10 $\times$ DreamTaqTM Green Buffer and 0.625 U DreamTaqTM Green DNA Polymerase (Fermentas, ON, Canada). PCR protocol was: 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 30 s. To clone full-length cDNA of *AbEXP*, 3'RACE and 5'RACE were carried out subsequently. For 3'RACE, two sets of PCR were performed with different primers and template. The first set of amplification was conducted in a

total volume of 50 μL containing 1 μL cDNA, 1 μL 10 mM dNTPs, 2 μL 10 mM 3'RACE11 and 3'RACE12 primers, 5 μL 10 $\times$ DreamTaqTM Green Buffer and 1 μL 5 U/L DreamTaqTM Green DNA Polymerase (Fermentas, Ontario, Canada). The second set of PCR was conducted in a total volume of 50 μL containing 1 μL product generated by the first set of amplification, 1 μL 10 mM dNTPs, 2 μL 10 mM 3'RACE21 and 3'RACE22 primers, 5 μL 10 $\times$ DreamTaqTM Green Buffer and 1 μL 5 U/L DreamTaqTM Green DNA Polymerase (Fermentas, ON, Canada). PCR protocol was: 94 °C for 5 min followed by 25 cycles of 94 °C for 40 s, 56 °C for 30 s and 72 °C for 1 min. For 5'RACE, terminal deoxynucleotidyl transferase (TdT) was used to add a poly(dG) tail to the first strand cDNA. The reaction mixture (50 μL) included 20 μL cDNA, 10 μL 5 $\times$ TdT buffer, 5 μL 0.1% BSA, 2.5 μL 10 mM dCTP and 15 U TdT and it was then incubated at 37 °C for 30 min. Then two sets of PCR were conducted as mentioned above with some modifications. For the first set of PCR, cDNA with a poly(dG) tail was added as the template and primers were 5'RACE11 and 5'RACE12. For the second set of PCR, the product generated by the first set of amplification was added as the template and primers were 5'RACE21, 5'RACE22. PCR protocol was 94 °C for 5 min followed by 25 cycles of 94 °C for 40 s, 60 °C for 30 s and 72 °C for 90 s. Sequences of all the primers used were listed in Table S1.

Bioinformatic analysis

The nucleotide sequence and deduced amino acid sequence were compared through database search using BLAST program (NCBI, <http://www.ncbi.nlm.nih.gov>) and the open reading frame (ORF) was analyzed (<https://www.ncbi.nlm.nih.gov/orffinder/>). Isoelectric point and molecular weight of AbEXP was calculated on line (http://web.expasy.org/compute_pi/). AbEXP and other expansins obtained from Genbank (<https://www.ncbi.nlm.nih.gov/genbank/>) were aligned and a phylogenetic tree was constructed on line (<http://www.phylogeny.fr>). Secondary structure prediction was performed with SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html). The three-dimensional (3-D) modeling was accomplished by Swiss-Model (<http://swissmodel.expasy.org/interactive>).

Real time quantitative PCR analysis of *AbEXP*

To investigate the *AbEXP* expression pattern in different tissues and under different abiotic stresses, total RNA of *A. bidentata* were extracted using RNeasy Pure Plant Kit (Qiagen Biotech, Beijing, China). cDNA was amplified from the total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, San Jose, USA) according to the instruction. The real time quantitative PCR

(RT-qPCR) contained 2 μ L five-fold diluted cDNA template, 1 μ L AbEXPF (10 mM), 1 μ L 10 mM AbEXPR (10 mM), 10 μ L FS Universal SYBR Green Master (Roche, LA, CA, USA) and 6 μ L ddH₂O. The reaction was performed using CFX96™ Real-Time System (Bio-Rad, Hercules, USA). The reaction conditions were: 94 °C for 4 min, and then subjected to 40 cycles of amplification (40 cycles of 94 °C for 1 min, 56 °C for 30 s and 72 °C for 15 s). The 18S ribosomal RNA (AB558157.1) was chosen as the internal reference. The primers designed for the validation of the target genes from *A. bidentata* were listed in Table S1.

20E extraction and analysis

20E in *A. bidentata* roots was extracted according to the method described before (Wang et al. 2014). High performance liquid chromatography (HPLC) analysis conditions: Agilent 1280 HPLC system equipped with 250 $\times$ 4.6 mm Agilent HC-C18 column; samples were eluted with 20: 80 (v/v) acetonitrile/water at a flow rate 1 mL/min and monitored at 242 nm. 20E was quantified with a genuine standard (Sigma, St. Louis, USA).

Results and discussion

Sequencing of full-length cDNA and bioinformatic analysis of *AbEXP*

A full-length cDNA encoding an expansin of *A. bidentata* was successfully amplified and sequenced in our research. The full-length cDNA of 1584 bp contained a 5'-UTR (untranslated region), a start codon ATG, 786 bp ORF, a stop codon TAA and 3'-UTR (Fig. S1). The 786 bp ORF encoded a protein of 261 amino acids with a calculated isoelectric point 9.77 and molecular weight 28.68 kDa. The results of BLASTP search in Genbank database (<https://www.ncbi.nlm.nih.gov/genbank/>) showed *AbEXP* had high homology with many other plant expansins such as *Coffea arabica* CaEXPA2, *A. thaliana* AtEXPA4, *Rephanus sativus* RsEXPA1, *Medicago truncatula* MtEXPA10 and *Theobroma cacao* TaEXPA4, suggesting that *AbEXP* belongs to the plant expansin superfamily (Fig. S2).

AbEXP contains previously identified highly conserved regions of EXPA and EXPB (Cosgrove 1998; Choi et al. 2006), including seven conserved C (Cys) residues in the N-terminal half of the protein, an HDF motif in the center region and five conserved W (Trp) residues towards the C-terminus (Fig. S2). As shown in Fig. S1, two domains were identified in *AbEXP* through BLASTP. Domain 1 is a double-psi beta-barrel (DPBB) and domain 2 is homologous to grass pollen allergens. This result was in accordance with

other expansins (Sampedro and Cosgrove 2005; Cosgrove 2015).

The secondary and 3-D structures of *AbEXP* were analyzed by Swiss-Model (<http://swissmodel.expasy.org/inter-active>). The result showed that *AbEXP* contains 17.24% alpha helices, 45.60% random coils, 24.90% extend strands and 12.26% beta turns (Fig. 1a). The 3-D structure of *AbEXP* had the highest similarity (36%) with the template 2hcz.1.A, beta-expansin 1a. The 3-D structure of *AbEXP* (Fig. 1b) was torpedo-shaped with two domains (domain 1 and 2), which was the canonical structure of plant expansins (Cosgrove 2015). In order to determine the evolutionary relationship of *AbEXP*, a phylogenetic tree for various expansins from different species was constructed on the website (<http://www.phylogeny.fr>). Expansins were sorted into EXPA, EXPB, EXLA and EXLB (Fig. S3). *AbEXP* has a closer relationship with EXPAs and has the closest relationship with *M. truncatula* α -expansin 10 (MtEXP10) (Fig. S3). The result suggests that *AbEXP* belongs to the α -expansin superfamily.

Expression profiles of *AbEXP* in different tissues

We performed RT-qPCR to analyze expression pattern of *AbEXP* in *A. bidentata* roots, stems, and leaves. As shown in Fig. 2, *AbEXP* expressed in all tissues at different levels. *AbEXP* had the highest expression in stems and the lowest expression in leaves. The expression level of *AbEXP* in *A. bidentata* stems was 3.98 and 6.18 times higher, respectively, than that in roots and leaves. It is not surprising that expansin genes exhibit different expression profiles by cell and organ specificity (Min et al. 2005). The tissue-specific or tissue-preferential expression pattern may suggest that different expansins play a specific role in growth and development. In our research, *AbEXP* with a stem-preferential expression pattern may imply its function in the growth and development of stems.

Expression changes of *AbEXP* in response to salinity stress and nutrient deficiency

To explore possible role of *AbEXP* in adaption to salinity stress, *A. bidentata* seedlings were treated with 25 mM NaCl. Figure 3a showed that the growth of *A. bidentata* seedlings was inhibited after salt treatment. The stem and root length of *A. bidentata* were decreased by 25.69 and 33.91% after NaCl treatment (Fig. 3b). Meanwhile, the fresh and dry weights of NaCl-treated seedlings were 41.82 and 37.21% lower than control group (Fig. 3c). For salt stress, *AbEXP* expression displayed no significant change in leaves. However, it was highly induced to be 51.7-fold of the control in roots but decreased in stems by 7.41-fold after 1 month of treatment (Fig. 3d). Changes in *AbEXP* expression implied that *AbEXP* was involved in salt stress response in

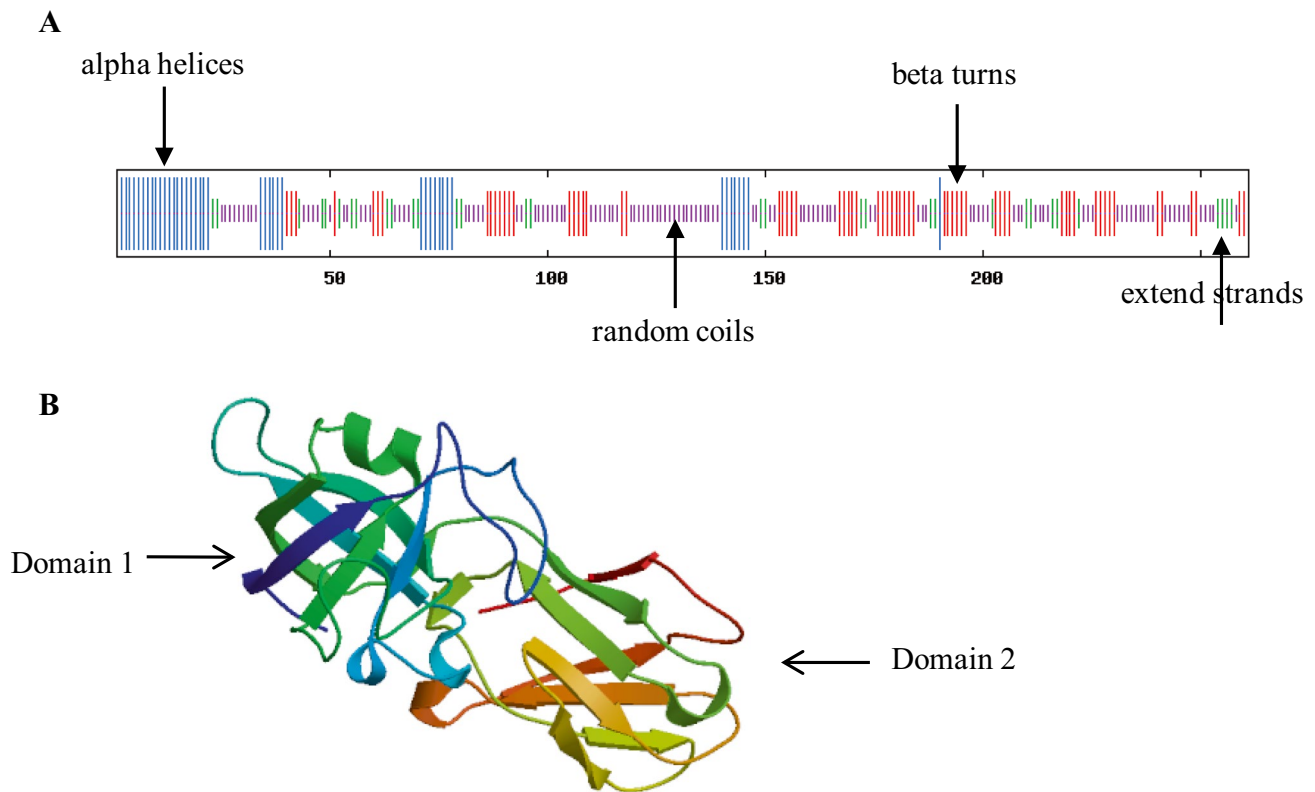


Fig. 1 Secondary and three-dimensional structures of the deduced AbEXP protein. **a** Secondary structure of AbEXP protein. **b** Three-dimensional structure of AbEXP protein

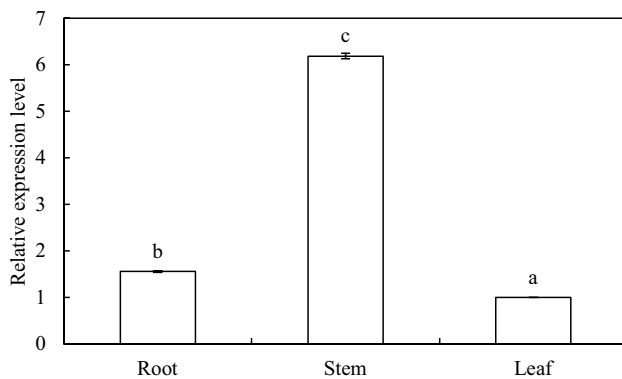


Fig. 2 Expression pattern of *AbEXP* in different *A. bidentata* tissues. Total RNA was extracted from 1-month-old aseptic *A. bidentata* seedlings. Data are expressed as mean $\pm$ standard deviation of three replicates. Different letters above the bars mean significant differences ($p < 0.05$)

A. bidentata. There is increasing evidence for the involvement of expansins in adaptation of plants to salt stress. The tobaccos overexpressing *TaEXPB2* showed higher germination rates and survival rates than wild-type plants under salt stress. The improved salt tolerance of transgenic tobacco was due to the improved water status, selective ion absorption

and enhanced antioxidant competence (Chen et al. 2016). Transgenic tobacco overexpressing *TaEXPB23* showed the improvement in the tolerance to salt stress by enhancing water retention ability and decreasing osmotic potential (Han et al. 2012). Genetic manipulation of *AbEXP* expression may lead to the development of novel strategies to improve *A. bidentata* tolerance to salt stress.

To investigate the response of *AbEXP* to nutrient deficiency, *A. bidentata* seedlings were treated with $-N$, $-P$, $-K$ and $-Fe$ conditions. Compared to the control, significant reduction in length of stem and root, and plant biomass were observed in plants under nutrient deficiency stress (Fig. 4a–c). Under N deficiency, *AbEXP* expression levels in roots and leaves were down-regulated by 9.95-fold and 4.58-fold, respectively, but had no significant change in stems (Fig. 5a). The expression of *AbEXP* in roots and stems increased to 10.4- and 27.5-fold of the control level under P deficiency while it was down-regulated in leaves (Fig. 5b). The treatment ($-K$) up-regulated *AbEXP* expression in roots and stems, but down-regulated that in leaves (Fig. 5c). *AbEXP* expression in roots under Fe deficiency was enhanced to 43.9-fold of the control level. However, no significant change of *AbEXP* expression was detected in stems and leaves (Fig. 5d). Our results showed the variations

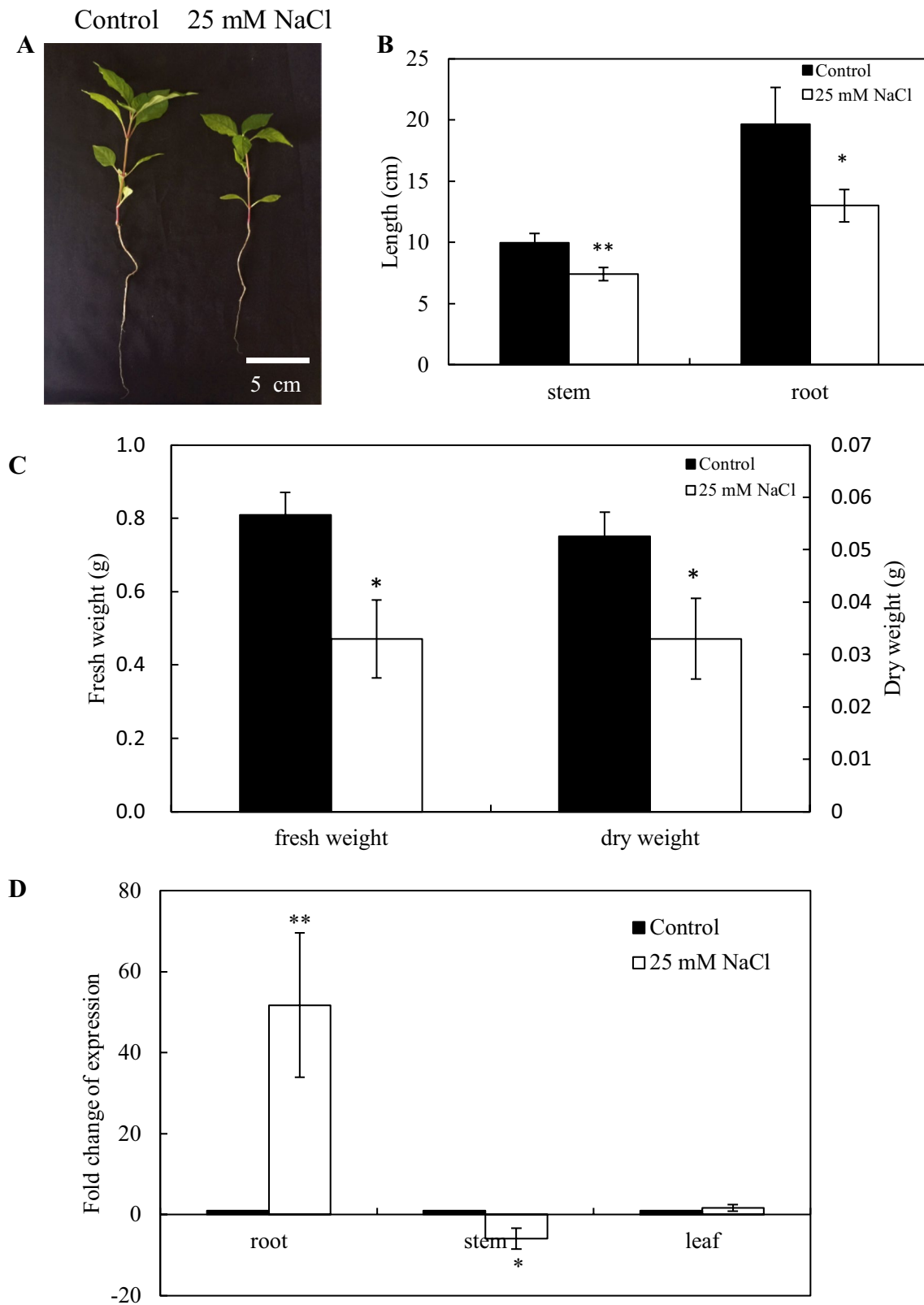


Fig. 3 Effect of NaCl stress on growth and *AbEXP* expression of *A. bidentata*. 10-day-old *A. bidentata* seedlings were treated with 25 mM NaCl for 1 month. Seedlings grown in the basic nutrient solution were used as control. **a** *A. bidentata* seedlings. **b** Effects on stem

and root length. **c** Effects on fresh weight and dry weight. **d** *AbEXP* expression response. Data are expressed as mean $\pm$ standard deviation of three replicates. Statistical significance in comparison with the corresponding control values is indicated by * $p < 0.05$, ** $p < 0.01$

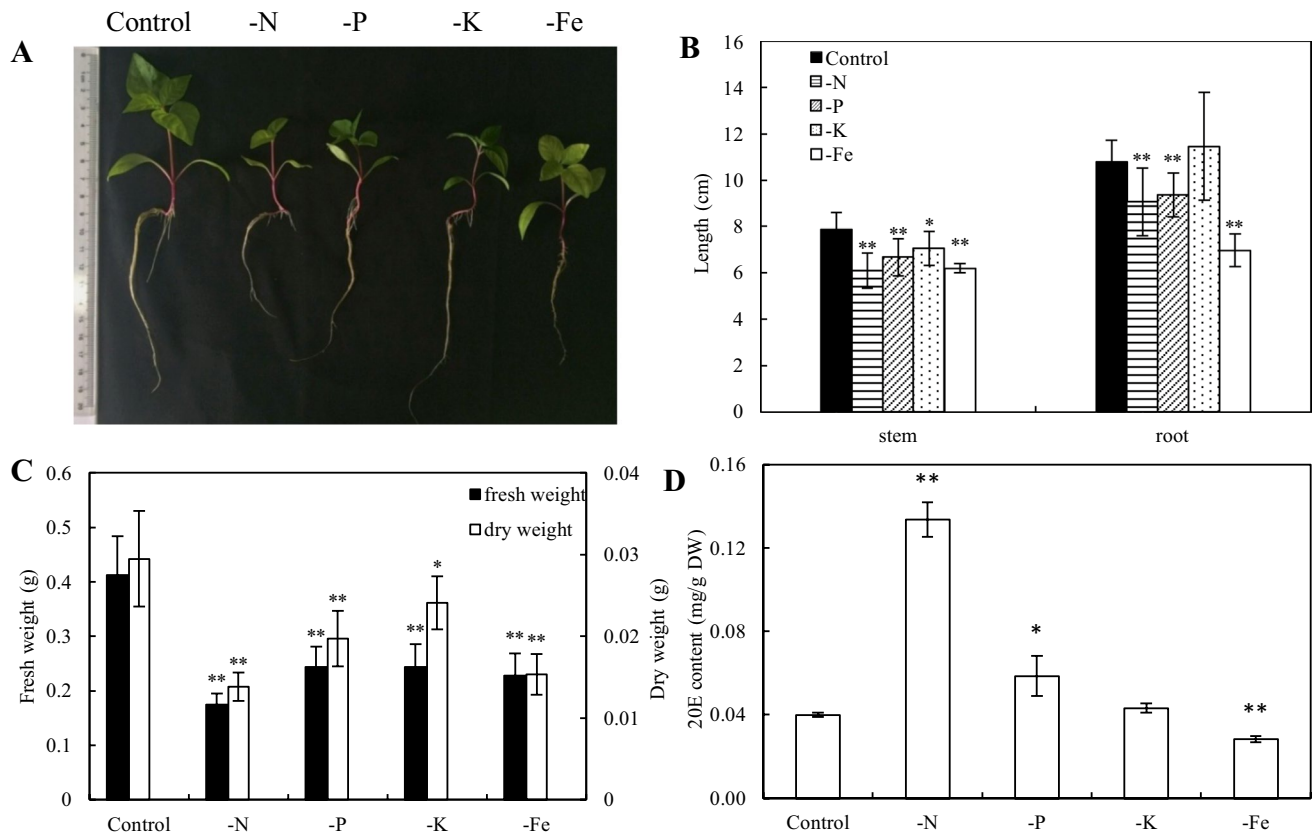


Fig. 4 Effects of nutrient deficiency on the growth and 20E accumulation in *A. bidentata* roots. 15-day-old *A. bidentata* seedlings were treated under low nitrogen (–N), low phosphorus (–P), low potassium (–K) or low iron (–Fe) for 15 days. Seedlings grown in the full-strength nutrient solution were used as control. **a** *A. bidentata* plants.

b Effects on stem and root length. **c** Effects on fresh weight and dry weight. **d** 20E accumulation in *A. bidentata* roots. Data are expressed as mean $\pm$ standard deviation of three replicates. Statistical significance in comparison with the corresponding control values is indicated by * $p < 0.05$, ** $p < 0.01$

of *AbEXP* expression might play a special role in response to different nutrient deficiency. Responsiveness to nutrient deficiency stresses varied among *GmEXPBs* in soybean (Li et al. 2014). *GmEXP2* in soybean roots specifically responded to –P stress. Overexpression of *GmEXP2* in soybeans promoted the leaf expansion and stimulated the root growth. Under low P condition, transgenic soybean had higher biomass and more P accumulation than that of wild-type, indicating the improved tolerance to P starvation in transgenic soybeans (Zhou et al. 2014). Genetic manipulation of *AbEXP* expression may improve *A. bidentata* tolerance to nutrient deficiency.

20E accumulation under salinity stress and nutrient deficiency

To explore the effects of salinity stress and nutrient deficiency on 20E accumulation, 20E content was quantified in *A. bidentata* with or without stress treatment. As shown in the Fig. S4, 20E production was not affected by 25 mM NaCl. On the contrary, 20E content was stimulated by –N

and –P treatment, reaching the value of 0.13 mg/g dry weight (DW) and 0.06 mg/g DW, respectively, about 3.25- and 1.50-fold of the control level (Fig. 4d). The accumulation of 20E was not affected after –K treatment. However, it was inhibited by 29.25% under Fe deficiency (Fig. 4d). It was reported that plant secondary metabolism showed a sensitive response to nutrient deficiency (Chishaki and Horiguchi 1997). N deficiency could stimulate the biosynthesis of phenylethanoid glycosides and flavonoids in *Castilleja tenuiflora* (Medina-Pérez et al. 2015). Deficiency of P could induce the transcript levels of genes involved in flavonol biosynthesis in tobacco, resulting in the increased accumulation of flavonols in tobacco leaves (Jia et al. 2015). Phenols concentration in *Pisum sativum* was enhanced under Fe deficiency (Jelali et al. 2010). In our research, 20E concentration in *A. bidentata* roots was elicited under N and P deficiency. It was reported that the transcription factor of cotton *EREBP* (ethylene response element binding protein) gene was transferred to *A. bidentata* for new cultivar breeding with enhance resistance to environmental stress (Duan et al. 2013). We believe that genetic modification of *AbEXP*

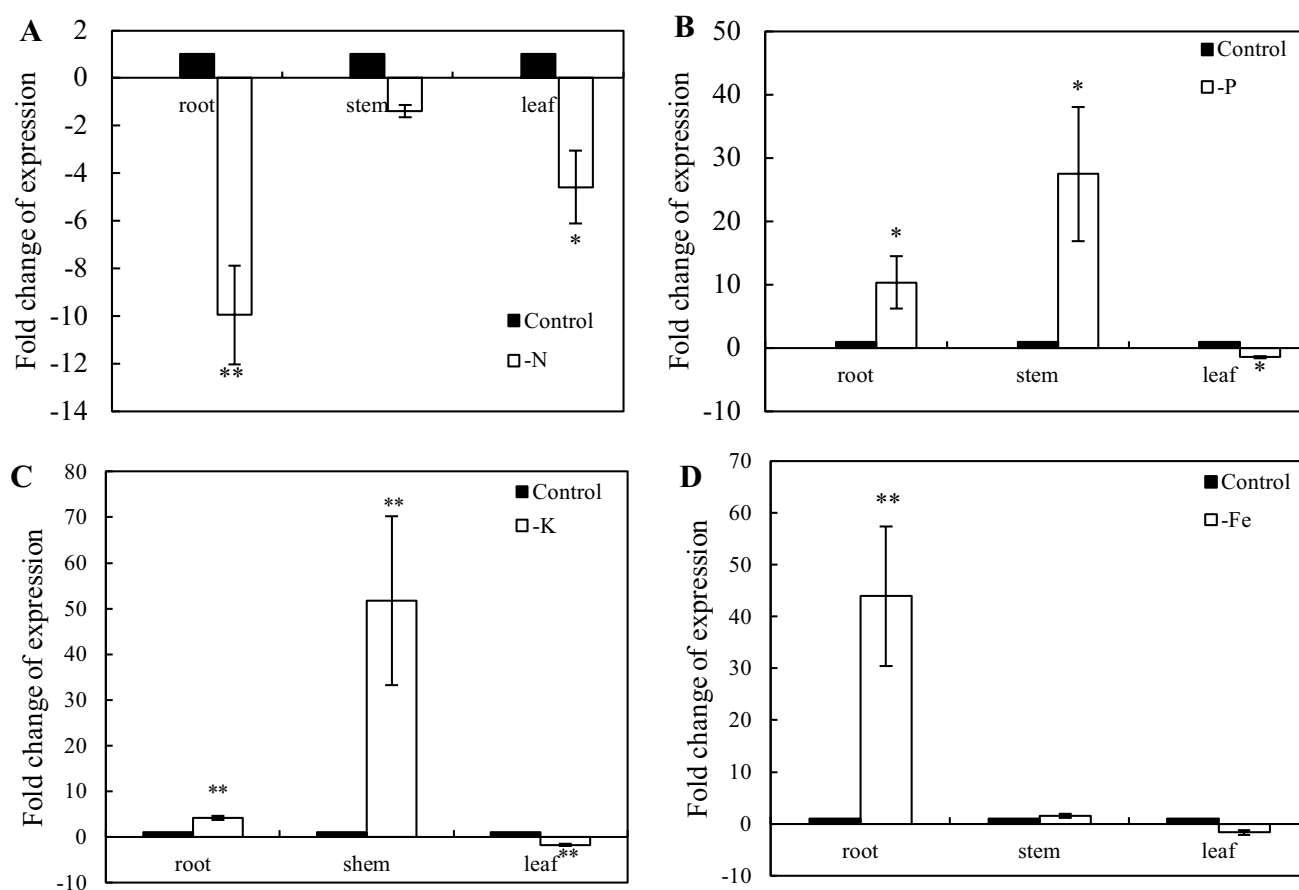


Fig. 5 Effects of nutrient deficiency on *AbEXP* expression. 15-day-old *A. bidentata* seedlings were treated with -N (a), -P (b) and -K (c) and -Fe (d) for 15 days. Seedlings grown in the full-strength nutrient solution were used as control. Data are expressed as mean $\pm$ standard

deviation of three replicates. Statistical significance in comparison with the corresponding control values is indicated by * $p < 0.05$, ** $p < 0.01$

expression might enhance the tolerance to environmental stress and nutrient deficiency. It would be a new strategy to cultivate transgenic *A. bidentata* plants under the harsh condition of soil for enhanced production of 20E.

Conclusion

In conclusion, we have successfully cloned and characterized an expansin gene *AbEXP* in *A. bidentata*. Bioinformatic analysis revealed that *AbEXP* had high identity to other plant expansins and contained conserved region of expansins. The 3-D model of *AbEXP* represented a typical special structure of expansins. *AbEXP* displayed a stem-preferential expression pattern in *A. bidentata*. *AbEXP* was responsive to salt stress and nutrient deficiency, suggesting that *AbEXP* was involved in adaption of *A. bidentata* to such stresses. Under N and P deficiency, the contents of bioactive 20E in *A. bidentata* roots could be enhanced significantly. Our work will be helpful to possible molecular engineering

of *AbEXP* for *A. bidentata* cultivation with enhanced 20E production under the harsh condition of soil.

Acknowledgements This work was supported by the National Natural Science Foundation of China (No. 81473183), Soochow Scholar Program (No. 14317363) and the Postgraduate Research & Practice Innovation Program of Jiangsu Province.

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Cloning and characterization of an expansin gene *AbEXP* from *Achyranthes bidentata*

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Electronic supplementary material

Table S1 Primer sequences used in this investigation

Name	Sequence (5'-3')
EXPF	ACCCAAGTATGCTTTGCCCA
EXPR	CTTGGGGCTTAAACCCTGAAAT
AbEXPF	AGGTGGCTCTTGGGAAACTG
AbEXPR	TCCTGTGTTACACCGTAGC
Ab18SF	GGGGAGTTCCCCCTTGATTG
Ab18SR	TCTTCATCGATGCGAGAGCC
5'RACE11	GGCCACGCGTCGACTAGTACGGGGGGGGGGGGGG
5'RACE12	CAGCACGGTACTTAGCAATCTTGAG
5'RACE21	GGCCACGCGTCGACTAGT
5'RACE22	GCATGGCGAGGTCGAAATGTGGGC
3'RACE11	GCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
3'RACE12	CTGGATGCCAATGAGTCGTAATTG
3'RACE21	GCAGTGGTATCAACGCAGAGTAC
3'RACE22	GGTTGGTCAGTCACTTTCTTTTAGG

Fig. S1

CCCCCCCCCCCC

CCCCCTCCACTAGAGCCAGAACTTATCCACACAACCAACACACTTTTCCAACCTTATCACC
CTATGCTTTTCTCTCACTAAAACTAAAACAAAGAGTGGGTTTTTTTAGAGAGAGAAAAAA
ATGAGAATGTTTAGCATTTTAAATATAGTTTTATTATTACATTATGGGTATTATCAACA
M R M F S I F N I V L L F T L W V L S T
TTAGTTGATGCTAGGATTCTGGAAATTACCAAGGTGGCTCTTGGGAACTGCTCATGCT
L V D A R I P G N Y Q G G S W E T A H A
ACTTTCTATGGTGGTGTGATGCTTCTGGCACCATGGGAGGGGCATGTGGATATGGGAAC
T F Y G G A D A S G T M G G A C G Y G N
TTGTATAGCCAAGGCTACGGTGTGAACACAGGAGCACTAAGCACAGCATTATTCAACAAT
L Y S Q G Y G V N T G A L S T A L F N N
GGGTACAGTTGTGGTGCATGTTTCGAACTCAAGTGTGTAACTACCCGGGTGGTGTCTA
G Y S C G A C F E L K C V N Y P G W C L
CCGGGTAGCCCATCAATCCTAATAACAGCRACCAATTTTTGCCACCCAACTATGCTTTG
P G S P S I L I T A T N F C P P N Y A L
CCCAATGACAATGGCGGGTGGTGCAACCCGCCCGCCACATTCGACCTCGCCATGCCC
P N D N G G W C N P P R P H F D L A M P
ATGTTCTCAAGATTGCTAAGTACCGTGCTGGCATTGTACCCGTTGCTTCCGAAGGATA
M F L K I A K Y R A G I V P V A F R R I
CCATGCCGTAAACGTGGAGGAATAAGGTTCACAATCAACGGTCACCGTTACTTTAACTTG
P C R K R G G I R F T I N G H R Y F N L
GTGTTGATCACCAACGTGGCAGGTGCAGGTGACATTGTGAAGACTTATGTTAAAGGATCA
V L I T N V A G A G D I V K T Y V K G S
AGGACTAACTGGATGCCAATGAGTCGTAATTGGGGGCAGAATTGGCAATCTAATGCTGTT
R T N W M P M S R N W G Q N W Q S N A V
TTGGTTGGTCAGTCACCTTCTTTTAGGGTGACAGCCAGTGACAAGAGATCATCCACTTCC
L V G Q S L S F R V T A S D K R S S T S
TGGAACATTGCTCCTTCTAACTGGCAATTTGGCCAACTTTTACTGGCAAGAATTTCAAG
W N I A P S N W Q F G Q T F T G K N F R
GTTTAAAGCCCCAAGAAAAAGTCCAAAAAAATATTATCATTCCCTCTAAATTTTAAAAA
V *

TTTTCTTTTAAAAATCCTTCTTTTAAATGGGTTTTTTTTTACCCCTTTTCCTTTTTTCTA
TTAAAAGGGAATTCGCTTGTGTTCTAGTTGAAAAATTAAGAACAGTTTGATGAGCGTATA
AGTTTGATAGGTTATTGTTAAGTTTACGTACGGTCAATTTTTTCGGCTTGTTTAGGGAAG
AATTTATTATTTTCTTGACCTTTAAATTGGGAAAAGTGAGAAGAGACTTTTCTTAATGTG
GGATTAGGAGGGAGGGTTAAATTTGAGATTTTAATTTTTTTTTTTTTTTTGGGTATTTTT
TTGGAGGTGTGAAATTGTAATTTTGGAGAAGGGTGAGGGGTATAGTGGGAAAAGAAGGGC
TGAAGAGGCTGCAAAAAAGGTGTGGCCCGCAGCCTAATGATCTTCTAATTATTAATATGA
AGGGAGGATTTTATGTTTAAAAATTGGTAAAAAGTATATGATATGATAATATAGTATTTGT
AGTAGTAGAGTGTGAATTGTGAAATGTTGCACTTGCACTCAACTTTGGTATTGTAATTTTG
TAACTCTTTTGATGTGGTTCAATTATAAGAACTTTATTTTCCTTTAAAAA

Fig. S1 The full-length cDNA sequence of *AbEXP* and the deduced amino acid sequence. The start codon (ATG) and the stop codon (TAA) are boxed. Two domains of AbEXP are underlined.

Fig. S2

AbEXP	MRMFSIFNIV	LL-FTLWVLS	TL—VDARIP	GNVQGGSWET	AHATFYGGAD	ASGTMGGACG
CaEXPA2	---MAAMGLT	CI-VALSLLL	TL—TEARIP	GVYTGDSWES	AHATFYGGSD	ASGTMGGACG
AtEXPA4	----MAIKLA	IL-FTTFVLF	SL—ADARIP	GIYSGGAWQN	AHATFYGGSD	ASGTMGGACG
RsEXPA1	---MAITKIA	IL-FTTFVLF	SL—ADAKIP	GVYSGGAWQT	AHATFYGGSD	ASGTMGGACG
MtEXPA10	---MGMVSMV	YV-VTLIMSL	TMMVDARIP	GVYNGGAWQT	AHATFYGGSD	ASGTMGGACG
TcEXPA4	MAAAATSSLT	VLCFASLLSF	SV-VVNARIP	GVYTGGSWES	AHATFYGGSD	ASGTMGGACG
AbEXP	YGNLYSQGYG	VNTGALSTAL	FNNGYS	CGAC	FELKCVNYPG	WCLPGSPSIL ITATNFCPPN
CaEXPA2	YGNLYSQGYG	VNTAALSTAL	FNNGFS	CGSC	FELKCADDPQ	WCHPGSPSIL ITATNFCPPN
AtEXPA4	YGNLYSQGYG	TNTAALSTAL	FNNGMS	CGAC	FELKCANDPQ	WCHSGSPSIL ITATNFCPPN
RsEXPA1	YGNLYSQGYG	TNTAALSTAL	FNSGMS	CGAC	FELKCANDPQ	WCHSGSPSIL ITATNFCPPN
MtEXPA10	YGNLYSQGYG	VNTAALSTAL	FNNGLS	CGSC	FELKCANDKQ	WCHSGSPSIF ITATNFCPPN
TcEXPA4	YGNLYSQGYG	VNTAALSTAL	FNNGLS	CGAC	FEIKCANDPR	WCHPGSPSIF VTATNFCPPN
AbEXP	YALPNDNGGW	CNPPRFHFDL	AMPMFLKIAK	YRAGIVPVAF	RRIPCRKRGG	IRFTINGHRY
CaEXPA2	YALPNDNGGW	CNPPRFHFDL	AMPMFLRIAE	YRAGIVPSY	RRVPCRKQGG	IRFQINGFRY
AtEXPA4	LAQPSDNGGW	CNPPREHFDL	AMPVFLKIAQ	YRAGIVPSY	RRVPCRKRGG	IRFTINGHRY
RsEXPA1	FAQASDNGGW	CNPPRAHFDL	AMPVFLKIAQ	YRAGIVPSY	RRVPCRKRGG	IRFTINGHRY
MtEXPA10	FAQPSDNGGW	CNPPRFHFDL	AMPMFLKIAE	YRAGIVPVAF	RRVPCRKRGG	IRFTINGFRY
TcEXPA4	YALPSDNGGW	CNPPRSHFDL	AMPMFLKIAE	YRAGIVPSF	RRVPCRKQGG	IRFTINGFRY
AbEXP	FNLVLITNVA	GAGDIVKTYV	KGSRTN	WMPM	SRNWGNWQS	NAVLVGQSLS FRVTASDKRS
CaEXPA2	FNLVLITNVA	GAGDIVQASV	KGSKTG	WITL	SRNWGNWQT	NAVLAGQSLS FRVRASDRRS
AtEXPA4	FNLVLITNVA	GAGDIVRASV	KGSRTG	WMSL	SRNWGNWQS	NAVLVGQALS FRVTGSDRRT
RsEXPA1	FNLVLITNVA	GAGDIVRTSV	KGSRTG	WMSL	SRNWGNWQS	NAVLVGQSLS FRVTGSDRRT
MtEXPA10	FNLVLISNVA	GAGDIVRTYV	KGTQTG	WMPM	SRNWGNWQS	NSVLVGQALS FRVTGSDRRT
TcEXPA4	FNLVLVTNVA	GAGDIVKVSF	KGKTG	WLSM	SRNWGNWQS	NAVLVGQALS FRVTGSDKRT
AbEXP	STSWNIAPSN	WQFGQTFTGK	NFRV			
CaEXPA2	STSWNIAPAN	WQFGQTFTGK	NFRV			
AtEXPA4	STSWNMVPSN	WQFGQTFVGK	NFRV			
RsEXPA1	STSWNIVPSN	WQFGQTYVGK	NFRV			
MtEXPA10	STSWNIAPHN	WQFGQTFTGK	NFRV			
TcEXPA4	STSWNVASAN	WQFGQTFTGK	NFRV			

Fig. S2 Multiple alignment of AbEXP with the following other plant expansin: CaEXPA2 (AD C35365), AtEXPA4 (At2g39700), RsEXPA1 (ATC67493), MtEXPA10 (KEH39447), TcEXPA4 (EOY07514). The highly conserved C (Cys) and W (Trp) residues and the HFD motif are boxed.

Fig. S3

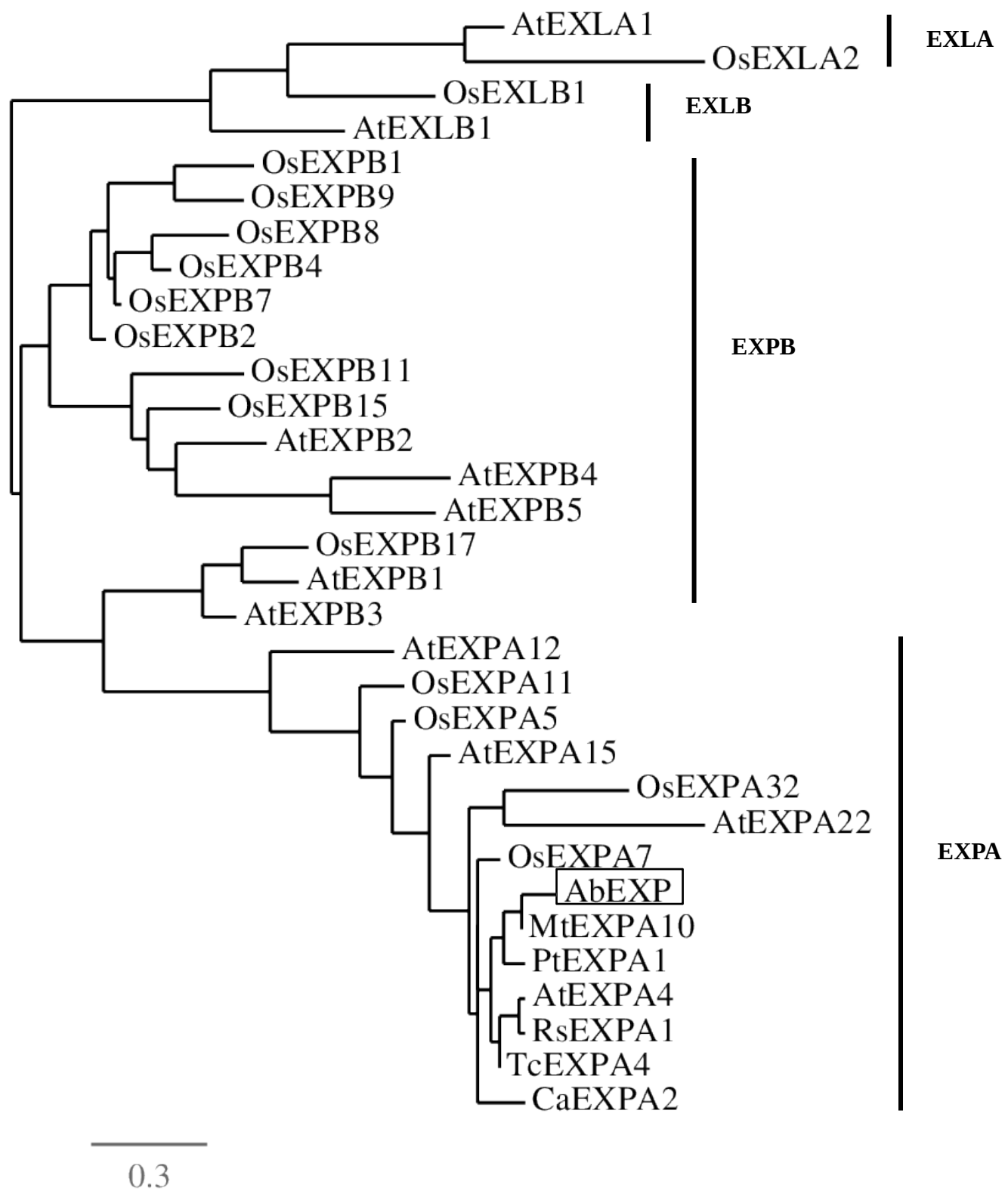


Fig. S3 Phylogenic tree analyzes of various expansins from different species.

Accession numbers: AtEXLA1 (Q9LZT4); OsEXLA2 (Q7XCL0); OsEXLB1 (Q850K7); AtEXLB1 (O23547); OsEXPB1 (Q40638); OsEXPB9 (Q7XCG7); OsEXPB8 (Q10T32); OsEXPB4 (Q94LR4); OsEXPB7 (Q9LD07); OsEXPB2 (O24230); OsEXPB11 (Q6H676); OsEXPB15 (Q7XT40); AtEXPB2 (Q9SHY6); AtEXPB4 (Q9SHD1); AtEXPB5 (Q9M203); OsEXPB17 (Q7X6J9); AtEXPB1 (Q9SKU2); AtEXPB3 (Q9M0I2); AtEXPA12 (Q9LDJ3); OsEXPA11 (Q4PNY1); OsEXPA5 (Q6ZGU9); AtEXPA15 (O80622); OsEXPA32 (Q6YYW5); AtEXPA22 (Q9FL80); OsEXPA7 (Q852A1); MtEXPA10 (KEH39447); PtEXPA1 (EEE83158); AtEXPA4 (O48818); RsEXPA1 (ACT67493); TcEXPA4 (EOY07514); CaEXPA2 (ADC35365).

Fig. S4

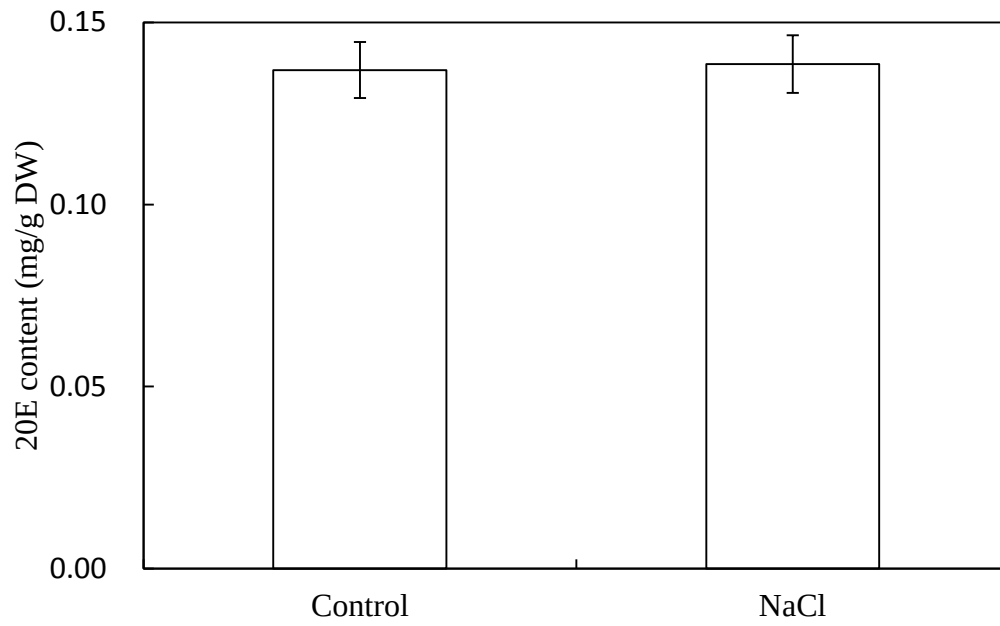


Fig. S4 Effect of NaCl stress on 20E content in *A. bidentata* roots. 10-day-old *A. bidentata* seedlings were treated with 25mM NaCl for one month. Seedlings grown in the basic nutrient solution were used as control. Data are expressed as mean $\pm$ standard deviation of three replicates.