



Overexpression of the Transcription Factor *NtNAC2* Confers Drought Tolerance in Tobacco

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Abstract

NAC proteins constitute one of the largest families of plant-specific transcription factors and play an important role in biological processes, including plant development and phytohormone homeostasis, and in responses to various environmental stresses. In this study, we isolated an NAC group A gene (named *NtNAC2*) from *Nicotiana tabacum* L. Quantitative RT-PCR (qRT-PCR) analysis indicated that *NtNAC2* was significantly upregulated under drought stress, which implied *NtNAC2* was important in tobacco under such conditions. Overexpression of *NtNAC2* in tobacco plants exhibited enhanced drought tolerance by means of improved seedling growth. Under drought stress, organic osmoprotectants were significantly accumulated in these plants. Additionally, the activities of antioxidant defense enzymes, like superoxide dismutase (SOD) and peroxidase (POD), which could effectively scavenge accumulated reactive oxygen species (ROS), increased in *NtNAC2*-overexpression transgenic tobacco plants compared with wild-type plants. The net photosynthetic rate was also significantly increased in *NtNAC2*-overexpression transgenic lines compared with wild-type plants, and the content of malondialdehyde (MDA) and proline was lower in *NtNAC2*-overexpression transgenic lines than that in wild-type plants ($P < 0.01$). Furthermore, the expression of *NtWRKY28*, a drought resistance gene, was significantly increased and the δ -OAT gene was downregulated in *NtNAC2*-overexpression plants relative to wild-type plants. Taken together, these results indicated that *NtNAC2* functions as a positive regulator of drought stress tolerance. This study provides a basis for further study of drought resistance conferred by the *NtNAC2* gene.

Keywords *NtNAC2* · Antioxidant defense · Drought resistance · *Nicotiana tabacum*

Introduction

Plant growth and development are often adversely affected by different stresses during growth and developmental processes, including drought, high temperature, cold, and salinity (Long et al. 2013). Among these, drought is one of the most significant abiotic stresses, limiting plant growth, development, and productivity (Thirumalaikumar et al. 2017). At the cellular

level, one of the first responses of plants to drought stress is the production of reactive oxygen species (ROS), thus affecting the equilibrium of the redox state and resulting in oxidative stress. Such disruptions to redox homeostasis manifest as severe cellular damage due to peroxidation, a decline in photosynthetic efficiency, reduced cell membrane stability, and leaf wilting (Carvalho 2008; Choudhury et al. 2013; Benjamin and Nielsen 2006; Hanin et al. 2011).

In order to adapt to the stresses conferred by water deficits, plants have developed molecular mechanisms that coordinate expression of numerous genes that serve to protect them from such environmental adversities (Agarwal et al. 2006). The different expression levels of regulatory genes induced under various stresses protect cells from stresses by promoting the appropriate stress response. Typical regulatory genes encode transcription factors (TFs) and protein kinases (Nakashima 2006; Umezawa et al. 2006). Numerous studies have demonstrated that TFs play vital roles in plant gene regulation by either activating or preventing target gene expression (Puranik et al. 2012; Nakashima et al. 2012). These TFs

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include members of the NAC, MYB, AP2/ERF, WRKY, and bZip families (Wang et al. 2017). The NAC (*NAM*, *ATAF*, and *CUC*) transcription factor (*NAC-TF*) genes belong to a large family of TFs that encode important regulatory proteins in plants (Wang et al. 2011). The main structural features of these transcription factors include an N-terminal containing a conserved NAC domain of approximately 150 amino acid residues and a C-terminal that functions as a highly variable transcriptional regulatory region (Olsen et al. 2005). NAC TFs play vital roles in regulating plant growth and developmental processes, including abiotic stress responses (Shao et al. 2015). A few NAC genes, such as *AtNAC072* (RD26), *AtNAC019*, and *AtNAC055* from *Arabidopsis* and *BnNAC* from *Brassica*, have been shown to be involved in the response to various environmental stresses. Transgenic plants overexpressing three different *Arabidopsis* NAC genes (*ANAC019*, *ANAC055*, and *ANAC072*) demonstrate significantly increased drought tolerance (Tran et al. 2004). Additionally, Liu et al. (2016a, b) reported that *CarNAC6* is induced by drought and functions to enhance drought tolerance in *Arabidopsis*. Some NAC genes, such as *ATAF1* and *ATAF2* from *Arabidopsis* and *StNAC* from potato, are induced by pathogen attack and wounding. Huang et al. (2015) cloned the novel NAC transcription factor gene *TaNAC29* from wheat, which was shown to be upregulated by high salinity, dehydration, ABA, and H_2O_2 treatments. *TaNAC29* enhances tolerance to high salinity and drought stress in transgenic *Arabidopsis* and exhibits hypersensitivity to ABA. Hifzur et al. (2016) reported that overexpression of the *NAC 67* transcription factor from finger millet conferred tolerance against salinity and drought stress in rice.

NAC proteins are plant-specific TFs, and the NAC family has been recently reviewed by Puranik et al. (2012). At present, a number of studies have presented bioinformatic analyses on the NAC transcription factors in tobacco. For example, Tian et al. (2009) cloned the NAC transcription factor *NtNAC1* from tobacco and analyzed its physicochemical properties, advanced structure, and biological functions. Die et al. (2011) cloned *NtNAC8* from tobacco, and bioinformatic analyses indicated that *NtNAC8* may act as a regulatory enzyme related to intermediate metabolism and related responses in plants. At present, the research regarding NAC transcription factors is still limited to structure identification and expression analysis, with the biological role of specific NAC transcription factors still requiring functional verification.

In this study, we assessed the role of NAC transcription factors in relation to drought resistance in tobacco, and a new NAC gene, *NtNAC2* (*Nicotiana tabacum NAC2*), was isolated and characterized. We found that *NtNAC2* was induced by drought stress, and over-expression of *NtNAC2* in transgenic plants conferred significantly improved drought tolerance under water-deficit conditions. Our results provide

a reference for further research regarding the biological function of *NtNAC2* and the signal transduction pathways influenced by this transcription factor. Our results also provide a starting point for screening and breeding new tobacco germplasm with high quality and drought tolerance with the goal of an overall improvement in crop stress resistance.

Experimental Procedures

Plant Material and Growth Conditions

Tobacco (*Nicotiana tabacum* ‘Xanthi’) was sown in pots containing a 3:1 mixture of nutrient soil:perlite (v/v) in a growth chamber under a 16-h light/8-h dark photoperiod, 25 °C, and 10–20% relative humidity and with photosynthetically active radiation of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were cultivated for 3–4 weeks before treatment.

Tobacco seeds were used for transformation in this study, and T4 *NtNAC2*-overexpressed transgenic tobacco lines were used for drought stress treatments. Tobacco seeds were surface sterilized with 75% ethanol for 30 s and H_2O_2 for 10 min and then rinsed four times with sterile water. Sterilized seeds were sown on nutrient agar plates containing $0.5 \times$ MS medium at pH 5.8 and containing 30% (w/v) sucrose and 0.8% (w/v) agar. Seedlings were grown in a chamber at 22/23 °C (day/night), with photosynthetically active radiation of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, a photoperiod of 16/8 h (day/night), and 60% relative humidity. Seedlings were grown on this medium for 20 days before being transplanted into nutrient soil.

Drought Tolerance Assays

For the drought treatment, the 3-week-old T_1 transgenic tobacco plants were grown with 20% polyethylene glycol (PEG) 6000 (w/v) for 7 days. The phenotype of the seedlings was recorded with photographs. A total of 50 surface-sterilized seeds from homozygous T_0 transgenic lines and non-transgenic (NT) tobacco plants were germinated on MS medium at pH 5.8 and containing 30%(w/v) sucrose and 0.8% (w/v) agar and containing different mannitol concentrations (0, 150, and 300 mmol/l) at 25 °C with a 16-h light/8-h dark photoperiod. Each experiment was repeated at least three times with similar results. The seedling growth was measured by calculating the root length of ten seedlings at the end of a 2-week germination.

Total RNA Isolation

Young fresh leaves (0.1 g) were harvested, and total RNA was extracted using a HP Plant RNA Kit (R6837-02; Omega, USA). Each reverse transcription reaction was performed with total RNA (1.5 μg) using a M-MLV reverse transcriptase kit

(TaKaRa, Dalian, China) according to the manufacturer's instructions.

Cloning of *NtNAC2* and Construction of Prokaryotic and Plant Expression Vectors

The cDNA fragment of the *NtNAC2* open reading frame was from *Nicotiana tabacum*. Using the reported tobacco NAC transcription factor, a Blastp search was performed in the GenBank database to obtain the cDNA and amino acids of tobacco homologs. Primers were designed based on the obtained cDNA sequences using the software Premier 5.0 (the primer sequences are shown in Table 1). Reverse transcription was performed to obtain a cDNA template. The PCR product was cloned into the pMD19-T vector (TaKaRa, Dalian, China) and sent for sequencing (Shanghai Ying Weijie Biotechnology Company, China). The plasmid pSH737 was digested with *Hind*III and *Xba*I, and the expression sequence of *Kpn*I and *Eco*RI double digested sites was digested with *Kpn*I and *Eco*RI. The expression vector pSH737-*NtNAC2* was obtained using T4 DNA ligase.

Identification of the *NtNAC2* Gene by *Agrobacterium*-Mediated Transformation of Tobacco and Molecular Identification

Tobacco seedlings (3 to 4 weeks old) were transformed with *Agrobacterium* strain EHA105 in which 35S::*NtNAC2* was harbored using the tobacco leaf disc transformation method (Horsch et al. 1985). After taking the leaves of tobacco seedlings, a DNA extraction kit (Biochemical Technology Co., Ltd., Beijing, China) was used to extract DNA for PCR detection.

Quantitative RT-PCR Analyses

Total RNA was isolated from the young fresh leaves of tobacco (NT and transgenic lines) under stress, and the cDNA was synthesized according to the above procedure. Quantitative RT-PCR was carried out using an ABI 7500 real-time PCR system (Applied Biosystems, USA). Detection of qRT-PCR products was performed via staining with a QuantiNova SYBR Green PCR kit (Cat. 208054; Qiagen, Germany). The primer sequences are shown in Table 2.

Table 1 Primers used for RT-PCR analysis in the present study

Primer name	Primer sequence (5'→3')
<i>NtNAC2-F</i>	TTCCACATTTCCCTACTTCCTAC
<i>NtNAC2-R</i>	CCCTGACCATCCACCACATTAT

Table 2 Primers used for RT-PCR analysis in the present study

Primer name	Primer sequence (5'→3')
<i>NtNAC2-F</i>	CCACATTTCCCTACTTCCTACCTC
<i>NtNAC2-R</i>	CCCTGACCATCCACCACATT
<i>actin-F</i>	TGGTTAAGGCTGGATTGCT
<i>actin-R</i>	TGCATCCTTTGACCCATAC
δ -OAT-F	GGCACGTTCTGGTCGATTGC
δ -OAT-R	CTCCGGCCTGAATGCAAAGC
<i>NtWRKY28-F</i>	GCTGATATGTCATCACAACAGTATGG
<i>NtWRKY28-R</i>	GAGGGAGGGATTATAGTGAGGGT

Determination of Physiological Indicators

Determination of Enzyme Activity and Related Products of the Oxidation Pathway

Plants were treated with simulated drought stress (7 days), and superoxide dismutase (SOD) and peroxidase (POD) were measured with a test kit (Suzhou Keming Biotechnology Co, Ltd., China). The contents of malondialdehyde (MDA) and proline were measured as per the kit's instructions.

Measurement of the Net Photosynthetic Rate and Transpiration Rate in Transgenic Tobacco

Using an L1-6400XT portable photosynthesis measurement system, mature leaves under the same growth conditions and at the same parts were selected, and the indexes were measured at 10:00–14:00 on sunny days. Each leaf position was measured three times.

Statistical Analysis

All data were expressed as mean \pm standard error. For root length, ten seedlings were measured; for physiological parameter assays, three replicates were measured; and for qRT-PCR, three biological replicates were assessed. Microsoft Excel and GraphPad Prism 5.0 software were employed for data analysis. One-way ANOVA post hoc Duncan's multiple range test was used for multiple variable comparisons. Any difference relative to the control at * $P < 0.05$ or ** $P < 0.01$ was considered significant.

Results and Discussion

Cloning and Characterization of *NtNAC2* in Tobacco

The open reading frame of *NtNAC2* was obtained from tobacco (GenBank accession#: XM_016579112). Based on the sequence information, the *NtNAC2* gene was predicted to be

1029 bp in length and encode 342 putative amino acids. The cloned *NtNAC2* sequence was consistent with the database coding sequence.

To reveal the evolutionary relationship of *NtNAC2* from various plant species, we constructed a phylogenetic tree using amino acid sequences derived from the GenBank database. Blastp analysis showed that *NtNAC2* shared a high degree of sequence similarity with *Solanum tuberosum* (XP_006364781.1) and *Capsicum annuum* (XP_016543940.1) during evolution (Fig. 1).

Overexpression of *NtNAC2* in Tobacco Regulates Root Growth under Drought Stress

To test whether *NtNAC2* was involved in drought tolerance in tobacco, we generated eight independent *NtNAC2*-overexpression transgenic lines of tobacco (designated as *NtNAC2* lines) (Fig. 2), and we selected three transgenic lines (5, 11, 15) with *NtNAC2* transcript levels for further investigation.

The root system is the main organ of the plant that serves to absorb nutrients and moisture (Yang et al. 2016) and is the earliest part of the plant that responds to drought. Therefore, enhancing the drought resistance of roots can effectively increase the rational distribution of water to plants, reduce the damage caused by drought, and increase the plants' survival rate under stress conditions (Tian 2016). To further understand the role of *NtNAC2* in the stress response, we collected the seeds of transgenic lines for root growth experiments. Seed germination and seedling growth of T_0 generation plants revealed that there was no significant difference between WT plants and the transgenic lines in the absence of mannitol treatment, though the root length of the control plants was longer than that of the transgenic plants (Fig. 3a). Under treatment with 150 mmol L^{-1} mannitol, the root lengths of the three transgenic lines respectively were 23, 22, and 17% longer than those of the WT plants. Following treatment with 300 mmol L^{-1} mannitol, the root lengths of the three transgenic lines were 28, 31, and 18% longer, respectively, than those of the WT plants (Fig. 3b). The root growth of the transgenic lines was significantly better than that of WT plants. This result indicated that the transgenic tobacco with the *NtNAC2* gene has a more developed root system than wild type to absorb water and nutrients to compensate for the arid environment. Therefore, overexpression of *NtNAC2* strengthened the drought resistance of tobacco roots.

Overexpressing *NtNAC2* in Tobacco Promotes Enhanced Drought Tolerance

Plants change in response to the external stress conferred by drought. The lack of water balance in plants leads to decreased cell water potential and turgor pressure, resulting in the occurrence of sagging and wilting in the stem and leaves. Prolonged drought duration leads to severe water loss from the plant cell

protoplasts, leading to plant death (Sheng et al. 2017). In order to observe phenotypic differences under control conditions and drought, we tested the growth of WT and *NtNAC2*-overexpression plants under simulated drought stress. A total of 31 positive transgenic lines were obtained, and two homozygous transgenic lines (TP5 and TP15) were selected for these drought stress assays. The results indicated that there was no significant difference in the growth status or phenotype between *NtNAC2*-overexpression plants and WT plants under the control conditions (Fig. 4a). However, after 7 days of water stress induced by 20% PEG 6000, the leaves of WT plants were dehydrated and seriously weathered and yellowed, while the leaves of the transgenic plants appeared normal and fresh (Fig. 4b), indicating that overexpressing *NtNAC2* in tobacco serves to maintain water balance in plants, helping the plant grow normally under such conditions.

NtNAC2 Influences the Expression of Stress-Responsive Genes

Plants adapt to stress conditions by regulating the expression of a large number of stress-related genes. Several stress-responsive transcription factors, such as those in the MYB, WRKY, NAC, and bZIP families, are involved in plant abiotic stress responses, and some transcription factor genes have also been engineered to improve stress tolerance in model plants and crops (Li et al. 2017).

To investigate the expression pattern of *NtNAC2* and related genes under abiotic stresses, we examined the transcript accumulation of *NtNAC2* and some typical stress response genes, including δ -OAT and *NtWRKY28*, via qRT-PCR. The expression of *NtNAC2* in *NtNAC2*-overexpressed transgenic tobacco lines (especially in TP-5) was 7.0 times higher than that in WT plants when exposed to PEG (Fig. 5a), and the expression of δ -OAT in *NtNAC2*-overexpressed transgenic tobacco was 0.29 times and 0.83 times lower than that in WT plants under these conditions (Fig. 5b). The expression of *NtWRKY28* was significantly increased in transgenic tobacco (in TP-5), reaching up to 3.3 times and 2.3 times higher than that in WT plants (Fig. 5c). These results indicated that overexpression of *NtNAC2* in tobacco could significantly induce the expression of *NtWRKY28* and decrease the expression of δ -OAT, suggesting that *NtNAC2* may be involved in the process of osmotic adjustment during drought stress and might function to improve drought tolerance in tobacco.

Effects of *NtNAC2* Overexpressed in Tobacco on the Activities of Antioxidant Enzymes and Osmoprotectants

Reactive oxygen species (ROS) homeostasis is a key concern for plants, and plants have evolved an active antioxidant defense, including SOD, POD, and the synthesis and

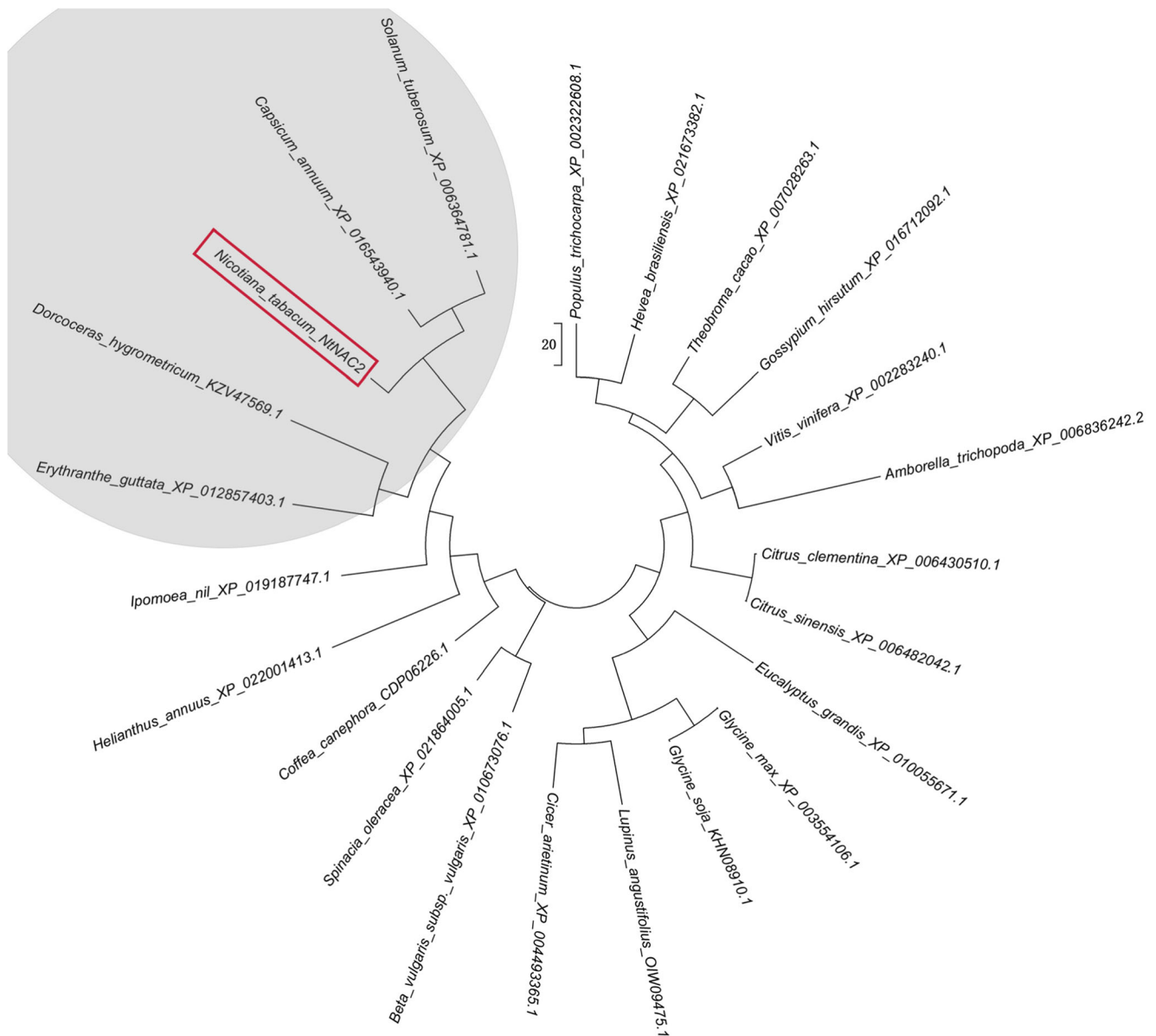


Fig. 1 Phylogenetic tree analysis of *NtNAC2* in tobacco and other species. Sequence alignment was performed using Clustal X software, and the phylogenetic tree was created and visualized using MEGA 5. Protein sequences used for alignment were as follow: *Capsicum annuum* (XP_016543940.1), *Solanum tuberosum* (XP_006364781.1), *Dorcoceras hygrometricum* (KZV_47569.1), *Erythranthe guttata* (XP_012857403.1), *Ipomoea nil* (XP_019187747.1), *Helianthus annuus* (XP_022001413.1), *Coffea canephora* (CDP_06226.1), *Spinacia oleracea* (XP_021864005.1), *Beta vulgaris subsp. vulgaris* (XP_010673076.1), *Cicer arietinum* (XP_004493365.1), *Populus trichocarpa* (XP_002322608.1), *Hevea brasiliensis* (XP_021673382.1), *Theobroma cacao* (XP_007028263.1), *Gossypium hirsutum* (XP_016712092.1), *Vitis vinifera* (XP_002283240.1), *Amborella trichopoda* (XP_006836242.2), *Citrus clementina* (XP_006430510.1), *Citrus sinensis* (XP_006482042.1), *Eucalyptus grandis* (XP_010055671.1), *Glycine max* (XP_003554106.1), and *Glycine soja* (KHN08910.1)

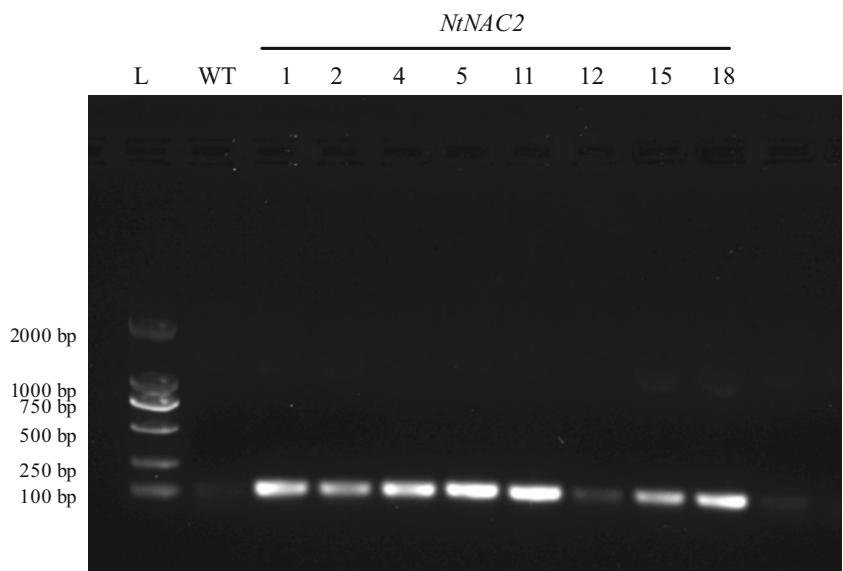
Beta vulgaris subsp. vulgaris (XP_010673076.1), *Glycine max* (XP_003554106.1), *Dorcoceras hygrometricum* (KZV_47569.1), *Glycine soja* (KHN_08910.1), *Spinacia oleracea* (XP_021864005.1), *Eucalyptus grandis* (XP_010055671.1), *Amborella trichopoda* (XP_006836242.2), *Lupinus angustifolius* (OIW_09475.1), *Hevea brasiliensis* (XP_021673382.1), *Helianthus annuus* (XP_022001413.1), *Theobroma cacao* (XP_007028263.1), and *Cicer arietinum* (XP_004493365.1)

accumulation of osmoprotectants such as proline (Ashraf and Foolad 2007; Molinari et al. 2007), to remove excessive ROS. SOD converts superoxide radicals ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2), and POD converts H_2O_2 to water on various substrates, such as electron donors. MDA is the final product of plant cell membrane lipid peroxidation and is one important marker of membrane system injury (Sun et al. 2010). Wang et al. (2009) found that the increase of alfalfa membrane

permeability was significantly and positively correlated with the accumulation of MDA, while MDA accumulation was negatively correlated with the amount of growth.

The activation of antioxidant systems is often related to transcription factors. To investigate the role of *NtNAC2* in antioxidant defense under stress conditions, the content of MDA and proline and the activities of the antioxidant enzymes SOD and POD were analyzed in *NtNAC2*-

Fig. 2 The expression level of *NtNAC2* in transgenic tobacco plants. The expression of *NtNAC2* was analyzed via RT-PCR in *NtNAC2* overexpression and WT plants grown under control conditions. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was amplified as a control for the amount of template. WT wild type; transgenic lines: 1,2,4,5,11,12,15, and 18



overexpression tobacco plants exposed to water stress. Under 20% PEG 6000 treatment, the SOD activities of the transgenic plants were 124.9, 130.3, and 105.6 U g⁻¹ (FW), respectively, and the average SOD activity of WT plants was only 65.73 U g⁻¹ (FW), indicating that the SOD activity of

transgenic plants was 82.99% higher than that of the control (Fig. 6a). The POD activities of transgenic plants were 393.9, 445.1, and 415.3 U g⁻¹ (FW), respectively, and that of WT plants was 312.4 U g⁻¹ (FW), indicating that the POD activity of transgenic plants was 33.81% higher than that of WT plants

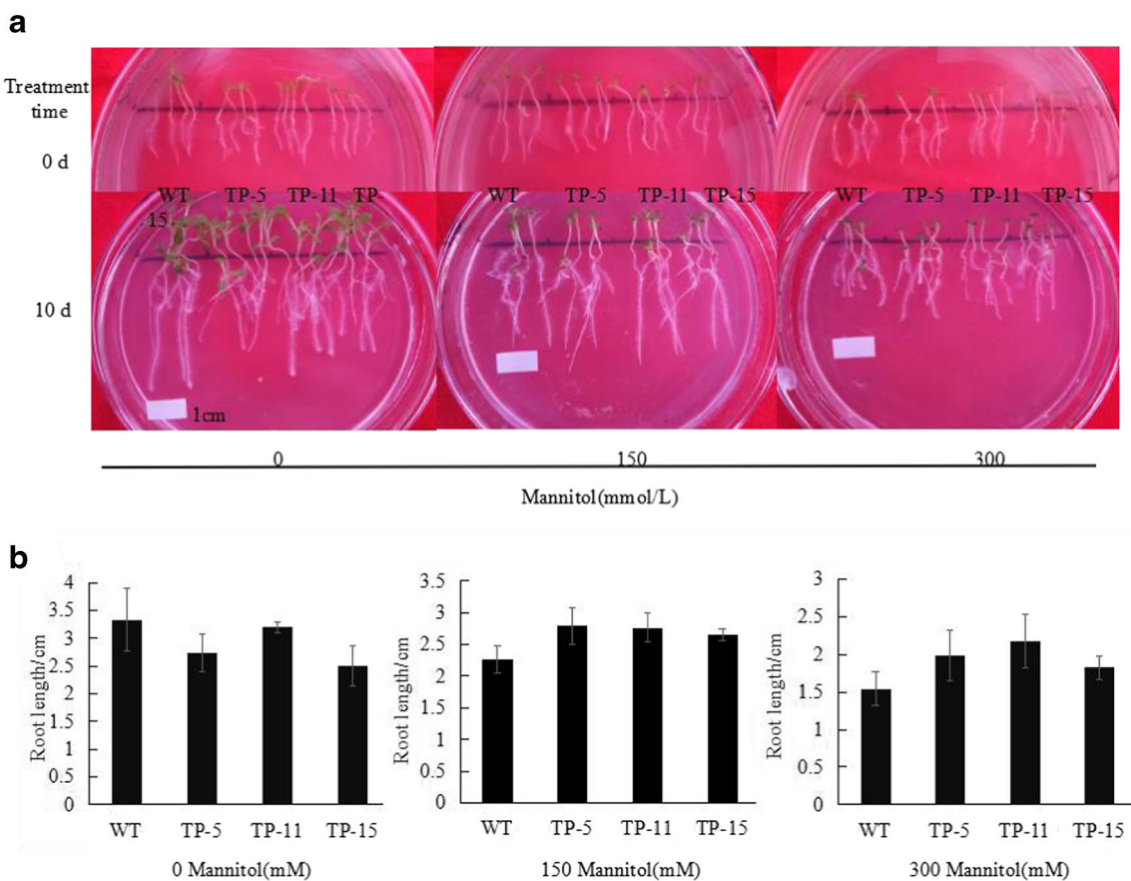


Fig. 3 **a** Root development of NT and T₀ transgenic lines on MS medium containing different concentrations of mannitol. Values are means ± SE of four (root) replicates. **b** Control and transgenic plant roots under different

concentrations of mannitol. WT wild type; transgenic lines TP-5, TP-11, and TP-15. The same is shown in Fig. 4

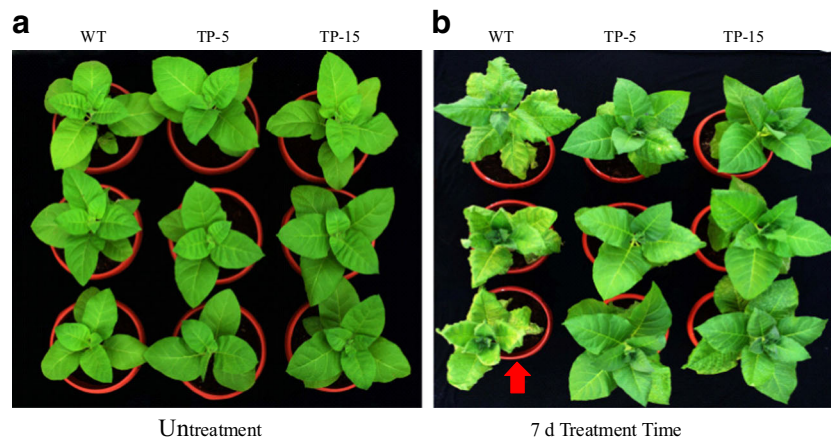


Fig. 4 Phenotypic differences between WT and transgenic tobacco seedlings under drought stress. **a** Photographs were taken under normal conditions. There was no significant difference in phenotypes between transgenic and control groups. Three-week-old seedlings grown at 22 °C under long days (16-h light/8-h dark). **b** Three-week-old seedlings watered with 20% PEG once a day. Photographs were taken 7 days after

drought stress treatment. Photographs of *NtNAC2*-overexpression plants and WT plants grown under PEG treatment. Transgenic plants maintained normal growth; the control group suffered environmental damage that led to wilted leaves. These observations indicated increased drought tolerance in *NtNAC2*-overexpression plants

(Fig. 6b). The MDA content in WT plants was $8.6 \mu\text{mol g}^{-1}$ on average, and in the transgenic plants, it was 4.1, 2.3, and $3.2 \mu\text{mol g}^{-1}$, respectively. As such, the MDA content in the transgenic plants was 57.1% lower than that in the control (Fig. 6c) (FW). The proline content of WT plants was $256.2 \mu\text{g g}^{-1}$, and in the transgenic plants, it was 35.1, 76.1, and $74.6 \mu\text{g g}^{-1}$ (FW), respectively, indicating that the proline content was 75.8% lower than that in WT plants (Fig. 6d). Proline is an important osmotic regulator in plants, and increasing proline content under drought stress helps maintain osmotic regulation, enhances cell membrane system stability, and scavenges ROS. When the proline content is low, this indicates that plants are not subject to minor drought stress and drought has less of an impact on the plant. The membrane system defense enzymes SOD and POD were overexpressed in the transgenic tobacco, serving to resist stress and senescence under drought conditions. The MDA content was lower in the transgenic tobacco than WT, indicating that the membrane system of the transgenic tobacco was intact, indicating

that the cell membrane of wild-type tobacco was seriously damaged. Together, the above results indicate that the transgenic tobacco plants were more resistant to drought than the WT plants.

Overexpression of *NtNAC2* in Tobacco Enhances the Net Photosynthetic Rate

Photosynthesis is an important metabolic process for crop growth and is a major source of material and energy for plant growth and development. Improvements in plant water use and efficiency under drought stress is an important approach for enhancing plant drought tolerance. Previous studies have suggested that improving water use efficiency in plants can be achieved by increasing the net photosynthetic rate and by inducing stomatal closure to reduce stomatal conductance. Under drought stress, stomatal opening is inhibited and water dissipation is reduced, so the final plant water retention capacity is increased, resulting in an increased tolerance of plants to

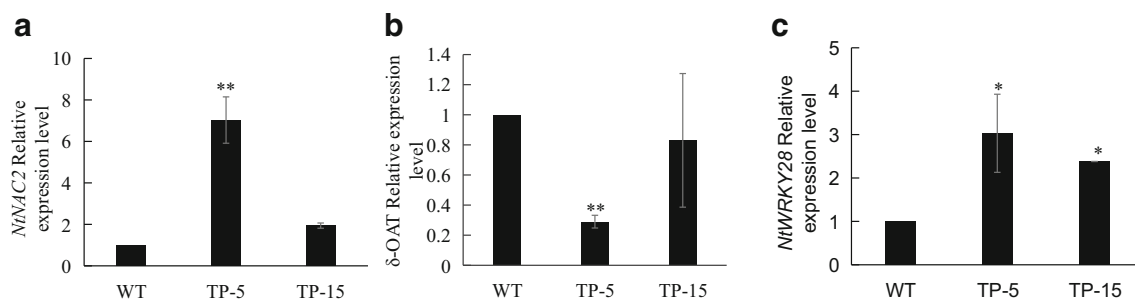


Fig. 5 Expression patterns of *NtNAC2* and some typical stress response genes, δ -OAT and *NtWRKY28*, in transgenic tobacco lines under drought stress, as assessed by quantitative RT-PCR. **a** Overexpression of *NtNAC2* in transgenic tobacco lines (especially in TP-5) was dramatically enhanced. **b** The expression of δ -OAT was decreased. **c** Overexpression

of *NtNAC2* in tobacco plants could significantly induce the expression of *NtWRKY28*. Total RNA was isolated from tobacco leaves at the indicated treatment time points. PEG polyethylene glycol 6000 treatment. Data are mean \pm SD calculated from three replicates

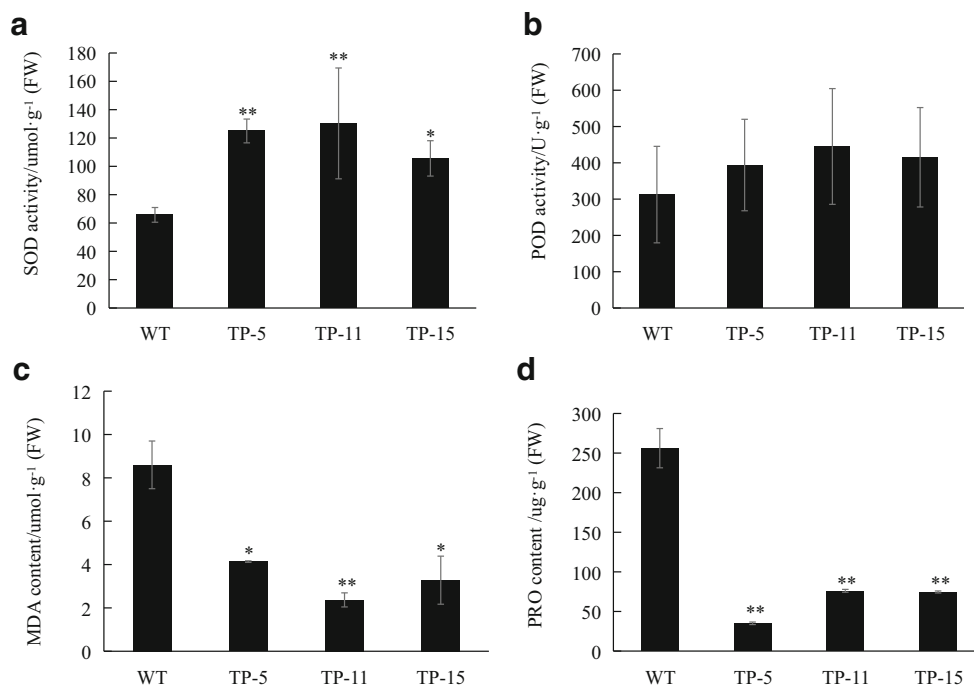


Fig. 6 The SOD and POD activity and MDA and proline (PRO) content of transgenic and wild-type tobacco plants under 20% PEG 6000 treatment. Analysis of SOD (**a**) and POD (**b**) activities in WT and transgenic lines (TP-5, TP-11, and TP-15) under drought conditions. Twenty-day-old tobacco plants were deprived of water for 7 days, and leaves were then collected to detect SOD and POD activities. SOD activity and POD activity in the transgenic lines were both significantly higher than those in the control. **c** Content of the MDA in transgenic lines

and WT tobacco under drought stress for 7 days. **d** Content of free proline in transgenic lines and WT tobacco under drought stress for 7 days. The contents of MDA and proline in the transgenic plants were significantly lower than in the control plants, indicating that the transgenic plants received less drought stress. Data are mean \pm SD calculated from three replicates. Asterisks indicate a significant difference between the WT and the two transgenic lines (* $P < 0.05$, ** $P < 0.01$). Three biological replicates were performed, which produced similar results

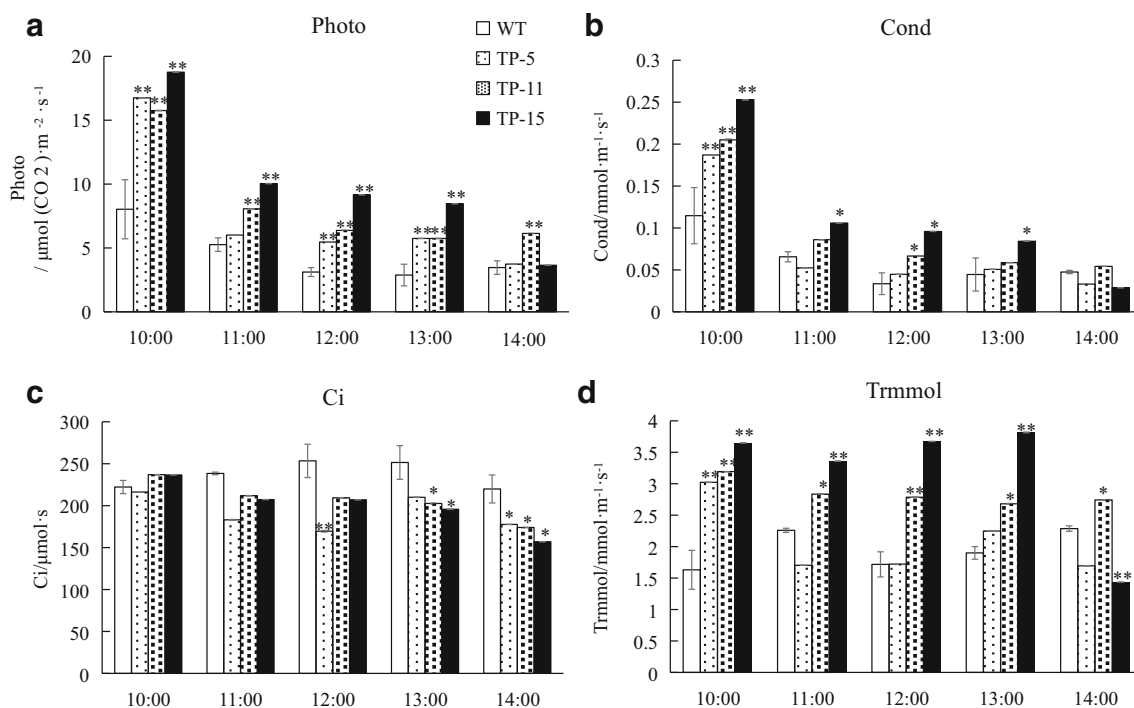


Fig. 7 Analysis of the net photosynthetic rate and the intercellular CO_2 concentration (C_i) in NT and transgenic lines in response to drought stress. **a** Photo net photosynthetic rate, **b** Cond stomatal conductance, **c** C_i carbon dioxide concentration, **d** Trmmol transpiration rate

drought (Guo et al. 2014). In this current study, the results of net photosynthetic rate analysis showed that this rate was higher in the transgenic plants compared to that in WT plants, with the net photosynthetic rate of the transgenic plants substantially higher than that of WT over the whole day (Fig. 7a). These results indicated that the expression of *NtNAC2* improved the photosynthetic characteristics in tobacco and helped maintain strong photosynthesis under drought conditions, serving to maintain plant growth and development. The level of CO₂ was 16.27% lower in the transgenic tobacco plants compared to WT (Fig. 7c), but the net photosynthetic rate was 107.45% higher in the transgenic tobacco plants compared to WT, which indicated that the transgenic tobacco plants had a better ability to maintain water than the WT plants.

Conclusions

NAC TFs act as pivotal transcriptional regulators in the adaptation of plants to environmental stresses. NAC genes have not been found in yeast, but they have been identified in dicotyledons and monocotyledons (Liu et al. 2018). A variety of information has been learned regarding NAC TFs since their discovery; however, research in this area is still in its infancy (Shao et al. 2015). Based on a review of the literature, few tobacco NAC genes have been verified to function in abiotic stress tolerance. To further study the function of the NAC TFs in tobacco, we isolated a NAC gene, named *NtNAC2*, from *Nicotiana tabacum* (K326) and demonstrated that *NtNAC2* actively regulates tolerance to drought stresses in transgenic tobacco plants.

Han et al. (2014) cloned the *NtNAC2* gene and analyzed its structure and tissue-specific expression. Construction of a phylogenetic tree suggested that all known NAC proteins involved in abiotic stress tolerance are closely related. The *NtNAC2* protein was extremely similar to the NAC proteins RD26, ANAC055, and ANAC019, which all belonged to the RD26 sub-group. The *RD26* gene was studied in *Arabidopsis thaliana* and reported to be induced by drought, and members of this sub-group are highly expressed when plants face conditions of biotic or abiotic stress (Fujita et al. 2004).

In this study, we selected three transgenic lines (5, 11, 15) for root growth experiments under drought stress and drought resistance in T₁ tobacco plants. We observed that the transgenic plants had stronger drought tolerance, which could be explained by various indicators. Under different levels of drought stress (150 and 300 mmol L⁻¹ mannitol), the roots of the transgenic plants were longer. This result is consistent with the results of Liu et al. (2016a). The roots of drought-resistant plants are longer than those of the control under drought stress. Our test results also show that the net photosynthetic rate and stomatal conductance of the transgenic plants were larger than

those of WT. In addition, the increased drought resistance of the transgenic plants could be explained by the increased expression of drought-resistant genes. Both the NAC and WRKY transcription factors play important roles to improve abiotic and biotic stress in transgenic plants (Erpen et al. 2018). Our results show that both *NtNAC2* and *NtWRKY28* expression are upregulated in transgenic tobacco, indicating that they played the active roles under drought stress. *NtNAC2*-overexpression tobacco plants showed higher activity levels of SOD and POD under drought stress conditions, and SOD and POD are beneficial to the maintenance of ROS levels during drought, and the content of MDA was lower in these transgenic plants than that in WT. Antioxidant enzyme activity results are consistent with Ning et al. (2017), which indicated that overexpression of *NtNAC2* gene enhances the tobacco stronger drought tolerance. The above observations indicate that *NtNAC2* may be involved in the process of osmotic adjustment in tobacco plants during drought stress and likely functions to improve plant drought tolerance.

Results of this study indicate that *NtNAC2* may serve as a novel target for engineering drought tolerance in crop plants and highlights important candidate genes involved in the stress response of crops. Additionally, by using genetic engineering methods, this study has opened up a possibility toward improving the drought resistance of tobacco and has provided a molecular basis for improving the drought resistant of other crops.

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Author Contribution Statement XX and YX designed and conducted the experiments. XX wrote the manuscript. LL contributed by helping with some experiments presented in the manuscript. LL helped to edit the manuscript. LL and ZD supervised the studies and revised the manuscript. All authors read and approved the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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