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Research paper

Transgenic poplar overexpressing the endogenous transcription factor *ERF76* gene improves salinity tolerance

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The ethylene response factor (ERF) family is one of the largest plant-specific transcription factor families, playing an important role in plant development and response to stresses. The *ERF76* gene is a member of the poplar ERF transcription factor gene family. First, we validated that the *ERF76* gene expressed in leaf and root tissues is responsive to salinity stress. We then successfully cloned the *ERF76* cDNA fragment containing an open reading frame from di-haploid *Populus simonii* × *Populus nigra* and proved that ERF76 protein is targeted to the nucleus. Finally, we transferred the gene into the same poplar clone by the *Agrobacterium*-mediated leaf disc method. Using both RNA-Seq and reverse transcription-quantitative polymerase chain reaction, we validated that expression level of *ERF76* is significantly higher in transgenic plants than that in the nontransgenic control. Using RNA-Seq data, we have identified 375 genes that are differentially expressed between the transgenic plants and the control under salt treatment. Among the differentially expressed genes, 16 are transcription factor genes and 45 are stress-related genes, both of which are upregulated significantly in transgenic plants, compared with the control. Under salt stress, the transgenic plants showed significant increases in plant height, root length, fresh weight, and abscisic acid (ABA) and gibberellin (GA) concentration compared with the control, suggesting that overexpression of *ERF76* in transgenic poplar upregulated the expression of stress-related genes and increased the ability of ABA and GA biosynthesis, which resulted in stronger tolerance to salt stress.

Keywords: ERF transcription factor, genetic transformation, *Populus simonii* × *P. nigra*, RNA-Seq, salt tolerance.

Introduction

In the natural environment, plant growth and development are often affected by drought, salinity, heat and other abiotic stresses. Physiologically, plants' exposure to stress can lead to osmotic stress, active oxygen damage, ion toxicity and photoinhibition, which lead to irreversible cell damage (Munns 2002, Widodo et al. 2009) and interruption of many important life processes (Darwish et al. 2009). Therefore, plants have evolved multifaceted mechanisms in response to these stresses at the morphological, physiological and biochemical levels (Vinocur and Altman 2005). Under stress conditions, it is observed that many stress-responsive functional genes and regulatory genes are upregulated and a variety of stress-related proteins are accumulated (Seki et al. 2002, Bhatnagar-Mathur et al. 2008). The overexpression of functional genes encoding enzymes for the synthesis of osmotic compounds, transporters and reactive oxygen species (ROS) scavengers can improve stress tolerance in plants (Umezawa et al. 2006).

Transcription factors (TFs) play important regulatory roles in stress responses by affecting their target genes via binding to the *cis*-acting elements (Yanagisawa 2004, Mizoi et al. 2012, Nakashima et al. 2012, Rushton et al. 2012). Transgenic plants overexpressing the TF genes could enhance their tolerance to various stresses including salinity (Liu et al. 1998, Kasuga et al. 1999, Sakuma et al. 2006). Among the TF genes, the ethylene response factor (ERF) genes encode plant-specific TF proteins, and are one of the largest gene families in the plant kingdom, sharing common structures within the conserved AP2/ERF domains (Wessler 2005, Mizoi et al. 2012). The ERF gene family is further divided into the ERF and the dehydration response

element binding (DREB) subfamilies, based on the features of the AP2/ERF domains (Sakuma et al. 2002). The DREB proteins have a high affinity for dehydration-responsive elements (DRE) or C-repeat elements found in genes involved in response to various abiotic stresses (Stockinger et al. 1997, Liu et al. 1998, Nakano et al. 2006), while ERFs bind to the GCC-box motifs and their variants, including the jasmonate and elicitor response element (Lorenzo et al. 2003).

Studies show that ERFs are involved in plant responses to various environmental conditions and in improving tolerance to multiple abiotic stresses, including high salinity, cold or high temperature, drought, disease, water deficit, etc. (Mizoi et al. 2012, Rehman and Mahmood 2015, Thirugnanasambantham et al. 2015). Several ERFs have been isolated from various plant species, including *Arabidopsis* thaliana (Abogadallah et al. 2011), *Nicotiana tabacum* (Guo et al. 2004), *Triticum aestivum* (Rong et al. 2014), *Oryza sativa* (Jin et al. 2013*b*), *Glycine max* (Zhai et al. 2013), *Solanum lycopersicum* (Pan et al. 2012), *Brassica rapa* (Seo et al. 2010), *Saccharum officinarum* (Trujillo et al. 2008), etc. These ERF genes are upregulated in response to salinity treatment and transgenic plants overexpressing the ERF genes have increased tolerance to salt stress, suggesting that the ERF genes play an important role in response to high salt stress.

Populus simonii × *Populus nigra* is widely distributed in the northern part of China. It grows fast and is adaptive to certain levels of cold, drought, salt and alkali stresses. However, increasing soil salinization in China has become one of the main environmental factors that challenges poplar growth. In order to identify key genes for salt resistance in poplar, 86 cDNA fragments in response to high salt stress were obtained using the cDNA-AFLP method (Wang et al. 2011). One of the cDNA fragments contains an AP2/ERF domain (GenBank accession number: GW672629), and shares high homology with the poplar TF *ERF76* gene (Wang et al. 2011). There are 209 members of the ERF gene family in the poplar genome (Jin et al. 2013*a*), and 33.52% of genes selected from the ERF gene family respond to high salt stress; the expression level of the *ERF76* gene is the most significant among the salt-inducible ERF genes (Wang et al. 2014). To further characterize the function of TF *ERF76* in *P. simonii* × *P. nigra*, we overexpressed the *ERF76* gene in di-haploid *P. simonii* × *P. nigra* by transgenic technology based on the *Agrobacterium*-mediated leaf disc method. We then assessed gene expression levels, plant growth and plant hormone concentrations under salt stress. Our results demonstrate that overexpression of *ERF76* significantly enhances salt tolerance in transgenic poplar. These studies have shed light on the roles of *ERF76* in response to salt stress in poplar.

Materials and methods

Plant materials

In order to characterize *ERF76* gene expression in wild-type poplar, we harvested twigs from the same clone of di-haploid *P. simonii* × *P. nigra* to minimize genetic variation. The twigs were planted in pots to regenerate new branches and roots in the greenhouse with 60–70% relative humidity, 16/8-h light/dark cycle and an average temperature of 25 °C. Two-month-old seedlings with new roots and leaves were then subjected to the following treatments: 150 mM NaCl for 24 h or no NaCl treatment (control).

To prepare the explants for gene transformation, sterilized seedlings were prepared from di-haploid *P. simonii* × *P. nigra* through leaf tissue culture and regeneration. This involved four steps: (i) sterilizing the leaves from 2-month-old plant twigs using sterilization solution (1% Cl, 0.05% TWEEN 20) for 10 min, followed by rinsing three times using sterilized water; (ii) cutting the sterilized leaves into 1 × 1 cm leaf discs and putting them on the precultural medium [1/2 Murashige and Skoog medium (MS) containing 0.5 mg l^{-1} 6-benzylaminopurine (6-BA) and 0.05 mg l^{-1} 1-naphthaleneacetic acid (NAA)] under 24 ± 1 °C and 16/8 h (light/dark) for ~30 days; (iii) regenerating shoots in shooting medium (1/2MS containing 0.1 mg l^{-1} 6-BA and 0.05 mg l^{-1} NAA) for ~30 days; and (iv) regenerating roots in rooting medium (1/2MS containing 0.2 mg l^{-1} indole-3-butytric acid) for 1–2 months.

Isolation of the ERF76 gene and vector construction

A candidate cDNA fragment (GenBank accession number: GW672629) was isolated previously from di-haploid *P. simonii* × *P. nigra*, which was proved to be responsive to high salt stress (Wang et al. 2011, 2014). This cDNA shared high homology with poplar *ERF76* cDNA (locus name: Potri.005G195000.1). The primers were designed to amplify the coding sequence of the poplar *ERF76* gene as follows: ERF-F1: 5′-ATGATGCAGAAAGATTTTAGCACTG-3′ and ERF-R1: 5′-TTAACCAGTGGAGGAAGGACGGCG-3′. The reverse transcription-polymerase chain reaction (RT-PCR) product was then purified and sequenced to confirm the identity of the fragments. To prepare the gene overexpression construct, cloning sites were introduced by PCR using a primer pair containing XbaI and SacI restriction sites, ERF-F2: 5′-GCGTCTAGAATGATGCAGAAAGATTTTAGCACTG-3′ and ERF-R2: 5′-GCGGAGCTCTTAACCAGTGGAGGAAGGACGGCG-3′ at the 5′ and 3′ terminals, respectively. The cDNA was then directly cloned into the pBI121 binary vector, to replace the position of the XbaI–SacI β-glucuronidase cassette. The cauliflower mosaic virus (CaMV) 35S promoter/nopalin synthase (NOS) terminator system and kanamycin-resistant gene (*NPTII*, neomycin phosphotransferase II) were used in the constitutive expression system. The expression vector was sequenced, confirmed and introduced into *Agrobacterium* EHA105 by an electroporation system.

Subcellular localization of ERF76 protein

The coding sequence of *ERF76* lacking the termination codon was fused in-frame into the 5′ terminus of the coding region of green fluorescent protein (GFP) and expressed under the control

of CaMV35S promoter in the pBI121 vector. The primers containing XbaI and SpeI restriction sites were designed for amplification—ERF-F3: 5′-GCTCTAGAATGTGCGTATTGAAGGTGGCG AACC-3′ and ERF-R3: 5′-GGACTAGTACCAGTGGAGGAAGGACG GCGACTAG-3′. The constructed 35S::ERF76-GFP fusion and 35S::GFP vector as control were introduced into *Agrobacterium tumefaciens* strain EHA105. The transgenic *Arabidopsis* lines expressing 35S::ERF76-GFP and 35S::GFP were obtained by the floral dip method (Zhang et al. 2006*b*). The GFP fluorescence signals of T_2 transgenic seedlings in roots were detected under microscopy with a fluorescein isothiocyanate filter (LSM 700, Zeiss, Germany).

Gene transformation and transgenic plants confirmation

The procedure for poplar transformation and regeneration was as follows. (i) Strong leaves from 1- to 2-month-old di-haploid *P. simonii* × *P. nigra* were cut into segments of 1.0 × 1.0 cm, and placed on the precultural medium under 24 ± 1 °C and 16/8-h light/dark cycle for 2 days. (ii) The leaves were soaked in the solution of *A. tumefaciens* for 10 min, and put on precultural medium in dark for coculture under 24 ± 1 °C for 2–3 days. (iii) The leaves were transferred to selection medium (precultural medium containing 50 mg l^{-1} kanamycin) under 24 ± 1 °C and cyclic light/dark of 16/8 h for shoot regeneration. (iv) The regenerated microshoots were excised and placed on root-induced medium (rooting medium with 50 mg l^{-1} kanamycin) for root regeneration. Finally, the transgenic seedlings were transferred into pots in the greenhouse.

After selection by kanamycin resistance, the putative transgenic poplar lines were subjected to PCR and RT-PCR assays. A primer pair from binary vector pBI121 near the opposite ends of *ERF76* cDNA, PBI-F: 5′-CCATCGTTGAAGATGCCTCTGC-3′ and PBI-R: 5′-CTCTTCGCTATTACGCCAGCTG -3′, was designed and used for the amplification of the insert fragment of the *ERF76* cDNA. A primer pair from the *NPTII* gene of binary vector pBI121, NPT-F: 5′-ATGATTGAACAAGATGGATTGCACG-3′, and NPT-R: 5′-TCAGAAGAACTCGTCAAGAAGGCG-3′, was used for the analysis of *NPTII* gene expression by RT-PCR. The fragments from PCR and RT-PCR were further confirmed by DNA sequencing.

Stress treatment for transgene characterization

Both the transgenic plants validated by PCR and RT-PCR and the nontransgenic plants were transplanted into pots containing regular water in the greenhouse with 60–70% relative humidity, 16/8-h light/dark cycle and an average temperature of 25 °C for 2 months. The seedlings with new branches and roots were then subjected to the following treatments: water (control) or 150 mM NaCl for 24 h. After treatment, young leaf and root tissues were harvested from three clonal seedlings of each line. The samples from the same lines per time point per tissue were pooled, frozen immediately in liquid nitrogen and stored at −70 °C for RNA isolation and RT-qPCR analysis.

Reverse transcription-quantitative polymerase chain reaction assay

To quantify expression of the *ERF76* gene in transgenic plants compared with nontransgenic plants under both salt treatment (150 mM NaCl) and control (water) conditions, we used both leaf and root tissues for RT-qPCR. Total RNA was extracted using Column Plant RNAout Kit (Tiandz, Beijing, China) and proceeded to cDNA synthesis using PrimeScript™ RT reagent Kit (Takara, Dalian, China), according to the manufacturer's instructions. The quality and quantity of total RNA were checked by agarose gel electrophoresis and NanoDrop 2000c Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The RT-qPCR was performed on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the SYBR Premix Ex Taq™ kit (Takara, Dalian, China), according to the manufacturer's instructions. The amplification curve was generated after analyzing the raw data, and the cycle threshold (C_t) value was calculated based on the fluorescence threshold as 0.01. The expression level of poplar actin (GenBank accession number: JM986590) amplified with primer pair Actin-F: 5′-ACCCTCCAATCCAGACACTG-3′ and Actin-R: 5′-TTGCTGACCGTATGAGCAAG-3′ was used as an internal control (Regier and Frey 2010). A pair of primers, ERF-F4: 5′-ATGTGCGTATTGAAGGTGGCG-3′ and ERF-R4: 5′-CCTCCACATGCCGCTGTATTGG-3′, was used for the analysis of the expression levels of *ERF76* mRNA. The relative expression level of target genes in different samples was calculated using $2^{-\Delta\Delta C_t}$ method, defined as: $\Delta\Delta C_t = (C_{t\text{-target}} - C_{t\text{-control}})_2 - (C_{t\text{-target}} - C_{t\text{-control}})_1$.

Gene expression characterization using RNA-Seq

Twigs cut from a transgenic plant and a nontransgenic plant with the same genetic background of the di-haploid *P. simonii* × *P. nigra* were grown in 1/2MS medium containing 35 mM NaCl for 1 month. Two nontransgenic plants and five transgenic poplar lines served as biological replicates. Secondary leaves from each of the replicates (seven samples) were collected and frozen immediately in liquid nitrogen, and stored at −70 °C for RNA isolation. Total RNA from each of the samples was shipped to the GENEWIZ Company (www.genewiz.com) for mRNA sequencing, by use of the Illumina HiSeq2000 platform. In brief, sequencing library construction included the following steps: RNA quality checking (Agilent Eukaryote Total RNA Nano Kit, Agilent Technologies, Santa Clara, USA), library construction (Illumina TruSeq RNA Sample Pre Kit, Illumina, San Diego, USA), library purification (Beckman AMPure XP beads, Brea, USA), insert fragments test (Agilent High Sensitivity DNA Kit, Agilent Technologies), quantitative analysis of library (ABI 7500 real-time PCR instrument, Life Technologies, Carlsbad, USA; KAPA SYBR green fast universal 2× qPCR master mix, KAPA Biosystems, Boston, USA) and cBOT automatic cluster (TruSeq PE Cluster Kit v3-cBot-HS, San Diego, USA).

RNA-Seq data processing includes the following steps: (i) checking data quality and removing excess adaptors;

(ii) mapping the high-quality short sequence reads to the poplar reference genome (ftp://ftp.jgi-psf.org/pub/compgen/phytozome/v9.0/Ptrichocarpa/assembly/Ptrichocarpa_210.fa.gz); (iii) transcript assembling using the TopHat software (version 2.0.9); and (iv) gene expression quantification (in fragments per kilobase transcript per million mapped reads or FPKM) using Cufflinks (version 2, Trapnell et al. 2012).

Gene expression profiling by RNA-Seq is a powerful approach to shedding light on the molecules that are regulated by the transgenic genes (Li et al. 2014). Identification of differentially expressed genes (DEGs) is innovative in this study. We compared overall expression difference between the transgenic plants and the nontransgenic plants (control) by online software known as Pop's Pipes: Poplar Gene Expression Data Analysis Pipelines (http://sys.bio.mtu.edu/), and edgeR was chosen as a robust method for identification of DEGs among four algorithms in Pop's Pipes package: edgeR, DEseq, vst-limma and voom-limma (Robinson et al. 2010, Li et al. 2014). False discovery rate was controlled at 0.05 for multiple tests correction in the Pop's Pipes processing. The fold change (FC) of each gene was the log transformation (base 2) of the specific value (transgenic line/wild-type) of FPKM. If $FC > 0$, the gene is upregulated and if $FC < 0$, the gene is downregulated.

Measurement of plant height, root length, fresh weight, and ABA and GA content

We also compared plant growth and physiological traits in transgenic plants versus nontransgenic plants under the NaCl treatment and control conditions. Regarding experimental design, we focused on comparison of one variable at a time, that is, genotype (transgenic vs nontransgenic plants) and NaCl treatment (with treatment vs control). We used Student's *t*-test to test for significance.

Seedlings of the five transgenic poplar lines and the nontransgenic lines (with uniform plant size) were transferred to 1/2MS medium containing 0 and 35 mM NaCl, respectively. After 30 days, the plant height, root length and fresh weight were measured, and the leaves were harvested and powdered in liquid nitrogen and 500 mg tissue homogenized in 5 ml extracting phosphate buffered saline overnight at 4 °C. The supernatants were collected after centrifugation at 4500*g* for 10 min, and were used for abscisic acid (ABA) and gibberellin (GA) analysis by enzyme-linked immunosorbent assay, using Phytodetek ABA and GA test kit in three biological replicates (Agdia, Elkhart, IN, USA). The data are presented as mean ± standard error.

Results

Isolation and expression of the ERF76 gene

The cDNA fragment of the TF *ERF76* gene was obtained by RT-PCR, which has 1537 nucleotides with an open reading frame (ORF) encoding 445 amino acid residues. There is an AP2/ERF domain in the ERF76 protein, which shares high homology with other salt stress-related ERF proteins (Figure 1a and b), such as AtERF1 from *Arabidopsis thaliana* (AT4G17500.1, Allen et al. 1998), OPBP1 from *N. tabacum* (NTU81157, Guo et al. 2004), TaERF3 from *T. aestivum* (EF570122, Rong et al. 2014), OsAP21 from *O. sativa* (NM_001048853, Jin et al. 2013*b*), GmERF7 from *G. max* (JN416602, Zhai et al. 2013), SlERF5 from *S. lycopersicum* (AY559315, Pan et al. 2012), BrERF4 from *B. rapa* (EX112088, Seo et al. 2010) and SodERF3 from *S. officinarum* (AM493723, Trujillo et al. 2008). The conserved AP2/ERF domain comprises a three-stranded antiparallel β-sheet and an α-helix packed approximately parallel to the β-sheet (Figure 1c). This domain shared up to 82.4% sequence homology with the well-characterized AP2/ERF domain of AtERF1 (AT4G17500.1, Allen et al. 1998, Figure 1c).

Evidence from RT-qPCR indicated that the expression level of *ERF76* in wild-type poplar increased significantly when treated with salt stress. Under the salt treatment (150 mM NaCl) for 24 h, the relative abundances of *ERF76* mRNA increased significantly ($P < 0.01$) by 11.2 and 86.9 times in leaves and roots of wild-type poplars, respectively (Figure 2), suggesting that *ERF76* from poplar is sensitive to high salt stress condition.

Subcellular localization analysis of ERF76 protein

To examine the subcellular localization of *ERF76* protein, the combined vectors containing the 35S::ERF76-GFP and the control 35S::GFP were transferred into *A. thaliana*. As shown in Figure 3, the fluorescence of 35S::ERF76-GFP fusion protein was localized exclusively to the nuclei of *Arabidopsis* cells, while the GFP protein was uniformly distributed throughout the entire cell. These results indicate that the *ERF76* protein is targeted to the nucleus.

Determination of transgenic poplar lines

Eight transgenic lines were selected and validated by PCR and RT-PCR. The PCR amplicons from the transgenic poplar lines were obtained and their sizes were congruent with the expected insert size of the *ERF76* gene amplified by PCR (Figure 4a), and with the expected size of the *NPTII* cDNA amplified by RT-PCR (Figure 4b). The amplified fragments were further confirmed by DNA sequencing of the purified amplicons. These results demonstrated that the cDNA fragment of *ERF76* was successfully integrated into poplar genome, and the *NPTII* gene was expressed under control of the NOS promoter.

Overexpression of *ERF76* in transgenic poplar plants was validated by both RNA-Seq and RT-qPCR. Evidence from RNA-Seq indicated that the abundance of *ERF76* mRNA in transgenic poplar leaves was 6.56 times higher (mean value) than that of the nontransgenic control by salt treatment (see Supplementary data, Excel file, available at *Tree Physiology* Online). Similarly, evidence from RT-qPCR indicated that *ERF76* expression was 2.1–6.0 times higher in leaves and 1.2–1.7 times higher in

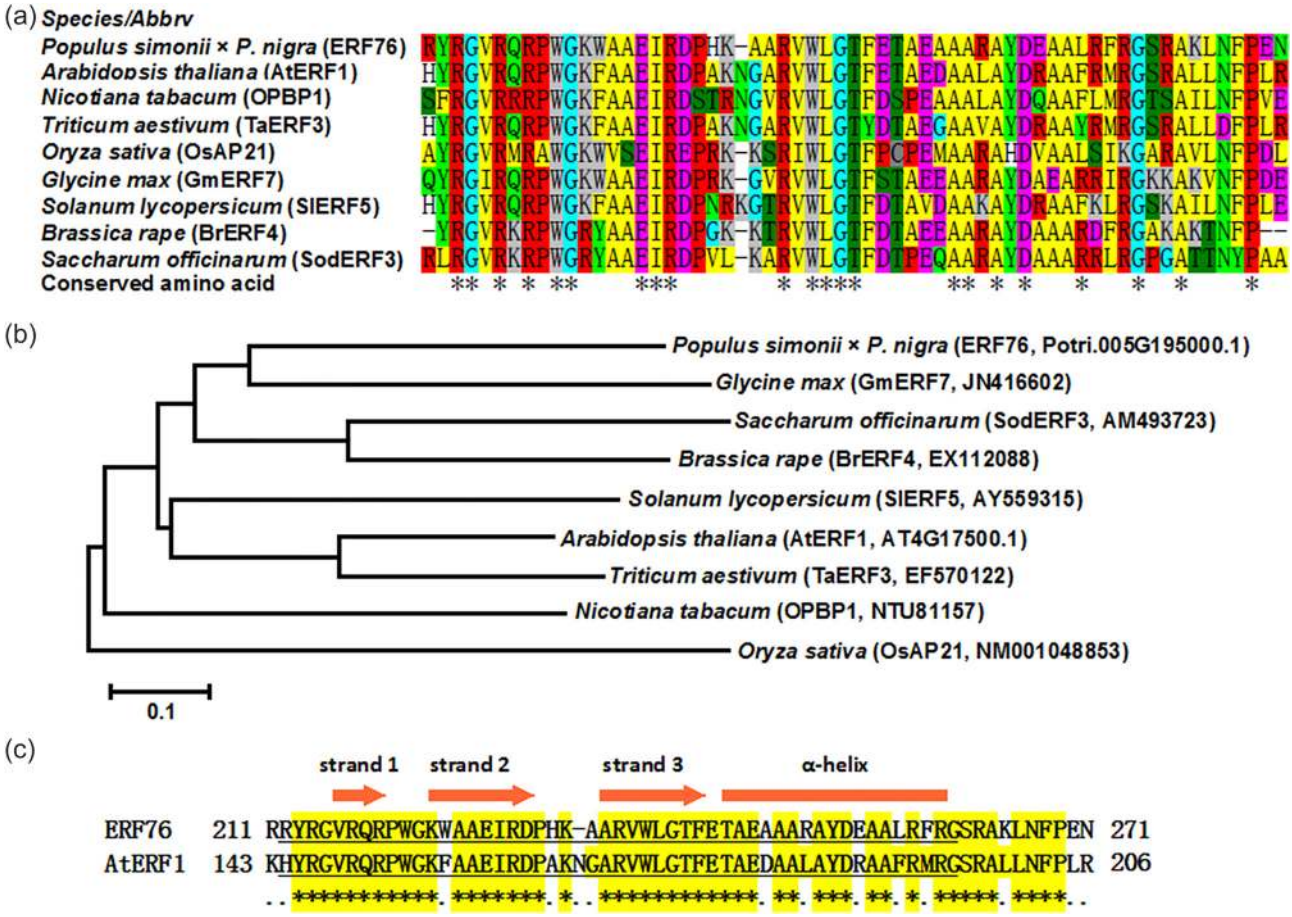

Figure 1. Alignment and phylogenetic tree analysis of the ERFs' amino acid sequences. (a) Alignment of amino acid sequences of ERFs from nine different plant species by Clustal_W. (b) Phylogenetic tree analysis of ERFs from nine different plant species by neighbor-joining method. (c) The AP2/ERF domains of *ERF76* (locus name: Potri.005G195000.1) and *AtERF1* (AT4G17500.1, GenBank accession number: AB008103) were aligned, and strongly conserved residues are highlighted in yellow. The arrangement of the secondary structural elements (top) and the consensus sequence (bottom) are shown.

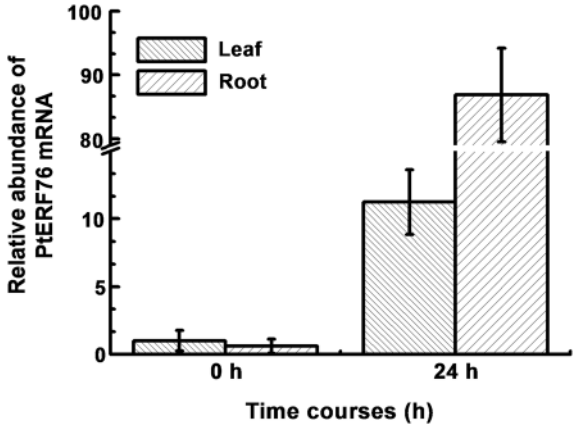

Figure 2. *ERF76* gene expression level in wild-type poplar under salt treatment. Two-month-old seedlings were subjected to water and 150 mM NaCl solution for 24 h, and leaves and root were harvested. Mean values and standard deviations were calculated from three independent RT-qPCR experiments.

roots, compared with that of the control, under salt stress conditions (Figure 5). Under normal conditions, the relative abundance of *ERF76* in transgenic poplar was 9.6–17.5 times higher in leaves and 28.7–47.5 times higher in root, compared with that of the nontransgenic poplar (Figure 5). These results indicate that *ERF76* is constitutively expressed under control of the 35S promoter, with or without salt stress treatment.

The effects of ERF76 gene on salt-tolerant adaption

We observed that leaf shape of the transgenic plants changed compared with that of the wild-type plants (Figure 6). The length-to-width ratios of the transgenic plants were 1.52 ± 0.02–1.62 ± 0.02, compared with that of 1.28 ± 0.02 in wild-type plants. The differences are significant at the $P \leq 0.01$ level (Table 1). These suggest that the concentration of plant hormones involved in plant growth and development, such as ABA and GA, may have changed in the leaves of transgenic plants.

In order to compare transgenic poplar with wild-type under normal and stress conditions, we measured plant height, root length, fresh weight, and ABA and GA concentrations. Results showed that the growth performance of both transgenic and nontransgenic plants was significantly inhibited under salt stress. Under both conditions, the transgenic poplar plants had larger plant height, root length and fresh weight than the nontransgenic plants.

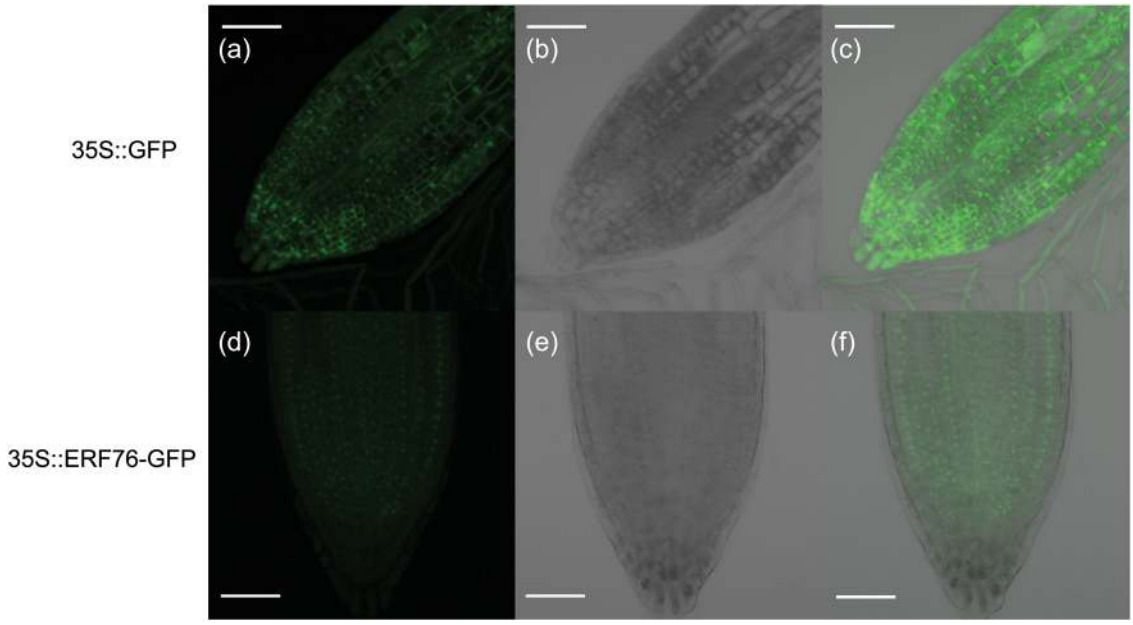

Figure 3. Subcellular localization analysis of *ERF76* protein. The GFP fluorescence signals in root tip cells from 35S::GFP control lines (a–c) and the fusion construct 35S::ERF76-GFP lines (d–f) were observed: (a and d) were observed in dark field for green fluorescence, (b and e) were observed in bright field and (c and f) were observed in combination. Scale bar = 20 μm.

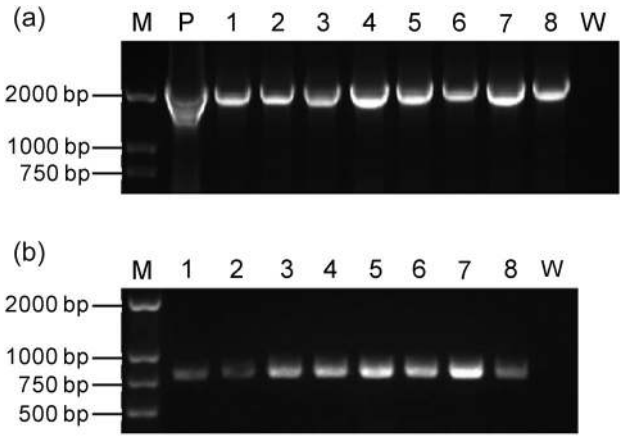

Figure 4. PCR (a) and RT-PCR (b) analyses of transgenic plants. (a) Amplification of the inserted fragment from the *ERF76* gene by PCR. (b) Amplification of the *NPTII* cDNA by RT-PCR. M, molecular marker; P, positive plasmid; 1–8, transgenic poplar; W, wild-type poplar.

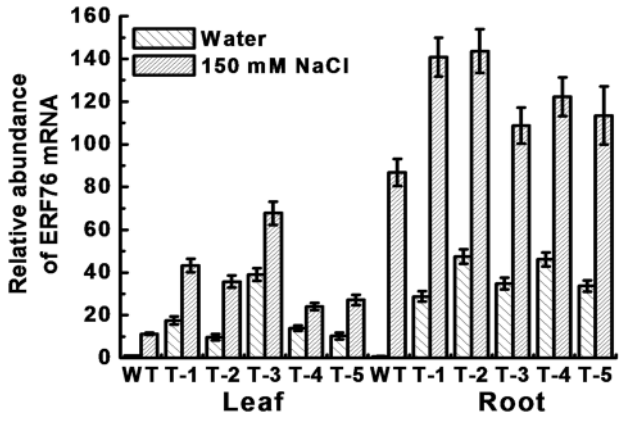

Figure 5. *ERF76* gene expression level in transgenic plants under salt stress treatment. Two-month-old seedlings were subjected to water and 150 mM NaCl solution for 24 h, and leaves and root were then harvested. WT, wild-type poplar; T-1 to T-5, transgenic poplar. Mean values and standard deviations were calculated from three independent experiments.

Plant height of the transgenic poplar was 27.0–46.1 and 17.5–57.9% higher than that of the nontransgenic plants under normal and stress conditions, respectively (Figure 7a). Root length of transgenic poplar was 21.2–33.3 and 15.7–43.9% higher than that of the nontransgenic plants under normal and stress conditions, respectively (Figure 7b). The fresh weight of the transgenic poplar was 7.97–36.7 and 13.4–63.6% higher than that of the nontransgenic plants under normal and stress conditions, respectively (Figure 7c).

Physiologically, ABA contents in leaves of transgenic poplar were 5.3–22.8 and 5.5–17.9% higher than those of nontransgenic plants under normal and stress conditions, respectively (Figure 8a). GA contents were 4.8–13.2 and 4.4–24.4% higher than those of nontransgenic plants under normal and stress conditions, respectively (Figure 8b).

Differentially expressed genes in transgenic poplar

Evidence from gene expression by RNA-Seq has identified a total of 375 DEGs in comparisons between transgenic and nontransgenic plants under NaCl treatment (see Supplementary data, Excel file, available at *Tree Physiology* Online). Among the DEGs, 268 genes were upregulated and 107 genes were downregulated. These two sets of genes were hypothesized to be regulated by

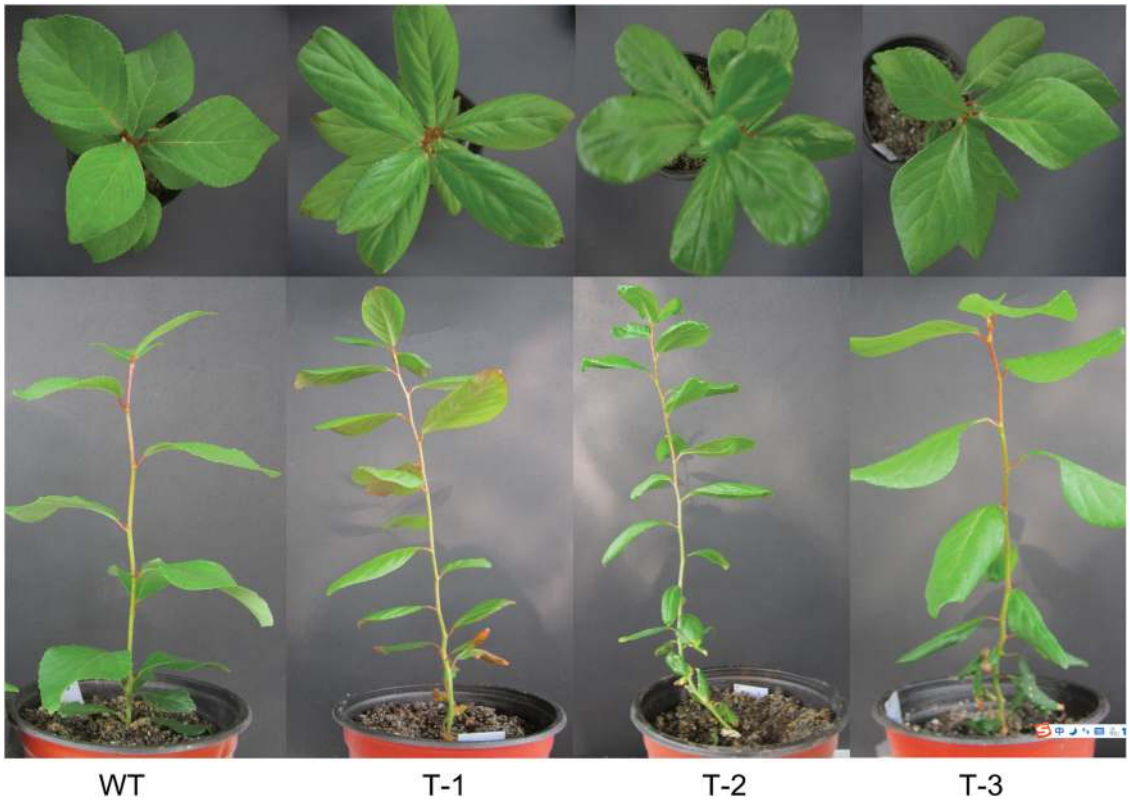

Figure 6. Comparison of leaf shapes of wild-type and transgenic poplar. WT, wild-type poplar; T-1 to T-3, transgenic poplar.

Table 1. Leaf length, width and length-to-width ratios of transgenic poplar lines compared with wild-type plants. Values are means ± SE of 10 separate leaves at the same location from 10 different lines. Confidence interval is 95%. WT, wild-type poplar; T-1 to T-5, transgenic poplar.

	WT	T-1	T-2	T-3	T-4	T-5
Length	5.66 ± 0.11	6.06 ± 0.14	6.28 ± 0.11	5.96 ± 0.14	6.02 ± 0.07	6.18 ± 0.11
Width	4.45 ± 0.13	3.76 ± 0.11	4.04 ± 0.09	3.92 ± 0.08	3.93 ± 0.05	4.04 ± 0.08
Length-to-width ratios	1.28 ± 0.02	1.62 ± 0.02	1.56 ± 0.02	1.52 ± 0.02	1.53 ± 0.02	1.53 ± 0.02

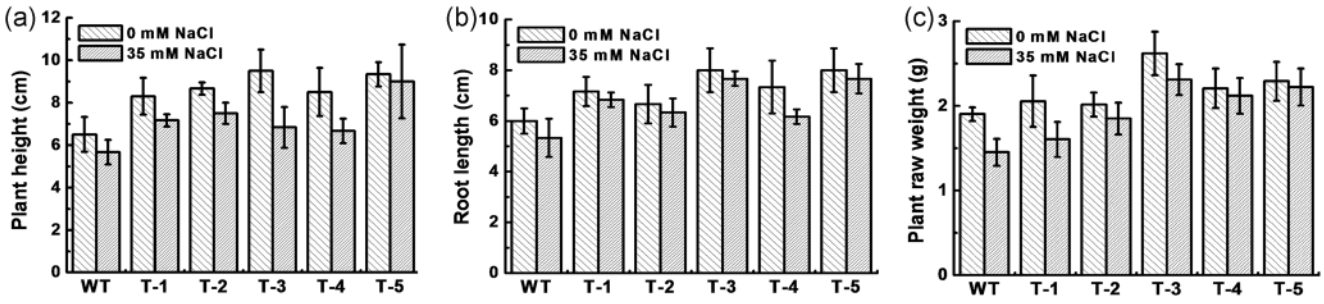

Figure 7. Comparisons of plant height, root length, and fresh weight between transgenic plants and nontransgenic plants. WT, wild-type poplar; T-1 to T-5, transgenic poplar. Mean values and standard deviations were calculated from three independent experiments.

ERF76 in the transgenic poplar. To assess whether promoters of the *ERF76*-activated genes contain putative *cis*-elements for *ERF76* binding, we searched for *cis*-elements in the 2-kb region upstream of the transcription starting sites of all the DEGs, using the phytozome database (http://www.phytozome.net/). We identified that 132 upregulated DEGs (49.3%) contain the DRE-related CCGAC core motif, ABRE (PyACGTGT/GC) or GCC-box. On the other hand, 27 downregulated DEGs (25.2%) harbor these *cis*-acting elements in the promoter regions (Table 2). These results show that upregulated DEGs contain the

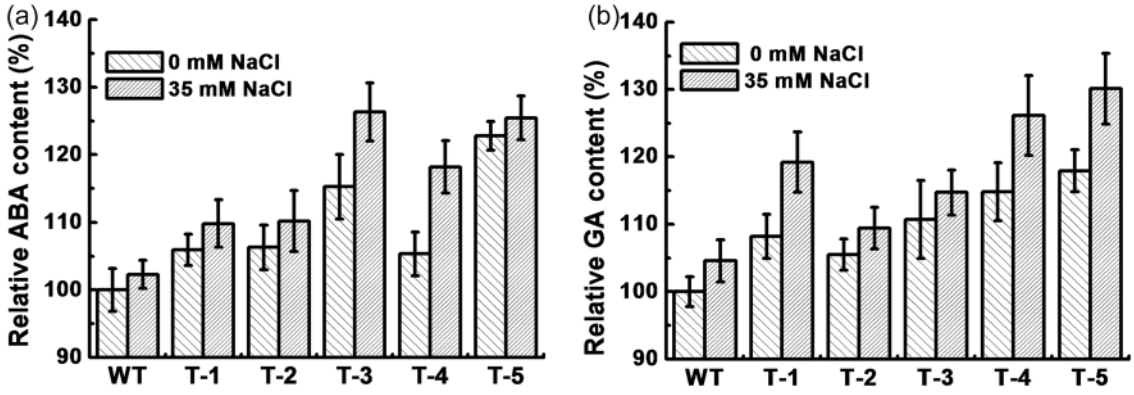

Figure 8. Comparisons of ABA and GA concentration between transgenic plants and nontransgenic plants. WT, wild-type poplar; T-1 to T-5, transgenic poplar. Mean values and standard deviations were calculated from three independent experiments.

Table 2. The number of up/downregulated DEGs in transgenic poplar. DREB represented gene contains the CCGAC core motif, ABRE represented gene contains the PyACGTGT/GC motif and GCC-box represented gene contains the AGCCGCC motif. Both represented gene contains both DREB and ABRE in the 2-kb promoter region upstream of ATG, respectively.

Up/downregulated	DEG numbers in transgenic poplar				
	Total numbers/with *cis*-element (%)	DREB (%)	ABRE (%)	GCC-box (%)	Both (%)
Up	268/132 (49.3)	94 (35.1)	58 (21.6)	6 (2.2)	26 (9.7)
Down	107/27 (25.2)	22 (20.5)	4 (3.7)	2 (1.8)	1 (0.9)

DRE-related CCGAC core motif, ABRE or GCC-box in their promoters at a higher frequency than downregulated DEGs do. However, since there is no clear correlation between the presence of these elements and stress-activated expression, other *cis*-acting elements are likely involved in the regulation of expression of these genes.

Out of the upregulated DEGs, 16 are TF genes and 45 are stress-related genes (Table 3). The TF genes include four ERF, five NAC, three WRKY, three MYB and one bHLH TF classes. They may be involved in further regulation of signal transduction and gene expression related to stress responses. The stress-related gene products can be classified into five groups. The first group comprises defense proteins including late embryogenesis-abundant (LEA) proteins, heat-shock proteins, detoxification enzymes, peroxidase, metal ion binding protein and hydroxyproline-rich glycoprotein (HRGP) family protein. Late embryogenesis-abundant proteins and heat-shock proteins have been shown to be involved in protecting macromolecules, such as enzymes and lipids (Goyal et al. 2005). Detoxification enzymes, such as glutathione *S*-transferase (GST) and peroxidase, are thought to be involved in protection of cells from active oxygens. The second group contains oxidases and oxygenase, including Fe(II)-dependent oxygenase, gibberellin 3-oxidase, cytochrome P450 oxidase, laccase and copper amine oxidase. The third group comprises stress-responsive proteins, including disease resistance-responsive protein, cold and salt responsive protein, multidrug resistance-associated protein, salt tolerance zinc finger and stress enhanced protein. The fourth group consists of phosphatases and kinases, such as phosphotyrosine phosphatases, mitogen-activated protein kinase kinase kinase (MAPKKK), protein-tyrosine phosphatase and PR5-like receptor kinase (PR5K). The PR5K is structurally related to a family of plant defense proteins. The fifth group contains GH3 family proteins, which catalyze the formation of a biologically active jasmonyl-isoleucine conjugate in the jasmonate signaling pathway.

In order to validate the hypothesized alternations of DEGs in transgenic plants, we also performed gene enrichment analysis of six representative DEGs related to ABA and GA signal pathways by RT-qPCR (Table 4, Figure 9). The result showed that the expressions of six DEGs were significantly higher in transgenic plants than those of nontransgenic plants, which are congruent with the results of RNA-Seq.

Discussion

ERF genes respond to abiotic stresses

Abiotic stresses can induce multiple pathway changes in a variety of plants, and the identification of pathways is critical to understanding plant response to abiotic stresses (Yamaguchi-Shinozaki and Shinozaki 2006). Research carried out in the past few years has been productive in identifying TFs that are important for regulating plant responses to abiotic stresses (Mizoi et al. 2012, Nakashima et al. 2012, Rushton et al. 2012). Ethylene response factors belong to plant-specific TF superfamily (Nakano et al. 2006), which influence a number of developmental processes and are important for adaptation to biotic or abiotic stresses, such as pathogen attack and salt/drought stress (Gu et al. 2002, Onate-Sanchez et al. 2007, Cela et al. 2011). The overexpression

Table 3. Stress-related DEGs in transgenic poplar.

Gene symbol	Functional category	Reference gene symbol	*cis*-element	FCs	*P*-value
Potri.005G195000	ERF TF	AT5G64750	DREB	6.56	1.47E–17
Potri.003G139300	ERF TF	AT1G64380		2.76	0.00276
Potri.001G067600	ERF TF	AT5G13330		1.78	0.02615
Potri.019G131300	ERF TF	AT4G27950		1.05	0.00882
Potri.005G069500	NAC TF	AT1G01720	DREB, ABRE	1.3	0.00037
Potri.002G081000	NAC TF	AT1G01720	ABRE	1.13	0.00249
Potri.005G103200	NAC TF	AT2G17040		1.75	0.00753
Potri.013G054000	NAC TF	AT3G04070	GCC-box	2.19	5.17E–07
Potri.010G166200	NAC TF	AT1G69490	DREB, ABRE	4.41	5.33E–0
Potri.003G111900	WRKY TF	AT2G30590		2.24	0.00446
Potri.005G085200	WRKY TF	AT5G64810	DREB, ABRE	2.15	0.00091
Potri.013G090300	WRKY TF	AT3G56400	DREB	1.39	0.00848
Potri.006G221800	MYB TF	AT4G38620		1.26	0.00141
Potri.007G067600	MYB TF	AT1G79180		4.16	0.00238
Potri.003G064600	MYB TF	AT5G14750		1.10	0.02927
Potri.009G081400	bHLH TF	AT4G37850	DREB	3.11	0.01057
Potri.T035000	GST	AT3G62760		1.60	0.00117
Potri.001G437400	GST	AT1G78380		2.00	0.00093
Potri.001G437200	GST	AT1G78380		2.00	0.00036
Potri.T149500	GST	AT2G29420		1.13	0.04131
Potri.016G132700	Peroxidase	AT5G05340		2.73	5.28E–08
Potri.013G154400	Peroxidase	AT5G05340		3.95	0.00022
Potri.016G084800	APX	AT3G09640		1.6	0.045285
Potri.007G053400	Peroxidase	AT5G67400	DREB, ABRE	1.11	0.03458
Potri.019G082600	HRGP	AT5G09520		3.74	0.008618
Potri.007G113700	HRGP	AT4G38080		3.44	0.007537
Potri.006G204300	HRGP	AT2G35980	ABRE	2.36	1.15E–09
Potri.016G071600	HRGP	AT2G35980	DREB	1.28	0.00065
Potri.016G046400	LEA protein	AT5G06760	DERB, ABRE	3.15	0.04518
Potri.T111300	LEA protein	AT5G06760	DREB, ABRE	3.91	0.01143
Potri.002G165000	LEA protein	AT2G46140	ABRE	2.03	0.04745
Potri.014G127700	LEA protein	AT4G02380	ABRE	1.64	0.00043
Potri.002G203500	LEA protein	AT4G02380	DREB, ABRE	0.9	0.00849
Potri.011G051600	Heat-shock factor	AT5G16820		1.95	0.00288
Potri.010G023600	2-Oxoglutarate (2OG) oxygenase	AT3G21420	DREB	2.66	0.00118
Potri.015G002800	2-Oxoglutarate (2OG) oxygenase	AT5G24530		1.18	0.028264
Potri.006G020000	Metal ion binding protein	AT4G16380		2.67	0.003899
Potri.006G020100	Metal ion binding protein	AT4G16380	GCC-box	3.4	0.025177
Potri.001G176600	Gibberellin 3-oxidase	AT1G15550		1.64	0.00019
Potri.001G118200	Copper amine oxidase	AT1G62810		1.64	0.009688
Potri.001G167900	Cytochrome P450 protein	AT5G07990	GCC-box	1.1	0.018695
Potri.003G066400	Cytochrome P450 protein	AT5G07990		0.99	0.036514
Potri.004G235400	Cytochrome P450 protein	AT4G19230		1.19	0.000636
Potri.015G086000	Cytochrome P450 protein	AT1G13080		0.81	0.049409
Potri.019G064200	Cytochrome P450 protein	AT5G24910	DREB, ABRE	1.78	0.008618
Potri.018G149300	Cytochrome P450 protein	AT5G36110		2.6	1.10E–07
Potri.006G094700	Cytochrome P450 protein	AT3G52970	ABRE	1.4	0.024892
Potri.009G043700	Cytochrome P450 protein	AT5G58860		2.76	0.027677
Potri.011G071100	Laccase	AT5G09360	DREB	5.65	0.041305
Potri.010G183600	Laccase	AT2G40370	ABRE	3.21	0.017257
Potri.008G073700	Laccase	AT2G40370	DREB, ABRE	2.72	0.03927
Potri.003G216200	Disease resistance-responsive protein	AT1G55210		1.24	0.009034
Potri.010G217200	Cold and salt responsive protein	AT2G38905	DREB	5.62	0.049864
Potri.014G130500	Multidrug resistance-associated protein	AT2G47800		3.11	0.029711
Potri.009G089400	Salt tolerance zinc finger	AT1G27730	DERB, ABRE	1.7	0.000358
Potri.005G084200	Stress enhanced protein	AT2G21970		1.43	0.0087
Potri.011G112400	Nine-*cis*-epoxycarotenoid dioxygenase 3	AT3G14440	ABRE	1.28	0.008825
Potri.014G159100	Phosphotyrosine phosphatases	AT1G05000	DERB, ABRE	1.53	0.028622
Potri.001G278600	MAPKKK	AT1G07150		1.9	0.004311
Potri.011G004000	PR5K	AT5G38280	DREB, ABRE	3.22	5.44E–05
Potri.014G095500	Auxin-responsive GH3 protein	AT2G46370	DREB	1.87	5.17E–07

Table 4. DEGs involved in ABA and GA signal pathways.

	Gene ID	FC	Function category	Forward primers	Reverse primers
ABA1	Potri.011G112400	1.28	NCED3 involved in ABA biosynthesis	CTGTCGTTCAAGATTTACCGG	GAACAAGCCTATTAGTCTCAG
ABA2	Potri.004G235400	1.19	(+)-ABA 8′-hydroxylase activity	GGTATGGCTCTATCTTCAAG	GAAGAGAGTCCTTGGCAATG
ABA3	Potri.009G037300	1.63	ABA induced protein phosphatase 2C	AACGAGAGTGTTAGTAGTCC	CATGTCTCTTCTCCTTCCAC
GA1	Potri.001G176600	1.64	GA 3-β-hydroxylase	GAGAACATTGAGAGTGCTAG	CAAGAGGAGAGCCAACTATG
GA2	Potri.002G213100	1.1	GA mediated signaling pathway	AAGGCTGTTGTGGTGTCTGT	TTACCACCTGAACTATCGCC
GA3	Potri.T155100	6.41	GA regulated protein	TCTTGTCGCATCTCTCCTTG	ATAATGGTTGCCGGAAGTGC

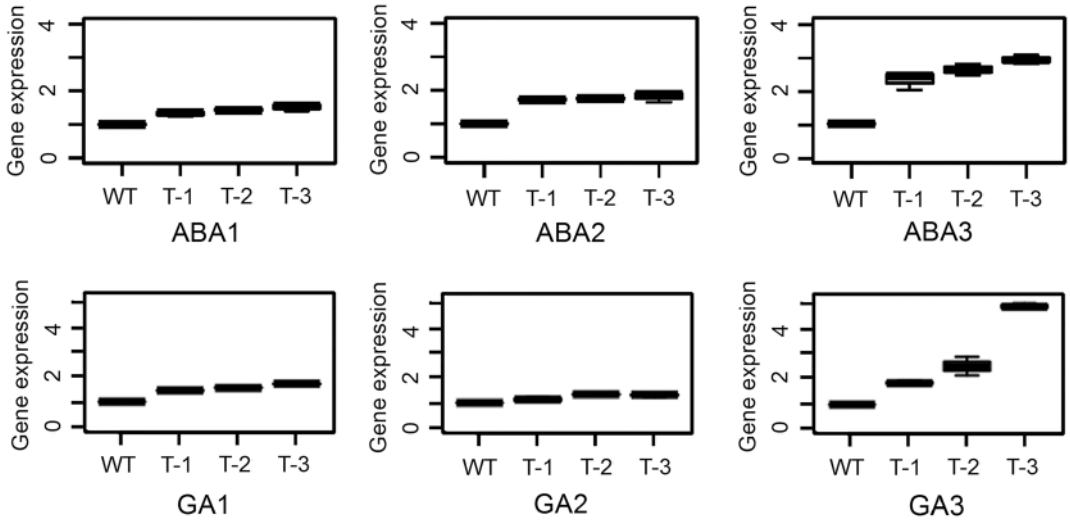

Figure 9. Gene expression level of DEGs related to ABA and GA signal pathways. WT, wild-type poplar; T-1 to T-3, transgenic poplar. Mean values and standard deviations were calculated from three independent RT-qPCR experiments.

of *HARDY*, an AP2/ERF gene from *Arabidopsis*, improves drought and salt tolerance in transgenic *Trifolium alexandrinum* L. (Abogadallah et al. 2011). The transgenic tobacco plants overexpressing the endogenous AP2/EREBP TF *OPBP1* enhance disease resistance and salt tolerance (Guo et al. 2004). The *TaERF3* from wheat promotes tolerance to salt and drought stresses in transgenic lines overexpressing *TaERF3* (Rong et al. 2014). *OsAP21* from rice increases salt/drought tolerance in transgenic *A. thaliana* (Jin et al. 2013*b*). *GmERF7*, isolated from soybean, enhances salt tolerance in transgenic tobacco plants (Zhai et al. 2013). *SlERF5*, obtained from tomato, promotes adaptation to drought and salt tolerance in transgenic tomato lines (Pan et al. 2012). Overexpression of *BrERF4* from *B. rapa* increases tolerance to salt and drought in transgenic *Arabidopsis* plants (Seo et al. 2010). *SodERF3* from sugarcane enhances salt and drought tolerance of transgenic tobacco plants (Trujillo et al. 2008). More ERF genes from multiple species are reported to be salt inducible and increase salt tolerance in transgenic plants. However, the function of *ERF76* from poplar in salt tolerance has not been characterized thoroughly.

Our previous studies indicated that 59 (33.52%) of 176 poplar ERF genes showed responses to high salt and drought stress in leaves. Of these 59 genes, 48 genes were upregulated and 11 were downregulated (Wang et al. 2014), suggesting that several poplar ERF genes might be related to the tolerance of salt and drought stress. Poplar *ERF76* gene is a member of the ERF TF superfamily and can be highly induced by salinity, drought and ABA in poplar (Wang et al. 2014). To further understand the roles of poplar *ERF76* in salt stress tolerance, we, for the first time, have isolated and cloned the full cDNA of *ERF76* from di-haploid *P. simonii* × *P. nigra*, followed by confirmation of nuclear localization signal of *ERF76* protein and characterization of the transgenic poplar lines overexpressing *ERF76*. Under salt stress condition, although the mRNA level of endogenous *ERF76* increased dramatically in wild-type poplar, the additive effect of native allele expression and novel 35S-driven allele of *ERF76* contributed to high expression level of *ERF76* in transgenic poplar. Vigorous growth of transgenic poplar was observed at the early development stages, resulting in significantly increased plant height, root length and fresh weight under salt stress, compared with nontransgenic plants.

Transgenic ERF76 enhances the expression of ABA and GA signal pathways

Plant hormones and plant hormone signaling pathways regulate complex signaling networks related to plant development and responses to environmental stresses (Bari and Jones 2009). Plant hormones, such as ABA, GA, Indole-3-acetic acid, salicylic acid (SA), etc., are essential for responses to changing environments by mediating growth, development, nutrient allocation and source/

sink transitions (Peleg and Blumwald 2011). ABA plays a vital role in regulating plant growth and development, and response to abiotic stress such as osmotic stress tolerance (Zeevaart and Creelman 1998, Achard et al. 2006, Zhang et al. 2006*a*). Plant abiotic stress responses are largely triggered by synthesis and transport of ABA, and are controlled through regulating genes involved in the ABA signal pathway (Zhu 2002, Cutler et al. 2010). In addition to major functions, such as seed germination, vegetative growth, flowering induction and fruit development (Sun and Gubler 2004), GA-responsive genes and exogenous addition of GAs are able to reverse the inhibitory effects of environmental conditions, such as salt, oxidative and heat stresses, in the germination and seedling establishment of *Arabidopsis* through modulation of SA biosynthesis (Magome et al. 2008, Alonso-Ramírez et al. 2009). The involvement of GA signaling in mediating growth and stress responses to abiotic stress is linked to DELLA proteins, enhancing stress tolerance by maintaining low ROS levels after either biotic or abiotic stress (Achard et al. 2006, 2008, Colebrook et al. 2014). The DEGs involved in the ABA and GA signal pathways, such as nine-*cis*-epoxycarotenoid dioxygenase 3 (NCED3) and gibberellin 3-oxidase, are detected to be significantly ($P < 0.05$) expressed in transgenic lines compared with nontransgenic plants, based on RNA-Seq and RT-qPCR. These indicate that overexpression of *ERF76* induces the increase of ABA and GA concentrations by the regulating DEGs involved in the ABA and GA signal pathways.

Transgenic ERF76 enhances the expression of defense-related genes

The present study indicates that some upregulated defense-related genes, such as LEA, GST and HRGP genes (Table 2), are induced by the transgenic *ERF76* gene. Late embryogenesis-abundant proteins are capable of suppressing desiccation-induced protein aggregation in water stress, in keeping with a role in water-stress tolerance (Goyal et al. 2005). Glutathione *S*-transferases induced by diverse environmental stimuli can maintain cell redox homeostasis, protect organisms against oxidative stress and detoxify endogenous plant toxins (Marrs 1996). Some plant GSTs play direct roles in reducing oxidative damage (Roxas et al. 2000) and enhancing tolerance to stresses (Edwards and Dixon 2005). The HRGPs are referred as distinguished plant cell wall component. Cross-linking of the HRGPs is an important process to strengthen the cell walls that contribute to plant defense reactions (Shailasree et al. 2004, Sujeeth et al. 2012).

Overexpression of ERF76 enhances the expression of oxidases and oxygenase genes

Certain oxidases, oxygenases and peroxidases also play significant roles in the regulation of stress tolerance. In the present study, related genes have been identified, including cytochrome P450 monooxygenase (P450) genes, laccase genes, ascorbate peroxidase (APX) genes and peroxidase. P450 genes catalyze the oxidation of various substrates through activation of molecular oxygen. A P450 gene from *Arabidopsis*, *CYP709B3*, is induced by salt stress and affects salt tolerance in *Arabidopsis* (Mao et al. 2013). The drought tolerance of transgenic *Arabidopsis* is enhanced by the overexpression of laccase gene (*AtLAC4*, Zhang et al. 2012). The tolerant fox-tail millet cultivar has higher total peroxidase activity and lower MDA content, compared with the susceptible variety, during salinity stress (Sreenivasulu et al. 1999). Ascorbate peroxidase plays an important role in the metabolism of hydrogen peroxide in higher plants, and transgenic tobacco lines overexpressing the *JctAPX* gene show higher tolerance to salt during seedling establishment and growth (Liu et al. 2014).

Overexpression of ERF76 enhances the expression of signal transfer-related genes

Stress signal could be detected by sensor or receptor proteins in the plasma membrane and transduced into activation of MAPKKK, MAPKK and MAPK in a kinase cascade. The expression of the *Arabidopsis AtPTP1* gene is regulated by stress factors, including high salt and cold stress (Xu et al. 1998). The gene also plays a role in a stress-responsive MAPK pathway (Huang et al. 2000). Our studies showed that some signal transfer-related genes, including phosphotyrosine phosphatases, MAPKKK and PR5K, were enhanced in transgenic poplar. These suggest that *ERF76* may be involved in stress-related signal transfer by regulation of PTPases genes in MAPK pathways.

ERF76 as a central regulator for tolerance of salt stress

ERFs can bind to both GCC-box and DRE elements (Gong et al. 2008). The DRE, which contains the core sequence A/GCCGAC, has been identified as a *cis*-acting promoter element regulating gene expression in response to drought, salt and cold stresses in *Arabidopsis* (Hao et al. 2002, Sakuma et al. 2002). *ERF1* (AT3G23240.1) from *Arabidopsis* belongs to the ERF subfamily, mostly binds to the DRE elements in the promoters of abiotic stress-responsive genes, and plays a significant role in the abiotic stress response (Cheng et al. 2013). We identified 268 upregulated genes in transgenic poplar overexpressing *ERF76* gene, such as LEA protein and laccase genes with physiological functions related to drought and salt stresses, and found DRE *cis*-elements in their promoter regions. All of the genes may contribute accumulatively to salt resistance phenotype caused by *ERF76* overexpression. The overall data support that *ERF76*, as a master regulator, contributes to salt stress tolerance.

Conclusions

The *ERF76* gene from polar is a salt-induced gene, and is significantly expressed in leaves and roots under salt stress conditions. Evidence from RNA-Seq indicates that under salt stress, the expression level of *ERF76* in transgenic poplar leaves is 6.56 times higher than that of nontransgenic poplar, and the 16 TF

genes and 45 stress-related genes are upregulated in transgenic plants. In addition, transgenic poplar plants showed higher salt tolerance, and had larger length-to-width ratio of leaves, and increased ABA and GA concentrations compared with the non-transgenic poplar. All the results indicate that the *ERF76* gene in transgenic poplar upregulated the expression of stress- and hormone-related genes, which resulted in stronger tolerance to salt stress.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Conflict of interest

None declared.

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