TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2014) 38: 1067-1079

doi:10.3906/bot-1403-105

Heterologous expression of *EsABA1* enhances salt tolerance with increased accumulation of endogenous ABA in transgenic tobacco

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Received: 31.03.2014 • **Accepted:** 18.07.2014 • **Published Online:** 17.11.2014 • **Printed:** 28.11.2014

Abstract: Salt stress can trigger several physiological responses in plants such as increased accumulation of ABA and antioxidant enzyme activities, which have been closely associated with the tolerance of plants to salt stress. In the present study a novel zeaxanthin epoxidase gene, designated *EsABA1*, was isolated from *Eutrema salsugineum*. *EsABA1* contained a full-length open reading frame (ORF) of 2001 bp, encoding 666 amino acids. Transcription of *EsABA1* rapidly accumulated after exposure to various abiotic stresses, suggesting that the *EsABA1* gene was involved in multiple stress responses and tolerance. To further investigate the roles of *EsABA1* under salt-stress conditions, transgenic tobacco lines that ectopically overexpressed *EsABA1* were obtained. These transgenic lines exhibited more tolerance to salt stress than wild-type (WT) plants, and overexpression of *EsABA1* increased the contents of ABA and proline as compared to WT plants under salt-stress conditions. Moreover, the expression levels of stress responsive genes and activities of antioxidant enzymes were much higher in the *EsABA1*-overexpressing lines than in WT plants. Therefore, our findings indicated that the elevated expression of *EsABA1* enhanced the tolerance of transgenic tobacco plants to high salinity stress.

Key words: *Eutrema salsugineum*, *EsABA1*, zeaxanthin epoxidase, salt stress, transgenic tobacco lines

1. Introduction

Salt stress is a major environmental problem limiting plant growth and productivity worldwide (Zhang et al., 2011) and leads to the accumulation of important metabolites such as abscisic acid (ABA), osmotically active compounds, and antioxidants. ABA plays an important role in plant responses to environmental stresses (Loik and Nobe, 1993). Increased ABA levels can improve the tolerance of plants to various abiotic stresses (Qin and Zeevaart, 2002; Yue et al., 2012). The above-cited studies suggest that overexpression of genes involved in ABA biosynthesis may be utilized to enhance plant tolerance to adverse conditions.

Salt stress can induce conditions of oxidative stress due to higher leakage of electrons towards O_2 during photosynthetic and respiratory processes, which results in significant increases in reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (.OH) (Ruelland et al., 2009; Abogadallah, 2010). To cope with the deleterious effects of ROS, ROS in plant cells can be modulated via important antioxidants such as the enzymes superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR) (Noctor and Foyer, 1998; Asada, 1999; Kausar et al., 2013; Gharari et al., 2014). Some recent reports have indicated that elevated ABA biosynthesis can significantly enhance the activities of antioxidant enzymes and increase the accumulation of osmolytes such as proline and malondialdehyde (MDA), which help plants tolerate stress by maintaining osmotic balance within the cells (Yue et al., 2012; Xian et al., 2014).

To date, much effort has been made to understand the regulatory mechanism of ABA biosynthesis. Some key genes involved in ABA biosynthesis have been isolated and characterized in tobacco, maize, and other plant species (Marin et al., 1996; Tan et al., 1997; Xian et al., 2014). Recent studies have provided insight into the regulatory and biosynthetic steps of ABA. The first step of the ABA biosynthesis pathway occurs in plastids, and it is the epoxidation of zeaxanthin and antheraxanthin to violaxanthin, which is catalyzed by a zeaxanthin epoxidase (ZEP) (Marin et al., 1996). After several structural modifications, violaxanthin is converted to 9-*cis*-

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epoxycarotenoid. The 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzes oxidative cleavage of the major epoxycarotenoid 9-*cis*-neoxanthin into a C_{15} intermediate, xanthoxin (Schwartz et al., 1997). Abscisic aldehyde oxidase (AAO) catalyzes the last step of ABA biosynthesis, whereby ABA aldehyde is converted to ABA (Seo et al., 2000).

Adverse environmental conditions such as drought, cold, and salt can greatly promote ABA biosynthesis in higher plants. Increased ABA levels under abiotic stress conditions may result mainly from enhanced de novo biosynthesis (Xiong and Zhu, 2003). Several key genes involved in ABA biosynthesis significantly increased when plants were subjected to abiotic stresses. In *Arabidopsis*, the expression of *AtZEP* was induced by drought, salt, and polyethylene glycol (Xiong et al., 2002). Similar results have been observed in other ABA biosynthetic genes, such as *NCED*s (Qin and Zeevaart, 1999; Xian et al., 2014). Moreover, a series of recent findings have shown that overexpression of the ABA biosynthetic genes in transgenic plants enhances abiotic stress tolerance under increased ABA levels. Park et al. (2008) reported that overexpression of *AtZEP* in *Arabidopsis* promotes ABA biosynthesis and enhances tolerance to salt and drought stress as compared with wild-type (WT) plants. The *Arabidopsis LOS5* gene encodes a molybdenum cofactor, which is essential for activating aldehyde oxidase. The transgenic cottons ectopically expressing *AtLOS5* exhibited more tolerance to drought stress than control plants (Yue et al., 2012). In addition, the overexpression of *NCED* homologs encoding the rate-limiting enzymes of ABA biosynthesis has been shown to improve the tolerance of transgenic plants to multiple abiotic stresses by increasing ABA levels (Qin and Zeevaart, 2002; Xian et al., 2014). Thus, identification of genes involved in pathways of ABA biosynthesis can be useful for improving the tolerance of transgenic plants to various abiotic stresses.

Eutremea salsugineum is an extremophile model plant with exceptionally high resistance to salinity, cold, and drought as well as oxidative stress (Inan et al., 2004). Hence, it is important to explore genes responsible for abiotic stress tolerance in *E. salsugineum* plants. In this study we investigated the role of the *EsABA1* gene from *E. salsugineum* in the salt-stress response by overexpressing the gene in tobacco (*Nicotiana tabacum*). Our results revealed that the activation of *EsABA1* expression in tobacco enhanced tolerance to salt stress under increased ABA levels. Furthermore, overexpression of *EsABA1* significantly promoted the accumulation of proline and enhanced the activities of 2 antioxidant enzymes (SOD and CAT). Taken together, our results indicated that the *EsABA1* gene could be utilized to improve salt tolerance in plants via genetic engineering.

2. Materials and methods

2.1. Plant materials and stress treatments

Seeds of tobacco (*Nicotiana tabacum*) and *Eutrema salsugineum* (Shandong ecotype) were germinated and grown in a greenhouse at 22 °C with a 16 h light/8 h dark photoperiod and 60%–80% relative humidity. To examine the expression patterns of *EsABA1* in response to various abiotic stresses and ABA treatment, 10-day-old *E. salsugineum* plants grown on Murashige and Skoog (MS) medium containing 0.8% agar were treated with cold (4 °C), NaCl (200 mM), PEG 6000 (30%), or ABA (100 μM) for different time periods (0, 3, 6, 12, and 24 h).

2.2. Isolation and cloning of *EsABA1*

Total RNA was extracted from 5-week-old *E. salsugineum* plants exposed to 200 mM NaCl for 12 h using the TRIzol reagent (Invitrogen, USA), and residual genomic DNA was digested by RNase-free DNase I (Invitrogen, USA). Based on the sequence (GenBank accession no.: XM_006393839) from *E. salsugineum*, a pair of gene-specific primers was designed to amplify the coding region of the *EsABA1* gene. The amplified PCR fragments were separated on a 1% agarose gel and then subcloned into pGEM-T vector (Promega, USA) for sequencing. DNA sequencing was performed by Invitrogen in Shanghai, China. The PCR amplification cycle was as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 7 min. Primers used in the above experiments are shown in Table 1.

Table 1. Primers used in this study.

Primer	Usage	Sequence
NtERD10C	qRT-PCR	5′-CGGTCTTTGAGTGATATC 5′-GAAGAAAGAGGAGCACA
NtP5CS	qRT-PCR	5’-TGATCGAAGATTAGCACTT 5’-TCCTACAGCACCTGAAGTC
EsABA1	qRT-PCR or RT-PCR	5’-CGACTTATTGCACGCGACA 5’-AATAGAATCCCCGAGCAGCG
EsABA1-overexpression	Subcloning	5’-ATGGGTTCAACTCCGTTTTG 5’-TTAAACTGCCTGAAGTAGTT

2.3. Bioinformatic analysis of *EsABA1*

Analysis of nucleotide sequences was carried out using the BLAST program at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST/). Analysis of amino acid sequences was done using the ClustalW2 program (www.ebi.ac.uk/Tools/msa/clustalw2). The phylogenetic relationship of sequences was constructed by the neighbor-joining method of MEGA 4.0. Analysis of conserved domains was done using the SMART program (http://smart.embl-heidelberg.de).

2.4. Vector construction and plant transformation

For the overexpression of *EsABA1*, the coding sequence of *EsABA1* was amplified with specific primers containing *SacI* and *KpnI* restriction sites. The amplified products were subcloned into pGEM-T vector (Promega, USA) for sequencing and were then digested. The digested fragments of *EsABA1* were ligated into a plant binary vector containing a super-promoter consisting of a trimer of the octopine synthase (OCS) transcriptional activating element affixed to the mannopine 2 synthase 2' (mas2') transcriptional activating element (Zhu et al., 2014).

The constructed plasmids were transferred into *Agrobacterium tumefaciens* strain *GV3101* and used to transform tobacco plants by a leaf-disc transformation method (Kang et al., 2005). Transformed tobacco plants were screened on MS agar medium containing 30 mg L^{-1} hygromycin. Furthermore, the *EsABA1* gene was amplified as the selection marker by PCR using genomic data from the hygromycin-resistant transformants. The PCR amplification conditions were as follows: 94 °C for 5 min, 28 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and a final elongation at 72 °C for 7 min.

2.5. Analysis of total chlorophyll content and salt-stress assays

Leaves of WT and 2 independent T2 transgenic tobacco lines (OE3 and OE5) were used for the chlorophyll leaching experiments. For the chlorophyll leaching assay, leaf discs (0.5 cm in diameter) were prepared from fully expanded tobacco leaves from WT and *EsABA1*-overexpressing lines. The discs were cut and floated on water and NaCl solutions (0, 400, and 600 mM). Their chlorophyll contents were determined spectrophotometrically after extraction using 80% acetone at 4 °C, as described by Lichtenthaler (1987). For the whole-plant salt-stress assay, 7-day-old tobacco plants from each line (WT, OE3, and OE5) were grown on MS medium with different concentrations of NaCl solution (0 and 250 mM) for 4 weeks.

2.6. Analyses of RT-PCR and qRT-PCR

Total RNA was extracted from plants using TRIzol reagent (Takara, Japan), and first-strand cDNA was synthesized from 500 ng of total RNA using reverse transcriptase ReverTraAce with oligo (dT) primers (Takara, Japan), according to the manufacturer's instructions. PCR was carried out with 1 μL of cDNA in a 20-μL reaction volume. *Nicotiana tabacum actin2* (*NtActin*) (Ziaf et al., 20011) was used as an internal control to normalize the RT mixtures. The PCR conditions were as follows: 95 °C for 5 min, 28 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 7 min.

Quantitative reverse transcription PCR (qRT-PCR) was performed using a SYBR Premix Ex Taq kit (TaKaRa, Japan), and the reactions were performed on a 7500 Real-Time PCR machine (ABI, USA) by an initial denaturation step at 95 °C for 30 s, 40 cycles of 95 °C for 15 s, and 60 °C for 30 s followed by standard dissociation protocol, according to the manufacturer's instructions. All quantifications were normalized using *EsActin2* (Zhou et al., 2012) or *NtActin* as internal reference. The calculations were performed using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001). PCRs were performed in triplicate with at least 3 independent samples. Primers used for analyses are listed in Table 1.

2.7. Biochemical analyses

The pigment compositions were determined using high-performance liquid chromatography (HPLC) with UV detection at 445 nm, as previously described (Park et al., 2008). Measurement of endogenous ABA levels was performed using the enzyme-linked immunosorbent assay (ELISA) method (Yang et al., 2001). Free proline content was determined according to the method of Bates et al. (1973). Activities of the antioxidant enzymes SOD, CAT, POD, and APX were measured according to the method described by Xie et al. (2008).

2.8. Determination of $O_2^{\cdot-}$ and H_2O_2 levels in the leaves of plants

Levels of H_2O_2 and $O_2^{\cdot-}$ in leaves of WT and transgenic plants exposed to high salinity conditions were detected by histochemical staining with DAB and NBT (Shulaev and Oliver, 2006). Leaf samples were vacuum infiltrated in 10 mL of DAB solution (1 mg mL^{-1}, pH 3.8) or NBT (1 mg mL^{-1}, pH 7.8) for 15 min at room temperature. To examine localization of H_2O_2 and $O_2^{\cdot-}$, immersed leaves were incubated in the dark for 8 h and in the light for 2 h, respectively. In addition, the H_2O_2 content was measured according to the method described by Mukherjee and Choudhuri (1983), and the $O_2^{\cdot-}$ content was determined as previously described by Liu and Pang (2010).

2.9. Statistical analysis

Each experiment was carried out in triplicate. Each column represents the mean of 3 independent experiments. The bars represent the standard deviation (SD) of the mean. Columns with different letters indicate significant differences between the means of WT and transgenic lines under control and salt treatments using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at $P < 0.05$.

3. Results

3.1. Cloning and sequence analysis of *EsABA1*

A full-length cDNA sequence of *EsABA1* from *E. salsugineum* was obtained by the NCBI program. Nucleotide sequence analysis indicated that the full-length cDNA was 2364 bp, which contained the coding sequence of 2001 bp, the 5' untranslated region (UTR) of 136 bp, and the 3' UTR of 227 bp. The ORF of *EsABA1* encodes a protein of 666 amino acids (aa) with an estimated molecular mass of 73.67 kDa and a theoretical pI of 5.86. The SMART analysis revealed that the EsABA1 protein contained 2 conserved flavin-adenine dinucleotide (FAD) binding domains (one between residues 80 and 268, the other between residues 360 and 436) and a forkhead-associated (FHA) phosphopeptide binding motif (between residues 558 and 634).

Multiple sequence alignment of ZEP homologs from different plant species was performed using the ClustalW2 program (Figure 1a). The results indicated that EsABA1 shared 61% amino acid similarity with GmZEP3, 67% with TaZEP, 72% with ZmZEP, 77% with CpABA1, and 92% with BnZEP. In order to gain insight into the evolutionary relationship, a phylogenetic tree was constructed based on the amino acid alignments using MEG 4.0 (Figure 1b). Several other ZEP proteins in different plant species were obtained from the NCBI database. The phylogenetic analysis indicated that the EsABA1 protein was similar to its counterparts in diverse plant species.

3.2. Expression patterns of *EsABA1*

To determine the spatial expression pattern of the *EsABA1* gene, qRT-PCR was used to analyze its mRNA levels in some major tissues of *E. salsugineum* including leaves, stems, flowers, roots, and siliques. As shown in Figure 2a, the results of transcription analyses indicated that *EsABA1* was expressed in all tested tissues, yet its expression levels varied among these tissues. The *EsABA1* gene was expressed more in leaves and stems than in siliques, while its lowest expression occurred in roots.

We then investigated whether the transcription of *EsABA1* was induced in response to various abiotic stresses such as cold, PEG, and NaCl. Our results revealed that all these stresses obviously stimulated expression levels of *EsABA1*. As shown in Figure 2b, the transcription of *EsABA1* increased gradually until 12 h after treatment, and the expression of *EsABA1* peaked within 12 h and then dropped when the seedlings were subjected to 200 mM NaCl. Upon cold treatment at 4 °C, the *EsABA1* mRNA quickly accumulated at 3 h and then continuously increased until 6 h after treatment, followed by a significant reduction after 24 h. Treatment with 30% PEG 6000 caused a quick induction of *EsABA1* transcripts at 6 h, followed by a significant reduction until 12 h after treatment. Upon ABA treatment, the transcription levels of *EsABA1* upregulated at 3 h and exhibited a gradual increase, reaching the highest level at 12 h. These data suggest that the expression of *EsABA1* was induced by various abiotic stresses and ABA.

3.3. Generation of transgenic tobacco lines overexpressing *EsABA1*

To investigate the physiological roles of *EsABA1* in response to salt stress, we generated transgenic tobacco plants ectopically expressing *EsABA1* driven by a super-promoter (Figure 3a), and 5 independent transgenic lines were selectively cultured on MS agar medium containing 30 mg L^{-1} hygromycin. The result of RT-PCR analysis showed that the *EsABA1* gene was overexpressed constitutively in several independent T2 transgenic lines compared with WT plants (Figure 3b). Among these transgenic lines, 2 independent transgenic lines highly expressing *EsABA1* (OE3 and OE5) were used for salt-tolerance analysis.

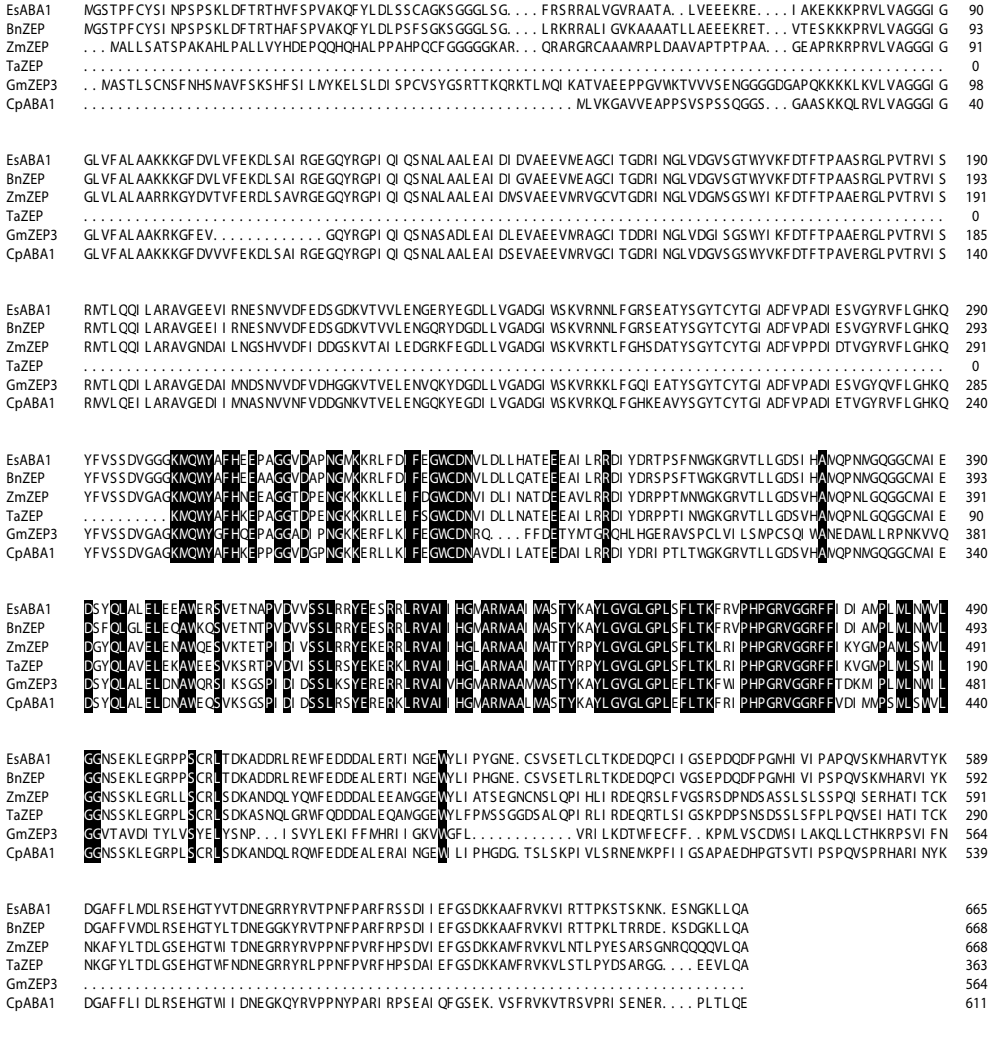
3.4. HPLC analysis of the pigment compositions

Previous studies have shown that *Nicotiana ABA2* and *Arabidopsis ZEP* encode putative zeaxanthin epoxidases which catalyze the conversion of zeaxanthin to violaxanthin. Thompson et al. (2000) reported that downregulated expression of *LeZEP1* promoted the accumulation of zeaxanthin in leaves of tomato. Moreover, zeaxanthin cannot be detected in transgenic *Arabidopsis* plants overexpressing *AtZEP*. These studies suggested that the content of zeaxanthin may be associated with the expression levels of *ZEP* genes in plants. In order to investigate the effects of *EsABA1* overexpression in transgenic tobacco plants, the pigment compositions were measured by HPLC (Table 2). The results revealed that zeaxanthin was undetectable in 2 independent transgenic lines highly expressing *EsABA1* (OE3 and OE5), whereas a small amount of zeaxanthin was detected in WT plants. Furthermore, these transgenic lines exhibited higher violaxanthin content than WT plants. Our results further indicated that the *EsABA1* gene played a pivotal role in the zeaxanthin cycles. Based on these data, 2 transgenic lines (OE3 and OE5) were selected for further studies.

3.5. Improved tolerance to salt stress in transgenic tobacco

To assess salt tolerance of plants, leaf disc assays were conducted to analyze the effects of *EsABA1* overexpression on the chlorophyll content. In this study, leaf discs were cut from leaves of both 5-week-old WT and transgenic lines. The leaf discs were then floated on NaCl for 3 days to determine the chlorophyll levels. As shown in Figures 3c and 3d, there were no significant phenotypic differences between WT and transgenic plants under control (no salt) conditions, whereas leaf discs from the 2 transgenic lines maintained higher chlorophyll content than WT plants upon exposure to NaCl. When treated with 400 mM

(a)



(b)

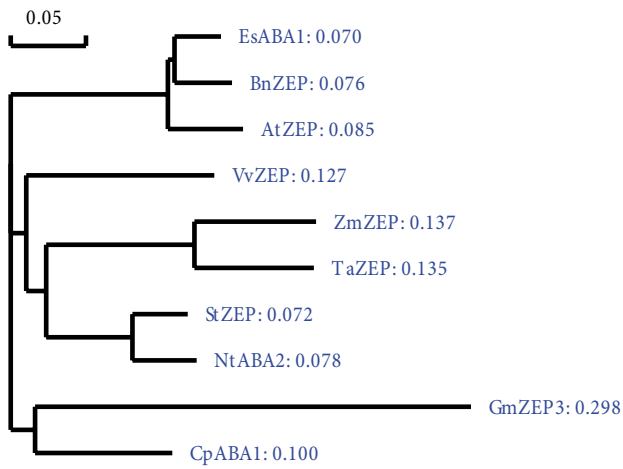


Figure 1. Multiple sequence alignment and phylogenetic analysis of EsABA1 and other ZEP proteins from several different plant species. (a) Alignment of EsABA1 and other plant ZEP homologs. Amino acids shaded in black indicate identical and similar residues, respectively. (b) Phylogenetic tree of EsABA1 and other ZEP homologs from other plant species using the neighbor-joining method by MEGA 4.0 software. The GenBank accession numbers of the amino acid sequences of all ZEP proteins used in this study are as follows: EsABA1 (AAV85824), AtZEP (NP_851285), BnZEP (ADC29517), VvZEP (AAR11195), ZmZEP (NP_001151443), StZEP (ADF28629), TaZEP (CAX36916), NtABA2 (BW97847), GmZEP3 (AEK69513), and CpABA1 (BAB11934).

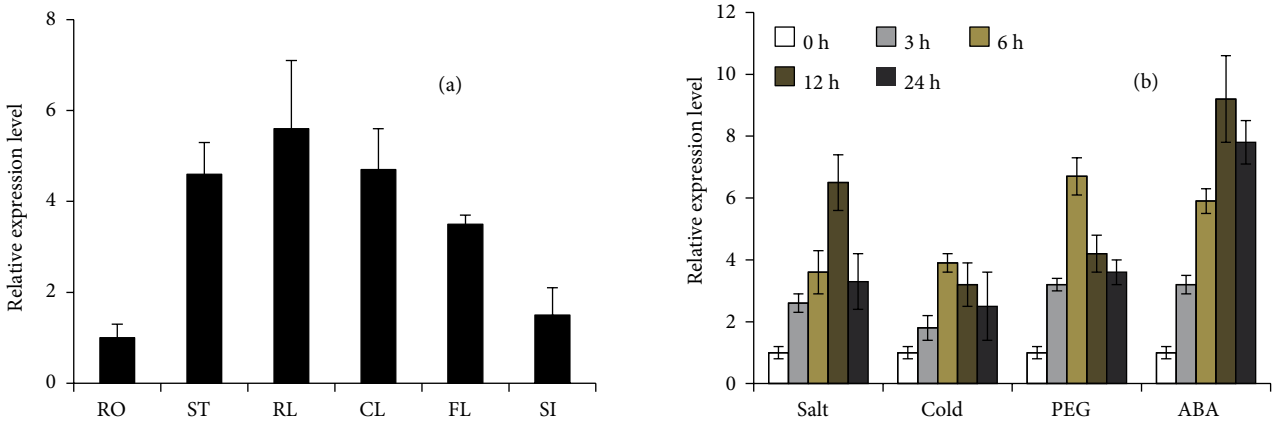


Figure 2. qRT-PCR analysis of the expression patterns of *EsABA1* in different tissues and under various abiotic treatments. (a) Transcription levels of *EsABA1* in roots (RO), stems (ST), rosette leaves (RL), cauline leaves (CL), flowers (FL), and siliques (SI) of *E. salsugineum*. (b) qRT-PCR analysis was carried out using total RNA extracted from 10-day-old *E. salsugineum* seedlings that were treated with cold (4 °C), NaCl (200 mM), PEG 6000 (30%), or ABA (100 μM) for different time periods (0, 3, 6, 12, and 24 h). *EsActin2* was used as internal control for gene expression normalization. Data represent means and standard errors of 3 replicates.

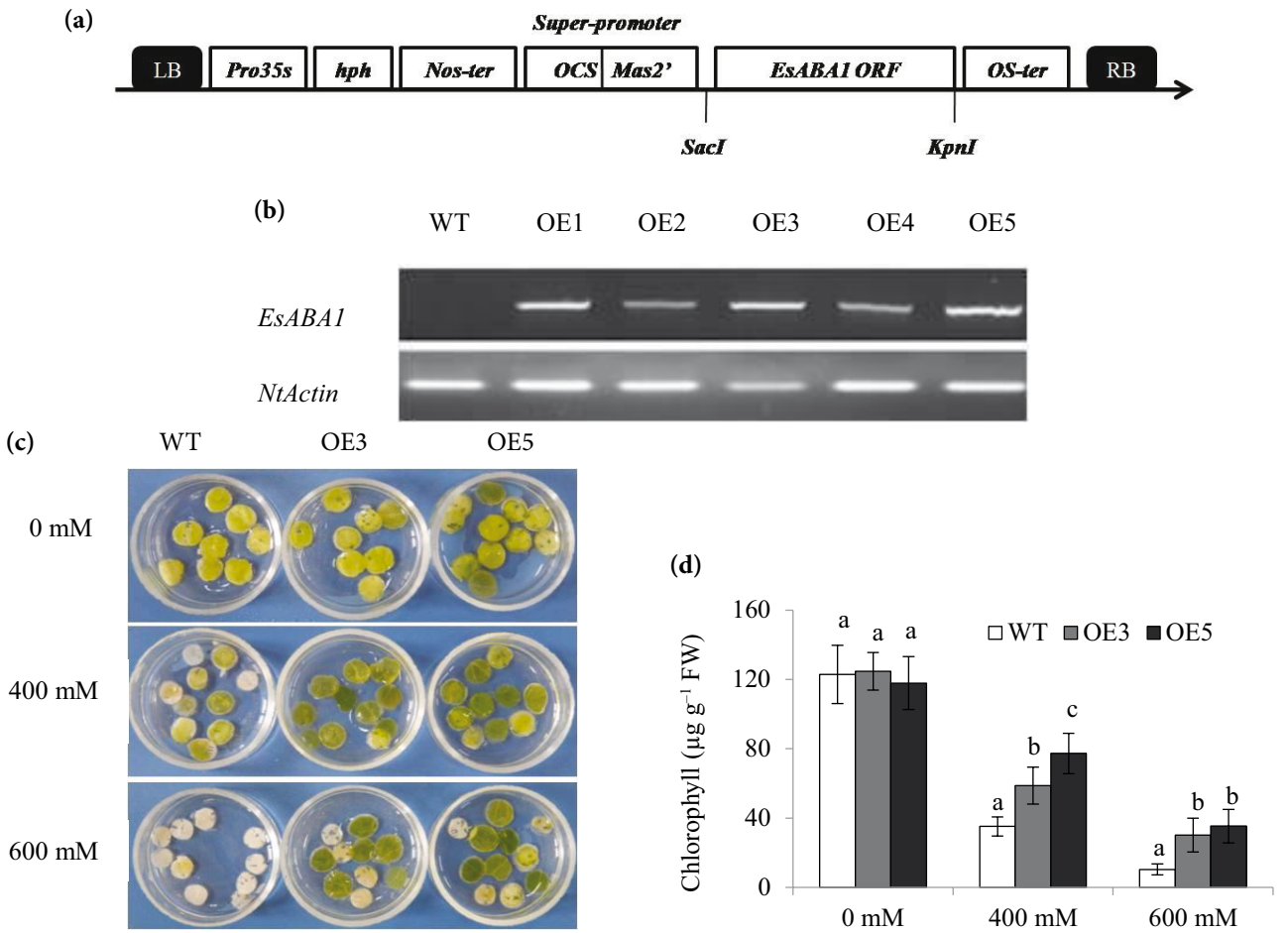


Figure 3. Characterization of *EsABA1*-overexpressing transgenic tobacco lines. (a) A physical map of the construct carrying *EsABA1* cDNA driven by the super-promoter. (b) RT-PCR analysis of the transcription levels of *EsABA1* in WT and 5 independent T2 transgenic tobacco lines. (c) Leaf-disc assay of WT and transgenic lines (OE3 and OE5) at different concentrations of NaCl (0, 400, and 600 mM) for 3 days. (d) Chlorophyll content in leaf discs of both WT and transgenic lines (OE3 and OE5) at different concentrations of NaCl (0, 400, and 600 mM).

Table 2. HPLC analysis of the pigment compositions of WT and 2 transgenic lines (OE3 and OE5). Five-week-old WT and transgenic lines were pretreated with darkness for 2 h. Zeaxanthin (Z) + antheraxanthin (A) + violaxanthin (V) = 100%. The errors represent standard deviations of 3 replicates.

Pigment composition (%)	WT	Transgenic lines	
		OE3	OE5
Z	6.8 ± 0.16	0 ± 0.00	0 ± 0.00
A	9.5 ± 0.82	3.5 ± 0.12	2.6 ± 0.24
V	83.7 ± 0.32	96.5 ± 0.12	97.4 ± 0.24

NaCl, chlorophyll contents of the transgenic lines (OE3 and OE5) decreased to 58.7 μg g^{-1} FW and 77.3 μg g^{-1} FW, respectively; however, content was still much higher than in WT plants (35.2 μg g^{-1} FW). When the concentration of NaCl was elevated to 600 mM, leaf discs of WT plants were almost totally bleached, while the chlorophyll contents of transgenic lines remained at 30.2 μg g^{-1} FW and 35.4 μg g^{-1} FW, respectively.

To further investigate whether overexpression of *EsABA1* enhanced salt tolerance in transgenic plants, we performed salt-stress experiments with WT and the 2 transgenic lines (OE3 and OE5). WT and the transgenic lines (OE3 and OE5) were grown in MS medium for 7 days without salt-stress treatments. Then these plants were transferred to MS medium containing 0 or 250 mM NaCl for 4 weeks. As shown in Figure 4, no difference

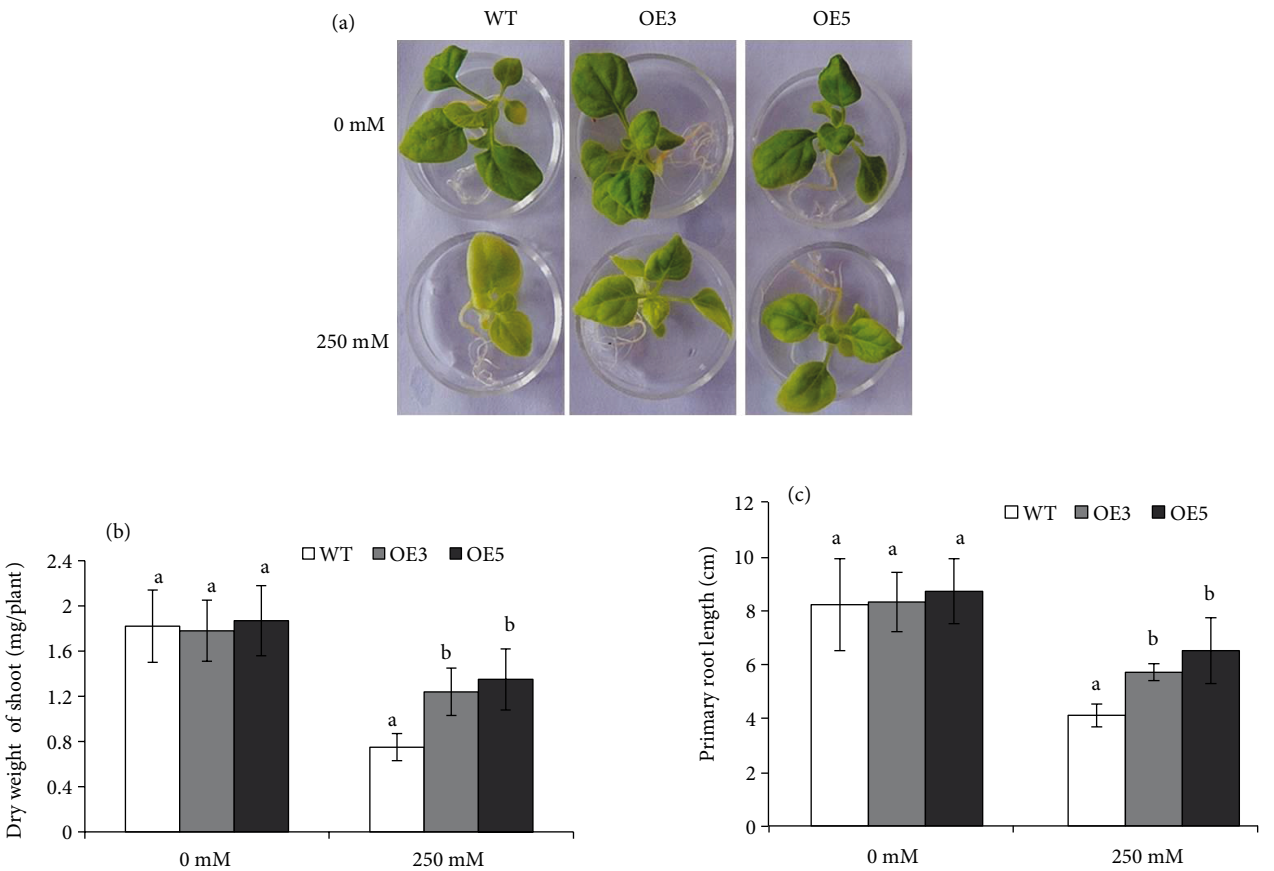


Figure 4. Comparison of the phenotype and growth parameters of WT and 2 transgenic lines (OE3 and OE5). (a) Morphological analysis of WT and transgenic lines cultivated on MS medium containing different concentrations of NaCl (0 and 250 mM), (b) shoot dry weight, and (c) primary root length.

between WT and transgenic lines was observed without the presence of 250 mM NaCl. However, transgenic lines OE3 and OE5 displayed a significant increase in shoot dry weight and primary root length relative to WT plants under high salt conditions. These observations indicated that the transgenic lines exhibited better growth than WT plants when subjected to salt stress.

3.6. Detection of $O_2^{\cdot-}$ and H_2O_2 in leaves of plants

To further examine ROS accumulation, the levels of H_2O_2 and $O_2^{\cdot-}$ in leaves of both untreated and treated plants were detected using histochemical staining with DAB and NBT, respectively. As shown in Figure 5, the leaves of untreated WT and transgenic lines (OE3 and OE5) exhibited similar staining. However, leaves of WT plants exhibited more staining spots than those of transgenic lines when subjected to salt stress. Moreover, the endogenous contents of H_2O_2 and $O_2^{\cdot-}$ were also measured. Our data showed that the contents of H_2O_2 and $O_2^{\cdot-}$ were higher in WT plants than in the transgenic lines. These results demonstrated that the transgenic plants accumulated lower H_2O_2 and $O_2^{\cdot-}$ content than WT plants after exposure to salt stress.

3.7. Analysis of the ABA and proline contents in transgenic tobacco

In our study, the *EsABA1* gene was found to promote the conversion of zeaxanthin to violaxanthin. Transgenic tobacco plants ectopically expressing *EsABA1* exhibited more salt tolerance than WT plants. These results suggested that the *EsABA1* gene was involved in the regulation of ABA biosynthesis and modulated ABA signal transduction. Previous studies have shown that elevated ABA levels result in a significant increase in proline levels in plants, and proline has been shown to improve the tolerance of plants to salt stress (Woodward and Bennett, 2005).

To explore the physiological characterizations for the improved salt tolerance of the 2 transgenic plants (OE3 and OE5), the contents of ABA and proline were measured. Under no salt-stress conditions, there were significant differences in ABA content between WT and the transgenic line (OE5), whereas the proline levels were not altered (Figure 6). After 10 days of exposure to salt stress, it was observed that salt stress greatly increased the accumulation of endogenous ABA and proline in the leaves

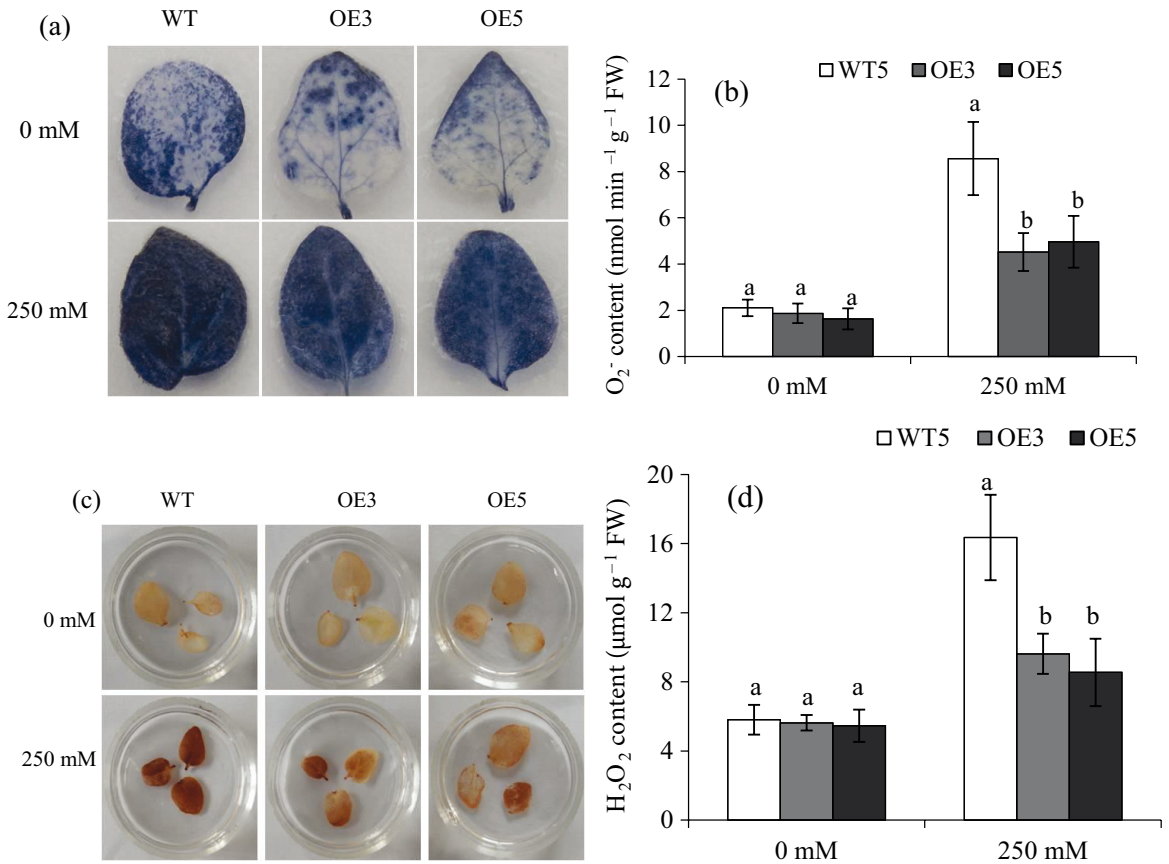


Figure 5. Analysis of localization and quantification of $O_2^{\cdot-}$ and H_2O_2 in leaves of WT and 2 transgenic lines (OE3 and OE5) under control (0 mM NaCl) and salt (250 mM NaCl)-stress conditions. (a) Localization of $O_2^{\cdot-}$ by NBT staining, (b) quantification of the $O_2^{\cdot-}$ content, (c) localization of H_2O_2 by DAB staining, and (d) quantification of the H_2O_2 content.

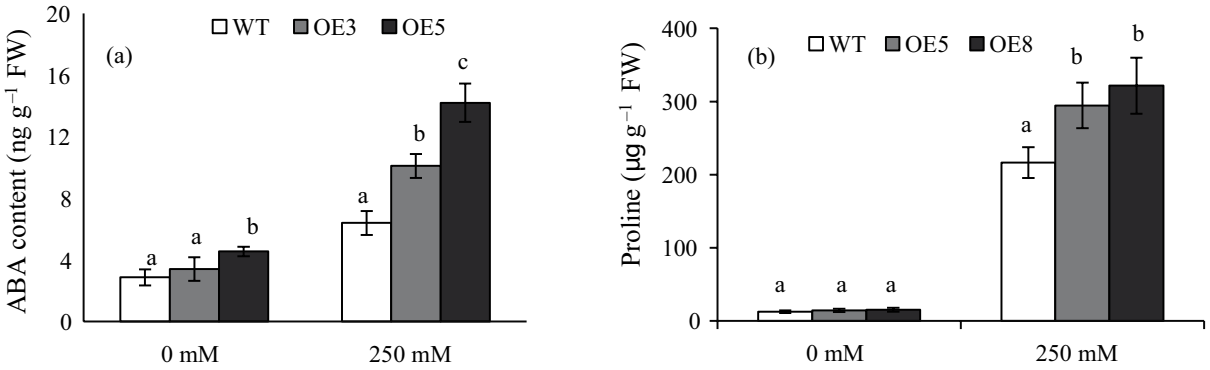


Figure 6. Determination of the ABA and proline content in WT and 2 transgenic lines (OE3 and OE5) under control (0 mM NaCl) and salt (250 mM NaCl)-stress conditions. (a) ABA and (b) proline.

of both WT and the transgenic lines, but the transgenic lines had significantly higher ABA and proline contents than the WT plants. There were also significant differences in ABA contents between the 2 transgenic lines.

3.8. Upregulated stress responsive genes and improved antioxidant enzyme activities in transgenic tobacco

Recent studies have indicated that increased ABA levels can enhance the activities of antioxidant enzymes and upregulate the expression of genes encoding stress responsive transcriptional activators such as *NtP5CS* and *NtERD10C* (Guo et al., 2012; Xian et al., 2014). In the present study we investigated the expression of 2 stress responsive genes and the activities of antioxidant enzymes. As shown in Figure 7, the transcription levels of *NtP5CS* and *NtERD10C* were much higher in the 2 transgenic lines (OE3 and OE5) than in the WT plants under salt-stress conditions. In addition, the activities of antioxidant enzymes including SOD, CAT, POD, and APX were measured (Figure 8). Under normal conditions there were no significant differences between WT and the transgenic lines in the activities of antioxidant enzymes. Under salt-stress conditions, the activities of 2 antioxidant enzymes (SOD and CAT) were markedly enhanced in the leaves of both WT and transgenic lines, but the transgenic lines showed higher activities of the 2 antioxidant enzymes than WT plants.

4. Discussion

Salt stress is one of the most serious environmental factors limiting plant growth and productivity. Plants have evolved a considerable strategy to cope with adverse environments. A number of studies have demonstrated that ABA is involved in plant response to various abiotic stresses and plays cardinal roles in the regulation of plant acclimation or adaptation to adverse environmental conditions. Exploring the biosynthetic genes underlying ABA metabolic pathways is, therefore, essential to understanding the basic mechanism of plant tolerance to abiotic stresses such as salt, drought, and cold. It has recently been reported that key genes such as *ZEP*, *NCED*, and *AAO* play important roles in the regulation of the ABA biosynthesis (Schwartz et al., 1997; Seo et al., 2000; Xiong et al., 2002). Moreover, the increased expression of these ABA biosynthetic genes can improve the tolerance of transgenic plants to abiotic stresses (Park et al., 2008; Xian et al., 2014).

Previous studies have indicated that ZEP proteins contain commonly conserved sequence motifs: 2 monooxygenase flavin-adenine dinucleotide (FAD) binding sites and 1 forkhead-associated (FHA) phosphopeptide binding motif. The FAD binding sites have been positively associated with zeaxanthin

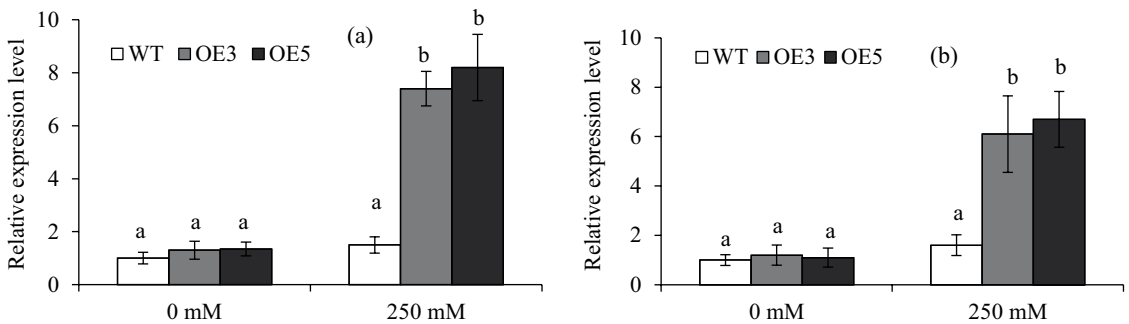


Figure 7. qRT-PCR analysis of the transcription levels of stress responsive genes including *NtERD10C* (a) and *NtP5CS* (b) in WT and 2 transgenic lines (OE3 and OE5) under control (0 mM NaCl) and salt (250 mM NaCl)-stress conditions.

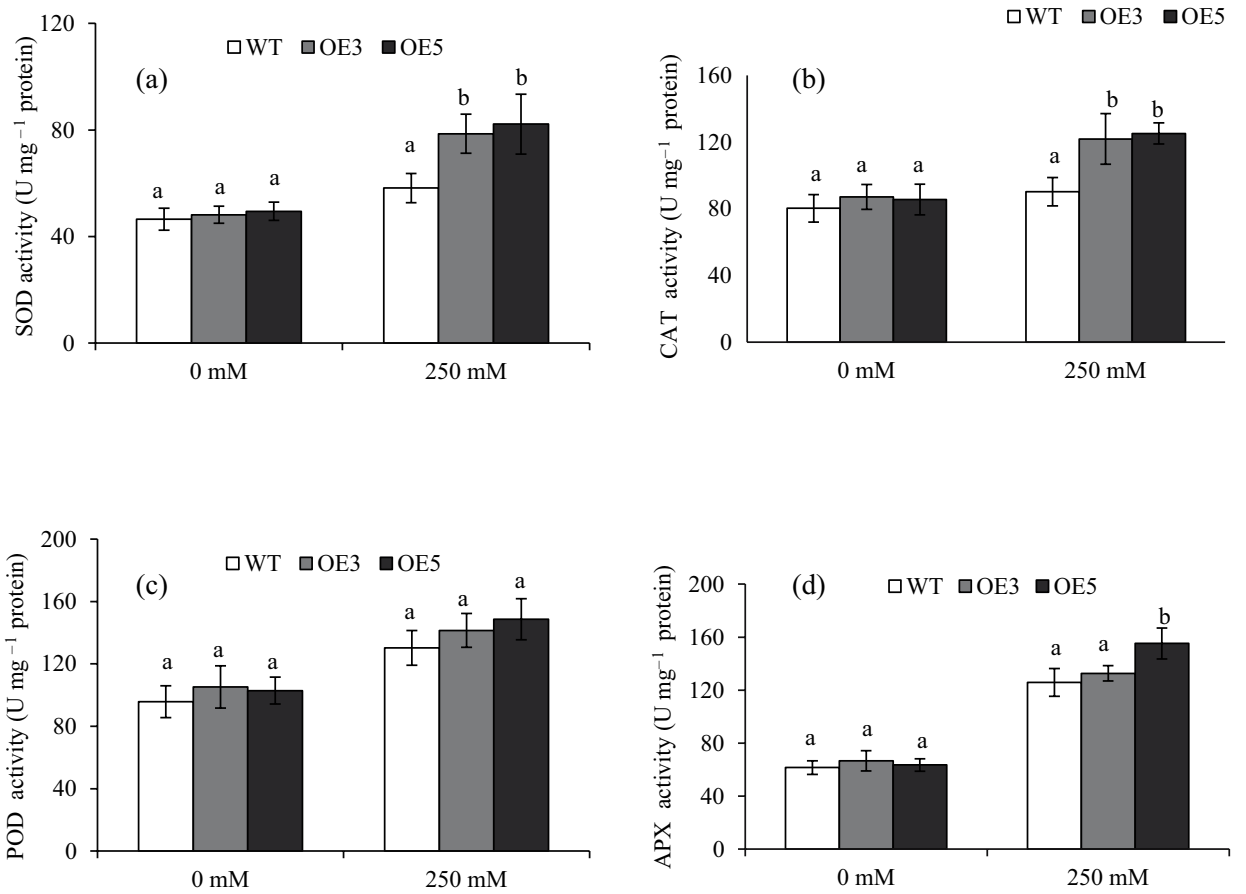


Figure 8. Overexpression of *EsABA1* enhances antioxidant enzyme activities in the 2 transgenic lines (OE3 and OE5) when subjected to 250 mM NaCl. The activities of antioxidant enzymes of WT and transgenic lines were determined under control (0 mM NaCl) and salt (250 mM NaCl)-stress conditions. (a) SOD, (b) CAT, (c) POD, and (d) APX.

epoxidase activity (Barrero et al., 2005). In the present study a novel zeaxanthin epoxidase gene, *EsABA1*, was isolated and characterized from *E. salsugineum*. Sequence analyses revealed that the EsABA1 protein contained 2 *FAD*-binding sites and 1 FHA binding domain, which showed a high level of sequence similarity to other plant species. Furthermore, a phylogenetic tree constructed using the *ZEP* genes of 10 different plant species showed that *EsABA1* was clustered in the same subgroup with BnZEP. These results suggested that the *EsABA1* gene was a potential zeaxanthin epoxidase, which plays important roles in ABA biosynthesis pathways.

Environmental factors influence the pathways of ABA biosynthesis (Xiong and Zhu, 2003). Previous studies have shown that light or darkness stimulates the backward reaction of the violaxanthin cycle and promotes the conversion of zeaxanthin back to violaxanthin in *A. thaliana* and *C. reinhardtii* (Förster et al., 2009). In addition, abiotic stresses, especially drought and salt, have been shown to dramatically activate ABA biosynthesis through transcriptional regulation of ABA biosynthetic genes such as *ZEP* and *NCED* (Qin and Zeevaart, 1999; Xiong and Zhu, 2003). However, regulation of these ABA biosynthetic genes shows variation depending on plant part and developmental stage. There are also significant differences among plant species in the expression patterns of the ABA biosynthetic genes. The *ZEP* genes involve the ABA biosynthetic pathway, and their expression and regulation have been investigated in different plant species (Xiong et al., 2002). In tobacco and tomato, the *ZEP* genes in roots of plants were upregulated under stress conditions, while the transcription levels of *ZEP* genes in leaves were not increased. However, the expression level of *AtZEP* greatly increases in both shoots and roots of *Arabidopsis* upon drought and salt stresses. Recently, a majority of stress-responsive genes from *E. salsugineum* were identified in response to salt, drought, and cold stresses; however, little is known about the physiological role of ABA biosynthetic genes in stress tolerance (Taji et al., 2008).

In this study, our data showed that the *EsABA1* was expressed in almost all major plant tissues, indicating that *EsABA1* may be involved in diverse physiological and developmental processes. Furthermore, the expression levels of *EsABA1* were also investigated under abiotic stress conditions. An abundance of *EsABA1* mRNA after 12 h of exposure to salt stress was observed in *E. salsugineum* seedlings, which indicated that it was an early salt-responsive gene. The salt-induced expression pattern of *EsABA1* was similar to the observations made in *ZEP* genes of other plant species (Xiong et al., 2002). In addition, the transcription of *EsABA1* was induced by exogenous ABA, suggesting that *EsABA1* was involved in ABA signaling pathways.

ABA is an important plant hormone and plays important roles in vegetative development in response to environmental stresses such as drought and high salinity conditions. It has been shown that increased ABA levels can improve drought and salt tolerance in some plant species (Guo et al., 2012). Overexpression of *LeNCED1* in transgenic tomato showed increased water-use efficiency with endogenous ABA accumulation at very high levels (Thompson et al., 2000). In *Arabidopsis*, the *LOS5/ABA3* gene plays important roles in the last step of ABA biosynthesis, and overexpression of this gene has been shown to improve tolerance to drought stress, with elevated ABA levels in transgenic plants (Yue et al., 2012). Moreover, overexpression of *AtZEP* enhances the ABA biosynthesis in transgenic *Arabidopsis*, and these transgenic plants exhibited more tolerance to salt and drought stresses than WT plants (Park et al., 2008). Consistent with these reports, ectopic expression of *EsABA1* promoted ABA biosynthesis, and the transgenic lines exhibited more vigorous growth than WT plants under high salt conditions. Our results further revealed slight differences in the ABA content between WT and the transgenic lines before salt-stress treatment; however, ABA levels of transgenic plants are much higher than those of WT plants under salt-stress conditions. This may be explained through a model for stress-inducible ABA biosynthesis in *Arabidopsis* plants.

ABA biosynthesis can be modulated by multiple enzymes of ABA biosynthesis (Xiong and Zhu, 2003). The first biosynthetic step in ABA biosynthesis is the conversion of zeaxanthin to all-trans-violaxanthin, a 2-step epoxidation process catalyzed by ZEP (Marin et al., 1996). These generated precursors then undergo a series of catalytic processes through some key enzymes, such as NCED and AAO, and eventually produce ABA (Schwartz et al., 1997; Seo et al., 2000). Thus, it was possible that overexpression of a solitary gene related to ABA biosynthesis could not cause a great increase in ABA biosynthesis under normal conditions. However, salt stress was shown to induce a large amount of ABA accumulation in *EsABA1*-overexpressing lines. It was postulated that abiotic stresses stimulated the expression of *NCED* (a limiting enzyme of controlling ABA biosynthesis) and *AAO* (a final step of the ABA biosynthetic enzyme). The *EsABA1* gene, in cooperation with other ABA biosynthesis genes such as *NCED* and *AAO*, leads to more ABA biosynthesis under salt-stress conditions. In addition, proline accumulation has been observed in both WT and the transgenic lines in response to salt stress, whereas the proline levels of transgenic lines were much higher than those of the WT plants. It has been indicated that the elevated endogenous ABA levels can enhance tolerance to multiple abiotic stresses with increased proline accumulation (Yue et al., 2012), suggesting that the ABA levels may influence proline biosynthesis in plants. In our experiments, the 2 transgenic lines (OE3 and OE5) exhibited more proline accumulation than the WT plants, which may result from differences in ABA contents between WT and transgenic lines.

Similar results were observed in the expression of stress responsive genes and antioxidant enzymes, which showed higher activity in the transgenic lines as compared with WT plants under salt-stress conditions. The increased expression of *EsABA1* upregulated the expression of stress responsive genes and enhanced the activities of 2 antioxidant enzymes (SOD and CAT) and the accumulation of proline, which may contribute to the efficient reduction of ROS accumulation caused by salt stress. In the present study 2 major types of ROS, levels of H_2O_2 and $O_2^{\cdot-}$, were lower in transgenic lines than in WT plants, indicating a reduced potential for oxidative damage evoked by salt stress. Enhanced tolerance could also result from the reduction in ROS damage as a consequence of *EsABA1* overexpression. Collectively, our finding suggested that the *EsABA1*-overexpressing lines had a more active ROS-scavenging system than the WT plants.

In conclusion, the *EsABA1* gene from *E. salsugineum* was isolated and expressed in all the tested tissues. The expression of *EsABA1* was upregulated by abiotic stresses and ABA. Overexpression of *EsABA1* in transgenic tobacco improved salt tolerance, which may be attributed to enhancement of the transcription of stress responsive genes and the activities of antioxidant enzymes as a result of increased ABA or proline accumulation. Therefore, the *EsABA1* gene may be utilized to improve plant salt tolerance by genetic manipulation.

Acknowledgments

We thank Jian Zhu and Zhixue Liu, professors at Tongji University, for their valuable suggestions. This work received support from the Anhui Science and Technology University (ZRC2014403).

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