Improved Drought and Salt Tolerance in Transgenic Arabidopsis Overexpressing a NAC Transcriptional Factor from Arachis hypogaea

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The NAC (NAM, ATAF, and CUC) proteins share a highly conserved NAC domain and constitute a large family of plant-specific transcriptional factors. We have isolated a drought-induced NAC gene from Arachis hypogaea, named AhNAC2 (Arachis hypogaea NAC2) but its specific role remains unknown. In this study, we found that transgenic Arabidopsis overexpressing AhNAC2 lines were hypersensitive to ABA in root growth, seed germination, and stomatal closure compared to wild type Arabidopsis. The transgenic lines exhibited enhanced tolerance to drought and salinity stress, and the expression levels of 12 stress-related genes in the AhNAC2 transformed plants were higher than in wild type Arabidopsis. These results indicate that AhNAC2 is a major player in the NAC gene family involved in ABA signaling. Its role as a candidate gene for engineering drought and salt tolerance in cultivated plants is discussed.

Key words: transcription factors; drought resistance; salt tolerance; transgenic Arabidopsis; Arachis hypogaea

Plants are exposed to various abiotic stresses such as drought and high-salinity during growth and development. They have developed physiological and biochemical strategies to cope with these stresses. An important step in plant stress resistance is the activation of stress-related gene expression, which is largely regulated by specific transcription factors. Over 5% of Arabidopsis genomic sequences are used for encoding more than 1,500 transcriptional factors, about 45% of which are from families specific to plants. Several homologous families of transcription factors have been reported to play roles in eliciting stress responses.

The NAC (NAM, ATAF, and CUC) family of proteins is one of the largest families containing plant-specific transcription factors. It is characterized by a conserved DNA-binding NAC domain located in the N-terminal region and a highly different C-terminal. A large number of NAC proteins have been identified in plants. Based on similarity to known NAC domains in plants, approximately 180 potential NAC genes from Oryza sativa and Arabidopsis have been classified into two groups. Group I is divided into 14 subgroups (TERN, ONAC022, SENU5, NAP, ATNAC3, ATAF, OsNAC3, NAC2, ANAC011, TIP, OsNAC8, OsNAC7, NAC1, and NAM), the remaining four subgroups (ANAC01, ONAC003, ONAC001, ANAC063) composing group II. NAC proteins in subgroups ATAF, NAP, AtNAC3, and OsNAC3 are predicted to be involved in stress responses and related functions due to a conserved subdomain E and diverse motifs in TARs (Transcriptional Activation Regions). Three stress-related NAC genes, ANAC (ANAC019), AtNAC3 (ANAC035), and RD26 (ANAC072), are induced by drought, salinity, or ABA in Arabidopsis, and transgenic plants overexpressing these genes showed improved drought tolerance compared to the wild type, while Arabidopsis overexpressing RD26 was hypersensitive to exogenous ABA. In Oryza sativa, SNAC1 (ONAC033) was induced by drought with transgenic plants overexpressing SNAC1 showing strong resistance to both drought and salt stress. A stress-responsive NAC gene SNAC2 (OsNAC6) has also been characterized in rice. The transgenic rice overexpressing SNAC2 showed significant improved tolerance to cold, salinity and dehydration stress, as well as increased sensitivity to ABA. In addition, AtNAC2 (ANAC092/OR1) is a known salt-responsive NAC gene that has been found to be involved in the salt stress response. NTL8 (membrane-bound NAC) is also predicted to play a role in salt tolerance. Despite numerous studies investigating the roles of these genes in model plant systems, the role of these genes in other crop plants has yet to be thoroughly investigated.

We have isolated a dehydration inducible NAC-like gene from dehydrated peanut leaves (Arachis hypogaea; AhNAC2), which shows a typical significant response to abiotic stresses and ABA treatment. Here, we further confirmed the role of the AhNAC2 gene in drought and salt tolerance in AhNAC2 transformed Arabidopsis. This study provides new insight into the role and function of AhNAC2 in drought and salt stress tolerance in an important crop species.

Materials and Methods

Plant materials. Seeds of peanut (Arachis hypogaea L. cv. ShanYou 523) were sown in growth medium (vermiculite, perlite and soil; 1:1:2) and grown in plastic pots in a growth chamber with a photoperiod of 16h light at 26°C and 8h of darkness at 22°C, as described previously. Seeds of Arabidopsis wild type (Columbia ecotype,
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Col-0 were surface sterilized in 70% ethanol for 2 min and in 1% sodium hypochlorite for 10 min. After five washes with sterile water, the seeds were sowed on MS (Murashige and Skoog) medium supplemented with 2% sucrose and 0.8% agar. After 2 d of vernalization at 4 °C, the seeds were germinated and grown in a growth chamber under a daily cycle of 16 h light and 8 h dark at 20 ± 2 °C. Seven d after sowing the seedlings were planted in plastic pots in a medium of vermiculite, peat moss, and perlite (1:2:1).

Stress treatment of peanut plants. To analyze the expression of AhNAC2 under water stress, peanut 2-week-old seedlings were removed from the soil and then dehydrated on Whatman 3 mm filter paper for 0, 0.5, 1, 1.5, 2, 5, 10, and 24 h at 26 °C at approximately 60% humidity under dim light, as previously reported. High salt and exogenous hormone treatments, 2-week-old seedlings were removed from the soil and then hydroponically grown in each of the following solutions: 300 mM NaCl, 100 mM ABA (Genview, USA), 50 μM 6-benzylaminopurine (6-BA, Sigma-Aldrich, Saint Louis, MO), 30% polyethylene glycol (PEG molecular weight 6000, Fluka, Buchs, Switzerland) or deionized water (H2O, control), at 26 °C under dim light for 0, 1, 5, and 10 h. After treatment, the leaves were collected and frozen immediately in liquid nitrogen for RNA extraction or stored at −80 °C until further use.

Northern blotting analysis. Total RNA was isolated from the leaf samples of peanut plants using a modified phenol–chloroform method. Approximately 10 μg of total RNA was separated on a 1.5% w/v agarose gel containing formaldehyde and then transferred onto a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) through blotting buffer (20 × SSC; 3 mM NaCl, 0.3 M sodium citrate, pH 7.0). The membrane was fixed by incubation at 80 °C for 2 h. The AhNAC2 RNA probe was made using 474 bp of the non-NAC domain in the 3′ end of the AhNAC2 ORF (Open Reading Frame), and was amplified by PCR with primers (Supplemental Table S1; see Biosci, Biotechnol, Biochem, Web site). The probe was labeled with digoxigenin according to the manufacturer’s protocol (DIG Northern Starter Kit, Mannheim, Germany). The transferred membrane was hybridized and washed with washing buffer (100 mM Maleic acid, 150 mM NaCl, 0.3% Tween20, pH 7.5). The hybridized probes were immunodetected with anti-digoxigenin-AP and visualized with CDP-Star chiluminescent substrates using a DIG Northern Starter Kit (Roche, Mannheim, Germany).

Plasmid construction and Arabidopsis transformation. Transgenic constructs were made with a binary vector pBI121-GUSless modified from vector pBI121 (BD Biosciences Clontech, Franklin, New Jersey) without the GUS gene. To generate the overexpression construct of the AhNAC2 coding region, full-length cDNA was generated by RT-PCR with the following primers: 5′-TGC TCTAGA GCA ATG GGA ATT TCA GGT GAA CCC GAA CCC AAC C-3′ (the underlined parts are XbaI and BamHI sites respectively). The PCR product, confirmed by DNA sequencing, was cloned into modified binary vector pBI121-GUSless between the XbaI and BamHI sites, under the control of the constitutive cauliflower mosaic virus 35S promoter to generate 35S::AhNAC2 frame (Fig. 2). The overexpression construct was introduced into Agrobacterium tumefaciens strain GV3101 and then transformed into Arabidopsis wild type (Columbia ecotype, Col-0) plants by the floral dip method. Transgenic T1 lines were selected on medium containing 50 μg/L kanamycin (Sigma-Aldrich, St. Louis, MO). T2 seeds from the selected transgenic plants were germinated on a medium with 50 μg/L kanamycin, and homozygous lines were selected. The homozygous T3 progeny were then examined for the expression of the target genes by Northern blotting analysis and One Step SYBR Premix RT-PCR kit (Takara Biotechnology, South San Francisco) in a reaction volume of 20 μL to generate the first-strand cDNA according to the manufacturer’s instructions. Real-time PCR was performed using an Optical 96-well Fast Thermal Cycling Plate with ABI PRISM 7300 real-time PCR system (Applied Biosystems, San Francisco). Each reaction contained 10 μL of 2 × SYBR Premix Ex Taq™ (Takara Biotechnology, Dalian), 20 ng of cDNA, and 0.1 μM gene-specific primers in a final volume of 20 μL. The thermal cycle used was 95 °C for 30 s, then 40 cycles at 95 °C for 5 s, and 60 °C for 31 s, depending on the optimal reaction temperatures of the different primers. Stress-related genes of AT5G52310, AT5G52300, AT5G66400, AT5G51960, AT2G47190, AT1G32640, AT2G42540, AT2G25490, AT1G2570, AT1G25455, AT1G45259, AT4G25000, and an internal control gene of AT3G18780 were used for quantitative PCR with gene specific primers (Supplemental Table S2). Relative expression levels were calculated using the relative 2−ΔΔCT method, and were determined as described previously.

Assays of seed germination and growth of green cotyledons and roots. Seed germination and root growth were treated with exogenous ABA and assayed as described previously. Sterilized seeds of transgenic Arabidopsis and the wild type were sowed on half-strength MS agar plates supplemented with various concentrations of ABA (0, 0.1, 0.2, and 0.5 μM). To score green cotyledons, germinated seeds with fully expanded green cotyledons were recorded on day 5 after sowing. Three independent experiments were performed (30 seeds in each line per test). Assays of root growth were carried out by transferring 4-d-old seedlings of transgenic Arabidopsis and the wild type under normal spraying onto half-strength MS medium agar plates with ABA treatments (0, 5, 10, and 15 μM). Primary root lengths of more than 20 seedlings in each line were measured on day 8 after the start of treatment, each with three independent repeats.

Measurement of stomatal aperture. In the stomatal aperture experiments, fully expanded leaves of 4-week-old plants of transgenic Arabidopsis and wild type plants were excised and incubated for 2 h in a stomatal opening solution containing 50 mM KCl, 50 μM CaCl2, and 10 mM MES (pH 6.15), and then transferred to ABA solution containing 0, 1, or 10 μM ABA for 1 h. The stomatal aperture was measured as described previously. Three independent experiments were performed, and more than 50 stoma from three samples were measured in each replicate.

Drought stress tolerance assays and water-loss measurements. Survivability tests under drought conditions were conducted on three independent transgenic lines and wild type Arabidopsis. Seeds were germinated and grown on half-strength MS agar medium for 1 week and then transferred to soil, as described above. At the rosette stage (4 weeks after sowing), half of the samples were subjected to drought stress by withholding irrigation for 10 d to evaluate drought tolerance visually (the leaves wilted). These samples were then rewatered (once a day) for 4 d. The remaining samples were grown under a standard irrigation regime (watering once a day) for 14 d as a control. The survival rates of the plants were calculated as the ratio of plants with green leaves to the total number of plants used. All experiments were repeated at least 3 times, and more than 32 plants of each line were used in each replicate. For water-loss measurements, the aerial parts of 5-week-old plants were separated from the roots, placed on weighing dishes, and allowed to dry slowly on a laboratory bench (26 °C, 60% relative humidity). The weights of the samples were recorded at regular intervals (0, 0.5, 1, 2, and 4 h). The water-loss rate was a percentage ratio of sample weight at each time point related to the sample weight of the 0 h treatment. Experiments were repeated at least 3 times, and more than 20 samples from each line were used in each replicate.

Salt tolerance assays and chlorophyll content. In survivability tests under salt stress conditions, transgenic lines and wild type Arabidopsis were germinated and grown on half-strength MS agar medium for 6 d. The seedlings were transferred onto Whatman paper with 300 or
500 mM NaCl solution for 4 d treatment. The total chlorophyll content in each sample (five seedlings each experiment, three replicates) was determined by spectrophotometry at 663 nm and 645 nm after extraction with 80% acetone by following formula: \[ \text{Chls a + b} = 20.21 \times A_{645} + 8.02 \times A_{663}. \] Absorbance at 663 and 645 nm are \( A_{663} \) and \( A_{645} \) in the equation respectively. For the salt tolerance experiments, 4-week-old soil cultured plants were watered for 12 d at 4 d intervals with increased concentrations of NaCl of 100, 150, and 250 mM. The survival rates of the 6-week-old plants were calculated as the ratio of plants with green leaves to the total number of plants (32 shoots each experiment, replicates) under 250 mM NaCl treatment.

Statistical analysis. Statistics analysis of all data was carried out at the \( \alpha = 0.05 \) level using SPSS (version 11.5, SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) was performed, and the means of the significant ANOVA effects were compared using Tukey’s HSD post hoc comparisons. The cDNA sequence of \( \text{AhNAC2} \) was submitted to the GenBank database under accession no. EU755023.

Results

Gene structure and sequence analysis of \( \text{AhNAC2} \)

Genomic sequencing indicated the general intron patterns of \( \text{AhNAC2} \) gene (Supplemental Fig. S1A). A DNA fragment (1.7 kb), with three exons and two introns, was obtained from the peanut genomic DNA by PCR with two specific primers (Supplemental Table S1). The full-length cDNA of \( \text{AhNAC2} \) consists of 1,565 bp nucleotides (\( \text{AhNAC2}, \) Genbank Accession no. EU755023), including 102 bp of the 5′-untranslated region (5′-UTR), 413 bp of the 3′-untranslated terminal (3′-UTR), and 1,050 bp of the ORF encoding a 349 amino acid protein with a calculated molecular mass of 39.14 kDa and an isoelectric point of 7.35. Phylogenetic analysis revealed that the \( \text{AhNAC2} \) protein belongs to the AtNAC3 subgroup (Supplemental Fig. S1B), all the subgroup proteins sharing a highly conserved sequence at the NAC domain in the N-terminal region and some unknown motifs in the C terminal region (Supplemental Fig. S2).

Effects of abiotic stress and hormone treatment on expression of the \( \text{AhNAC2} \) gene in peanut plants

We examined the expression of \( \text{AhNAC2} \) under dehydration, salt, and ABA treatment in peanut plants by northern blotting analysis. When peanut seedlings were treated under dehydration stress, the mRNA of the \( \text{AhNAC2} \) gene was quickly induced at 0.5 h, and remained high until 24 h (Fig. 1). The \( \text{AhNAC2} \) transcript in the peanut seedlings treated with 300 mM NaCl or 30% PEG was upregulated at 5 and 10 h. \( \text{AhNAC2} \) expression in the seedlings was detected at 1 h during treatments with 100 \( \mu \)M exogenous ABA, but there were no obvious changes under the 50 \( \mu \)M 6-BA or the water (H\(_2\)O) treatment (Fig. 1). Thus \( \text{AhNAC2} \) is a dehydration- and salt-induced gene in the peanut, and it is also shows a response to ABA.

\( \text{AhNAC2} \) overexpression increased sensitivity to ABA in transgenic Arabidopsis

The overexpression construct with \( \text{AhNAC2} \) under the control of the CaMV 35S promoter was transformed into wild type Arabidopsis (ecotype Col-0; Fig. 2A). Three independent lines (\( \text{AhNAC2-OX1}, \text{AhNAC2-OX2}, \) and \( \text{AhNAC2-OX3} \)) were generated and verified by expres-

![Fig. 1.](image)

**A.** Northern blotting analysis of expression of the \( \text{AhNAC2} \) gene in 14-d-old peanut seedlings under dehydration (plants from the soil were dehydrated on Whatman 3 mm filter paper at 26 °C). B. Salt (300 mM NaCl), PEG (30% PEG6000, w/v), ABA (100 \( \mu \)M), 6-BA (50 \( \mu \)M) and control (H\(_2\)O) treatment. Each lane was loaded with 10 \( \mu \)g of total RNA prepared from treated samples. \( rRNA \) was visualized by ethidium bromide staining as a loading control.

Evaluation of \( \text{AhNAC2} \) overexpressed Arabidopsis to drought and salt tolerance under greenhouse conditions

Strong induction of \( \text{AhNAC2} \) under water stress led to a functional test of \( \text{AhNAC2} \) under drought conditions. Three independent transgenic lines (\( \text{AhNAC2-OX1}, \text{AhNAC2-OX2}, \) and \( \text{AhNAC2-OX3} \)) and the wild type were planted in soil in a greenhouse. After drought treatment, the 35S::\( \text{AhNAC2} \) transgenic plants performed significantly better than the wild type in terms of survival rates (Fig. 4A) and greenness of leaves (Fig. 4C) under drought stress. We also observed that more than 70% (\( \text{AhNAC2-OX1} \), 81.25 \( \pm \) 3.60%; \( \text{AhNAC2-OX2} \), 75.00 \( \pm \) 4.08%; \( \text{AhNAC2-OX3} \), 87.50 \( \pm \) 3.66%) of the transgenic plants in the three independent lines remained vigorous,
while the wild type plants had more than 70% fatality (wild type, 22.56 ± 7.44%) (Fig. 4A). The rate of water loss from the 35S::AhNAC2 plants was lower than that from the wild type plants, as measured by the fresh-weight loss of detached shoots (Fig. 4B). Independent transgenic lines (AhNAC2-OX1, AhNAC2-OX2, and AhNAC2-OX3) were then selected to explore the salt tolerance of AhNAC2 overexpressing Arabidopsis. The total chlorophyll contents dropped dramatically in the wild type plants (from 5.871 ± 0.254 mg/g FW to 2.387 ± 0.169 mg/g FW under 300 mM NaCl, and to 2.450 ± 0.390 mg/g FW under 500 mM NaCl), but remained at higher levels in the AhNAC2 transgenic line (AhNAC2-OX1, from 6.253 ± 0.095 mg/g FW to 3.524 ± 0.154 mg/g FW under 300 mM NaCl, and to 3.132 ± 0.133 mg/g FW under 500 mM NaCl) under salt stress (Fig. 5B). The shoots and roots of all the transgenic seedlings continued to grow and their leaves remained green during stress treatment, while the leaves of the wild type seedlings were thin and yellow (Fig. 5A). The survival rates of the 6-week-old transgenic plants were higher than the wild type plants in the salt tolerance experiments (Fig. 5C). In addition, the wild type plants had white or yellow leaves and less root biomass than the transgenic seedlings (data not shown). This indicates that overexpression of AhNAC2 improved the drought and salt tolerance of the transgenic plants.

Expression of stress-related genes in AhNAC2 overexpress Arabidopsis

We predict that AhNAC2 acts as an upstream activator of some stress-related genes by increasing stress-related signaling. Fifteen stress-related genes were selected and analyzed by quantitative real-time PCR. Eleven genes (RD29A, RD29B, RAB18, AtMYB2, AtMYC2, ERD1, COR47, COR15a, KIN1, AREB1, and CBF1) were significantly upregulated (Fig. 6) in AhNAC2 the overexpression transgenic line (AhNAC2-OX3) compared with wild type under normal conditions (p < 0.05). In addition, AMY1 was slightly upregulated, while the expression of CBF2, CBF3, and CBF4 showed little change (Fig. 6). These results indicate AhNAC2 overexpression can affect the expression levels of stress-related genes in Arabidopsis.

Discussion

Peanuts (Arachis hypogaea) are one of the most important economic crops in the world. In China, peanut distribution is mainly in arid and semiarid regions, and frequently faces drought stress which directly limits its growth and production. Transcriptional factors play an important role in the response to abiotic stresses, especially the NAC family transcription factors. We have isolated five putative NAC genes from A. hypogaea, AhNAC1, AhNAC2, AhNAC3, AhNAC4 and AhNAC5, and found that AhNAC2 may be involved in ABA signal transduction and drought response. Phylogenetic characterization and sequence analysis revealed that AhNAC2 is a stress-related NAC protein that belongs to the AtNAC3 subgroup. Subcellular localization and a yeast transactivation assay indicated that the AhNAC2-GFP fusion protein is localized in the nucleus and that the C terminal region of the protein has transcriptional activity. Moreover, the AhNAC2 protein can bind to specific NACRE (NAC Response Elements) in vitro (Liu X et al., unpublished results).

We have noticed that AhNAC2 is induced by dehydration and ABA. Here we found that the abundance of AhNAC2 mRNA was quickly upregulated at 0.5 h, and kept at high levels for up to 24 h of drought stress. Meanwhile, we detected upregulation of the AhNAC2 gene in the peanut seedlings treated with 300 mM NaCl or 30% PEG for 5 h, as well as 100 μM exogenous ABA for 1 h. Together, these data imply that AhNAC2 is a dehydration- and salt-induced gene in the peanut, and responds to ABA. Previous studies have indicated that several members of the AtNAC3 subgroup of NAC proteins (such as ANAC019, ANAC055, and ANAC072) are thought to be related to drought, salt and other stresses. All of these genes have similar motifs outside the NAC domain. These data provide evidence com-
firming that the AtNAC3 subgroup shares a conserved role in the response to stress stimuli and is a potential genetic resource for the improvement of crop stress tolerance.

Numerous reports have suggested that overexpression of stress-inducible transcription factors, such as CBF3 (DREB1A),26 CBF1 (DREB1B),27 DREB2A,28 ABF2,29 ABI5,30 and CBF4 (DREB1D),31 can increase tolerance to drought, salinity, and low temperature in Arabidopsis and other plant species. In this study, we overexpressed AhNAC2 in Arabidopsis and found that the root growth of the transgenic plants was gradually inhibited by ABA, and that the percentages of green cotyledons and the stomatal apertures of the transgenic lines were significantly lower than the wild type. These data suggest that overexpression of AhNAC2 in Arabidopsis results in increased sensitivity to ABA. In addition, the lower water losses under drought stress and higher chlorophyll contents under salt stress in the transgenic leaves compared to the wild type leaves further confirm that the transgenic plants obtained enhanced drought and salt tolerance.

The transgenic lines had later flowering and smaller leaves than the wild type plants, and the plant height and leaf numbers of the transgenic plants were lower than the wild type, however plant weight and main root length were not significantly different between the transgenic lines and the wild type (Supplemental Table S3). These phenotypes are similar to previously reported transgenic plants, such as overexpression of DREB1A and the constitutive active form of DREB2A,26,28 RD26 (ANAC072) overexpression transgenic Arabidopsis.

![Graph A: Comparison of primary root length of transgenic and wild type Arabidopsis seedlings treated with various concentrations of ABA (0, 5, 10, and 15 μM).](image)

Fig. 3. Transformation of AhNAC2 Enhanced ABA Sensitivity in Root Growth, Stomatal Apertures, and Green Cotyledons in Arabidopsis.

A, Comparison of primary root length of transgenic and wild type Arabidopsis seedlings treated with various concentrations of ABA (0, 5, 10, and 15 μM). Data represent means for 20 plants, vertical columns are means (±SD). WT, wild type; OX1, AhNAC2 transformed line AhNAC2-OX1; OX2, AhNAC2 transformed line AhNAC2-OX2; and OX3, AhNAC2 transformed line AhNAC2-OX3. B, ABA inhibition of root growth of transgenic and wild type Arabidopsis seedlings. Six-d-old plants were treated in half-strength MS medium supplemented with 0 or 10 μM ABA for 8 d. Yellow bars represent 1 cm. C, Effects of ABA treatment on seed germination of transgenic and wild type plants of Arabidopsis. Seedlings were treated in half-strength MS medium supplemented with 0.2 μM ABA for 7 d. D, Green cotyledon rates of AhNAC2 transformed and wild type Arabidopsis. Seeds were treated with ABA at concentrations (0, 0.1, 0.2, and 0.5 μM) for 5 d. Data represent means ± SD (n = 30). E, Effects of ABA on stomatal apertures of transgenic and wild type plants of Arabidopsis. Leaves of Arabidopsis were treated with 0 μM (control), 1 μM, or 10 μM ABA for 1 h. F, Stomatal aperture transgenic lines and wild type plants of Arabidopsis in response to 0, 1, and 10 μM ABA. Bars represent SD (n = 50, 50 stoma per data measurements). Asterisks indicate significant differences between AhNAC2 transgenic plants and the wild type plants (p < 0.05).
Fig. 4. Overexpression of AhNAC2 Enhanced Drought Tolerance in Arabidopsis.
A. Survival rates of independent AhNAC2 overexpressing lines (AhNAC2-OX1, AhNAC2-OX2, and AhNAC2-OX3) and wild type (WT) Arabidopsis plants under drought treatment. Columns are means ± SD (n = 32), and asterisks indicate significant differences between the wild type and AhNAC2 overexpress plants (p < 0.05). B. Water loss rates of AhNAC2 overexpress lines (AhNAC2-OX1, AhNAC2-OX2, and AhNAC2-OX3) and wild type (WT) Arabidopsis plants under water-loss treatment. Columns are means ± SD (n = 20), and asterisks indicate significant differences between the wild type and AhNAC2 overexpress plants (p < 0.05). C. Transgenic and wild type Arabidopsis plants treated under drought stress. Three-week-old plants of AhNAC2 overexpression lines (AhNAC2-OX1, AhNAC2-OX2, and AhNAC2-OX3) and wild type (WT) plants were grown for 10 d without watering and then watered for 4 d.

Fig. 5. Overexpression of AhNAC2 Enhanced Salt Stress Tolerance in Arabidopsis.
A. Arabidopsis transgenic and wild type seedlings subjected to salt stress. Six-d-old seedlings of AhNAC2 overexpress lines and the wild type were treated on filter paper with 300 mM and 500 mM NaCl for 4 d. B. Total chlorophyll contents in transgenic and wild type seedlings of Arabidopsis under salt stress. C. Survival rates of AhNAC2 overexpress lines (AhNAC2-OX1, AhNAC2-OX2, and AhNAC2-OX3) and wild type Arabidopsis plants under salt stress. The 4-week-old plants were watered for 12 d at 4 d intervals with increasing concentrations of NaCl, 100, 150, and 250 mM. The results were recorded on the 14th day after treatment. Columns are means ± SD (n = 32), and asterisks indicate significant differences between the wild type and AhNAC2 overexpress plants (p < 0.05).
showed improved drought tolerance compared to the wild type, and the homologous genes ANAC (ANAC019) and AtNAC3 (ANAC035) also showed a similar response. AhNAC2 has a motif comparable to these NAC proteins in amino acid sequence. One possible explanation is that AhNAC2 replaces or enhances the function of some Arabidopsis NAC proteins.

We examined the expression levels of 15 stress-related genes in AhNAC2 overexpress transgenic Arabidopsis, and found that 12 were upregulated (RD29A, RD29B, RAB18, AtMYB2, AtMYC2, ERD1, COR47, COR15a, KIN1, AREB1, CBF1, and AMY1). RD29A and RD29B are located close to one another on the Arabidopsis genome. The promoter of the RD29A gene contains both DRE (Drought Response Elements) and ABRE (ABA Response Elements), but RD29B has only ABRE. The RD29A is a marker gene in response to drought and ABA, whereas the expression of RD29B is ABA-dependent and responds only to ABA. This further establishes that AhNAC2 is involved in the ABA-dependent pathway due to upregulation of RD29B and RD29A. AtMYB2 and AtMYC2 are significant stress-related transcription factors, and they function as transcriptional activators in the ABA signal transduction pathway under drought stress in plants. More importantly, the expression of COR47, COR15a, KIN1, and CBF1 in transgenic plants was higher than in the wild type plants which the role of these four genes under cold stress tolerance. CBF1 may cause the expression of the CORs genes by an independent ABA pathway, improving freeze stress tolerance. Although the cold tolerance of transgenic plants calls for further research, the AhNAC2 gene may be involved in the regulation of ABA-independent pathway by upregulation of related target genes. This provides evidence of the involvement of AhNAC2 in complex transcriptional networks in Arabidopsis.

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