Three stress-responsive NAC transcription factors from *Populus euphratica* differentially regulate salt and drought tolerance in transgenic plants

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Stress-responsive *NAM*, *ATAF1/2* and *CUC2* (*SNAC*) genes are being used to alter stress tolerance in *Arabidopsis* or grasses through genetic engineering. However, limited reports are available about the functional characteristics of *SNAC* in trees. In this study, three putative NAC proteins were identified from *Populus euphratica*. PeNAC034 and PeNAC045 were classified into the ATAF subgroup andPeNAC036 into the ANAC072 subgroup. These three SNAC transcription factors were localized in the nucleus and contained the transcription activation domain in their *C*-terminal. Under drought and salt stresses, *PeNAC036* was strongly induced in the whole plant, but *PeNAC034* was significantly suppressed in the roots and stems, and *PeNAC045* was inhibited in the roots. *PeNAC036* overexpression in *Arabidopsis* wild-type (WT) (*OEPeNAC036*) and *PeNAC036* complementation in mutant *anac072* (*anac072/PeNAC036*) lines increased tolerance to salt and drought, whereas *PeNAC034* overexpression in WT (*OEPeNAC034*) and *PeNAC034* complementation in mutant *ataf1* (*ataf1/PeNAC034*) lines enhanced salt and drought sensitivity. After drought and salt treatments, the expression levels of *COR47*, *RD29B*, *ERD11*, *RD22* and *DREB2A* were up-regulated in *OEPeNAC036* and *anac072/PeNAC036* lines, but were down-regulated in *OEPeNAC034* and *ataf1/PeNAC034* plants. Compared with WT and Vector lines, *PeNAC045* overexpression in poplar WT (*OEPeNAC045*) led to a significant decrease in the net photosynthesis rate, stomatal conductance and transpiration rate under salinity and drought conditions. These results suggest that *P. euphratica* can adapt to the environment of high-salinity and drought, which may be related to the differential expression patterns of *SNAC* genes.

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Abbreviations — NAC, NAM (no apical meristem), ATAF1/2 (*Arabidopsis* transcription activation factor 1/2) and CUC2 (cup-shaped cotyledon); TFs, Transcription factors; *SNAC*, Stress-responsive *NAC*; GFP, Green fluorescent protein; RSMC, relative soil moisture content; qRT-PCR, Quantitative reverse transcriptase PCR; ORF, Open reading frame; WT, Wild-type; SD, synthetic dropout; Trp, Tryptophan; His, Histidine; Ade, Adenine

Introduction

Plant growth and productivity are affected by environmental stresses, such as drought and high salinity. To deal with these stresses, plants have evolved adaptive molecular mechanisms, including the expression of a number of genes that alter plant morphology, and physiological and metabolic processes. During these processes, stress-response genes and downstream machinery are regulated via signal transduction pathways (Nakashima et al. 2009, Shinozaki et al. 2003). The core signaling components include kinase, phosphatases, and transcription factors (TFs) (Bray 2002, Shinozaki and Yamaguchi-Shinozaki 1997, Zhu 2001). TFs play crucial roles during abiotic stress responses, which function as important players by binding to *cis*-elements in the promoter regions of target genes, or other functional modular structures. Plant genomes contain a large number of TFs, such as DREB, bZIP, AP2/ERF, WRKY, MYB and NAC, which directly or indirectly regulate plant responses to various stresses (Agarwal et al. 2006, Alves et al. 2013, Martin and Paz-Ares 1997, Mizoi et al. 2012, Nakashima et al. 2012, Rushton et al. 2010).

The NAC superfamily is one of the largest plant-specific TF families (Shao et al. 2015). Large numbers of *NAC* genes have been detected in many sequenced species at the genome-wide scale, including 112 in *Arabidopsis thaliana* (Lamesch et al. 2012), 140 in *Oryza sativa* (Ouyang et al. 2007), and 169 in *Populus trichocarpa* (Tuskan et al. 2006). Phylogenetic analyses suggest that 837 NAC proteins are divided into 21 subfamilies, most of which possess the conserved *N*-terminal DNA-binding NAC domain (~150 amino acids) and highly variable *C*-terminal transcriptional activation regulatory domains (Aida et al. 1997, Souer et al. 1996, Zhu et al. 2012). NAC proteins serve important functions in plant developmental processes, such as in embryo and flower formation (Kunieda et al. 2008, Souer et al. 1996), shoot apical meristem and cotyledon formation (Aida et al. 1999, Hibara et al. 2003), secondary wall formation (Kubo et al. 2005, Mitsuda et al. 2007, Mitsuda et al. 2005, Zhong et al. 2006), xylary fiber development (Ko et al. 2007, Yamaguchi et al. 2010), wood formation (Ohtani et al. 2011, Zhong et al. 2010), fruit ripening and anthocyanin biosynthesis (Ma et al. 2014, Zhou et al. 2015, Zhu et al. 2014), leaf senescence (Guo and Gan 2006, Kim et al. 2009), and lateral root development (He et al. 2005, Xie et al. 2000).

Moreover, NAC TFs play vital roles in responses to various biotic and abiotic stresses, such as disease (Jensen et al. 2008, Wang et al. 2009a), drought, salt and cold (Mao et al. 2014, Sakuraba et al. 2015). Stress signaling is mediated by a subfamily of stress-responsive NAC TFs (Takasaki et al. 2015). The same subfamily frequently regulates similar processes in different species, for example, nearly all SNAC proteins are involved in stress responses (Zhu et al. 2012). Although most SNAC TFs contain a closely homologous NAC domain, their stress-induced expression patterns and protein structures are largely diversity (Fang et al. 2008). Whether each subfamily represents a class of genes with a similar function remains to be established in plants. *SNAC* subfamily genes *ANAC019, ANAC055*, and *ANAC072/RD26* are all up-regulated in response to drought, salt and ABA and overexpression of any one of these genes improves tolerance to drought stress in *Arabidopsis* (Tran et al. 2004). *SNAC1* overexpression enhances drought resistance and salt tolerance in rice (Hu et al. 2006). *OsNAC5* overexpression enlarges roots in rice and enhances drought tolerance (Jeong et al. 2013, Song et al. 2011). *SNAC2/OsNAC6* overexpression enhances tolerance to multiple abiotic stresses (Hu et al. 2008, Nakashima et al. 2007). However, *ATAF1* overexpression increases plant sensitivity to ABA and salt (Wu et al. 2009). The *ataf1* knockout mutant enhances drought and ABA tolerance (Jensen et al. 2008, Lu et al. 2007). Furthermore, *ATAF1* is a negative regulator of defense response against different types of pathogens (Wang et al. 2009a). *ATAF2* functions as a repressor of pathogenesis-related proteins in *Arabidopsis* (Delessert et al. 2005). These studies suggest that the SNAC TF subfamily participates in the regulation of responses to different abiotic and biotic stresses. NAC-domain proteins are the key regulators of plant responses to stress, and the features of the same *SNAC* subfamily genes are in direct contrast with the mode of regulation in *Arabidopsis* and grasses. However, the functions of the *SNAC* subfamily in response to environmental stresses in trees have not yet been elucidated. Trees have a long life span, therefore, they may be able to cope with excess salt and drought for extended periods of time (Brinker et al. 2010). The first genome sequenced tree was that of the black cottonwood (*P. trichocarpa* Torr. & Gray), which is a rapidly growing tree species (Tuskan et al. 2006). Thus, previous research on NAC TFs mainly focused on SND (Secondary wall-associated NAC domain protein) subfamily members, such as WNDs (Wood-associated NAC domain TFs), VNDs (Vascular-related NAC domain TFs), NSTs (NAC Secondary wall thickening TFs), SMBs (SOMBRERO-related domain TFs) and BRNs (BEARSKIN-related domain TFs), to improve plant biomass production (Ohtani et al. 2011, Zhong and Ye 2010, Zhu et al. 2012). However, the functional characteristics of the SNAC subfamily members in poplar have yet to be elucidated. Most species of poplar trees are drought or salt sensitive, but *P. euphratica* Olivier grows in dry deserts and can tolerate high salinity (Chen and Polle 2010, Ma et al. 2013).

P. euphratica maintains higher growth and photosynthetic rates than other congeners at high salinity (Janz et al. 2012, Wang et al. 2007). Therefore, *P. euphratica* is an ideal model species for research on abiotic tolerance (e.g., responses to salinity or drought stress) of trees (Li et al. 2013, Li et al. 2011, Ma et al. 2013, Tang et al. 2013). Our previous studies have revealed that many SNAC TFs exhibit different expression patterns under salt or drought stress in the leaves of *P. euphratica* (Table S1) (Li et al. 2013, Tang et al. 2013). On the basis of these studies, the functions of *SNAC* subfamily members in *P. euphratica* need further investigation. In the present research, *SNAC* genes *PeNAC034*, *PeNAC045* and *PeNAC036* were cloned and identified from *P. euphratica*. PeNAC034 and PeNAC045 belong to the ATAF subgroup, and PeNAC036 is classified into the ANAC072 subgroup. The expression of *PeNAC036* was induced in the whole plant by drought and salt, but *PeNAC034* and *PeNAC045* were inhibited in the roots and stems. *PeNAC034* overexpression in *Arabidopsis* wild-type (WT) or mutant *ataf1* (SALK_057618) plants and *PeNAC045* overexpression in *P. tomentosa* WT plants enhanced drought and high-salt sensitivity, whereas *PeNAC036* overexpression in *Arabidopsis* WT or mutant *anac072* (SALK_083756) plants increased drought and salt tolerance. These results suggest that these three *Pe-SNAC*s，which are differentially expressed during abiotic stresses, differentially regulate salt and drought tolerance in transgenic plants.

Materials and methods

Plant materials and stress treatments

One-year-old *Populus euphratica* plants were obtained from the Xinjiang Uygur Autonomous Region of China. The seedlings were planted in 5 l pots containing loam and were watered with 1 l of full-strength Hoagland nutrient solution every 2 weeks in a greenhouse for 2 months prior to the experiments at Beijing Forestry University. For drought stress treatment, uniformly grown *P. euphratica* plants were subjected to soil water deficiency at four relative soil moisture content (RSMC) levels (Group A control: 70%–75% RSMC; Group B moderate drought: 50%–55% RSMC; Group C moderate drought: 35%–40% RSMC and Group D severe drought: 15%–20% RSMC) for 2 months before harvesting (Li et al. 2011). For salt stress treatment, uniformly developed *P. euphratica* plants were watered using 1 l of 200 m*M* NaCl solution to each pot and every pot was placed on a tray to prevent the solution from flowing away (Li et al. 2013). The plants were harvested at 0, 4, 8 24, 48 and 96 h. All samples were collected and frozen immediately in liquid nitrogen, then stored at −80 °C for later use.

Arabidopsis thaliana ecotype Columbia (*Col*-0) and the SALK T–DNA insertion mutants

ataf1 (SALK_057618 (Jensen et al. 2008, Wang et al. 2009a)) and *anac072* (SALK_083756 (Zheng et al. 2012)) were obtained from the Arabidopsis Biological Resources Center (ABRC). The homozygous mutants were screened via PCR through a three-primer PCR strategy in a single step using two gene-specific primers and LBb1 (http://signal.salk.edu/tdnaprimers.2.html). Transgenic lines were identified using 1/2 Murashige and Skoog (MS) medium containing 50 mg l^{-1} kanamycin.

Plantlets of the triploid white poplar (*P. tomentosa* 'YiXianCiZhu B385') (Zhu et al. 1998) were cultured in vitro on solid MS medium and used for genetic transformations. Regenerated plantlets were acclimatized in pots with a 16/8 h light/dark photoperiod at 25 °C and then transferred to the greenhouse.

Multiple sequence alignment and phylogenetic analysis

The protein (primary transcript only) sequences of *Physcomitrella patens*, *Selaginella moellendorffi*, *Oryza sativa*, *A. thaliana* and *P. trichocarpa* were obtained from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). *Picea abies* and *P. euphratica* proteins were retrieved from Picea_abies_v1.0 (ftp://plantgenie.org/Data/ConGenIE/Picea_abies/v1.0/) and PopEup_1.0 (https://www.ncbi.nlm.nih.gov/genome/?term=populus+euphratica), respectively. To identify the NAC proteins, a HMM profile for NAC domain NAM (PF02365) was downloaded from Pfam (http://pfam.xfam.org/). HMMER (Eddy 1998) was used to screen the database containing the protein sequences of the seven plant genomes. Members of the SNAC subfamily were selected for further study. Multiple sequence alignment of SNAC proteins across moss (*P. patens*), spikemoss (*S. moellendorffi*), conifer (*P. abies*), monocot (*O. sativa*), eudicots (*A. thaliana* and *P. trichocarpa*) was performed using MAFFT v7.0 (Katoh and Standley 2013) with L-INS-I strategy (Ahola et al. 2006). And then, an unrooted phylogenetic tree was generated by the Neighbor-Joining (NJ) method with 1000 bootstrap replications using MEGA v6.0 software (Tamura et al. 2013). Multiple sequence alignment of amino acid sequences of *P. euphratica* SNAC proteins with other known SNAC subfamily proteins was performed using ClustalW program by DNASTAR software. Phylogenetic relationship between *P. euphratica* SNAC proteins and members of other known SNAC subfamily was also constructed based on NJ method using MEGA with 1000 bootstrap replications.

Isolation and sequence analysis

Total RNA was extracted from *P. euphratica* using the EASYspin Plus Plant RNA Kit (AidLab, Beijing, China) in accordance with the manufacturer's protocol. And then, cDNA synthesis was performed using the TIANGEN FastQuant RT Kit (Qiagen, Hilden, Germany). The full-length cDNA sequences of *PeNAC034* (XM_011005132), *PeNAC045* (XM_011024560) and *PeNAC036* (XM_011031134) (Ma et al. 2013) were amplified from *P.*

euphratica via PCR. The sequences of the primers used are shown in Table S2. Functional regions of PeNAC034, PeNAC045 and PeNAC036 were analyzed by InterPro (http://www.ebi.ac.uk/interpro/).

Quantitative real-time PCR (qRT –PCR) and reverse transcription PCR (RT-PCR) analyses

Total RNA for qRT-PCR and RT-PCR analyses was extracted from each *P. euphratica*, white poplar or *Arabidopsis* sample via the EASYspin Plus Plant RNA Kit (AidLab, Beijing, China). The quality and quantity of RNA were assessed with a NanoDrop2000 spectrophotometer (Thermo, Waltham, MA, USA) by determining the OD260/OD280 and OD260/OD230 ratios, respectively. Exactly 2 μg of total RNA (with gDNAse) was reverse-transcribed using the TIANGEN FastQuant RT Kit (Qiagen, Hilden, Germany). The expression levels of *PeNAC034*, *PeNAC045* and *PeNAC036* in *P. euphratica* under different treatments, that of *PeNAC045* in transgenic white poplar and that of abiotic stress-responsive genes in transgenic *Arabidopsis* were determined via qRT-PCR analysis using the SYBRGreen PCR Kit (Qiagen, Hilden, Germany) in 96-well plates with a StepOne Plus PCR System (Applied Biosystems, Carlsbad, CA, USA) under following default cycling conditions (40 cycles of 15 s at 95 °C and 1 min at 60 °C). The relative quantification value was calculated using the $2^{-\Delta\Delta Ct}$ method (Chen et al. 2009, Livak and Schmittgen 2001). *Ptr-18S* rRNA (AY652861) (Wang et al. 2014) and *At-Actin8* (AY063089) were used as the housekeeping genes in Poplar and *Arabidopsis*, respectively. RT-PCR was carried out to investigate the expression levels of *PeNAC034* and *PeNAC036* in transgenic *Arabidopsis* plants. PCR amplification (30 s at 95 ℃, 30 s at 60 °C and 1 min at 72 °C) was performed for 30 cycles. *At-Actin8* was used as the housekeeping gene in *Arabidopsis*. Each PCR assay was replicated for three biological replicates. All the primers used for qRT-PCR and RT-PCR analyses are shown in Table S2.

Subcellular localization analysis

Subcellular localization of PeNAC034, PeNAC045 or PeNAC036 was analyzed via ProtComp v9.0 (http://linux1.softberry.com/berry.phtml?topic=protcomppl&group=programs&subgroup=proloc). The amplified coding regions of *PeNAC034* and *PeNAC036* were inserted into the *Xba* I and *Xma* I sites while that of *PeNAC045* was inserted into the *Xba* I and *Bam*H I sites of the modified PBI121 (Clontech, Mountain View, CA, USA) vector under control of the cauliflower mosaic virus (CaMV) 35S promoter to generate *35S:PeNAC034-GFP*, *35S:PeNAC045-GFP* and *35S:PeNAC036-GFP* constructs, respectively. The constructs and PBI121 vector were transformed into *Arabidopsis Col*-0 via the floral dip method (Zhang et al. 2006) using *Agrobacterium tumefaciens* GV3101. The transgenic *Arabidopsis* lines were

screened on half-strength MS plate containing 50 mg l^{-1} kanamycin. Subcellular localization of the control GFP, PeNAC034-GFP, PeNAC045-GFP and PeNAC036-GFP fusion proteins was observed under a confocal laser scanning microscope (DN16000 CS; Leica, Wetzlar, Germany). Primers for PCR amplification are provided in Table S2.

Transcriptional activation analysis in yeast

Full-length (FL) open reading frame (ORF), *N*-terminal NAC domain (ΔC) and the fragment encoding *C*-terminal (ΔN) of *PeNAC034* were inserted into the *Nde* I and *Bam*H I sites, those of *PeNAC045* were inserted into the *Eco*R I and *Bam*H I sites, those of *PeNAC036* were inserted into the *Nco* I and *Xma* I sites of pGBKT7 (Clontech, Mountain View, CA, USA) to construct an in-frame fusion with the GAL4 activation domain, respectively. The empty vector (Vector) was used as a negative control. These different constructs were transformed into yeast strain AH109. The yeast liquid cultures were dropped on the Synthetic Dropout (SD)/Trp- and SD/Trp-/His-/Ade- agar media. After incubation at 28 °C for 3 days, the transcriptional activation activity was evaluated in accordance with the growth and *β*-galactosidase (*β*-gal) filter lift assay (Yeast Protocols Handbook; Clontech, Mountain View, CA, USA). Primers for PCR amplification are provided in Table S2.

Vector construction and plant transformation

To obtain transgenic plants, the entire coding region of *PeNAC034* was inserted into the *Bgl* II and *Bst* EII sites of the pCAMBIA-1301 vector for *Arabidopsis Col*-0 and mutant *ataf1* plants via floral dip method (Zhang et al. 2006), using *A. tumefaciens* strain GV3101. The coding region of *PeNAC036* was inserted into the pCAMBIA-1301 vector using the *Bgl* II and *Bst* EII sites for *Arabidopsis Col*-0 and mutant *anac072* plants by floral dip method using *A. tumefaciens* strain GV3101 (Zhang et al. 2006). The coding region of *PeNAC045* was cloned into the *Bgl* II and *Pml* I of the pCAMBIA-1301 vector for triploid white poplar plants via leaf disc method (Li et al. 2012a), using *A. tumefaciens* strain GV3101. Positive transgenic *Arabidopsis Col*-0 and mutant lines overexpressing *PeNAC034* and *PeNAC036* were screened on 1/2 MS plates containing 50 mg l^{-1} kanamycin and then identified by RT-PCR. The T3 homozygous transgenic lines were selected for further analysis. The transgenic poplar plantlets containing *PeNAC045* were selected on a medium containing 10 mg l^{-1} hygromycin and identified through PCR at the DNA level and qRT-PCR at the mRNA level. Primers for PCR amplification are provided in Table S2.

Abiotic stress assays of transgenic plants

The assays of root growth were carried out by transferring 7-day-old seedlings of *Arabidopsis* transgenic lines, mutants and *Col*-0 onto vertical square 1/2 MS medium agar plates supplemented with NaCl (0, 100 and 150 m*M*) and mannitol (0, 200 and 300 m*M*). Seven days after the start of treatment, primary root lengths of more than 15 seedlings in each line

were measured, each with three independent repeats.

Arabidopsis plants were germinated and grown on 1/2 MS plates for 3 days after 4 °C vernalization for additional 7 days and then transferred to 7 cm square pots filled with a mixture of peat moss, organic substrate and vermiculite (2:2:1) for stress treatment. Drought stress was induced by withholding water until the lethal effect of dehydration was observed. After rewatering for 3 days, the number of plants that survived was counted. Salt stress was induced by watering with 200 m*M* NaCl solution every 7 days until the plants showed evident salt-stressed phenotypes.

Poplar WT and transgenic plants were transferred to 10 cm square pots filled with a mixture of peat moss, organic substrate and vermiculite (2:2:1) and grown well-watered for 2 months before exposure to abiotic stress. For the drought stress assay, the plants were cultured without watering. For the salt stress assay, the plants were watered with 200 m*M* NaCl solution every 7 days. The net photosynthetic rate (P_N), stomatal conductance (g_s) and transpiration (E) rate were measured via Li-6400 Photosynthesis System (Li–Cor Biosciences, Lincoln, NE, USA).

Statistical analysis

All data were presented as mean ± standard errors. Each data set was compared with the control data set separately to determine whether they are significantly different from each other by Student's *t*-test (**P* < 0.05, ***P* < 0.01).

Results

NAC proteins and SNAC subfamily present in major groups of land plants

NAC proteins in the complete genome of the eudicots *A. thaliana* and *P. trichocarpa*, the monocot *O. sativa*, the conifer *P. abies*, the lycophyte *S. moellendorffii* and the moss *P. patens* were identified (Table, S3). The same NAC subfamily frequently regulates similar processes in different species, which indicating that all *SNAC* subfamily members are involved in stress responses (Zhu et al. 2012). A phylogenetic tree was created from the multiple sequence alignment of all 39 SNAC proteins, which are divided into two distinct subgroups (Fig. 1A). Among them, ATAF1, ANAC032, ATAF2 and ANAC102 belong to the ATAF subgroup. ATAF1 functions as a negative regulator in drought signal transduction pathways (Lu et al. 2007) and ATAF2 negatively regulates the expression of pathogenesis-regulated genes (Delessert et al. 2005). However, another ANAC072 subgroup comprising ANAC019, ANAC055 and ANAC072 could increase expression of stress-inducible genes and improve drought tolerance when overexpressed in transgenic plants (Tran et al. 2004). PNAC030, PNAC034, PNAC043 and PNAC045 are clustered in the ATAF subgroup, whereas PNAC036

and PNAC044 are clustered in the ANAC072 subgroup (Fig. 1A). During evolution, a large number of gene families contribute to segmental duplication and tandem duplication in plants (Cannon et al. 2004). *Populus* genome had undergone at least three rounds of genome-wide duplications (Tuskan et al. 2006). Poplar *SNAC* genes (*PNAC030* and *PNAC034*; *PNAC043* and *PNAC045*; *PNAC036* and *PNAC044*) are three pairs of paralogous genes in the terminal nodes (Fig. 1A). Three segmental duplication events of poplar *SNAC* genes can be found in the Plant genome duplication database (http://chibba.agtec.uga.edu/duplication/index/home): (Fig. 1B).

The SNAC subfamily comprises six genes of *P. euphratica*, namely, *PeNAC030*, *PeNAC034*, *PeNAC043*, *PeNAC045*, *PeNAC036* and *PeNAC044*, which show high homology with *PNAC030*, *PNAC034*, *PNAC043*, *PNAC045*, *PNAC036* and *PNAC044* of *P. trichocarpa* successively (Fig. 1A). *P. euphratica SNAC genes* (*PeNAC030* and *PeNAC034*; *PeNAC043* and *PeNAC045*; *PeNAC036* and *PeNAC044*) are also three pairs of paralogous genes (Fig. 2A). A Similar sequence indicates a similar function. Thus, each pair has overlapping expression patterns. Phylogenetic analysis showed that PeNAC030 and PeNAC034 are clustered in the ATAF1 clade; PeNAC043 and PeNAC045 belong to the ATAF2 clade; and PeNAC036 and PeNAC044 fall into the ANAC072 clade (Fig. 2A). NAC proteins clustered in the same subgroups indicate functional similarities among members of the same subgroup.

Sequences characterization of *PeNAC034, PeNAC045* and *PeNAC036*

According to the high-throughput sequencing in leaves of *P. euphratica* response to salt and drought stresses, many *SNAC* subfamily genes exhibited different expression patterns (Li et al. 2013, Tang et al. 2013). Three *SNAC* subfamily genes, namely, *PeNAC034*, *PeNAC045* and *PeNAC036*, were cloned from *P. euphratica* for further characterization. A detailed illustration of the exon/intron structures showed that *PeNAC034*, *PeNAC045* and *PeNAC036* genes contain three exons and two introns (Fig. 2B). The first two exons encode the *N*-terminal region and the last one encodes the *C*-terminal domain. Structural analyses of the deduced proteins of PeNAC034, PeNAC045 and PeNAC036 using InterPro suggested that they all have a typical NAC domain (IPR003441) (File, S2).

To further analyze the NAC domain, the predicted amino acid sequences of PeNAC034, PeNAC045 and PeNAC036 were compared with other known SNAC proteins from *A. thaliana*, *O. sativa*, *Glycine max*, and *Solanum lycopersicum*. The overall sequence alignment indicates that the five conserved subdomains (A–E) in the *N*-terminal region comprised the plant-specific NAC domain and a highly variable *C*-terminal transcriptional regulation domain with the nuclear localization sequence (File, S1).

Subcellular localization and transcriptional activation activity of PeNAC034, PeNAC045 and PeNAC036

Prediction of subcellular localization using ProtComp v9.0 software suggested that PeNAC034, PeNAC045 and PeNAC036 are all typical nuclear localization proteins. To determine the subcellular localization of PeNAC034, PeNAC045 and PeNAC036 in plant cells, the *35S:PeNAC034-GFP*, *35S:PeNAC045-GFP* and *35S:PeNAC036-GFP* fusion constructs, and the *35S:GFP* empty vector were transformed into *Arabidopsis Col*-0, and then the expression in the root tips was observed under confocal laser-scanning microscopy. The PeNAC034-GFP, PeNAC045-GFP and PeNAC036-GFP fusion proteins were targeted to the nucleus (Fig. 3A, II–IV), whereas the control GFP protein was located in the cytoplasm (Fig. 3A, I). Therefore, PeNAC034, PeNAC045 and PeNAC036 interacted with the cell nucleus, consistent with their function as transcriptional regulator.

Many NAC TFs exhibit transactivation activity (Fujita et al. 2004, Lu et al. 2007, Yu et al. 2015). The yeast one-hybrid system was used to examine the transcription activation activity of PeNAC034, PeNAC045 and PeNAC036 proteins. The FL, ΔC and ΔN of *PeNAC034*, *PeNAC045* and *PeNAC036* were fused in frame with the GAL4 DNA-binding domain in the pGBKT7 vector. Fusion plasmids and Vector were transformed into yeast strain AH109. All of these yeast cells grew well on SD/Trp- medium (Fig. 3B, I). The yeast cells containing the FL and ΔN of *PeNAC034*, *PeNAC045* or *PeNAC036* grew well on SD/Trp-/His-/Ade- medium, whereas the cells containing ΔC and empty vector did not grow (Fig. 3B, II). In addition, the yeast cells grown on the SD/Trp-/His-/Ade- medium turned blue in the presence of *β*-gal (Fig. 3B, III). Therefore, the full-length and *C*-terminal part of PeNAC034, PeNAC045 and PeNAC036 possess obvious transcriptional activation capacities, whereas the N-terminal part that includes the NAC domain sequence does not.

Expression profiles of *PeNAC034, PeNAC045* and *PeNAC036* under drought and salt stresses

The expression patterns of *PeNAC034*, *PeNAC045*, and *PeNAC036* in *P. euphratica* seedling roots, stems and leaves under drought or salt stress were analyzed via qRT-PCR. Under drought treatment, the relative expression levels of *PeNAC034* and *PeNAC045* significantly decreased in the roots and stems, but not decreased in the leaves. At the severe drought group, the transcripts level of *PeNAC036* was substantially increased in the whole plant, particularly in the roots and leaves (Fig. 4A). During seedlings treatment with 200 m*M* NaCl, the expression levels of *PeNAC034* and PeNAC045 were significantly suppressed in the roots, but *PeNAC036* was induced within 8 h after application of NaCl solution. Under salt treatment, the transcripts level of *PeNAC034* and *PeNAC036* were increased after 4 h and then decreased quickly after 24 h in the stems, but *PeNAC045* showed no significant difference. The expression of *PeNAC045* and *PeNAC036* were distinctly activated under salt stress in the leaves, but that of *PeNAC034* was suppressed (Fig. 4B). These results indicate

that *PeNAC034* was inhibited by drought and salt stresses but *PeNAC036* was induced, whereas *PeNAC045* was suppressed by drought stress but induced in the leaves by salt stress. Therefore, *PeNAC034*, *PeNAC045* and *PeNAC036* were all involved in plant responses to abiotic stress but exhibited diverse expression patterns.

The expression levels of *PeNAC034*, *PeNAC045* and *PeNAC036* in *P. euphratica* seedling roots, stems and leaves under normal growth conditions were detected via qRT-PCR. The three *NAC* genes exhibited different expression patterns in different tissues. The expression of *PeNAC034* was the highest in the leaves and the lowest in the roots, and that of *PeNAC045* was the highest in the stems and the lowest in the leaves, while *PeNAC036* was the highest in the stems (Fig. S1A).

Phenotype of the transgenic *Arabidopsis* plants overexpressing *PeNAC034* and *PeNAC036*

To analyze the functions of *PeNAC034* and *PeNAC036* in vivo, transgenic *Arabidopsis* lines *OEPeNAC034* and *OEPeNAC036* (overexpressed in *Arabidopsis Col*-0), *ataf1/PeNAC034* and *anac072/PeNAC036* (complementation in mutant *ataf1* and *anac072*) controlled by the cauliflower mosaic virus 35S promoter were generated. The T-DNA insertion *Arabidopsis* mutant lines of *ataf1* and *anac072* were obtained from the ABRC, and the homozygous mutants were verified via PCR using a three-primer PCR strategy (Fig. S2). Transgenic plants were screened in kanamycin-containing medium and were confirmed via PCR and RT-PCR using specific primers. *PeNAC034* was expressed in *OEPeNAC034* and *ataf1/PeNAC034* lines, and *PeNAC036* was expressed in *OEPeNAC036* and *anac072/PeNAC036* lines (Fig. 5A, 5D).

The growth phenotype of *OEPeNAC034*, *ataf1/PeNAC034*, *OEPeNAC036* and *anac072/PeNAC036* lines grown under well-watered conditions were observed in different development stages. Fourteen days after sowing, the differences in the primary root length were not significant (Fig. 5B, 5E). For the 40-day-old seedlings grown in soil, the difference in plant height between *Col*-0 and *OEPeNAC034* lines was not significant, but the plant height of *ataf1* mutant line was higher than those of *Col*-0 and *ataf1/PeNAC034* lines (Fig. 5C). Forty-day-old seedlings of *Col*-0, *OEPeNAC036*, *anac072* and *anac072/PeNAC036* grown in soil, *anac072* mutant line had the highest stem elongation and *OEPeNAC036* line showed bolting time delay (Fig. 5F). Thus, transgenic *OEPeNAC036* and *anac072/PeNAC036* lines delayed the shooting time, but this phenotype was not observed in *OEPeNAC034* and *ataf1/PeNAC034* lines.

Performance of the transgenic *Arabidopsis* plants overexpressing *PeNAC034* and *PeNAC036* under drought or salt stress

To evaluate the effect of *PeNAC034* and *PeNAC036* ectopic overexpression on osmotic and

salinity tolerances, 7-day-old seedlings of *Col*-0, *OEPeNAC034*, *ataf1*, *ataf1/PeNAC034*, *OEPeNAC036*, *anac072* and *ananc072/PeNAC036* were transferred to 1/2 MS agar medium with mannitol and NaCl. After 7 days, the root growth of *Col*-0, *OEPeNAC034*, *ataf1*, *ataf1/PeNAC034*, *OEPeNAC036*, *anac072* and *anac072/PeNAC036* plants were inhibited by mannitol and NaCl. On medium containing 200 m*M* mannitol and 100 m*M* NaCl, the primary root lengths of two independent lines of *OEPeNAC034* plants were shorter than those of *Col*-0 plants, and the primary root lengths of two independent lines of *ataf1/PeNAC034* plants were also shorter than those of *ataf1* mutant plants (Fig. 6A). However, on medium containing 300 m*M* mannitol and 150 m*M* NaCl, the primary root growths of transgenic and *Col*-0 plants all were inhibited to a greater extent than those of *ataf1* mutant plants (Fig. 6A). On medium containing 200 m*M* mannitol and 100 m*M* NaCl, the primary root lengths of *anac072* mutant plants were the shortest compared with transgenic and *Col*-0 plants (Fig. 6B). Moreover, on medium containing 300 m*M* mannitol and 150 m*M* NaCl, the primary root lengths of two independent lines of *OEPeNAC036* plants were longer than those of *Col*-0 plants, and the primary root lengths of two independent lines of *anac072/PeNAC036* plants were also longer than those of *anac072* mutant plants (Fig. 6B). These results demonstrate that high *PeNAC034* expression contributes to osmotic and salt hypersensitivity in transgenic *Arabidopsis* but high level of *PeNAC036* can enhance plant tolerance to osmotic and salt conditions.

To examine the long-term effect of *PeNAC034* and *PeNAC036* on drought and salt stresses 20-day-old soil-planted seedlings of *Col*-0, *OEPeNAC034*, *ataf1* and *ataf1/PeNAC034* and 25-day-old soil-planted seedlings of *Col*-0, *OEPeNAC036*, *anac072* and *anac072/PeNAC036* were subjected to salt treatment by watering with 200 m*M* NaCl solution every 7 days and were exposed to drought treatment by withholding water. During treatment with progressively increasing concentration of NaCl, the lethal effect was observed. Survival rates were examined 15 days after watering with NaCl solution. Approximately 83.33% of *Col*-0 plants survived, but only 37.48% of *OEPeNAC034*#1 and 29% of *OEPeNAC034*#2 plants survived. A total of 97.92% of *ataf1* mutant plants survived, but only 8.23% of *ataf1/PeNAC034*#1 and 6.25% of *ataf1/PeNAC034*#2 plants survived (Fig. 7A). A total of 81.17% of *Col*-0 plants survived, but more than 93.65% of *OEPeNAC036*#1 and 93.82% of *OEPeNAC036*#2 plants survived. About 6.23% of *anac072* mutant plants survived, but more than 96% of *anac072/PeNAC036*#1 and 93.75% of *anac072/PeNAC036*#2 plants survived (Fig. 7B). After 15 days of drought, the lethal effect was observed. Survival rates were examined 3 days after rewatering. A total 100% of *Col*-0 plants survived, but only 12.33% of *OEPeNAC034*#1 and 6% of *OEPeNAC034*#2 plants survived. Nearly 100% of *ataf1* mutant plants survived, but only 6% of *ataf1/PeNAC034*#1 and 4% of *ataf1/PeNAC034*#2

plants survived (Fig. 7A). A total of 56.15% of *Col*-0 plants survived, but 98% of *OEPeNAC036*#1 and 100% of *OEPeNAC036*#2 plants survived. Approximately 6.25% of *anac072* mutant plants survived, but more than 93.75% of *anac072/PeNAC036*#1 and 96% of *anac072/PeNAC036*#2 of plants survived (Fig. 8B). These results show that *PeNAC034* overexpression in transgenic plants enhanced drought and salt sensitivity, whereas *PeNAC036* overexpression in transgenic plants improved drought and salt tolerance.

Expression of the stress-related genes in transgenic *Arabidopsis* plants of *PeNAC034* and *PeNAC036*

Morphological assays showed that *PeNAC034* in transgenic *Arabidopsis* plants reduced tolerance to drought and salt stresses, whereas *PeNAC036* in transgenic *Arabidopsis* plants enhanced tolerance to drought and salt stresses. PeNAC034 and PeNAC036 are NAC TFs that function as transcriptional regulators. Therefore, the expression levels of stress-responsive marker genes were analyzed in *Col*-0, *OEPeNAC034*#1, *ataf1*, *ataf1/PeNAC034*#1, *OEPeNAC036*#1, *anac072* and *anac072/PeNAC036*#1 plants under drought and salt conditions. Under normal conditions, the expression levels of *COR47*, *ERD11* and *DREB2A* genes were lower in *ataf1* plants than *Col*-0, *OEPeNAC034* and *ataf1/PeNAC034* lines, and the expression levels of *DREB2A*, *ERD11*, *RD22* and *RD29B* genes were higher in *OEPeNAC034* plants than in other lines (Fig. 8A). After drought and salt treatments, the transcripts of *COR47*, *ERD11*, *DREB2A* and *RD29B* accumulated to a high level in *Col*-0 and *ataf1* plants, and the expression levels of these marker genes were reduced in *OEPeNAC034* and *ataf1*/*PeNAC034* lines (Fig. 8A). The expression levels of *COR47*, *DREB2A*, *ERD11*, *RD22* and *RD29B* genes were the lowest in both non-stressed and stressed *anac072* plants than in other lines, and the transcripts of these five stress-responsive genes were dramatically increased in *OEPeNAC036* and *anac072*/*PeNAC036* lines compared with *Col*-0 or *anac072* after drought and salt treatments (Fig. 8B). Therefore, *PeNAC036* may be correlated with the stress-regulation pathway or directly regulated downstream stress-response genes, as did *ANAC072* positively regulate to drought (Fujita et al. 2004, Tran et al. 2004). In addition, *PeNAC034* negatively regulated the expression of stress-responsive genes under drought and salt stresses, as did *ATAF1* negatively regulate responses to drought and salinity (Jensen et al. 2008, Lu et al. 2007).

Phenotype of the transgenic poplar plants overexpressing *PeNAC045*

To investigate the functions of *PeNAC045*, an overexpression construct with *PeNAC045* gene under the control of the CaMV 35S promoter was transformed into poplar. Transgenic plants were selected in hygromycin-containing medium and were confirmed via PCR and qRT-PCR using specific primers. Two independent *OEPeNAC045* lines produced the expected amplification band and elevated *PeNAC045* transcript levels compared with WT and Vector

plants (Fig. 9A, 9C). Vector lines were also verified via PCR using specific primers and produced the expected amplification band (Fig. 9B). Transgenic lines derived from regenerated plantlets were used as samples at the same time WT line derived from regenerated plantlets was used as a control. No significant differences in phenotype were noted among WT, Vector and *OEPeNAC045* plants (Fig. 9D).

Performance of transgenic poplar plants overexpressing *PeNAC045* under drought and salt stresses

To understand the physiological role of *PeNAC045* overexpression on drought and salt stresses, seedlings of *OEPeNAC045* were transferred into soil and grown for 2 months under normal conditions were exposed to drought treatment by withholding water or were subjected to salt treatment by watering with 200 m*M* NaCl solution every 7 days. During withholding-water treatment, the leaves of two independent lines of *OEPeNAC045* were already wilting on day 8, whereas those of WT and Vector plants remained turgid (Fig. 10A). On day 10, the leaves of *OEPeNAC45* plants were completely wilted, but those of WT and Vector plants were just beginning to wither (Fig. 10A). The P_N, g_s and E curves of plants during 10 days of drought stress showed that two independent *OEPeNAC045* lines began to rise rapidly and then fell sharply, whereas those of WT and Vector plants began to rise slightly and then gradually decreased (Fig. 10A). The P_N, g_s and E of *OEPeNAC045* plants were higher than that of WT and Vector on day 4, whereas they were lower compared with that of WT and Vector on day 6 (Fig. 10A). During treatment with progressively increasing NaCl concentration, the leaves of two independent lines of *OEPeNAC045* fell off earlier than WT and Vector at the bottom (Fig. 10B). The P_N, g_s and E curves of plants during the 15 days of salt stress indicated that *OEPeNAC045* plants had a steep decline compared with the minimal decline of WT (Fig. 10B). This observation indicates that WT plants maintained higher P_N, g_s and E than *OEPeNAC045* lines. Therefore, *PeNAC045* overexpression in plants reduced drought and salt tolerance, and PeNAC045 may be a negative regulator of stress responses, as did *ATAF2* negatively regulate responses to wounding (Delessert et al. 2005).

Discussion

Water deficit and high soil salinity are severe threats to plant growth and productivity. Plants adapt to these stresses through the expression of numerous stress-induced genes. TFs regulate stress-inducible genes by binding *cis*-acting elements in the promoter (Yamaguchi-Shinozaki and Shinozaki 2006). The NAC family is present in major lineages of land plants, including mosses, ferns, conifers, monocots and eudicots (Zhu et al. 2012). NAC TFs serve important functions not only in plant development but also in abiotic stress responses (Nakashima et al.

2012). The ANAC072 subgroup already existed in early-diverged land plants including moss, fern, conifer and eudicots, except for monocot, but the ATAF subgroup contains only the spermatophyte plants (Fig. 1A). This finding implies that the ATAF subgroup evolved later among land plants than the ANAC072 subgroup. Most abiotic stress studies of *SNAC* subfamily genes have focused on *Arabidopsis* (Fujita et al. 2004, Lu et al. 2007, Tran et al. 2004), soybean (Hao et al. 2011), tomato (Ma et al. 2013) and grasses, such as rice (Hu et al. 2006, Hu et al. 2008, Nakashima et al. 2007) and wheat (Mao et al. 2012, Tang et al. 2012), but few studies investigated woody plants. *P. euphratica* is a model species for studying abiotic stress (Ma et al. 2013). In general, NAC proteins clustered in the same subgroups and share similar motif sequences, indicating functional similarities among members of the same subgroup. Based on the high-throughput sequencing results, *P. euphratica SNAC* subfamily genes response to salt and drought stresses (Li et al. 2013, Tang et al. 2013).

In the present study, we characterized three *Pe-SNAC* genes, namely, *PeNAC034*, *PeNAC045* and *PeNAC036*. Sequence analysis showed that PeNAC034, PeNAC045 and PeNAC036 contain a highly conserved NAC-binding domain, which can be divided into five subdomains (A–E) and can bind both DNA and protein, in the N-terminal region; they also contain a highly variable *C*-terminal transcriptional regulation domain for the activation or suppression of downstream target genes (Fig. 2C) (Ernst et al. 2004, Ooka et al. 2003). We conducted a transactivation activity analysis using a yeast system and found that PeNAC034, PeNAC045 and PeNAC036 function as transcriptional activators and contain transcription regulatory regions in their *C*-terminal (Fig. 3B). Subcellular localization analysis indicated that the PeNAC034-GFP, PeNAC045-GFP and PeNAC036-GFP fusion proteins are localized in the nucleus (Fig. 3A). These data are consistent with that of other NAC TFs, that is, ATAF1, ATAF2, ANAC072, SNAC1, SNAC2/OsNAC6, OsNAC5 and TaNAC2 which are all nuclear localized proteins with transcription activation activities in the *C*-terminal (Fujita et al. 2004, Hu et al. 2006, Hu et al. 2008, Lu et al. 2007, Mao et al. 2012, Takasaki et al. 2010, Wang et al. 2009b).

The expression patterns of *PeNAC034*, *PeNAC045* and *PeNAC036* in different tissues were varied. The expression level of *PeNAC034* was the highest in the leaves, whereas *PeNAC045* and *PeNAC036* were highly expressed in the stems (Fig. S1A). In rice, *SNAC2* was expressed at relatively high levels in the roots, stems and leaves (Kikuchi et al. 2000), and accumulation of *OsNAC5* mRNA was high in the leaves and panicles and low in the stems and roots (Sperotto et al. 2009). *ATAF2* expression was the highest level in the roots and leaves, and the lowest in flower buds and bolt stems in *Arabidopsis* (Delessert et al. 2005).

PeNAC034 transcription was suppressed in the roots and stems by drought and salt

stresses, but was induced in the leaves by ABA (Fig. S1B). After osmotic and salt treatments, the root growth of *OEPeNAC034* and *ataf1/PeNAC034* plants was inhibited to a greater extent than that of *Col*-0 and *ataf1* plants (Fig. 6A). Under osmotic stress, *OEPeNAC034* and *ataf1/PeNAC034* seedlings had shorter root system, which hampered water absorption from deep soils and aggravated drought sensitive. The whole-plant survival of *OEPeNAC034* and *ataf1/PeNAC034* plants was also significantly reduced compared with that of *Col*-0 and *ataf1* plants under salt and drought stresses (Fig. 7A). In the same subgroup, *ATAF1* is a negative regulator of stress responsive genes under drought stress and is strongly induced by dehydration and ABA treatment. In addition, *ataf1-1* (SALK_067648) and *ataf1-2* (SALK_057618) mutants improved drought tolerance (Lu et al. 2007). *ATAF1* overexpression increases ABA levels and *ataf1-1* mutants are ABA-hyposensitive during seedling development and germination (Jensen et al. 2013). *ATAF1* is also a positive regulator of senescence, *ATAF1* overexpression plants promoted developmental senescence, but *ataf1-4* (GK565H08) mutant plants have delayed senescence (Garapati et al. 2015). Complementation of *PeNAC034* in mutant *ataf1* plants indicated that *ataf1*/*OEPeNAC034* plants could enhance sensitivity to drought and salt conditions (Fig. 6A, 7A). These results suggest that *PeNAC034* has a similar function to *ATAF1* and may be a negative regulator of drought and salt responses.

PeNAC036 expression was strongly induced not only by drought and salt conditions but also by ABA treatment (Fig. S1B). *PeNAC036* mRNA accumulated to higher levels in the leaves and roots experiencing severe drought rather than under salt stress, suggesting that the role of *PeNAC036* is associated mainly with the plant response to dehydration. The seedlings of *OEPeNAC036* and *anac072/PeNAC036* plants had shorter stem elongation than those of *Col*-0 and *anac072* plants (Fig. 5F). Under osmotic and salt stresses, *OEPeNAC036* and *anac072/PeNAC036* plants had longer primary root lengths compared with *Col*-0 and *anac072* plants (Fig. 6B). Mutant *anac072* (SALK_083756) showed lower tolerance to salt stress than WT (Li et al. 2012b). Under osmotic stress, *OEPeNAC036* and *anac072/PeNAC036* seedlings had long root system, which facilitated water absorption from deep soils and strengthened drought tolerance. Under salt and drought stresses, the whole-plant survival of *OEPeNAC036* plants was higher than that of *Col*-0, in particular, the survival rate of *anac072/PeNAC036* lines was significantly higher than that of *anac072* mutant lines (Fig. 7B). In the same subgroup of *PeNAC036*, *ANAC019*, *ANAC055* and *ANAC072* in *Arabidopsis* were mainly induced by drought and salt stresses (Tran et al. 2004). Transgenic plants overexpressing either *ANAC019*, *ANAC055*, or *ANAC072* showed significantly increased drought tolerance (Tran et al. 2004). *ANAC072* was also induced by ABA, and the plants overexpressing *ANAC072* were highly sensitive to ABA (Fujita et al.

2004). ANAC019 may be a senescence activator that serves a function in activating flavonoid and anthocyanin biosynthesis (Hickman et al. 2013). In *anac055* (Salk_011069) mutant, early down-regulation of chloroplast-related genes hints at accelerated senescence (Hickman et al. 2013). *ANAC019*, *ANAC055* and *ANAC072* have overlapping expression patterns (Hickman et al. 2013). Complementation of *PeNAC036* in mutant *anac072* plants enhanced drought and salt tolerance. These results suggest that *PeNAC036* may serve a similar function to *ANAC072* and plays a positive regulatory role in drought and salt stresses.

PeNAC045 mRNA expression was significantly suppressed in the roots and stems by drought and salt treatment but induced in the leaves (Fig. 4). Previously, we have shown that transgenic *Arabidopsis* overexpression (*OEPeNAC045*) and complementation (*ataf2/PeNAC045*) lines possess shorter primary root length and lower seedlings height than *Col*-0 and *ataf2* (SALK_136355C) mutant plants under salt treatment (Zhang et al. 2015). To confirm the functions of *PeNAC045* in woody plants, we generated transgenic overexpression poplar plants (*OEPeNAC045*). With strengthening of drought stress, the leaves of *OEPeNAC45* plants completely wilted earlier and displayed significantly lower levels of photosynthesis and stomatal conductance compared with WT and Vector plants (Fig. 10A). During treatment with progressively increasing concentration of NaCl, the leaves of *OEPeNAC045* fell off earlier at the bottom and showed significantly lower levels of photosynthesis and stomatal conductance than WT and Vector plants (Fig. 10B). In the same clade with *PeNAC045*, *ATAF2* repressed pathogenesis-related proteins and was induced by salt and wounding stresses but not ABA (Delessert et al. 2005). *ATAF2* expression was suppressed by both brassinosteroids and light (Peng et al. 2015). However, *ATAF2* overexpression led to an increased biomass and yellowing of the leaves (Wang and Culver 2012). These results suggest that *PeNAC045* plays a similar function to *ATAF2* and may be a negative regulator of stress response.

In our research, the opposite phenotypes of drought and salt response in transgenic plants of three *Pe-SNAC*s were observed. *PeNAC036* overexpression conferred salt and dehydration stress tolerance in transgenic plants, whereas *PeNAC034* or *PeNAC045* overexpression enhanced salt and dehydration sensitivity (Fig. 7, 10). These results are consistent with the stress-response patterns of three genes under drought and salt stresses in *P. euphratica* (Fig. 4). *PeNAC036* was strongly induced by severe drought and salt stresses. Although *PeNAC034* was slightly induced in the stems and *PeNAC045* was induced in the leaves by salt stress, both genes were suppressed in the roots and stems by drought stress, and were also suppressed in the roots by salt stress. In order to adapt to the environment of salt and drought, *PeNAC036* was up-regulated but *PeNAC034* and *PeNAC045* were down-regulated in *P. euphratica*. These results support the close relationship between the stress induction of genes

and the stress tolerance of the corresponding transgenic plants.

Gene regulation by osmotic stress (dehydration and salt) has been thought to involve both ABA-dependent and ABA-independent pathways (Shinozaki and Yamaguchi-Shinozaki 1997). Previous studies demonstrated that ectopic expression of *ANAC019*, *ANAC055*, *ANAC072*, *ANAC096*, *GmNAC20*, *CarNAC4* and *TaNAC67* led to the induction of the stress-responsive genes *COR47*, *RD29B*, *ERD11*, *DREB2A* and *RD22* (Hao et al. 2011, Jensen et al. 2010, Mao et al. 2014, Tran et al. 2004, Xu et al. 2013, Yu et al. 2015). To further understand the molecular basis of *PeNAC034*- and *PeNAC036*- mediated response to drought and salt stresses, the expression profiles of several stress-responsive genes were investigated in transgenic *Arabidopsis* (Fig. 8). The enhanced tolerance of *OEPeNAC036* and *anac072/PeNAC036* transgenic *Arabidopsis* lines to osmotic stresses can be attributed to the increased expression of ABA-responsive genes *COR47* (Gilmour et al. 1992) and *RD29B* (Yamaguchi-Shinozaki and Shinozaki 1993a), stress-responsive genes *ERD11* (Kiyosue et al. 1993) and *RD22* (Yamaguchi-Shinozaki and Shinozaki 1993b) and ABA-independent gene *DREB2A* (Sakuma et al. 2006). However, the osmotic stress-induced expression of *COR47*, *RD29B*, *ERD11*, *RD22* and *DREB2A* genes was impaired in *OEPeNAC034* and *ataf1/PeNAC034* transgenic *Arabidopsis* lines, which decrease drought and salt tolerance. The subgroup ATAF and subgroup ANAC072 in *SNAC* subfamily may act as negative and positive regulators of stress-responsive genes expression, respectively.

NAC TFs could recognize a conserved consensus sequence (NAC recognition sequence, NACRS) (Tran et al. 2004). Different NAC groups have differential binding affinity for NACRS (Xu et al. 2013). *SNAC* subfamily genes may recognize different NACRS at the promoter region of different target genes in *Arabidopsis*. ATAF1 regulates the accumulation of ABA by binding to TTGCGTA in the promoter region of *NCED3*, a key enzyme in ABA biosynthesis under dehydration stress (Jensen et al. 2008, Jensen et al. 2013). ATAF2 could bind a 25-bp A/T-rich consensus sequence of a *DEFL* gene promoter region to induce transcriptional changes in response to pathogen attack (Huh et al. 2012). ATAF2 binds a 36-bp A/T-rich sequence of the *NIT2* promoter region to regulate *NIT2* gene expression involved in auxin biosynthesis (Peng et al. 2015). ATAF2 also binds the A/T-rich EE/CBS elements of the *BAS1* and *SOB7* promoters regions to suppress *BAS1* and *SOB7* expression (Peng et al. 2015). The recognition sequence of ANAC019/055/072 contains CATGT and harbors the core DNA-binding site CACG in the *ERD1* promoter (Tran et al. 2004). *Pe-SNAC* genes act as opposite regulators of stress-responsive genes expression under osmotic stress, which may be due to the recognition of different NACRS. Thus, additional experiments, such as the identification of the full range of target genes regulated by PeNAC036, PeNAC034 and PeNAC045, will be required to understand the exact mechanism through which *Pe-SNAC*

genes intervene in osmotic stress responses in woody plants.

In conclusion, we identified major roles of three *Pe-SNAC*s under drought and salt stresses. *PeNAC036* could be induced in the whole plant under drought and salt conditions, whereas *PeNAC034* and *PeNAC045* were inhibited in the roots and stems. The significantly enhanced drought and salt tolerance of *PeNAC036*-overexpressing *Arabidopsis* plants suggests PeNAC036 plays a positive role in abiotic-stress responses. Conversely, PeNAC034 and PeNAC045 act as negative regulators of plant abiotic-stress. Transgenic plants with overexpressed *PeNAC034* and *PeNAC045* were sensitive to drought and salt. *P. euphratica* can adapt to the environment of high-salinity and drought, which may be related to the differential expression patterns of *SNAC* genes in different tissues.

Author contributions

XL, XX, and WY designed the experiments. XL, XZ, HD, and CL performed experiments. All authors discussed the results. XL and XX wrote the article.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. *P. euphratica SNAC* genes response to drought and salt stresses.

Table S2. Primers used in this research.

Table S3. Number of the *NAC* family and *SNAC* subfamily in various plant species.

File S1. Amino acid alignment of Pe-SNACs with other known SNAC proteins. A-E indicates the five conservative subdomains of NAC domains. Predicted nuclear localization sequence is indicated by a double-headed arrow. Identical amino acids are shaded in black, and similar amino acids are shaded in gray. Alignments were performed using ClustalW program by DNASTAR software.

File S2. Nucleotide and amino acid sequences of *PeNAC034*, *PeNAC045* and *PeNAC036*.

Fig. S1. (A) The expression profiles of *PeNAC034 PeNAC045* and *PeNAC036* transcripts in the different tissues under normal conditions. The $2^{-\Delta\Delta Ct}$ method was used to measure the relative expression levels of the target gene in the different tissues. Results are relative to expression in the roots. Poplar *18S* rRNA was used as an endogenous control. (B) The expression patterns of *PeNAC034* and *PeNAC036* in the leaves under ABA treatment. The plants were treated with 1 L of 100 μ*M* ABA solution to each pot. The level of expression of each gene at CK (ABA 0 h) is set to 1. The $2^{-\Delta\Delta Ct}$ method was used to measure the relative expression levels of the target gene in stressed and non-stressed tissues. Poplar *18S* rRNA was used as an endogenous control.

Data are means ± SE of three independent experiments. *Asterisks* indicate a significant difference compared to the non-treatment controls. Student's *t*-test (**P* < 0.05, ***P* < 0.01).

Fig. S2. Identification of the T-DNA insertion mutants *ataf1* and *anac072*. (A) Identification of the T-DNA insertion mutant *ataf1*. Illustration of *ATAF1* (AT1G01720) gene structure and location of mutant T–DNA insertions. Exons are indicated as thick lines and introns as thin lines. The T-DNA insertion is located in the third exon of the *ATAF1* gene. Genotyping for homozygous of *ataf1* was identified via PCR using T-DNA–specific primer LBb1 as well as the gene-specific primers LP and RP. (B) Identification of the T-DNA insertion mutant *anac072*. Illustration of *ANAC072* (AT4G27410) gene structure and location of mutants T–DNA insertions. Exons are indicated as thick lines and introns as thin lines. The T-DNA insertion is located in the 5' UTR of the *ANAC072* gene. Genotyping for homozygous of *anac072* was identified via PCR using T-DNA–specific primer LBb1 as well as the gene-specific primers LP and RP.

M, DNA marker III; LP, Forward primer; RP, Reverse primer; LBb1, primer specific to T – DNA left border.

Figure Legends

Fig. 1. Phylogenetic tree of the SNAC subfamily proteins across six plant species and distribution of *P. trichocarpa SNAC* genes on poplar chromosomes. (A) Phylogenetic analysis of SNAC proteins across six plant species. The amino acid sequences were aligned using MAFFT and the phylogenetic tree was constructed using MEGA 6.0 software by the neighbor-joining method with bootstrap analysis of 1000 replicates. The phylogenetic tree was divided into the ATAF subgroup and the ANAC072 subgroup. Members of SNAC proteins from six species are labeled: ○, *Physcomitrella patens* (moss); □, *Selaginella moellendorffi* (spikemoss); ∆, *Picea abies* (conifer); ●, *Oryza sativa* (monocot); ■, *Arabidopsis thaliana* (eudicot, herb); ▲, *Populus trichocarpa* (eudicot, woody). (B) Distribution of *P. trichocarpa SNAC* genes on poplar chromosomes. Chromosome numbers are indicated at the right of each bar. Straight lines connect the genes presented on duplicated chromosomal segments. *Ptr-SNAC* genes are highlighted in red.

Fig. 2. Phylogenetic analysis and multiple sequence alignment of *P. euphratica* SNAC proteins with other known SNAC proteins. (A) Phylogenetic tree analysis of Pe-SNACs with other known SNAC proteins. The neighbor-joining method of MEGA 6.0 software was used to generate the unrooted phylogenetic tree of SNAC proteins. Bootstrap analyses were computed with 1000 replicates. PeNAC034, PeNAC045 and PeNAC036 are labeled with a *black dot*. The gene names and GenBank accession numbers were as follow: *A. thaliana*, *ATAF1* (CAA52771), *ANAC032* (AEE35979), *ANAC102* (AED97798), *ATAF2* (CAA52772), *ANAC019* (AEE32864), *ANAC055* (AEE75683), *ANAC072* (AEE85335); *O. sativa*, *OsNAC5* (BAA89799), *SNAC2* (CBX55846), *OsNAC52* (AAT44250), *OsNAC3* (BAA89797), *OsNAC4* (BAA89798), *SNAC1* (AIQ84858); *P. euphratica*, *PeNAC030* (XP_011027492), *PeNAC034* (XP_011003434), *PeNAC043* (XP_011031100), *PeNAC045* (XP_011022862), *PeNAC036* (XP_011029436), *PeNAC044* (XP_011042499); *Glycine max*, *GmNAC20* (ACC66314), *GmNAC4* (AAY46124), *GmNAC3* (AAY46123), *GmNAC2* (AAY46122); *Solanum lycopersicum*, *SlNAC1* (NP_001234482), *JA2L* (NP_001306107), *SlNAC4* (AGH20612), *JA2* (NP_001233972). (B) Structures of *PeNAC034*, *PeNAC045* and *PeNAC036*. Exons are denoted by black boxes. Introns are denoted by lines. The translation initiation and termination codons are shown.

Fig. 3. Subcellular localization and transactivation assay of PeNAC034, PeNAC045 and PeNAC036. (A) Subcellular localization of PeNAC034, PeNAC045 and PeNAC036 in *Arabidopsis* cells. The control 35S:GFP, 35S:PeNAC034-GFP, 35S:PeNAC045-GFP and

35S:PeNAC036-GFP fusion proteins were expressed in root tips of *Arabidopsis* seedlings. Seven days after sowing, root tips were detected under a fluorescent or light field by a fluorescence microscope. Scale bar = 25 μm. (B) Transactivation assay of PeNAC034, PeNAC045 and PeNAC036 in yeast. The FL, ΔC and ΔN of *PeNAC034*, *PeNAC045* and *PeNAC036* were fused in frame with the GAL4 DNA-binding domain in the pGBKT7 vector. The empty pGBKT7 was expressed in yeast as a negative control. All plasmids were transformed into yeast strain AH109. The transformed yeast culture was incubated for 3 days at 28 °C and dropped onto SD/Trp- and SD /Trp-/His-/Ade- agar media. Plates were incubated for 3 days and subjected to the *β*-gal activity assay by X-Gal staining. All results were repeated for three times independently. SD, synthetic dropout; Trp, Tryptophan; His, Histidine; Ade, Adenine.

Fig. 4. The expression patterns of *PeNAC034 PeNAC045* and *PeNAC036.* (A) The relative expression level of *PeNAC034 PeNAC045* and *PeNAC036* under drought treatment. The expression level of each gene at Group A is set to 1. (B) The relative expression level of *PeNAC034 PeNAC045* and *PeNAC036* under salt treatment. The expression level of each gene at the non-treatment control is set to 1. Drought treatment, the plants were subjected to soil water deficiency at four RSMC levels (Group A control: 70%–75% RSMC; Group B moderate drought: 50%–55% RSMC; Group C moderate drought: 35%–40% RSMC and Group D severe drought: 15%–20% RSMC) for 2 months. Salt treatment, the plants were watered using 1 L of 200 m*M* NaCl solution. The $2^{-\Delta\Delta Ct}$ method was used to calculated the relative expression levels of the target genes. Poplar *18S* rRNA was used as an endogenous control.

Data are means ± SE of three independent experiments. *Asterisks* indicate a significant difference compared with the non-treatment controls (Groups A and Salt 0 h, respectively). Student's *t*-test (**P* < 0.05, ***P* < 0.01).

Fig. 5. Phenotype of transgenic *Arabidopsis* plants overexpressing *PeNAC034* and *PeNAC036*. (A) Molecular analysis of *PeNAC034* transgenic plants via PCR and RT-PCR using the specific primers (35S-F and RT-PCR-PeNAC034-R for PCR; RT-PCR-PeNAC034-F and RT-PCR-PeNAC034-R for RT-PCR). *Arabidopsis Actin8* was amplified as an internal control. (B) The primary root length of *Col*-0, *OEPeNAC034*, *ataf1* and *ataf1/PeNAC034* seedlings were measured at 7 day after transfer under normal conditions. Plants grown on 1/2 MS plates for 7 days and then transferred to vertically 1/2 MS square plates for additional 7 days. (C) The growth phenotypes of 40-day-old *Col*-0, *OEPeNAC034*, *ataf1* and *ataf1/PeNAC034* plants under well-watered conditions. Ten-day-old seedlings were transferred from 1/2 MS agar plates to pots for additional 30 days under well-watered conditions. (D) Molecular analysis of *PeNAC036* transgenic plants via PCR and RT-PCR

using the specific primers (35S-F and RT-PCR-PeNAC036-R for PCR; RT-PCR-PeNAC036-F and RT-PCR-PeNAC036-R for RT-PCR). *Arabidopsis Actin8* was amplified as an internal control. (E) The primary root length of *Col*-0, *OEPeNAC036*, *anac072* and *anac072/PeNAC034*6 seedlings were measured at 7 days after transfer under normal conditions. Plants grown on 1/2 MS plates for 7 days were transferred vertically to 1/2 MS square plates for additional 7 days. (F) The growth phenotypes of 40-day-old *Col*-0, *OEPeNAC036*, *anac072* and *anaco72/PeNAC036* plants under well-watered conditions. Ten-day-old seedlings were transferred from 1/2 MS agar plates to pots for additional 30 days under well-watered conditions.

Error bars indicate SE, and *asterisks* indicated significant difference between *Col*-0 and overexpression transgenic plants, between mutant and complementation transgenic plants, between *Col*-0 and mutant plants, respectively. Student's *t*-test (**P* < 0.05, ***P* < 0.01).

Fig. 6. The primary root length assays of *Arabidopsis Col*-0, mutants and transgenic plants under osmotic and salt stresses. (A) Morphological differences and the primary root length of *Col*-0, *OEPeNAC034*, *ataf1* and *ataf1/PeNAC034* seedlings on 1/2 MS agar plate containing indicated concentration of Mannitol and NaCl. (B) Morphological differences and the primary root length of *Col*-0, *OEPeNAC036*, *anac072* and *anac072/PeNAC036* seedlings on 1/2 MS agar plate containing indicated concentration of Mannitol and NaCl. Plants grown on 1/2 MS plates for 7 days were transferred vertically to 1/2 MS square plates supplemented with salt (100 and 150 m*M*) and mannitol (200 and 300 m*M*). The primary root length was measured 7 days after transfer.

Error bars indicate SE, and *asterisks* indicated significant difference between *Col*-0 and overexpression transgenic plants, between mutant and complementation transgenic plants, between *Col*-0 and mutant plants in response to osmotic and salt stress. Student's *t*-test (**P* < 0.05, ***P* < 0.01).

Fig. 7. Phenotype of *Arabidopsis Col*-0, mutants and transgenic plants under salt and drought stresses. (A) Phenotype and the survival rate of transgenic *OEPeNAC034* and *ataf1/PeNAC034*, mutant *ataf1*, control *Col*-0 plants under salt and drought stresses. Plants grown on1/2 MS plates for 10 days were transferred to pots and grown for additional 10 days before exposure to salt and drought stresses. (B) Phenotype of transgenic *OEPeNAC036* and *anac072/PeNAC036*, mutant *anac072*, control *Col*-0 plants under salt and drought stresses. Plants grown on 1/2 MS plates for 10 days were transferred to pots, and grown for additional 15 days before exposure to salt and drought stresses. Salt stress was induced by watering the plants with 200 m*M* NaCl solution every 7 days until the plants showed evident salt-stressed phenotypes. Drought stress was induced by withholding water until the lethal effect of dehydration was observed.

To quantify the survival rate, 12 plants of each plant type were used in each experiment, and four independent experiments were performed. *Error bars* indicate SE, and *asterisks* indicated significant difference between the *Col*-0 and overexpression transgenic plants, between the mutant and complementation transgenic plants, between the *Col*-0 and mutant plants, respectively. Student's *t*-test (**P* < 0.05, ***P* < 0.01).

Fig. 8. Expression profiles of the abiotic stress-responsive genes in *Col*-0, mutants and transgenic plants in response to drought and salt stresses. (A) Expression profiles of the abiotic stress-responsive genes in *Col*-0, *OEPeNAC034*#1, *ataf1*, and *ataf1/PeNAC034*#1 plants under drought and salt stresses. (B) Expression profiles of the abiotic stress-responsive genes in *Col*-0, *OEPeNAC036*#1, *anac072*, and *anac072*/*PeNAC036*#1 under drought and salt stresses. Ten-day-old seedlings were transferred from 1/2 MS agar plates to pots for additional 11 days before exposure to salt and drought treatments.

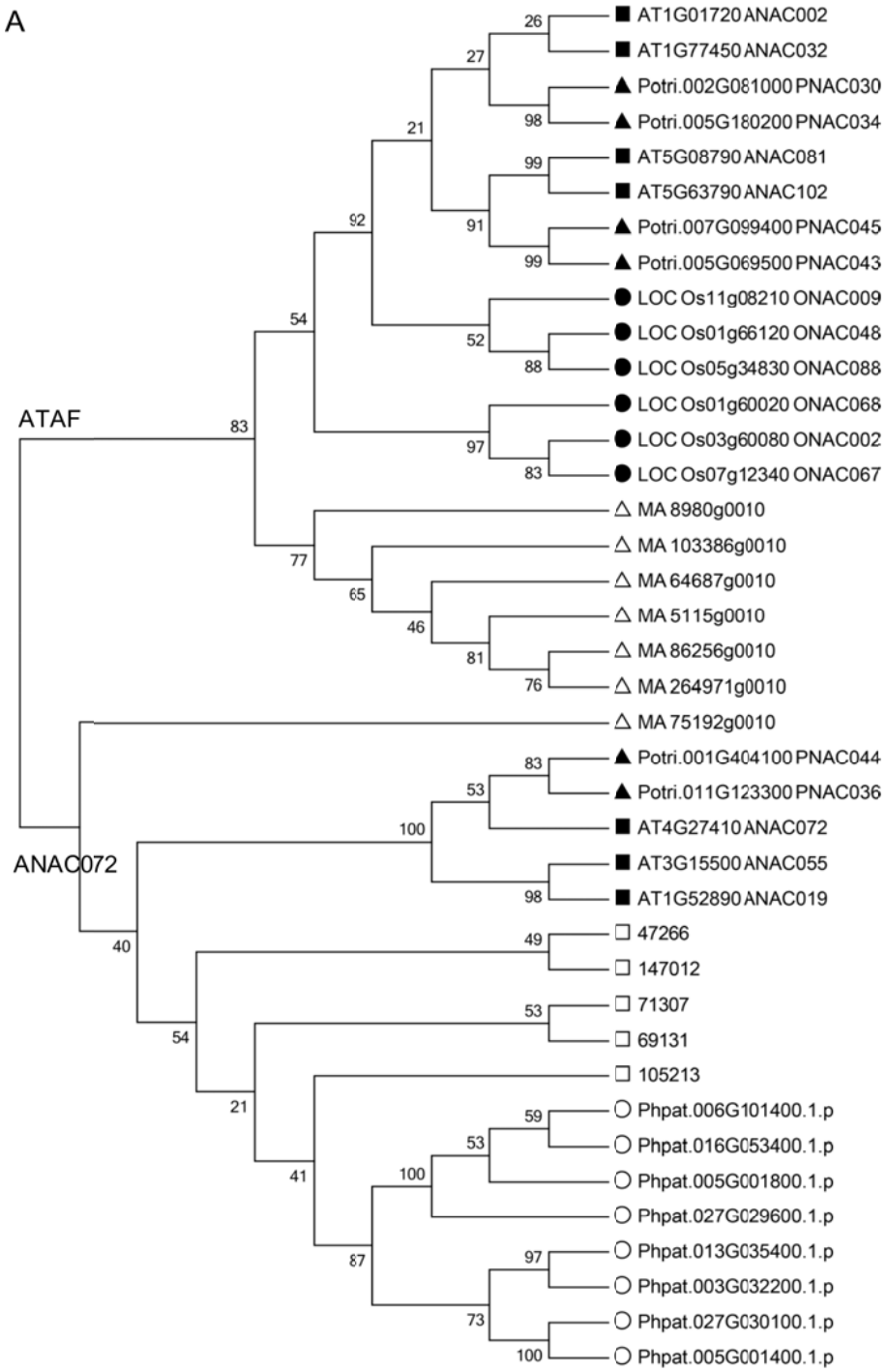
Drought treatment was induced by withholding water for 5 day. Salt treatment was induced by watering with 200 m*M* NaCl solution for 1 day. To quantify the abiotic stress-responsive genes transcript levels, 9 plants of each line were used for qRT-PCR analysis. The $2^{-\Delta\Delta Ct}$ method was used to measure the relative expression levels of the abiotic stress-responsive genes. *Arabidopsis Actin8* was amplified as a normalization control. Data are mean ± SE (n = 3 experiments), and *asterisks* indicated significant difference between *Col*-0 and overexpression transgenic plants, between mutant and complementation transgenic plants, between *Col*-0 and mutant plants in response to drought and salt stress. Student's *t*-test (**P* < 0.05, ***P* < 0.01).

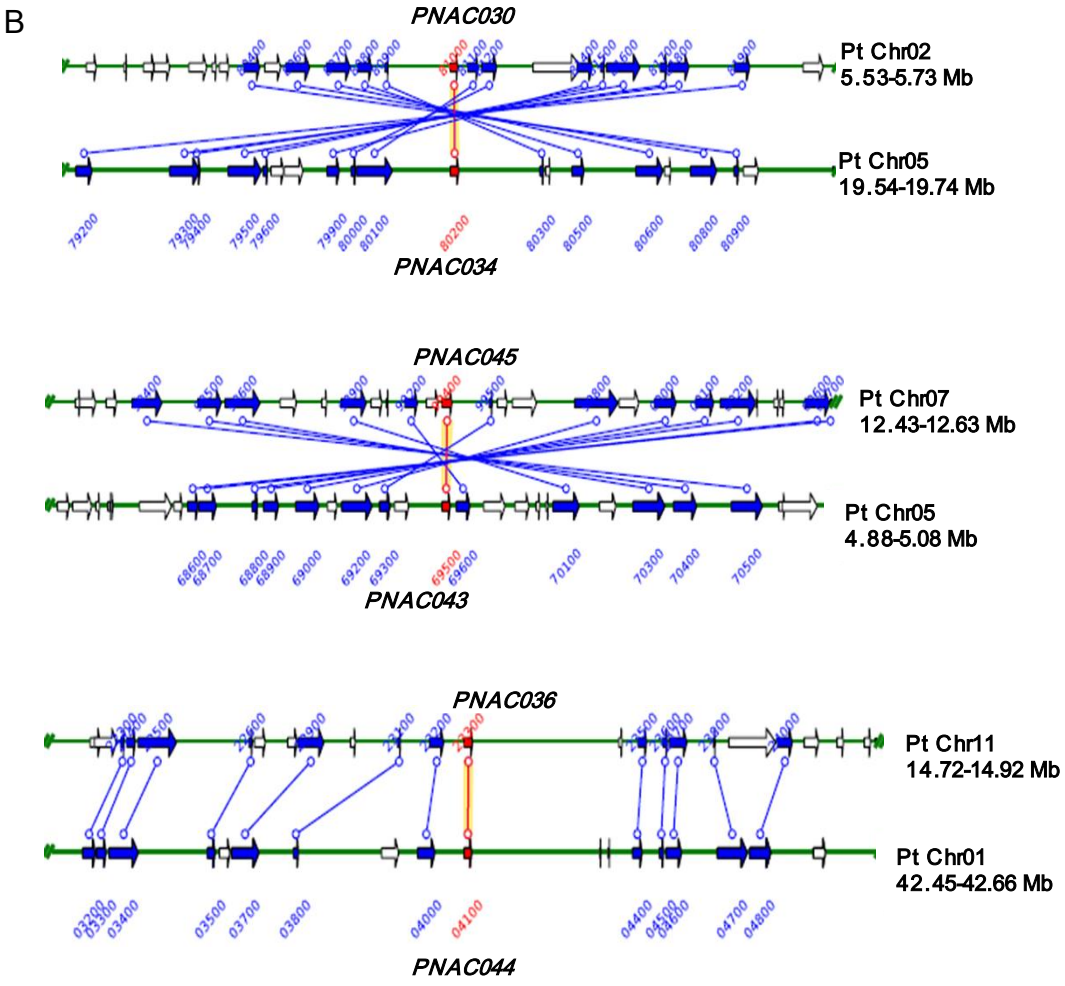
Fig. 9. Phenotype of transgenic poplar plants. (A) Molecular analysis of *PeNAC045* transgenic plants via PCR using the specific primers (35S-F and RT-PCR-PeNAC045-R). (B) Molecular analysis of Vector plants via PCR using the specific primers (35S-F and GUS-R). (C) Analysis of *PeNAC045* transcript level in WT, Vector and two transgenic lines via qRT-PCR. The $2^{-\Delta\Delta Ct}$ method was used to measure the relative expression levels of *PeNAC045*. Poplar *18S* rRNA was used as an endogenous control. Data are means ± SE of three independent experiments. *Asterisks* indicate a significant difference compared to the non-treatment controls. Student's *t*-test (**P* < 0.05, ***P* < 0.01). (D) Phenotype of WT, vector and *OEPeNAC045* plants under well-watered conditions. Regenerated plantlets were transferred from 1/2 MS agar medium to pots for one month.

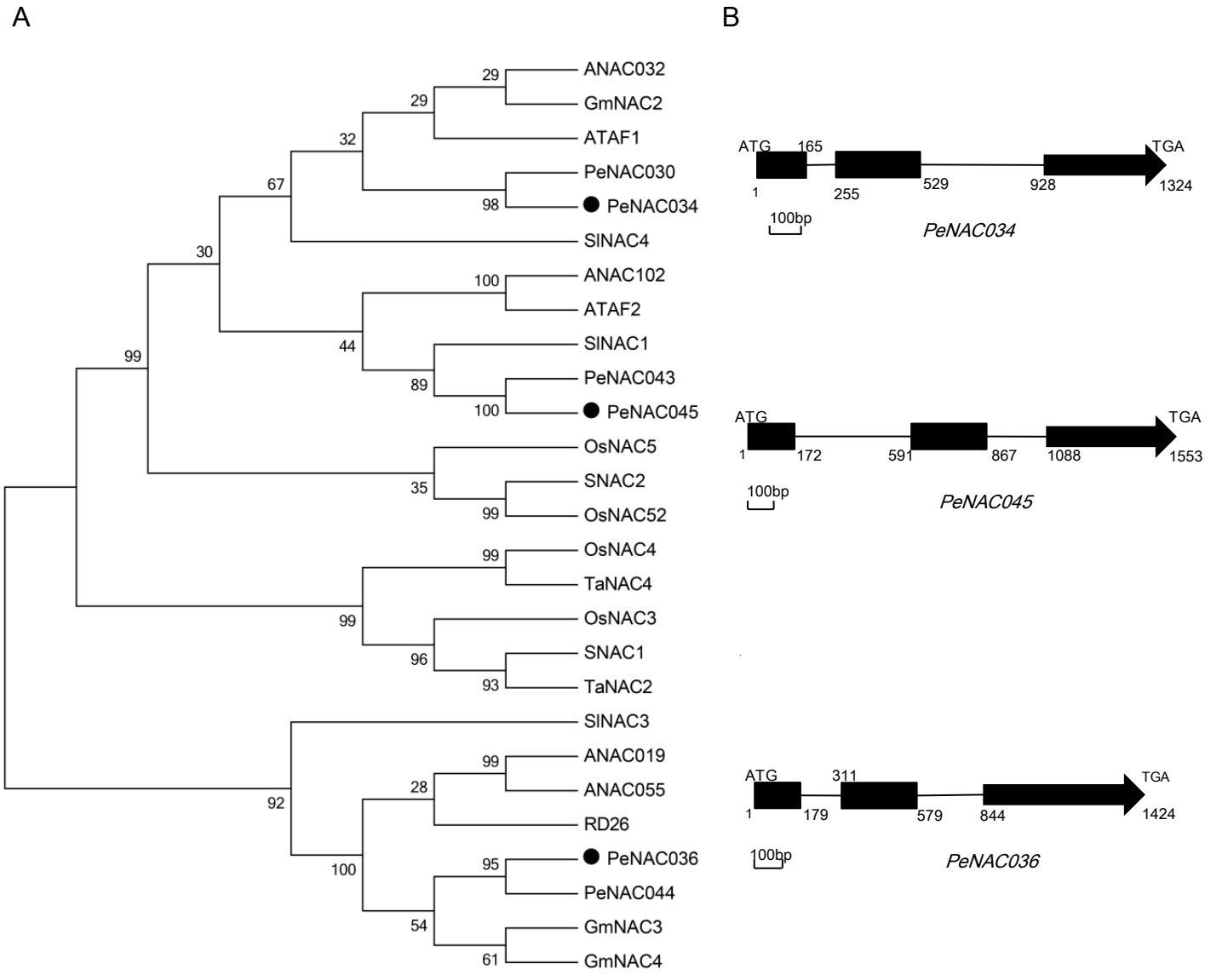
Fig. 10. Phenotype of poplar WT, Vector and *OEPeNAC045* plants under drought and salt stresses. (A) Performance of poplar WT, Vector and *OEPeNAC045* plants following drought stress. Drought stress was induced by withholding water. (B) Performance of poplar WT, Vector and *OEPeNAC045* plants following salt stress. Salt stress was induced by watering with 200 m*M* NaCl solution every 7 days. The net photosynthetic (P_N) rate, stomatal

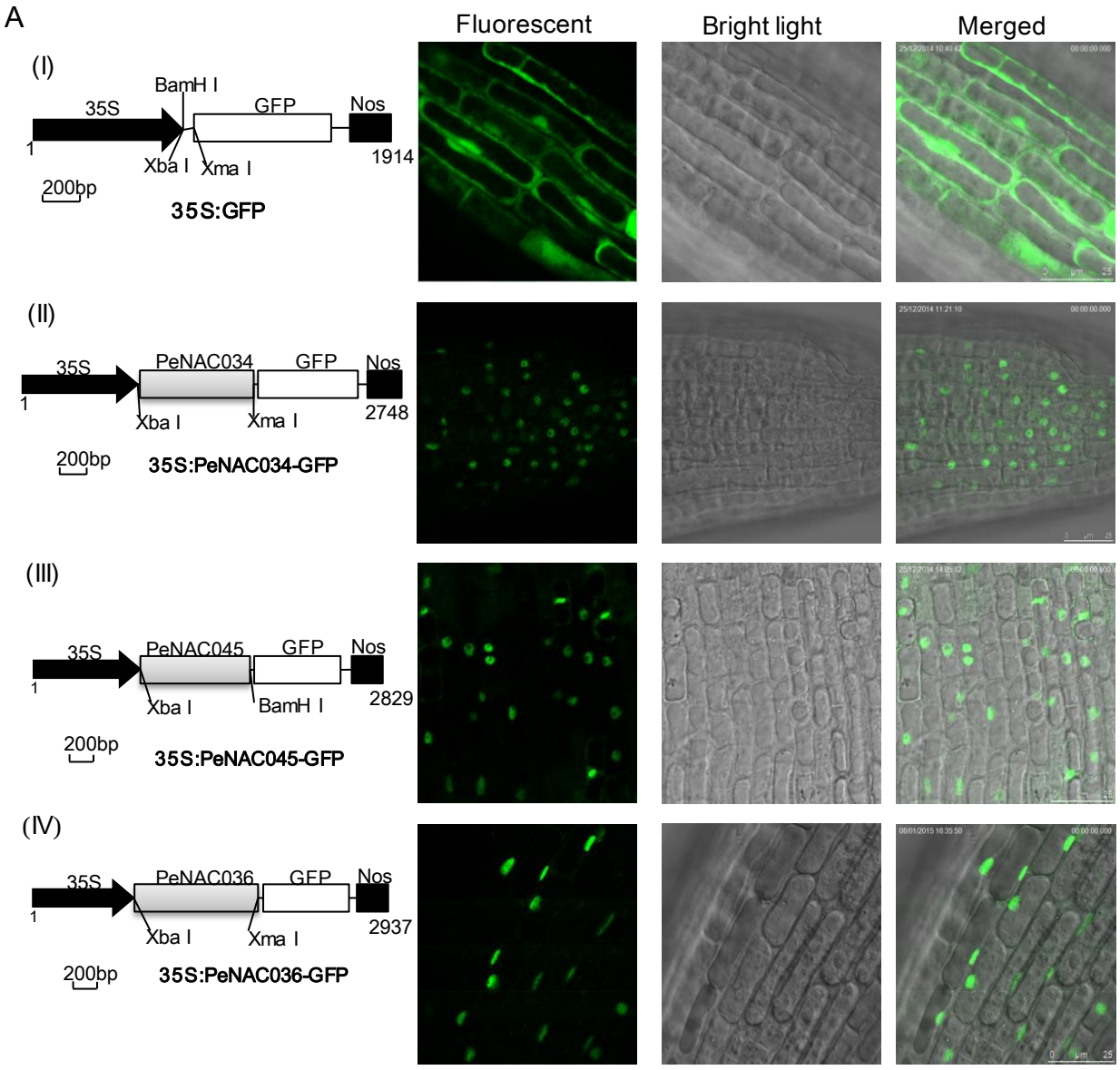
conductance (g_s) and transpiration rate (E) cures of poplar leaves following drought and salt stresses were measured using the LI-6400 Photosynthesis System.

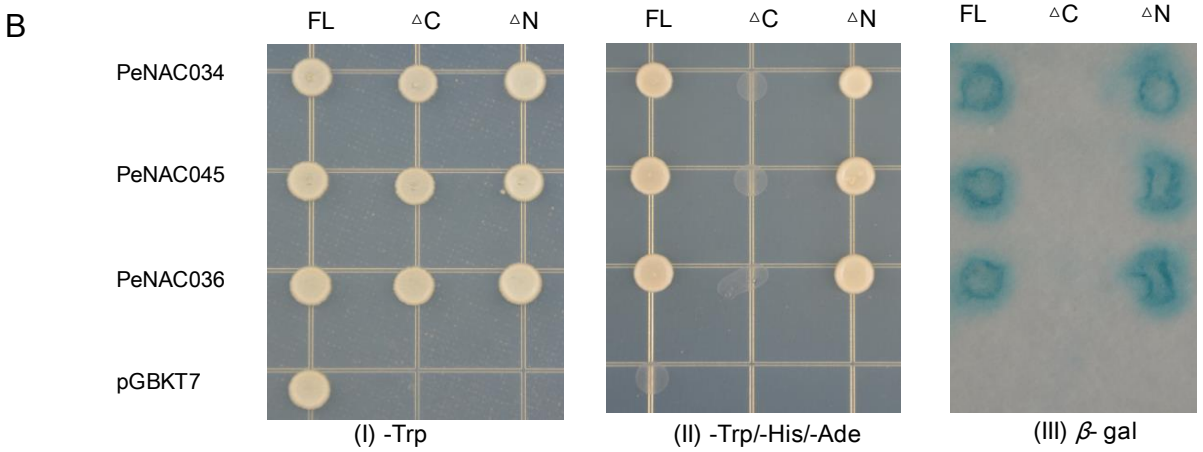
Data are mean ± SE (n=6). *Asterisks* indicated significant difference between WT and overexpression transgenic plants, between WT and Vector plants, respectively. Student's *t*-test (**P* < 0.05, ***P* < 0.01).

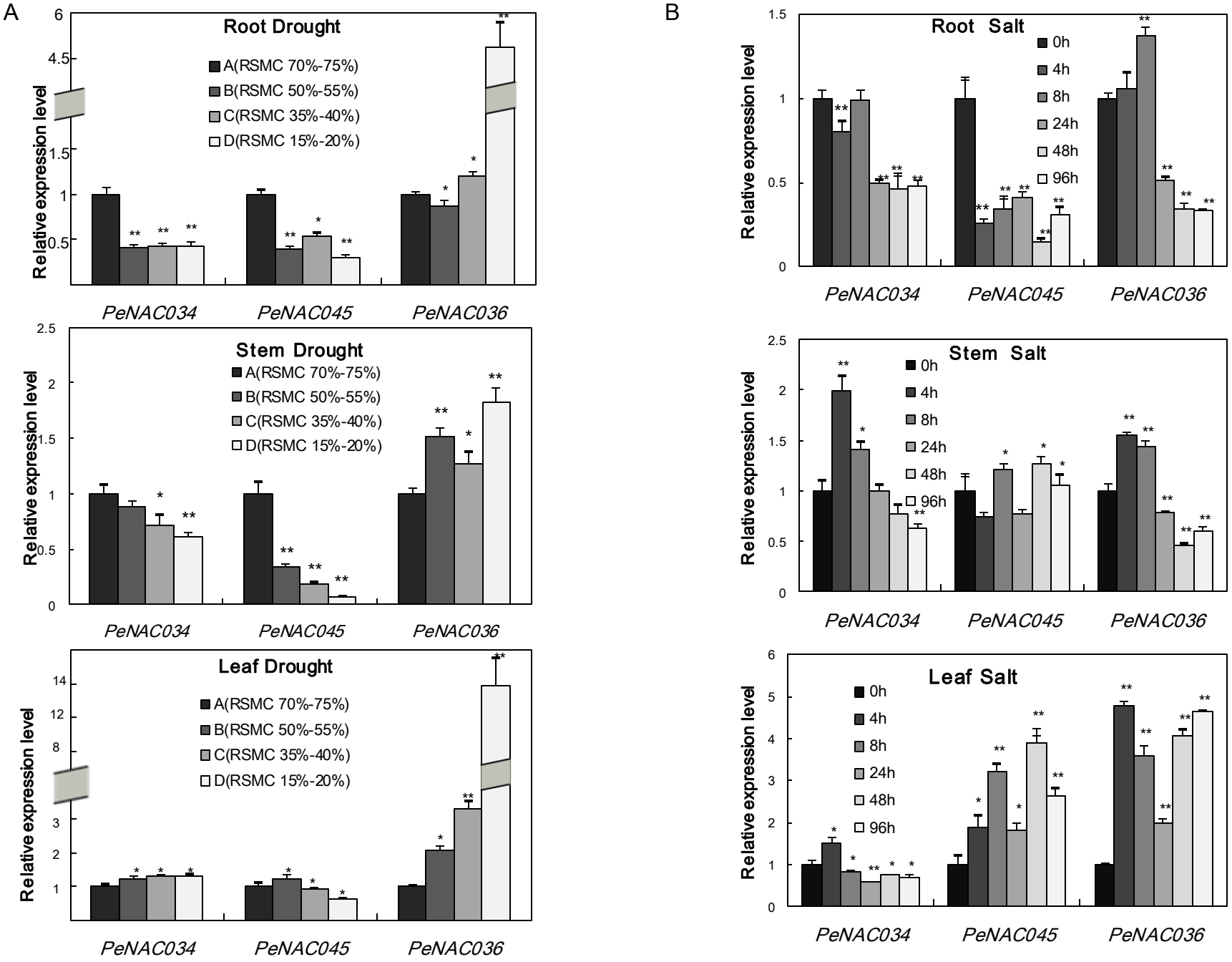


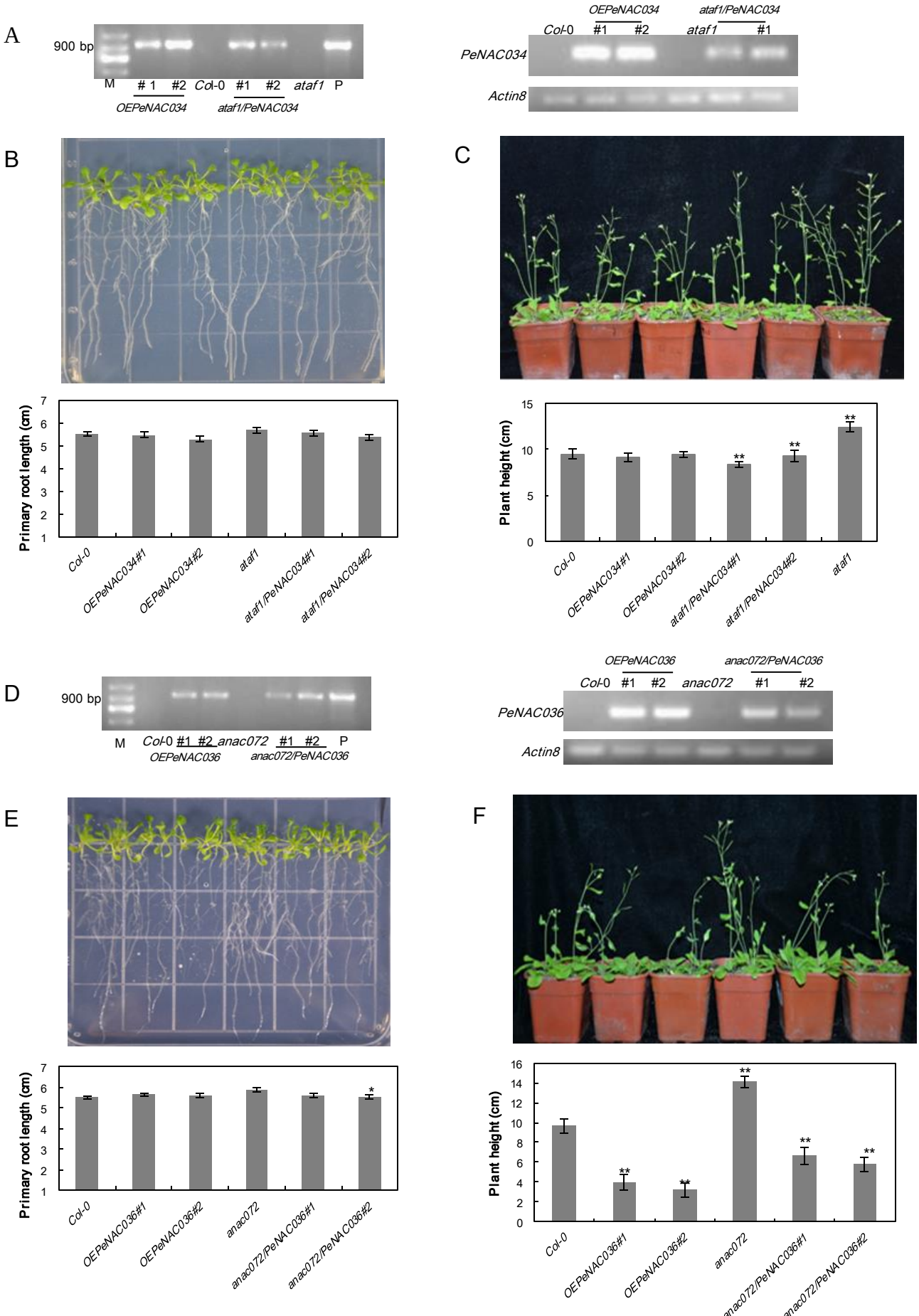


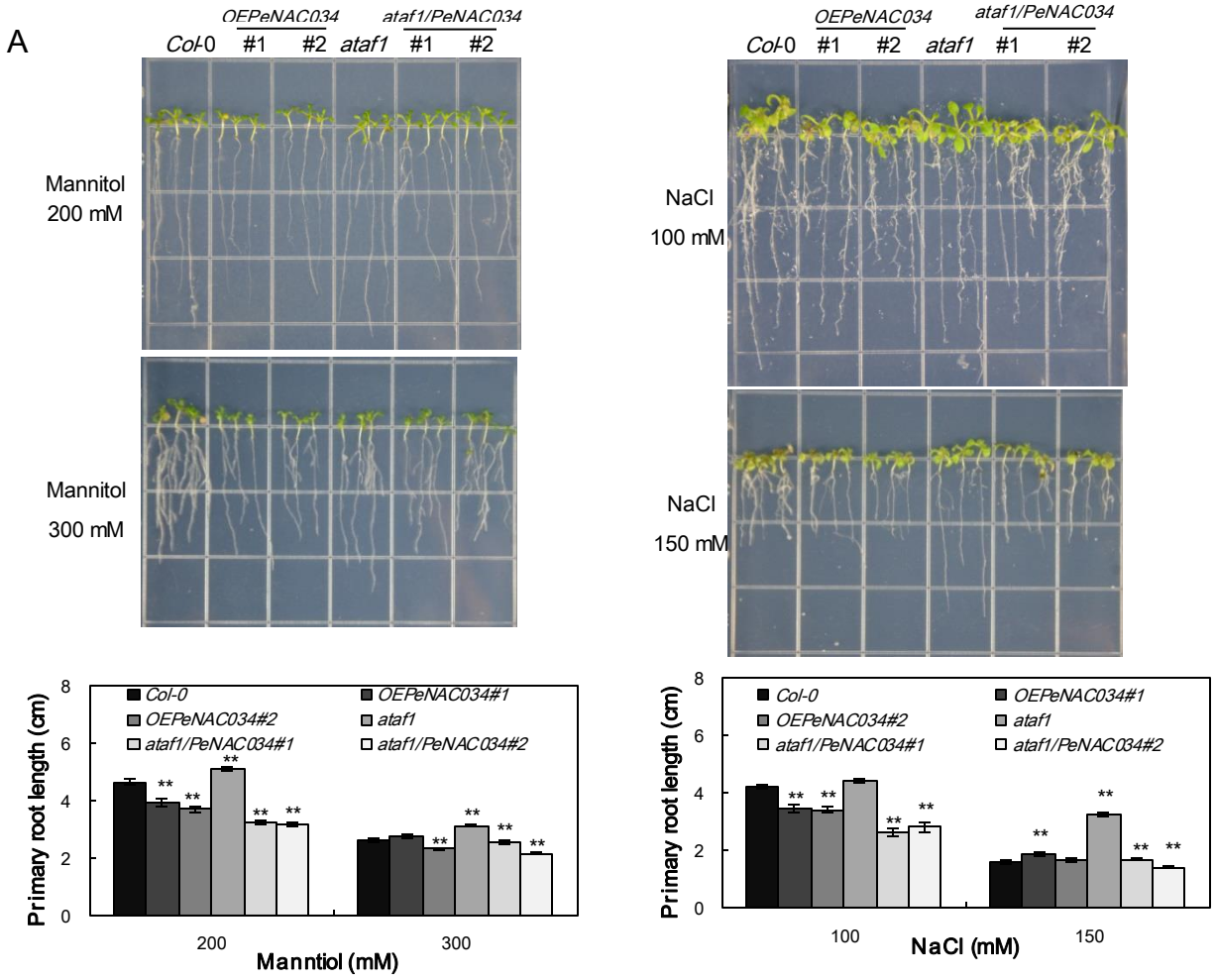












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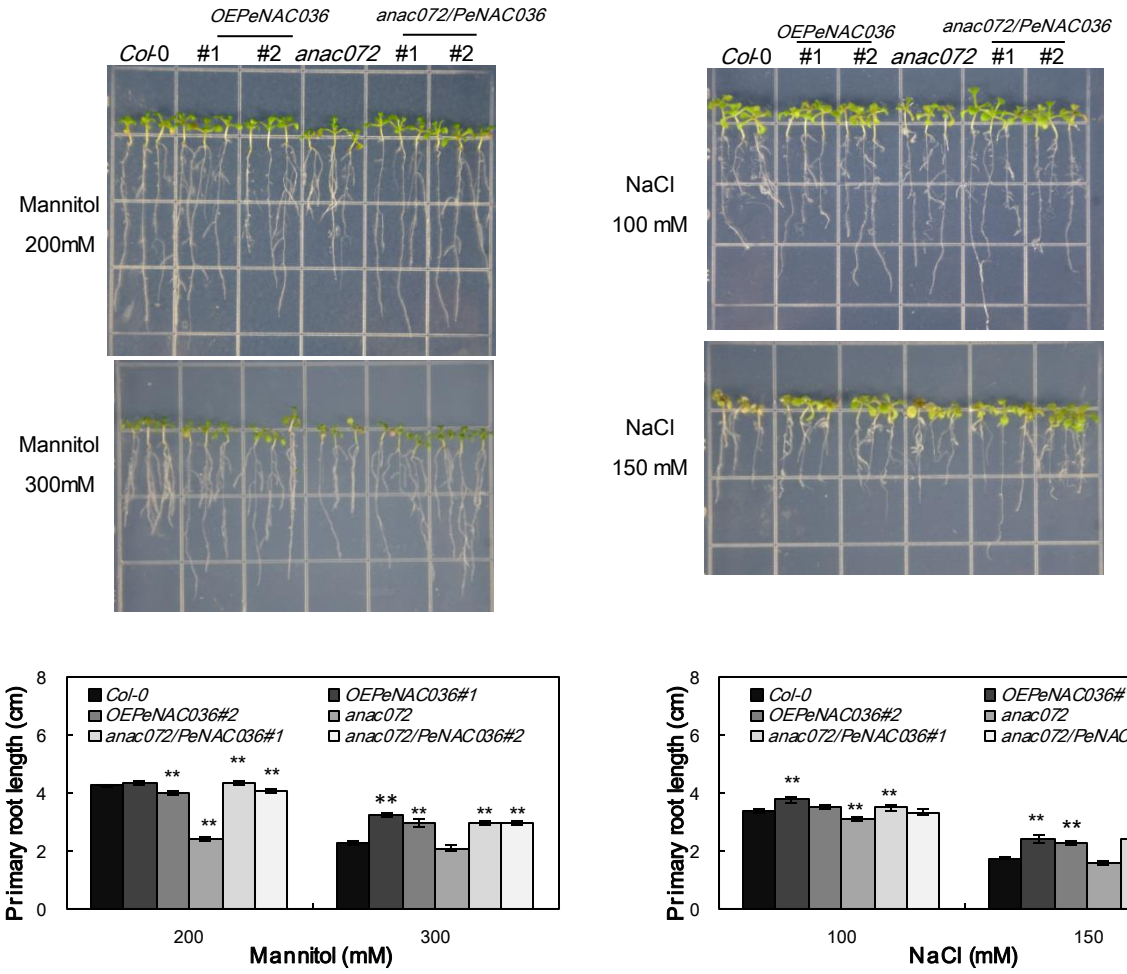

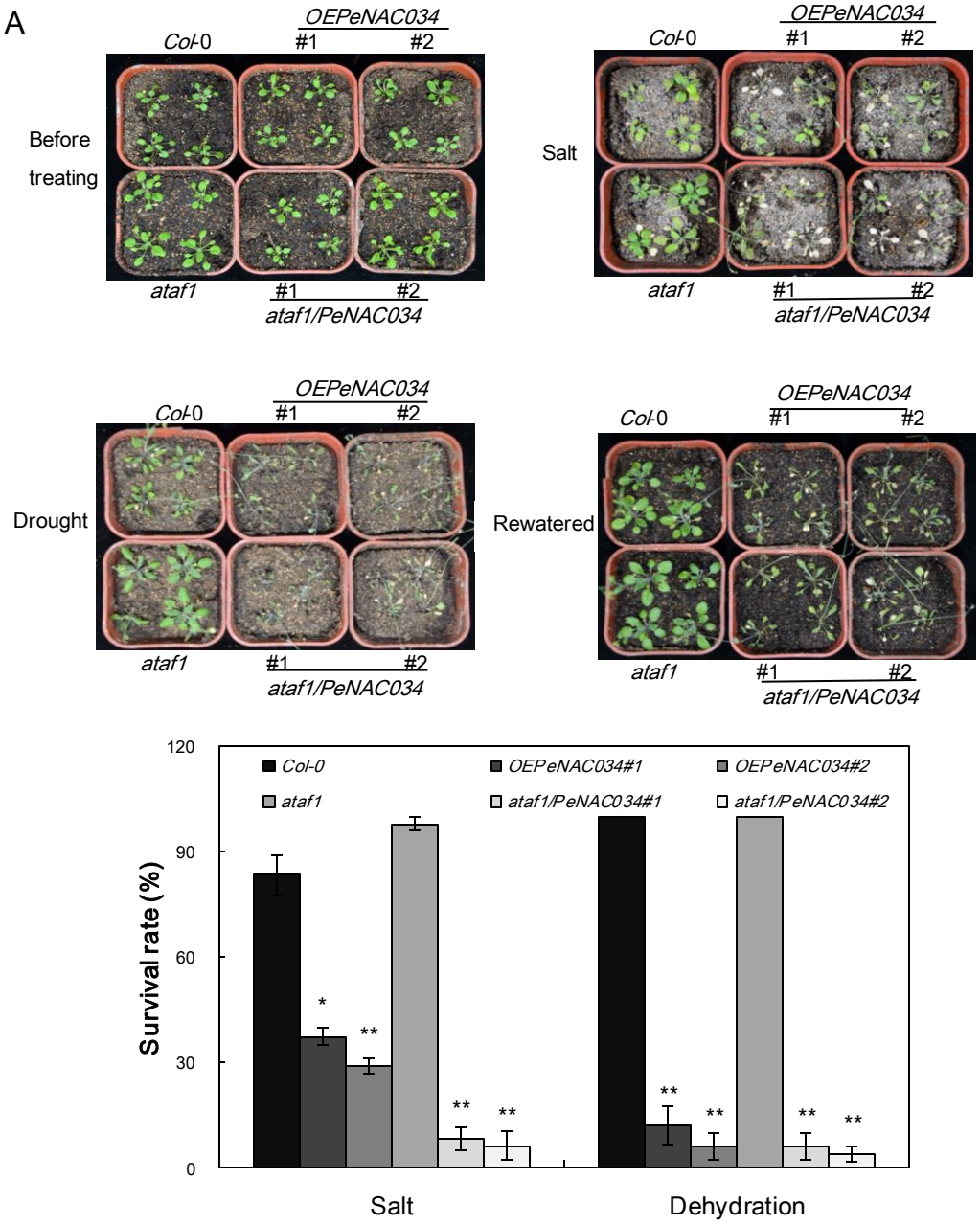

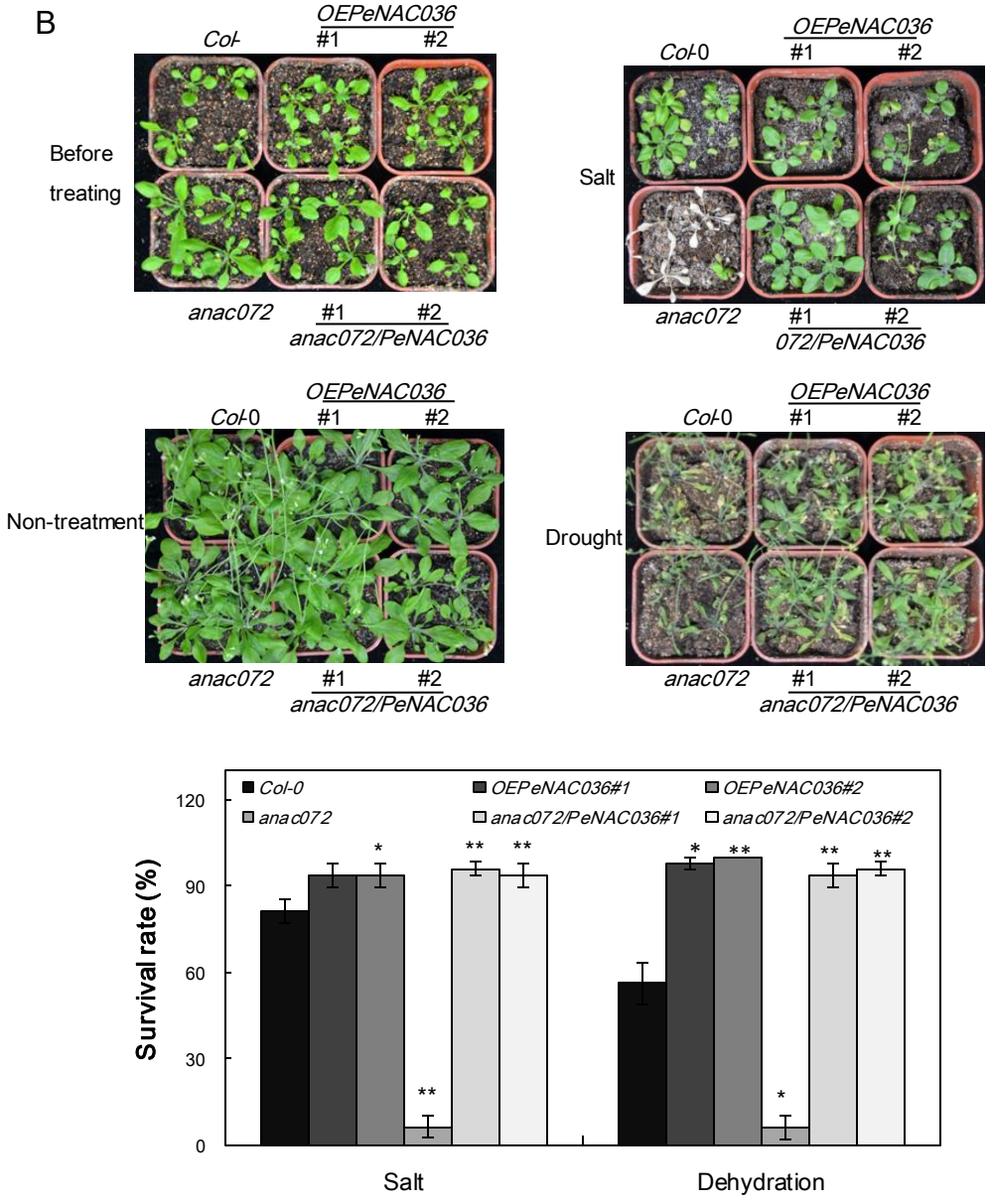

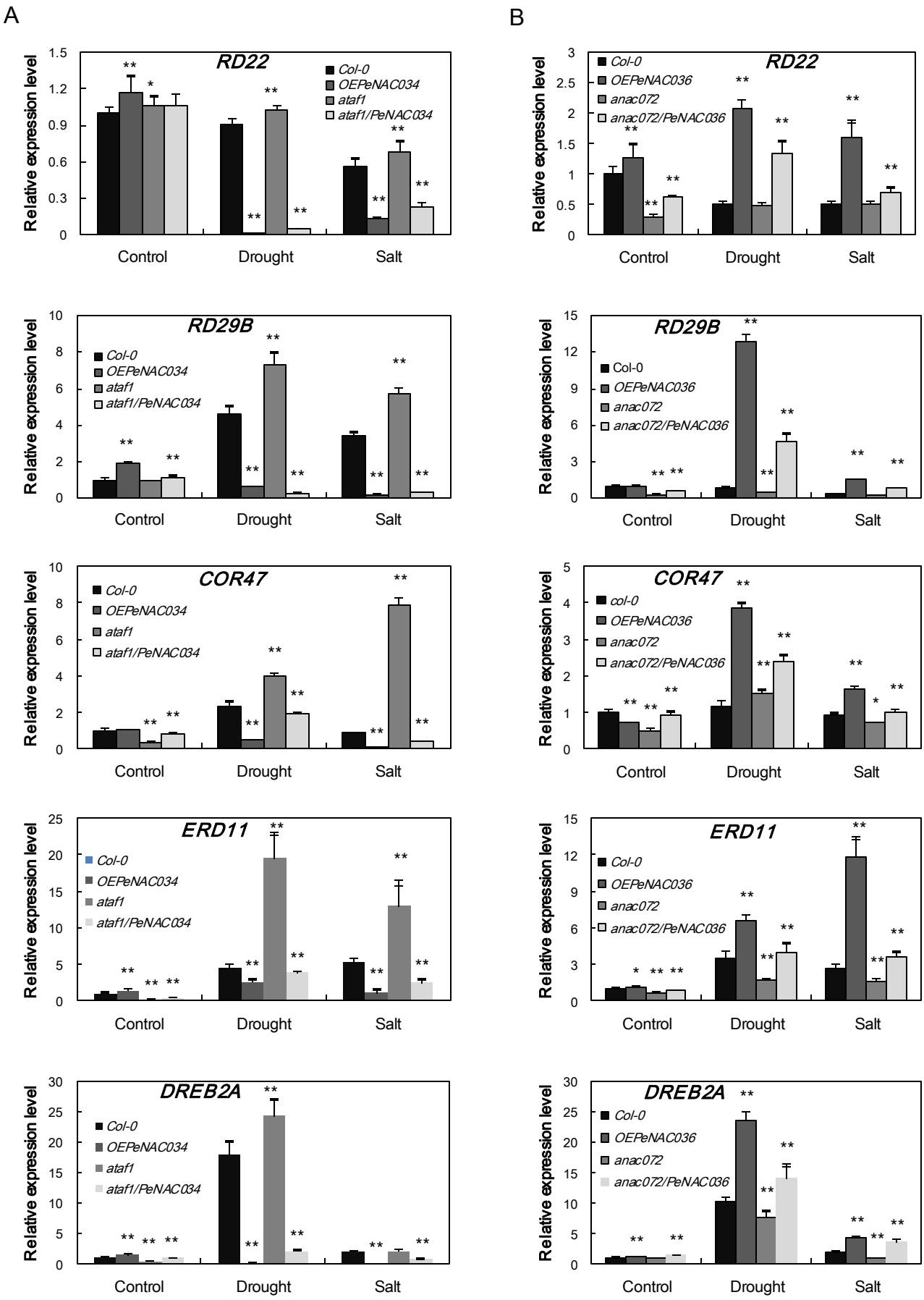

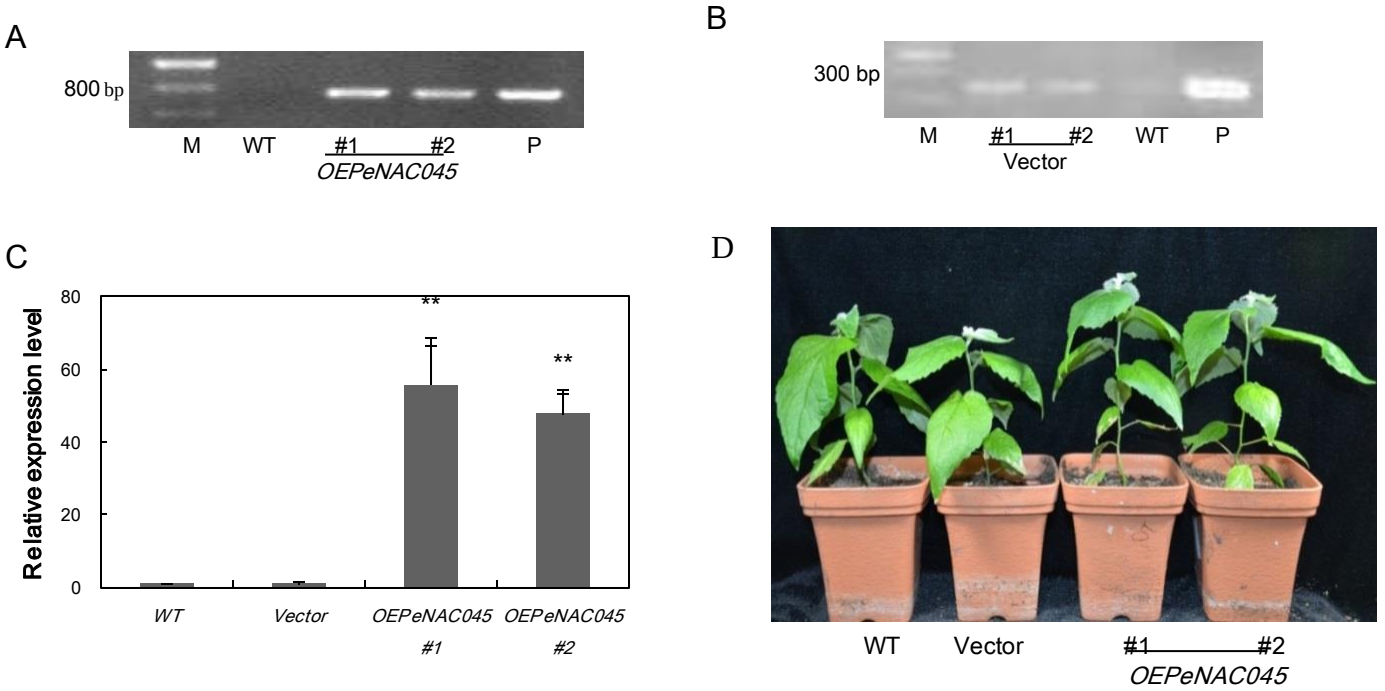

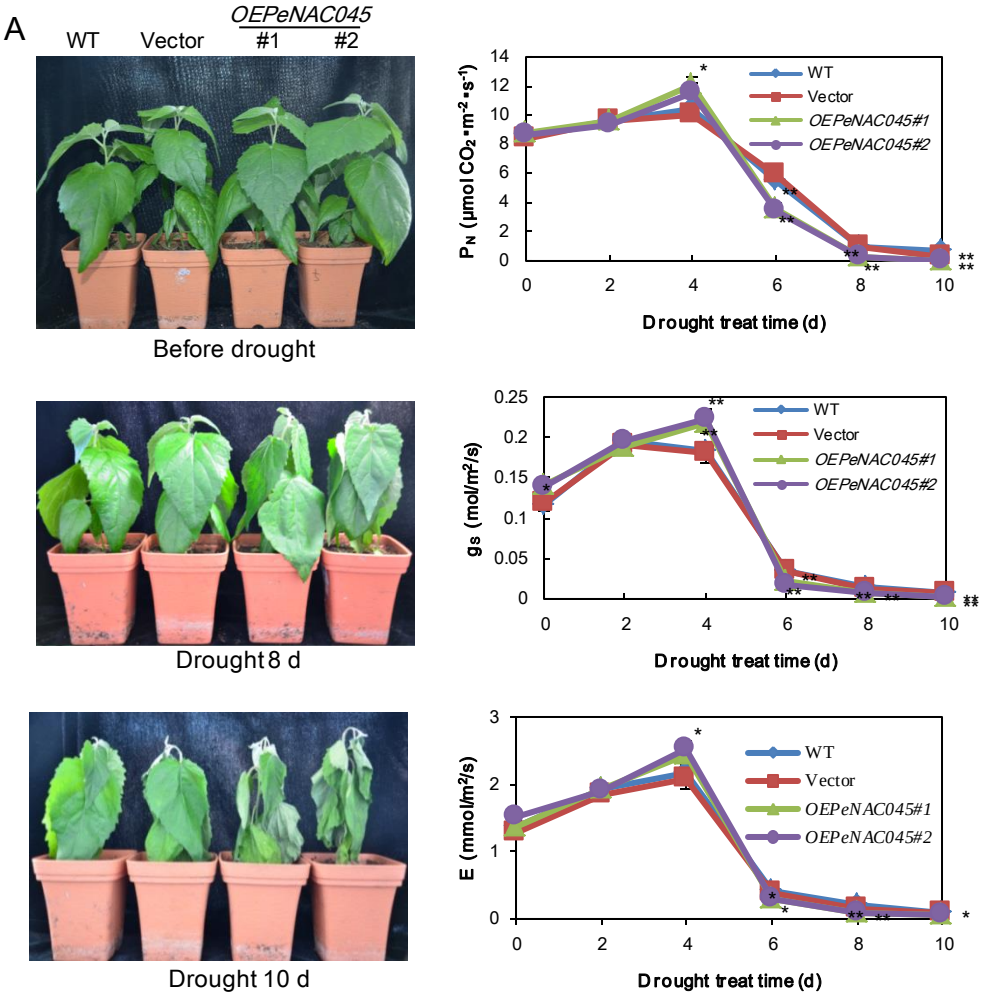

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