Improved salt tolerance in transgenic tobacco by over-expression of poplar *NAC13* gene

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Abstract

Background: 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.

Methods: In this study, we cloned a salt-inducible NAC transcription factor gene (*NAC13*) from a poplar variety 84K, followed by transforming it into both tobacco and Arabidopsis.

Results: Stable expression analysis of 35S::NAC13-GFP fusion protein in Arabidopsis indicated that *NAC13* was localized to the nucleus. We also obtained five transgenic tobacco lines. Evidence from morphological and physiological characterization and salt treatment analyses indicated that the transgenic tobacco enhanced salt tolerance, suggesting that *NAC13* gene may function as a positive regulator in tobacco responses to salt stress. Furthermore, 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.

Discussion: *NAC13* gene plays an important role in response to salt stress in tobacco. 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, which will provide a valuable theoretical basis for forest genetic breeding and resistant breeding.

Keywords NAC, transcription factor, gene over-expression, transgenic tobacco, salt tolerance

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. This family member was first found in *Petunia hybrid* (*Zhang 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 *Populous trichocarpa* and 145 NAC TFs are found in *Populous euphratica,* according to the PlantTFDB (http://planttfdb.cbi.pku.edu.cn/family.php?sp=Ptr&fam=NAC). NACs contain a highly conservative 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 branching growth (*Chuanzao et al. 2010*), 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 (*Hussey et al. 2011; Chai et al. 2015)*. Studies have indicated that transgenic Arabidopsis over-expressing *ANAC046* exhibits premature senescence and significantly reduces chlorophyll content (*Oda-Yamamizo et al. 2016*). Over-expression of *PaNACo3* in *Norway spruce* showed reduced flavonol biosynthesis and aberrant embryo development (*Dalman et al. 2017*). In poplar, the expression of *PtNAC068* and *PtNAC154* is associated with secondary 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*) and colleagues 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*).

Plants cells are always hypersensitive to abiotic stresses and then affected by induced reactive oxygen species (ROS) production (*Helene et al. 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, in order 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. 2017)*. Peroxidase (POD) is mainly present in cell walls, vacuoles and chloroplasts (*Rácz et al. 2018*). It is closely related to plant respiration and photosynthesis and often used as a physiological indicator of tissue aging. 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 (*Wang et al. 2017; Hu et al. 2018*); that is, the lower level of MDA, the less lipid peroxidation and the better cell membrane integrity (*Wang et al. 2017*). Recent research demonstrates that transgenic tobaccos with over-expression of *MsNAC2* from Alfalfa (*Medicago sativa L.*) had much lower MDA content than WT in the treatment of high salinity, PEC6000, and low temperature (*Shen et al. 2015*). *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*).

Previously we reported that transgenic poplar over-expressing *NAC13* can significantly improve salt tolerance *(Zhang et al. 2019*). In the present study, we isolated 1032 bp cDNA fragment of *NAC13* gene from the 84K poplar (*Populus alba* × *P. glandulosa*), followed by constructing a vector pBI121-NAC13 that over-expresses *NAC13* gene. The transgenic tobacco lines displayed enhanced salt tolerance, based on morphological and physiological analyses. Furthermore, we validated the hypothesis that the NAC13 protein functions as a transcriptional activator.

Materials and methods

Plant materials

The wild type tobacco (*Nicotiana tabacum L. cv. Petit Havana SR-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 sucrose. The plates were transferred to greenhouse at an average temperature of 25℃ and 16/8-h light/dark cycles. Two-week-old seedlings were used for stress treatment.

Cloning and characterization of NAC13 gene

Fresh leaves from the 84K poplar seedling were collected and frozen in liquid nitrogen for RNA isolation. Total RNA was extracted from the leaves by RNA Extraction Kit (Takara, China), and 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 RT-PCR with a pair of primers NAC13F1 and NAC13R1 (Supplemental Table 1). According to the cDNA sequence, the gene structure and conserved domain were analyzed by the online software of

Gene Structure Display Server (http://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 sequences homologous to the NAC13 protein. The sequences were then used to construct a phylogenetic tree, by use of MEGA5.0 with neighbor-joining methods 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 (Supplemental Table 1) which contain the restriction site *Spe* I. It was then fused into the pBI121-GFP vector with the CaMV35S promoter. 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

We performed protein-protein interaction analyses, using the yeast two-hybrid method (*Lin et al. 2017*). First, the cDNA fragment encoding the full length of *NAC13* was amplified with primers NAC13F3 and NAC13R3 (Supplemental Table 1), containing the *EcoR* I and *Sal* I restriction sites. Then, it was cloned into the pGBKT7 vector, in order to generate bait vector pGBKT7-NAC13. Furthermore, to explore the activation

region, we also cloned two different segments of the NAC13 cDNA (*Zhang et al. 2019*), followed by inserting them respectively into the pGBKT7, with two pairs of primers NAC13aF and NAC13aR, NAC13bF and NAC13bR (Supplemental Table 1). 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 Y2H 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 et al. 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 *Xba* I and *Sac* I (Supplemental Table 1). 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. 2016*). The transgenic tobacco seedlings was screened by means of resistance to kanamycin (Kana, 100 mg/L), followed by PCR validation with primers NAC13F1 and NAC13R1.

Stress tolerance assays of transgenic tobacco

In order 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,

166 respectively. The germination rates were recorded after 7 d under 16/8-h light / dark
167 cycle at 25℃.

168 For the root length assays, the seeds of WT and transgenic lines were cultured in the
169 MS medium for one week; the seedlings were then transferred to MS medium
170 supplied with 0, 75, and 150 mM NaCl, respectively. Five days later, we measured the
171 root length of each seedling. One month later, we measured plant height, raw weight
172 and root length. Three replicates were measured for each treatment.

173

174 **Measurement of SOD, POD, MDA and RWC**

175 The activity of SOD was determined according to inhibiting the reduction of
176 nitrotetrazolium blue chloride (NBT) by superoxide dismutase. POD activity was
177 measured by the method of (*Sun et al. 2013*). To measure the content of MDA, we
178 conducted the experiment with thiobarbituric acid (TBA) (*Feng et al. 2013*). REC was
179 measured as the method described by (*Yao et al. 2016*). Three biological replicates
180 were measured for each experiment.

181

182 **Histochemical detection of H_2O_2 and O_2^-**

183 Histochemical detection of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) was
184 conducted by use of 3, 3-diaminobenzidine (DAB), NBT and Evans Blue. DAB can
185 be dehydrogenated and oxidized under the catalysis of POD to produce brownish
186 substance (*Khokon et al. 2011*). NBT is one of the alkaline phosphatase substrates and
187 produces an insoluble blue product catalyzed by alkaline phosphatase (*Khokon et al.*
188 *2011*). Evans Blue (*Batchvarova et al. 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 under normal conditions for two weeks. Then the tobacco plants were subjected to 200 mM NaCl treatment for 15 days. We observed and recorded the phenotypic changes.

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 (Supplemental Figure 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 (Supplemental Figure 1B) and sequence alignment (Supplemental Figure 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. 1, the GFP 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.

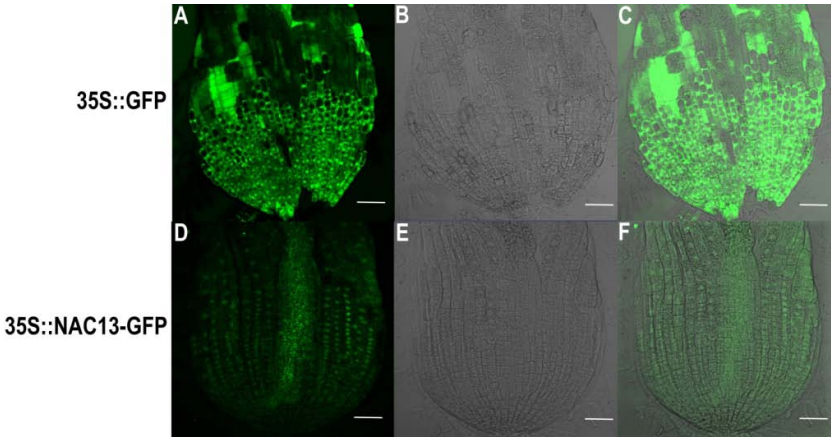


Figure 1 Subcellular localization analysis of NAC13 protein in the root tip cells of *Arabidopsis thaliana*. (A) and (D)were observed in dark field for green

fluorescence; (B) and (E) were observed in bright field; C and F were observed in combination. Scale bar=20μm.

Transcriptional activation of NAC13 protein by yeast two-hybrid

In order to test self-activation activity of the gene and find the activation fragment of NAC13 protein, we constructed the following bait 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. 2A). Evidence from yeast two-hybrid analyses indicated that NAC13 protein functions as a transcriptional activator and the activation domain is located in the C-terminal region (Fig. 2B).

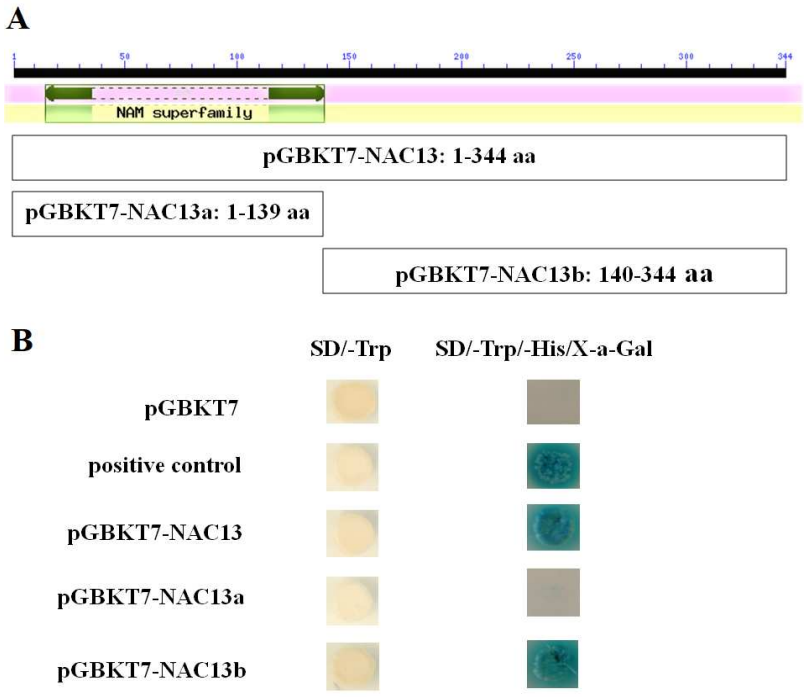


Figure 2 Transcriptional activation of NAC13 protein. (A) Bait vectors pGBKT7-NAC13 with full length of NAC13 protein; pGBKT7-NAC13a with the conserved

domain NAM; and pGBKT7-NAC13b with the remaining amino acid sequence. (B) Transcriptional activation assay. 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.

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. Evidence from both phenotypic screening by Kana (Fig. 3A) and PCR detection is shown in Fig. 3B. Compared to non-transgenic plants that cannot grow roots in the medium supplemented with 100 mg/L Kana, the transgenic plants grow normally. In addition, when grown on the medium without Kana, plant height and root system of the transgenic plants are significantly better than that of wild type (Fig. 3A). Furthermore, RNAs 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 be amplified only in the transgenic lines, but not in the wild type.

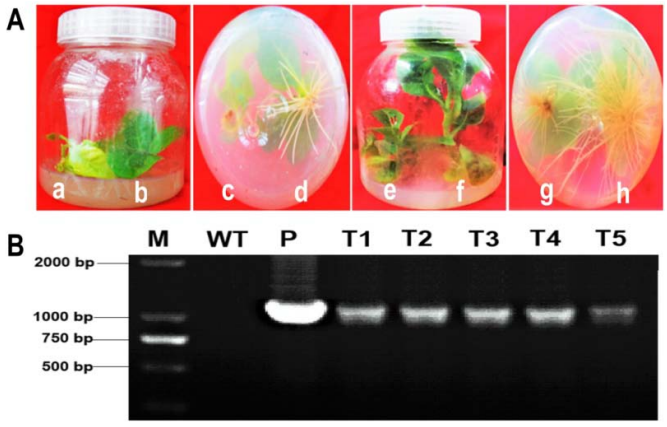


Figure 3 Identification of transgenic tobacco lines. (A) The phenotype of transgenic tobacco, (a) and (c) are non-transgenic plants; (b) and (d) are transgenic plants in rooting medium with 100 mg/L Kana; (e) and (g) are non-transgenic plants; f and h are transgenic plants in rooting medium without antibiotics. (B) Molecular identification of transgenic tobacco lines by PCR with primers NAC13F1 and NAC13R1. WT, wild type; P, positive control; T1-T5, transgenic tobacco lines. M, 2000 DNA marker.

Germination rate test of transgenic tobacco seeds under salt stress

Both Wild type and transgenic tobacco lines T1, T2 and T3 were subjected to salt stress. The seeds were sown on MS medium containing 0, 75 and 150 mM NaCl, respectively (Fig. 4A). Under the control conditions, no significant difference was observed between WT and the transgenic lines. However, the germination rate of transgenic tobacco under salt stress was significantly higher, compared to wild type. The germination rate of transgenic lines was over 80% and 50% under 75 and 150 mM NaCl, respectively. However, it was only 58% and 13% for wild type. These results indicate that transgenic tobacco over-expressing *NAC13* gene can enhance germination rate under salt stress.

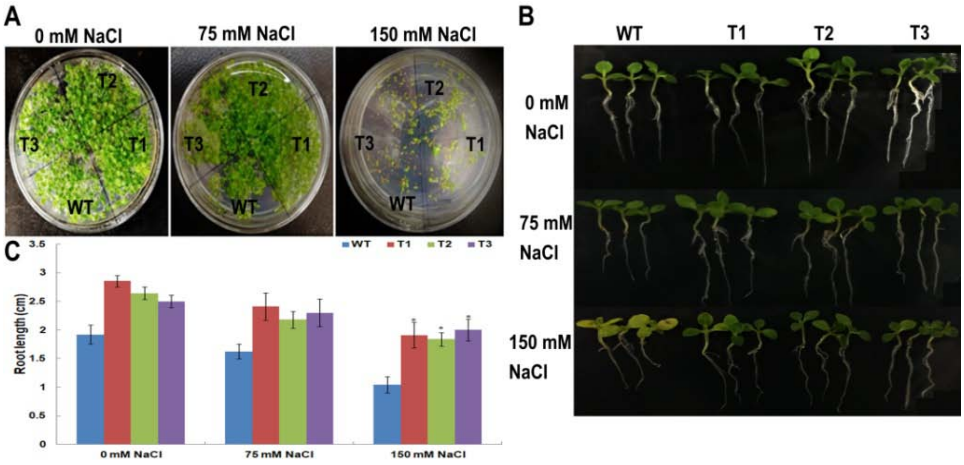


Figure 4 Phenotype of transgenic and WT tobacco seedlings under salt treatments. (A) Seed germination rates of transgenic tobacco; WT, wild type; T1-T3, transgenic tobacco lines; (B) Phenotype of transgenic and WT tobacco seedlings; (C) The root length of 5 days' tobacco seedlings under 0, 75 and 150 mM NaCl treatments, respectively.

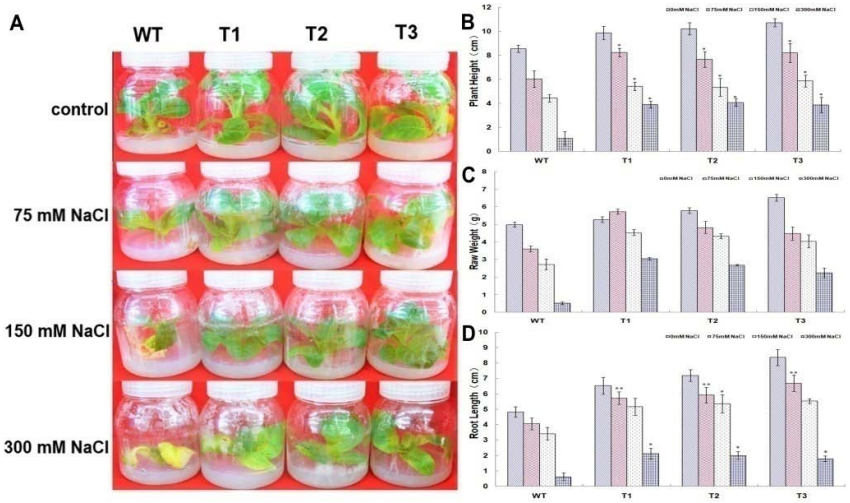
Root length test of transgenic tobacco under salt stress

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. 4B and 4C). The results showed that root length of the transgenic lines is 1.41±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 1.85±0.05 folds,, respectively. This indicated over-expression of *NAC13* gene can enhance salt tolerance at the early growing stage in tobacco.

296 **Morphological analysis of transgenic tobacco under salt stress**

297

298 Plant height, root length, and fresh weight of tobacco seedlings were measured, after
299 the plants were growing on the MS medium containing respective 0, 75, 150, 300 mM
300 NaCl for 30 days. Under the control condition, plant height, root length, and fresh
301 weight of the transgenic plants were 1.2±0.05, 1.54±0.19 and 1.18±0.13 folds,
302 respectively, higher than that in wild type (Fig. 5). When challenged with 75 mM salt
303 stress, the corresponding folds turned to be 1.32±0.05, 1.53±0.12, and 1.41±0.17,
304 respectively, indicating that the transgenic plants can grow better under the salt stress.
305 Furthermore, when treated with 150 mM NaCl, the folds changed to 1.26±0.06,
306 1.56±0.06 and 1.57±0.09, respectively. The wild tobacco plants were short and the
307 leaves became yellow, while the transgenic tobacco lines grew normally with dark
308 green leaves. When treated with 300 mM NaCl, wild tobacco could not survive, but
309 the transgenic plants were still able to grow (Fig. 5A).



310

311 **Figure 5 Growth of transgenic tobacco plants under salt stresses.** (A)
312 Comparisons between WT and transgenic lines under salt stress. WT: wild type; T1-3:
313 transgenic lines. (B) Plant height of WT and transgenic lines; (C) Plant weight; (D)
314 Root length.

315

316 **Physiological analysis of transgenic tobacco under salt stress**

317 The physiological parameters were determined under respective 0 and 150 mM NaCl
318 treatments (Fig. 6A). Under normal condition, results showed that, SOD, POD, and
319 relative water content (RWC) of the transgenic lines were 1.32±0.05, 1.51±0.13 and
320 1.17±0.09 folds, respectively, higher than that of wild type. But there was no obvious
321 difference in MDA content. Under 150 mM NaCl treatment, the folds corresponding
322 to the first three parameters became 1.27±0.05, 1.52±0.09 and 1.31±0.56, respectively.
323 Conversely, MDA content in wild type increased significantly, reaching 1.20±0.03
324 folds compared to the transgenic lines. These lines of evidence indicate that *NAC13*-
325 overexpressing transgenic plants have better salt tolerance than wild type.

326 Evens blue, DAB and NBT staining were used to analyze the accumulation of
327 superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) in wild type and transgenic
328 tobacco lines. Results indicated that the staining area of transgenic and wild type
329 leaves was similar under normal condition (Fig. 6B). After 150 mM NaCl treatment
330 for 24 h, the staining of wild type was significantly deeper than that of transgenic
331 lines, indicating that transgenic plant cells have a stronger ability to remove reactive
332 oxygen species including O_2^- and H_2O_2, thereby reducing cell damage and enhancing
333 plant tolerance.

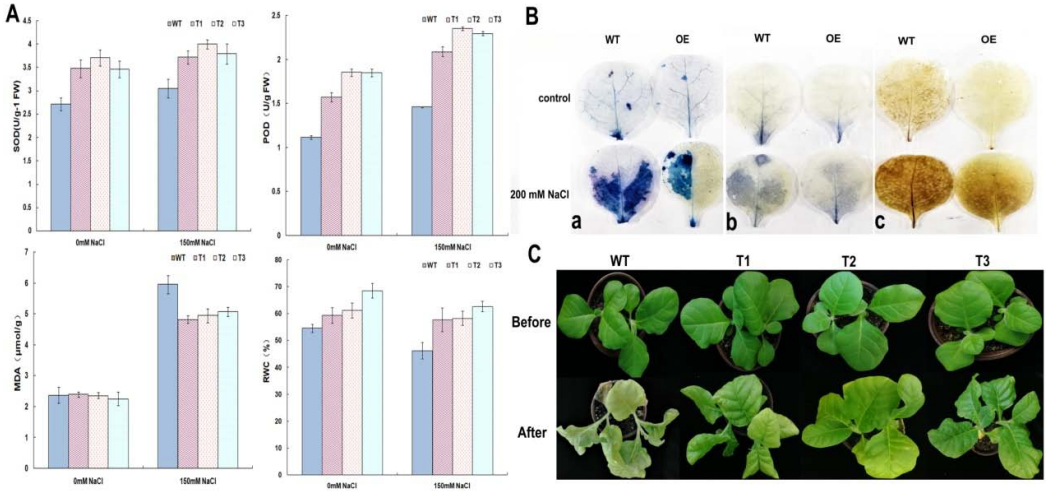


Figure 6 Physiological analyses of WT and transgenic tobacco. (A)The physiological parameters include SOD, POD, MDA content, and relative water content of WT. The transgenic lines and wild type were compared under respective 0 and 150 mM NaCl conditions. WT: wild type; T1-T3: transgenic lines. (B) Histochemical staining with (a) Evans blue, (b) DAB and (c) NBT, respectively. OE, transgenic tobacco; (C) Growth comparison in soil between WT and transgenic lines with 200 mM NaCl irrigation for a month.

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. 6C), indicating that *NAC13*-overexpressing transgenic plants have greater salt tolerance, compared to wild type.

Discussion

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*). 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 NAM domain 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 (*Miki et al. 2010*). 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 (*Miki et al. 2010*). These lines of evidence suggest that transcription activation of the NAC13 protein may require a specific tertiary structure other than the conserved NAM domain. Further studies are needed to validate this hypothesis.

NACs are one of the largest plant-specific transcription factor families, which play significant roles in plant growth and development, as well as in biotic and abiotic stresses. Over-expression 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 osmolytes contents and reduced H_2O_2 and O_2 contents (*Li et al. 2016*). Transgenic Arabidopsis plants over-expressing *ATAF1* can enhance drought tolerance (*Wu et al. 2010*). 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 this study, we cloned *NAC13* transcription factor from the 84K poplar, which is highly homologous to the gene of *Potri.001G404100.1* in *Populus trichocarpa*; *of*

376 *OAO97067.1* (*RD26*) in Arabidopsis*;* and of *NP_001312702.1* in tobacco. *RD26* gene
377 is inducible by dehydration, NaCl, and ABA stresses. In addition, Arabidopsis plants
378 over-expressing *RD26* displayed hyper-sensitivity to abscisic acid (*Miki et al. 2010;*
379 *Shabala et al. 2012*). This implies that poplar *NAC13* gene may be responsive to ABA
380 stress.

381 NAC TFs are closely related to the plant growth and lateral root development
382 (*Nuruzzaman et al. 2010*). Over-expression of *AtNAC2* in Arabidopsis can promote
383 lateral root development and increase the number of lateral root (*Zhang et al. 2018*),
384 which has been verified that *AtNAC2* gene may play a significant role in the lateral
385 root development according to participate in the ethylene and auxin signaling
386 pathways under salt treatment (*He et al. 2010*). A membrane-bound NAC TF *NTL8*
387 can regulate seed germination which is linked to salt signaling affects ion homeostasis
388 independently of ABA (*Sang-Gyu et al. 2010*). In our study, we screened 5 transgenic
389 tobacco lines by Kana resistance. According to phenotypic observation, *NAC13*-over-
390 expressing transgenic tobaccos grow better than wild type, due to a significantly
391 stronger root system. In addition, the germination rate is much higher compared to
392 wild type on the MS with salt treatment. These lines of evidence indicated that
393 *NAC13* gene played a potential role in the signaling pathways under adverse stress
394 conditions.

395 We measured SOD, POD, MDA and RWC of transgenic tobacco lines, respectively.
396 The activities of SOD, POD and the content of RWC were significantly higher than
397 WT. MDA was lower than the control under high salt treatment. These indicate that
398 *NAC13* gene may play an important role in the ROS scavenging pathways to protect
399 itself from the stress damage. In addition, we planted the transgenic and wild type
400 tobaccos in the greenhouse condition, and watered with 200 mM NaCl solution for 15

days, apparently, transgenic lines grew significantly better than the WT. Collectively, *NAC13* gene plays an important role in response to salt stress in tobacco, which is consistent with our previous studies in poplar (*Zhang et al. 2019*). 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-over-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.

Additional Information and Declarations

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Competing Interests

The authors have no conflicts of interest to declare.

Author contributions

TJ and BZ designed research. XZ conducted experiments, data analysis and wrote the manuscript. ZC and KZ performed in data analysis. RL revised the manuscript. All authors read and approved the manuscript.

Supplemental Information

Supplemental Table 1. Prime names and sequences

Supplemental Figure 1. The characteristics of NAC13 gene in 84K poplar.

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