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REGULAR PAPER – PHYSIOLOGY/BIOCHEMISTRY/MOLECULAR AND CELLULAR BIOLOGY

Ectopic expression of a poplar gene *NAC13* confers enhanced tolerance to salinity stress in transgenic *Nicotiana tabacum*

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Abstract
NACs are one of the major transcription factor families in plants which play an important role in plant growth and development, as well as in adverse stress responses. In this study, we cloned a salt-inducible NAC transcription factor gene (*NAC13*) from a poplar variety 84K, followed by transforming it into both *Nicotiana tabacum* and *Arabidopsis thaliana*. Stable expression analysis of 35S::NAC13-GFP fusion protein in Arabidopsis indicated that NAC13 protein was localized to the nucleus. We also obtained five transgenic tobacco lines. Evidence from morphological and physiological characterization and salt treatment analyses indicated that in the transgenic tobacco the salt tolerance was enhanced, suggesting that *NAC13* gene may function as a positive regulator in tobacco responses to salt stress.

Keywords Ectopic expression · *NAC13* gene · Salt tolerance · Transcription factor · Transgenic tobacco

Introduction

High salinity is a major abiotic stress that affects plant growth and development, resulting in reduced survival, photosynthetic rate, mineral element uptake rate and productivity (Nakashima et al. 2012). Therefore, molecular breeding has become a major means to develop stress-tolerant new plant varieties.

NACs are one of the important transcription factor (TF) gene families in plants. Members of this family were first found in *Petunia hybrid* (Li et al. 2018), and then successfully cloned in Arabidopsis (Shahnejat-Bushehri et al. 2017), rice (Nuruzzaman et al. 2010), and soybeans (Mochida et al. 2009). Currently, 170 NAC TFs are identified in *Populus trichocarpa* and 145 NAC TFs are found in *Populus euphratica,* according to the PlantTFDB (https://planttfdb.cbi.pku.edu.cn/family.php?sp=Ptr&fam=NAC). NACs contain a highly conservative deoxyribonucleic acid (DNA) binding domain which includes approximately 160 amino acid residues at the N-terminal of protein (Hu et al. 2010), a nuclear localization signal site, and a variable C-terminal domain (Hu et al. 2010; Jensen et al. 2010). The NAC TF family can be divided into three subfamilies, including no apical meristem (NAM), Arabidopsis transcription activation factor (ATAF), and Cup-shaped cotyledon (CUC) (Zhang et al. 2018).

The NACs play a vital role in transcription regulation in a series of biological processes, including branch growth (Mao et al. 2007), floral morphogenesis (Hendelman et al. 2013), leaf senescence (Kim et al. 2016), lateral root formation (Li et al. 2018), embryonic development (Larsson et al. 2011), cell division (Kim et al. 2006), and cell wall development (Chai et al. 2015; Hussey et al. 2011). Studies have indicated that transgenic Arabidopsis overexpressing *ANAC046* exhibits premature senescence and significantly reduces chlorophyll content (Oda-Yamamizo et al. 2016). Over-expression of *PaNACo3* in *Picea abies* showed reduced flavonol biosynthesis and aberrant embryo development (Dalman et al. 2017). In poplar, the expression of *PtNAC068* and *PtNAC154* is associated with secondary

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growth and vascular tissue development (Han et al. 2012). In addition, NAC transcription factors are also involved in plant responses to biotic and abiotic stress processes, including high salt (Movahedi et al. 2015), drought (Nguyen et al. 2018), freezing (Yu-Jun et al. 2011), and viral infection (Wang et al. 2009). For example, over-expression of the chrysanthemum *DgNAC1* gene in tobacco can increase salt tolerance (Liu et al. 2011). Furthermore, (Huang et al. 2015) indicated that transgenic plants over-expressing wheat *TaNAC29* gene showed improved tolerance to high salinity and dehydration; The transgenic plants accumulated less malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) under salt or dehydration stresses, but activities of superoxide dismutase (SOD) and catalase (CAT) were significantly improved. Transgenic poplar plants (*Populus deltoides* × *P. euramericana 'Nanlin895'*) over-expressing *CarNAC3* displayed enhanced drought and salt tolerance, with increased proline and photosynthetic pigment levels (Movahedi et al. 2015).

Plant cells are always hypersensitive to abiotic stresses and then affected by induced reactive oxygen species (ROS) production (Helene and Andrea 2014), including H_2O_2, O_2^-, OH^-, and OH_2 (Movahedi et al. 2015). SOD functions a main antioxidant enzyme and the key ROS scavenger to catalyze H_2O_2 and O_2^- in plants (Azarabadi et al. 2017). The activity of SOD can increase in plant cells under stress conditions such as drought, high light and salinity, to ensure the growth of plants (Leonowicz et al. 2018). For example, transgenic Arabidopsis plants over-expressing *ThNAC13* gene from *Tamarix hispida* had markedly elevated SOD activity, and the transcription level of SOD gene was significantly increased (Wang et al. 2017a). Peroxidase (POD) is mainly present in cell walls, vacuoles and chloroplasts (Rácz et al. 2018). Studies indicated that transgenic plants with *OsNAC45*-over-expression can more efficiently scavenge superoxide than wild type, suggesting a possible relationship between the gene and the elevated level of POD activity (Yu et al. 2018). MDA content is an important parameter for detecting lipid peroxidation in plant cell membranes (Hu et al. 2018; Shao et al. 2018; Wang et al. 2017b); that is, the lower level of MDA, the less lipid peroxidation and the better cell membrane integrity (Wang et al. 2017b). Recent research demonstrates that transgenic Arabidopsis with over-expression of *OoNAC72* from *Oxytropis ochrocephala* had much lower MDA content than WT in the treatment of high salinity and drought (Guan et al. 2019). *SNAC3* TF from rice was induced by drought, salinity and high temperature, *SNAC3*-OE transgenic plants showed significant lower MDA content which was involved in modulation of ROS scavenging pathways (Yujie et al. 2015). RWC is usually used to measure the water status of plants (Tanentzap et al. 2015), and often used as an important index to assess the stress tolerance or adaptation in plants (Arndt et al. 2015).

In our previous studies, we found that poplar *NAC13* gene had a high expression under salt stress (Yao et al. 2016b), so we speculated that *NAC13* gene was involved in plant response to abiotic stress, next we isolated 1032 bp gene fragment of *NAC13* gene from the 84K poplar (*Populus alba* × *P. glandulosa*), followed by constructing a vector pBI121-NAC13 that over-expresses *NAC13* gene. We validated that the NAC13 protein functions as a transcriptional activator. Furthermore, the transgenic tobacco lines displayed enhanced salt tolerance, based on morphological and physiological analyses. In this study, physiological and biochemical methods were used to determine the function of *NAC13*, which could provide a potential contribution to improve the salt tolerance of plants.

Materials and methods

Plant materials

The wild type tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR-1) and Arabidopsis (*Arabidopis thaliana* (L.) Heynh., Columbia-1) seeds were sterilized with 20% bleach for 15–20 min, and then washed 3–5 times with sterile water. The seeds were evenly spread on MS medium plates which contain 30 g L^{-1} sucrose. The plates were transferred to greenhouse at an average temperature of 25 °C and 16/8-h light/dark cycles. Two-week-old seedlings were used for the stress treatment.

SOD reaction solution (prepared first): Methionine 0.387964 g, nitrogen blue tetrazole (NBT) 0.0103 g, 0.5 M ethylene diamine tetraacetic acid (EDTA) 0.04 mL, riboflavin mother liquor 0.02 mL, mixed up with 0.05 M phosphate buffer saline (PBS) (200 mL), Stored at 4 °C.

Cloning and characterization of NAC13 gene

Fresh leaves from the 84K poplar seedling were collected and frozen in liquid nitrogen for ribonucleic acid (RNA) isolation. Total RNA was extracted from the leaves by RNA Extraction Kit (Takara, China), and complementary deoxyribonucleic acid (cDNA) synthesis was performed according to the instruction of Prime Script RT reagent kit (Takara, China).

NAC13 gene was cloned from the 84K poplar by Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-PCR) with a pair of primers NAC13F1 and NAC13R1 (Table S1). According to the cDNA sequence, the gene structure and conserved domain were analyzed by the online software of Gene Structure Display Server (https://gsds.cbi.pku.edu.cn/) and NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). In addition, using the NCBI database, we also blasted other protein conserved domain sequences

homologous to the NAC13 protein. The sequences were then used to construct a phylogenetic tree, by use of MEGA5.0 with Neighbor-Joining method after multi-sequence alignment with Bioedit (Liu et al. 2012).

Subcellular localization of NAC13 protein

The cDNA sequence of *NAC13* encoding region without stop codon was amplified by RT-PCR with primers NAC13F2 and NAC13R2 (Table S1) which contains the restriction site *SpeI*. It was then fused into the pBI121-GFP vector with the CaMV35S promoter and green fluorescent protein (GFP). The recombinant construct 35S::NAC13-GFP and the control vector 35S::GFP were respectively transferred into *Agrobacterium tumefaciens* GV3101 for stable transformation of Arabidopsis, by the floral dip method (Xiuren et al. 2006). The root tips of T3 transgenic Arabidopsis seedlings were used for detecting the GFP fluorescence signals with a confocal laser scanning microscope (LSM 700, Zeiss, Germany).

Transcriptional Activation assay of NAC13 protein

First, the cDNA fragment encoding the full length of *NAC13* was amplified with primers NAC13F3 and NAC13R3 (Table S1), containing the *EcoRI* and *SalI* restriction sites. Then, it was cloned into the pGBKT7 vector with GAL4 DNA binding domain and T7 promoter, to generate bait vector pGBKT7-NAC13. Furthermore, to explore the activation region, we also cloned two different segments of the NAC13 cDNA, followed by inserting them respectively into the pGBKT7, with two pairs of primers NAC13aF and NAC13aR, NAC13bF and NAC13bR (Table S1). The empty pGBKT7 vector was used as a negative control and the pGBKT7-p53 vector as a positive control. Finally, we transferred the vectors into the Y_2H Gold yeast strain, respectively, according to the method of standard LiCl transformation protocol (Wang et al. 2018). Transformants were grown for 3–5 days on selective medium without *Trp* and *His* plates. *β*-Galactosidase assays were then performed on filter lifts of the colonies to detect activation of the *lacZ* reporter gene (Nilles 2017).

Transgenic tobacco generation

The cDNA fragment of *NAC13* encoding region from 84K poplar was amplified by RT-PCR, using a pair of primers NAC13F4 and NAC13R4 with restriction sites *XbaI* and *SacI* (Table S1). It was then introduced into the binary vector pBI121 driven by the CaMV35S promoter. The recombinant plasmid was transferred into GV3101 for the tobacco transformation (Yao et al. 2016a). The transgenic tobacco seedlings was screened by means of resistance to Kanamycin (Kan, 100 mg L^{-1}), followed by PCR validation with primers NAC13F1 and NAC13R1.

Stress tolerance assays of transgenic tobacco

To investigate germination rates, we placed 100 seeds of each T3 transgenic line and wild type in the MS medium containing 0, 75, and 150 mM NaCl, respectively. The germination rates were recorded after 7 days under 16/8-h light/dark cycle at 25 °C.

For the root length assays, the seeds of WT and transgenic lines were cultured in the MS medium for one week; the seedlings were then transferred to MS medium supplied with 0, 75, and 150 mM NaCl, respectively. Five days later, we measured the root length of each seedling. One month later, we measured plant height, raw weight and root length. Each line had at least 10 seedlings, and three replicates were measured for each treatment.

Measurement of SOD and POD

We weighed about 0.2 g of fresh leaves, grinded them to a homogenate with 2 mL of pre-chilled 0.05 M PBS, and centrifuged at 10,000*g* for 10 min at 4 °C, with supernatant as the enzyme extract. The activity of SOD was determined by inhibiting the reduction of NBT. First, pipetted 1 mL of SOD reaction solution into a 2 mL centrifuge tube, added 1 mL of enzyme extract diluted 50 times and 1 mL 0.05 M PBS, respectively, and then let them reacted at 30 °C, 4000 Lx light incubator for 10 min, next measured the absorbance of the reaction solution at 560 nm respectively. The non-light treatment of 2 mL 0.05 M PBS was used to zero. And the SOD content was measured based on the formula: $(\Delta A_{560}\% \times V)/(50\% \times W \times T \times v)$. ΔA_{560} was the absorbance at 560 nm, V was the total volume of enzyme extract, W was the weight of samples, T was reaction time, v was the reaction volume of enzyme extract. POD activity was measured by the method of (Sun et al. 2013). First, the 0.5 mL plant enzyme extract was mixed up with 0.5 mL 0.1 M PBS, 0.5 mL 0.8% H_2O_2 solution and 0.5 mL 0.1 M guaiacol solution, and got them heated at 30 °C for 15 min. Then quickly determine the absorbance of the reaction solution at 470 nm. The reaction system with 0.5 mL ddH_2O instead of 0.8% H_2O_2 solution was used for zero adjustment. The POD content was measured based on the formula: $100 \times (A_{470} \times V)/(0.5 \times W \times T)$, A_{470} was the absorbance at 470 nm, V was the volume of plant enzyme extract, W was the weight of samples, T was reaction time. Three biological replicates were measured for each experiment.

Measurement of MDA and RWC

To measure the content of MDA, we conducted the experiment with thiobarbituric acid (TBA) (Feng et al. 2013). First, we weighed about 0.2 g of fresh leaves, grinded them to a homogenate with 1.5 mL of 10% TCA, and centrifuged at 10,000*g* for 10 min. Then, we mixed 1 mL of supernatant and 1 mL of 0.6% TBA, and heated them at 100 °C for 15 min. After the mixture was cooled to room temperature, we measured the absorbance at 532 nm, 600 nm and 450 nm respectively. The MDA content was measured based on the formula: $6.452\times(A_{532}-A_{600})-0.56\times A_{450}$. A_{532}, A_{600}, A_{450} represent the absorbance values of the reaction solution at 532 nm, 600 nm and 450 nm respectively. RWC was measured as the method described by (Yao et al. 2016a). First, we weighed the fresh individual leaves weight (Wf), immersed the leaf in ddH2O for several hours to constant weight, took out the water on the surface of the leaf with absorbent paper after taking it out and weighed the leaf full water weight (Wt). Then we dried the blade at 80 ℃ to constant weight and weighed the dry weight of the blade (Wd), the RWC content was measured based on the formula: $(Wf-Wd)/(Wt-Wd)\times 100\%$. Wf was the fresh weight of samples, Wd was the dry weight of samples, Wt was the full water weight of samples. Three biological replicates were measured for each experiment.

Histochemical detection of H_2O_2 and O_2^-

Histochemical detection of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) was conducted by use of 3, 3-diaminobenzidine (DAB), NBT and Evans Blue. DAB can be dehydrogenated and oxidized under the catalysis of POD to produce brownish substance (Khokon et al. 2011). NBT is one of the alkaline phosphatase substrates and produces an insoluble blue product catalyzed by alkaline phosphatase (Khokon et al. 2011). Evans Blue (Batchvarova and Yakimova 2009) is commonly used to detect cell membrane integrity and cell survival. Live cells are not stained blue, and dead cells are dyed light blue. One-month-old WT and transgenic tobacco plants were treated with 0, 150 mM NaCl for 24 h, the leaves were then immersed in DAB dye solution, NBT solution and Evans Blue solution for 12 h in the dark, respectively. Finally, the leaves were decolorized with decolorizing solution (ethanol: acetic acid, v/v, 3:1).

Salt treatment of transgenic tobacco in soil

For salt tolerance assays, 2-week-old WT and transgenic tobacco seedlings were planted in soil containing vermiculite and perlite (5:3:2, v/v/v) under normal conditions for 2 weeks. Then the plastic pots containing WT and transgenic tobacco were submerged in 0 mM and 200 mM NaCl treatment for 15 days, respectively. Each line had at least 10 seedlings. Then observed and recorded the phenotypic changes.

Statistical analysis

We used ANOVA to identify significant differences between transgenic lines and wild type lines. The error bars represent standard deviation of three independent replicates. The fold relationship that in this study was the ratio of three transgenic lines average value and the wild type. All fold data were shown in Online Resource.

Results

Characterization of *NAC13* gene from 84K poplar

NAC13 gene has 1032 bp open reading frame (ORF) that encodes a protein with 344 amino acids residues. It contains two introns and three exons (Fig. 1a), based on gene structure prediction. Evidence from sequence alignment indicated that the cDNA sequence of *NAC13* gene from the 84K poplar shares 99% identity with *Potri.001G404100.1* from *Populus trichocarpa*. Phylogenetic tree analyses (Fig. 1b) and sequence alignment (Fig. 1c) showed that *NAC13* gene from the 84K poplar contained a highly conserved domain NAM (NO APICAL MERISTEM). The domain consists of 126 amino acids that share high homology with counterparts from other species, such as *Populus trichocarpa* (100%, *Potri.001G404100.*1, XP_006370304.2), *Populus tomentosa* (100%, APA20125.1), *Populus euphratica* (98%, XP_011042499.1), *Quercus suber* (94%, POF12555.1), *Gossypium barbadense* (94%, PPS09923.1), *Durio zibethinus* (94%, XP_022717045.1), *Theobroma cacao* (93%, XP_007021328.2), *Vaccinium corymbosum* (94%, *NAC072*, AYC35383.1), *Catharanthus roseus* (94%, AWS00950.1), *Nicotiana tabacum* (94%, NP_001312702.1) and *Arabidopsis thaliana* (92%, *RD26*, OAO97067.1).

Subcellular localization of NAC13 protein

To address the subcellular localization of NAC13 protein, we developed 35S::NAC13-GFP construct against 35S::GFP, and transferred them into *Arabidopsis thaliana*, respectively. As shown in Fig. 2, the 35S::NAC13-GFP gene fluorescence is observed only in the nucleus of root tip cells, while the GFP gene in the positive control is expressed in all parts of the cells. This indicates that NAC13 protein is localized to the nucleus.

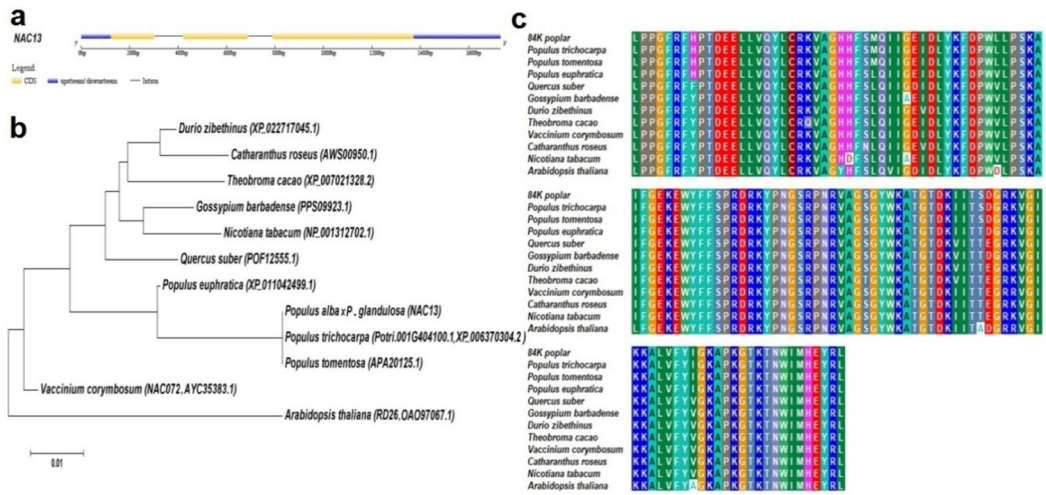

Fig. 1 The characteristic of *NAC13* gene in 84K poplar. **a** Gene structure of *NAC13* as predicted by Gene Structure Display Sever; **b** Phylogenetic tree analysis of NACs from different plants by MEGA 5.1 using Neighbor-Joining method; Alignment of protein conserved domain sequences of NACs by Clustal W

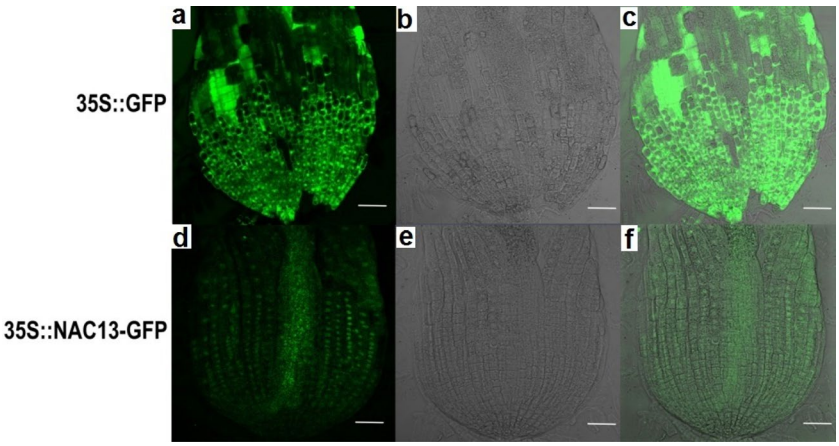

Fig. 2 Subcellular localization analysis of NAC13 protein in the root tip cells of *Arabidopsis thaliana*. **a**, **d** Were observed in dark field for green fluorescence; **b**, **e** were observed in bright field; **c**, **f** were observed in combination. Scale bar = 20 μm

Transcriptional activation of NAC13 protein

To test self-activation activity of the gene and find the activation fragment of NAC13 protein, we constructed the following vectors: (1) pGBKT7-NAC13 with full length of NAC13 protein; (2) pGBKT7-NAC13a with the conserved domain NAM; and (3) pGBKT7-NAC13b with the remaining amino acid sequence (Fig. 3a). We found that all fusion plasmids can grow on SD/-Trp medium, but only pGBKT7-NAC13, pGBKT7-NAC13b, and positive control can produce blue strain on SD/-Trp/-His/X-a-Gal medium (Fig. 3b). The results indicated that NAC13 protein functions as a transcriptional activator and the activation domain is located in the C-terminal region.

Molecular validation of transgenic tobacco

Through construction of the vector over-expressing *NAC13* gene and transforming it into tobacco, we finally obtained five transgenic lines. Compared to non-transgenic plants that cannot grow roots in the medium supplemented with 100 mg L^{-1} Kan, the transgenic plants grow normally (Fig. 4a). In addition, when grown on the medium without Kan, plant height and root system of the transgenic plants are significantly better than that of wild type (Fig. 4a). Furthermore, DNAs were extracted from WT and the transgenic tobacco leaves and then RT-PCR was conducted with primers NAC13F1 and NAC13R1. Evidence from the recombinant plasmid (positive control) indicated that the gene can

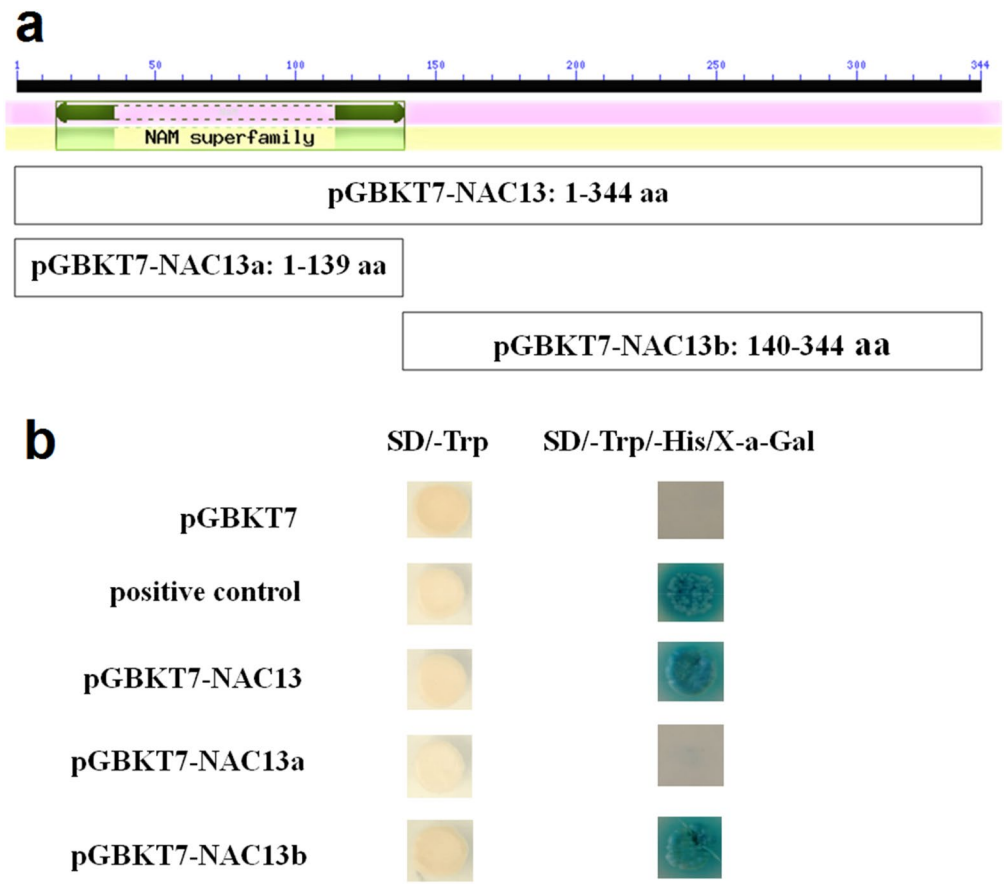

Fig. 3 Transcriptional activation of NAC13 protein. **a** Bait vectors pGBKT7-NAC13 with full length of NAC13 protein; pGBKT7-NAC13a with the conserved domain NAM; and pGBKT7-NAC13b with the remaining amino acid sequence. **b** Transcriptional activation assay. The pGBKT7 vector was used as a negative control; the transformants were incubated on SD/-Trp and SD/-Trp/-His/X-a-Gal to test for β-galactosidase activity

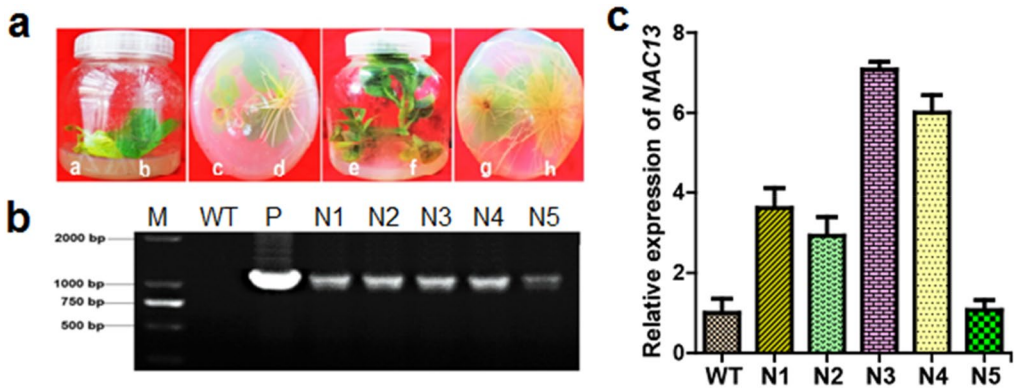

Fig. 4 Identification of transgenic tobacco lines. **a** The phenotype of transgenic tobacco, **a**, **c** are non-transgenic plants; **b**, **d** are transgenic plants in rooting medium with 100 mg L^{-1} Kan; **e**, **g** are non-transgenic plants; f and h are transgenic plants in rooting medium without antibiotics. **b** Molecular identification of transgenic tobacco lines by PCR with primers NAC13F1 and NAC13R1. **c** Expression of *NAC13* gene in wild type and five homozygous transgenic lines. *WT* wild type, *P* positive control, *N1–N5* transgenic tobacco lines. M, 2000 DNA marker. The error bars represent standard deviation of three independent replicates

be amplified only in the transgenic lines, but not in the wild type (Fig. 4b). Similarly, the expression level of the *NAC13* gene in the five transgenic tobacco lines was higher than that in the WT tobacco. The three lines N1, N3 and N4 with the highest expression levels were used to next studies. The three lines were renamed to T1, T2 and T3.

Germination rate test of transgenic tobacco seeds under salt stress

Both Wild type and transgenic tobacco were subjected to salt stress. The seeds were sown on MS medium containing 0, 75 and 150 mM NaCl, respectively (Fig. 5a). Under 0 mM NaCl stress, there was no significant difference between WT and the transgenic lines, they all had a great growth conditions. However, the germination rate of transgenic tobacco under 75 and 150 mM NaCl stresses was significantly higher, compared to wild type. We found that the germination rate of transgenic lines was over 80% and 50% under 75 and 150 mM NaCl stresses, but it was only 58% and 13% for wild type, respectively. These results indicate that transgenic tobacco ectopically expressing *NAC13* gene can enhance germination rate under salt stress.

Root length test of transgenic tobacco under salt stress

To test root length changes of transgenic tobacco under salt stress, 10-d seedlings of WT and transgenic tobaccos were transferred onto MS medium with 0, 75, and 150 mM NaCl, respectively. After five days, we measured root length (Fig. 5b, c). The results showed that root length of the transgenic lines is 1.39 ± 0.09 fold longer than that of WT on the normal condition. When the seedlings were subjected to respective 75 and 150 mM NaCl treatments, root length changed to 1.41 ± 0.07 and 1.84 ± 0.08 folds, respectively. This indicated ectopically expression of *NAC13* gene can enhance salt tolerance at the early growing stage in tobacco.

Morphological analysis of transgenic tobacco under salt stress

Plant height, root length, and fresh weight of tobacco seedlings were measured, after the plants were growing on the MS medium containing respective 0, 75, 150, 300 mM NaCl for 30 days. Under the control condition, plant height, root length, and fresh weight of the transgenic plants were 1.2 ± 0.05, 1.52 ± 0.19 and 1.18 ± 0.13 folds, respectively, higher than that in wild type (Fig. 6). When challenged with 75 mM salt stress, the corresponding folds turned to be 1.33 ± 0.05, 1.51 ± 0.13, and 1.38 ± 0.18, respectively, indicating that the transgenic plants can grow better under the salt stress. Furthermore, when treated with 150 mM NaCl, the folds changed to 1.25 ± 0.06, 1.56 ± 0.05 and 1.57 ± 0.09, respectively. The wild tobacco plants were short and the leaves became yellow, while the transgenic tobacco lines grew normally with dark green leaves. When treated with 300 mM NaCl, wild tobacco could not survive, but the transgenic plants were still able to grow (Fig. 6a).

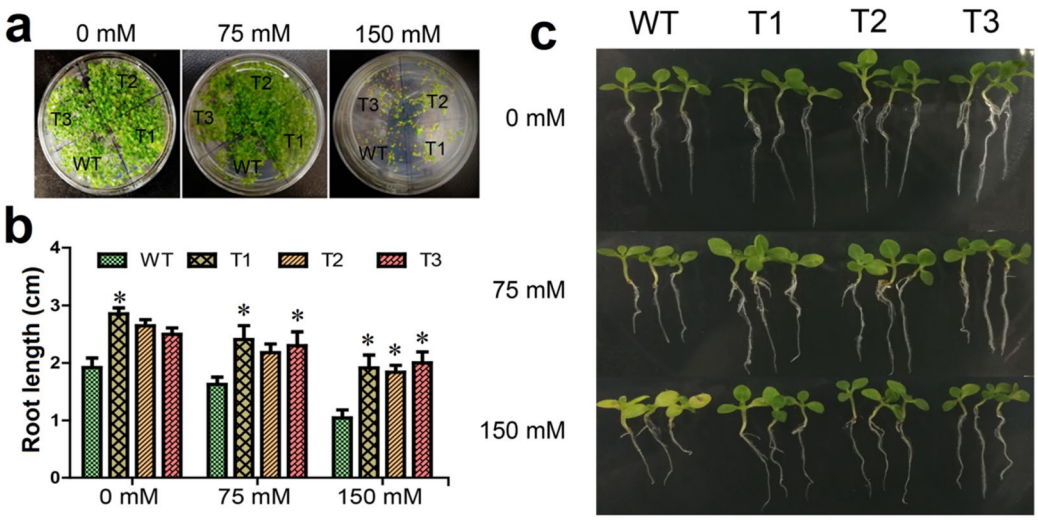

Fig. 5 Phenotype of transgenic and WT tobacco seedlings under salt treatments. **a** Seed germination rates of transgenic tobacco; *WT* wild type, *T1–T3* transgenic tobacco lines; **b** the root length of 5 days' tobacco seedlings under 0, 75 and 150 mM NaCl treatments, respectively; **c** phenotype of transgenic and WT tobacco seedlings. The error bars represent standard deviation of three independent replicates, asterisks indicate significant differences between transgenic lines and wild type lines (ANOVA, $*P < 0.05$)

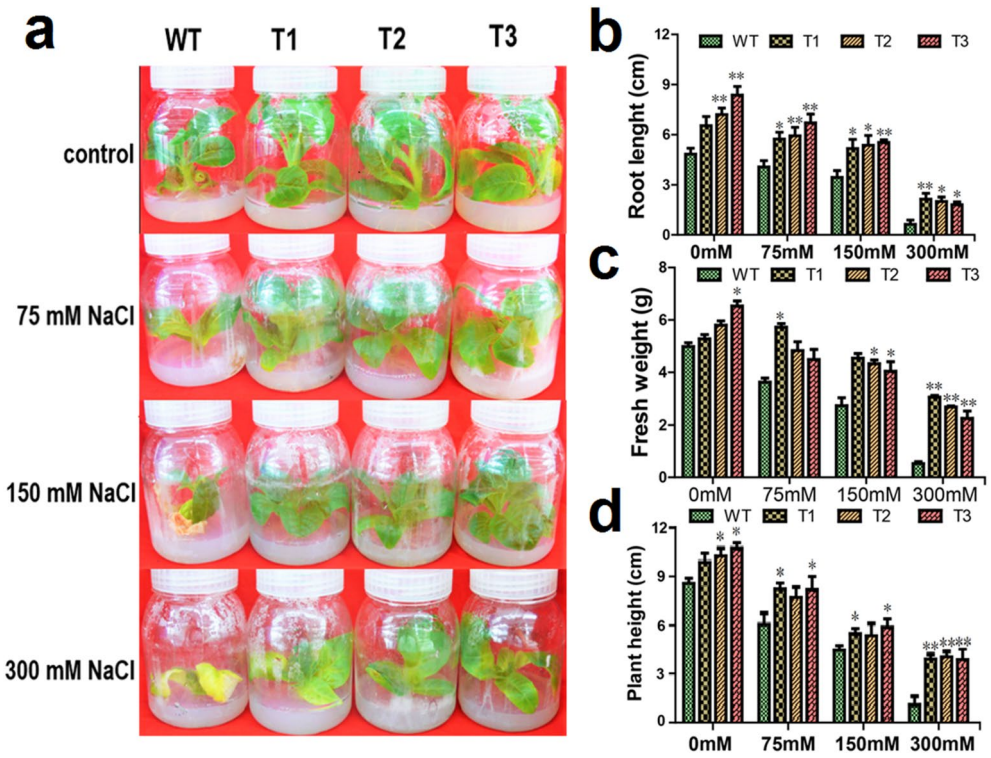

Fig. 6 Growth of transgenic tobacco plants under salt stresses. **a** Comparisons between WT and transgenic lines under salt stress. *WT* wild type, *T1–T3* transgenic lines. **b** Plant height of WT and transgenic lines; **c** fresh weight of WT and transgenic lines; **d** root length of WT and transgenic lines. The error bars represent standard deviation of three independent replicates, asterisks indicate significant differences between transgenic lines and wild type lines (ANOVA, $*P<0.05$, $**P<0.01$)

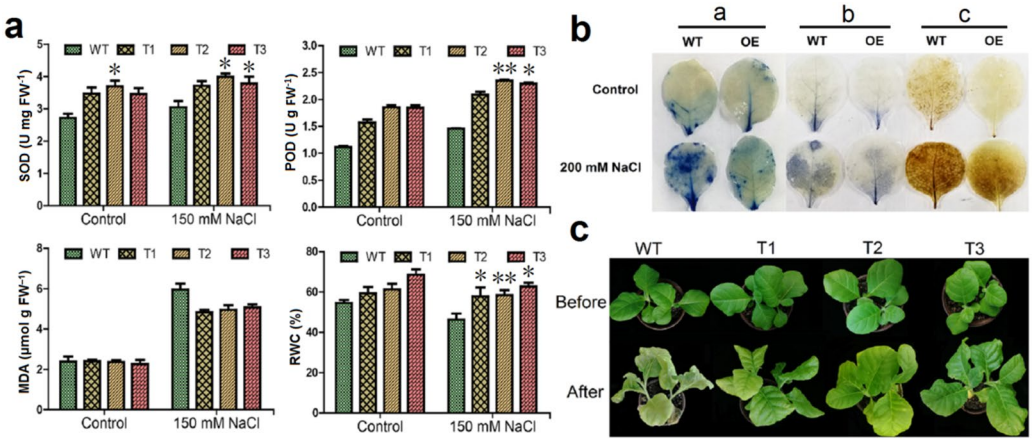

Fig. 7 Physiological analyses of WT and transgenic tobacco. **a** The physiological parameters include SOD, POD, MDA content, and relative water content (RWC) of WT. The transgenic lines and wild type were compared under respective 0 and 150 mM NaCl conditions. *WT* wild type, *T1–T3* transgenic lines. U: active unit, the size of the enzyme activity; **b** Histochemical staining with **a** Evans blue, **b** DAB and **c** NBT, respectively; OE,T2 transgenic line; **c** Growth comparison in soil between WT and transgenic lines with 200 mM NaCl irrigation for one month. The error bars represent standard deviation of three independent replicates, asterisks indicate significant differences between transgenic lines and wild type lines (ANOVA, $*P<0.05$, $**P<0.01$)

Physiological analysis of transgenic tobacco under salt stress

The physiological parameters were determined under respective 0 and 150 mM NaCl treatments (Fig. 7a). Under normal condition, results showed that, SOD, POD, and relative water content (RWC) of the transgenic lines were 1.31 ± 0.05, 1.58 ± 0.14 and 1.16 ± 0.09 folds, respectively, higher than that of wild type. But there was no obvious difference in MDA content. Under 150 mM NaCl treatment, the folds corresponding to the first three parameters became 1.26 ± 0.05, 1.49 ± 0.14 and 1.29 ± 0.05, respectively. Conversely, MDA content in wild type increased significantly, reaching 1.20 ± 0.03 folds compared to the transgenic lines. These lines of evidence indicate that ectopically expression *NAC13* gene transgenic plants have better salt tolerance than wild type.

Evens blue, DAB and NBT staining were used to analyze the accumulation of superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) in wild type and transgenic tobacco lines. Results indicated that the staining area of transgenic and wild type leaves was similar under normal condition (Fig. 7b). After 150 mM NaCl treatment for 24 h, the staining of wild type was significantly deeper than that of transgenic lines, indicating that transgenic plant cells have a stronger ability to remove reactive oxygen species including O_2^- and H_2O_2, thereby reducing cell damage and enhancing plant tolerance.

To test salt tolerance under natural condition, two-week-old transgenic plants and wild type were irrigated with 200 mM NaCl solution for 15 days. Leaves of wild type wilted to death, but the transgenic lines grew well (Fig. 7c), indicating that transgenic plants of ectopically expression *NAC13* gene have greater salt tolerance, compared to wild type.

Discussion

NACs are one of the largest plant-specific transcription factor families. NACs contain a highly conservative DNA binding domain which includes approximately 160 amino acid residues and consists of several spiral structures that surround a β-fold to form a new type of folded structure at the N-terminal of protein (Hu et al. 2010), and followed by a nuclear localization signal site, and a variable C-terminal domain (Hu et al. 2010; Jensen et al. 2010). In this study, we cloned a salt-induced *NAC13* transcription factor gene from the 84K poplar, which is highly homologous to the gene of *Potri.001G404100.1* in *P. trichocarpa*; *of OAO97067.1* (*RD26*) in Arabidopsis*;* and of *NP_001312702.1* in tobacco. *RD26* gene is inducible by dehydration, NaCl, and ABA. In addition, Arabidopsis plants over-expressing *RD26* displayed hyper-sensitivity to ABA (Fujita et al. 2004). This implies that poplar *NAC13* gene may be responsive to ABA stress. Evidence from sub-localization and trans-activation assays indicated that NAC13 protein is a nuclear protein that functions as a transcriptional activator. The NAC13 protein also has a conserved NAC domain named NAM in the N-terminal region from 15 to 139 aa. But our results from yeast two-hybrid experiments indicated that this domain showed no activation capacity. These are consistent with previous studies on *RD26* gene in Arabidopsis (Fujita et al. 2004). However, our studies indicated that the transcription activation domain is localized in the C-terminal region, which is congruent with the same study in Arabidopsis (Fujita et al. 2004). These lines of evidence suggest that transcription activation of the NAC13 protein may require a specific tertiary structure other than the conserved NAC domain. Further studies are needed to validate this hypothesis.

NAC TFs are closely related to the plant growth and lateral root development (Nuruzzaman et al. 2010). Overexpression of *AtNAC2* in Arabidopsis can promote lateral root development and increase the number of lateral root (Li et al. 2018), which has been verified that *AtNAC2* gene may play a significant role in the lateral root development according to participate in the ethylene and auxin signaling pathways under salt treatment (He et al. 2010). A membrane-bound NAC TF *NTL8* can regulate seed germination which is linked to salt signaling (Kim et al. 2008).

In this study, we screened 5 transgenic tobacco lines by Kan resistance. According to phenotypic observation, *NAC13*-ectopically-expressing transgenic tobaccos grow better than wild type, due to a significantly stronger root system. In addition, the germination rate is much higher compared to wild type on the MS with salt treatment. These lines of evidence indicated that *NAC13* gene played a potential role in the signaling pathways under adverse stress conditions.

NACs play an important role to respond to abiotic stress in plants. Accumulated evidences suggested that overexpression of stress-inducible NAC genes can improve stress tolerance of plants. For example, *SLNAM1* transgenic tobacco plants have higher tolerance to chilling stress which obtained improved osmolyte contents and reduced H_2O_2 and O_2 contents (Guo et al. 2016). Transgenic Arabidopsis plants over-expressing *ATAF1* can enhance drought tolerance (Wu et al. 2009). Studies showed that *CarNAC3* and *CarNAC6* from *Cicer arietinum* were integrated into the genome of poplar and all the transgenic lines could survive under higher salt stress while wild type plants withered and stopped growing (Movahedi et al. 2015). In the field of plant biotechnology, changing gene expression activity is a new direction for regulating plant growth, development, and improving plant tolerance.

In this work, we planted the transgenic and wild type tobaccos in the greenhouse condition, and watered with

200 mM NaCl solution for 15 days, apparently, transgenic lines grew significantly better than the WT. In addition, we measured SOD, POD, MDA and RWC of transgenic tobacco lines, respectively. The activities of SOD, POD and the content of RWC were significantly higher than WT. MDA was lower than the control under high salt treatment. We speculated that under normal conditions, the *NAC13* gene of poplar did not directly increase the content of POD and SOD in transgenic tobacco. Similarly, the content of ROS was no significant change in tobacco. But under the salt stress, *NAC13* gene could promote the production of POD and SOD by physiological or biochemical reactions, which promoted the removal of ROS. Therefore, under normal condition, there was no significant difference in physiological changes between WT and transgenic lines. These all indicate that *NAC13* gene may play an important role in the ROS scavenging pathways to protect itself from the stress damage. Future studies are needed to shed light on molecular mechanisms of gene regulation and gene networks related to *NAC13* gene in response to salt stress.

Conclusions

In summary, we chose *NAC13* gene which belongs to NAC transcription factor in the 84K poplar and confirmed that NAC13 protein was localized to the nucleus. Further, evidence from yeast two-hybrid screening demonstrated that NAC13 protein functions as a transcriptional activator, with an activation domain located in the C-terminal region. NAC13-ectopically-expressing transgenic tobacco plants proved that the gene can improve the salt tolerance of plants. This study will provide a valuable theoretical basis for forest genetic breeding and resistant breeding.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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