Research Square

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Isolation and functional analysis of the Larix olgensis LoNAC3 transcription factor gene

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Abstract

Background

Larch is an important timber tree species. The traditional methods of tree genetic breeding have been progressing slowly. It is necessary to carry out gene function analysis and genetically modified breeding research. The NAC transcription factor family is a plant-specific transcription factor family with various biological functions, as shown in recent research. However, there are few studies on the NAC gene among gymnosperm coniferous species.

Results

LoNAC3 with complete cds was identified and isolated from the cDNA of Larix olgensis based on transcriptome data. The cDNA length of *LoNAC3* is 1185bp, encoding 394 amino acids, with a conserved NAM domain located at the N-terminus, and subcellular localization in the nucleus. The results of real-time quantitative PCR analysis showed that the relative expression level of *LoNAC3* was higher in the needles of *L. olgensis*, and the response to salt and drought treatment was more obvious. In addition, *LoNAC3* was induced to express by methyl jasmonate (MeJA) and abscisic acid (ABA). By observing the phenotype of overexpressed *LoNAC3* tobacco, it was found that overexpressed tobacco is shorter and blooms earlier than wild-type tobacco. Under abiotic stress, overexpressed tobacco has lower germination rates and poorer growth status. Transgenic tobacco under stress treatment has a higher malondialdehyde (MDA) content than wild-type tobacco, while peroxidase (POD) activity is lower than wild-type tobacco.

Conclusions

Through the analysis of *LoNAC3* sequence and promoter expression, it can be concluded that *LoNAC3* is involved in the drought and salt stress response processes of *L. olgensis*, and is induced by ABA and MeJA expression. Overexpression of *LoNAC3* leads to stunted tobacco growth and negatively regulates its tolerance to drought and salt stress through the reactive oxygen species pathway. The preliminary analysis of the expression pattern and function of the *LoNAC3* can provide a theoretical basis and high-quality materials for genetic improvement of larch in later stages.

Background

Abiotic stresses such as drought and salt can lead to a decrease in the yield and quality of agricultural and forestry crops[1]. Drought can lead to a decrease in plant enzyme activity, which affects the growth rate of plants, especially in the seedling stage[2], leading to physiological disorders in plants; Salt can cause changes in soil osmotic potential, plant water potential, and ion toxicity, thereby affecting the growth and development process of plants[3], because the impact of changing osmotic potential on

plants is similar to that of drought stress. Research has found that drought can cause changes in plant enzyme activity, thereby affecting its physiological functions. It mainly affects two aspects of plant photosynthesis and respiration. Drought stress can directly lead to changes in chloroplast structure, reducing photosynthetic pigment content and leading to a decrease in photosynthetic rate[4]. Mild drought can increase crop respiration rate, which gradually decreases with increasing drought severity[5]. Non biological stresses such as drought and salt can directly lead to the accumulation of proline in plants, such as cell membrane lipid peroxidation, accumulation of malondialdehyde in cells, resulting in the destruction of biofilm structure and loss of function[6]. It can also cause protein denaturation, DNA strand breakage, and other harmful cellular effects, leading to cellular dysfunction[7].

NAC transcription factors are a class of plant specific transcription factors widely distributed in terrestrial plants, and are also one of the gene families with the most transcription factors in the plant genome. In the early stage, 117 transcription factors of the NAC family have been found in *Arabidopsis* [8]. With the deepening of sequencing work, 163 and 79 NAC family members were found in poplar and rice, respectively [9, 10]. In early research, the NAC family was divided into three subfamilies: ATAF, NAM and, OsNAC3 [11]. With the deepening of research and the completion of model plant genome sequencing work, researchers conducted a comprehensive analysis of the NAC family genes in rice and *Arabidopsis*, dividing NAC family proteins into two major groups including 18 subgroups [12]. NAC genes are generally composed of three exons and two introns. The first two exons form a highly conserved special structural domain at the N-terminus of NAC proteins, while the third exon forms a diverse transcriptional activation domain at the C-terminus, which is an important recognition feature of NAC protein structure [13]. The special structural domain of NAC protein at the N-terminus is generally composed of about 150 amino acids, which can be divided into five subdomains: A, B, C, D, and E. Among them, the three subdomains A, C, and D belong to highly conserved domains, and the remaining two subdomains with weak conservation are only conserved in some NAC subgroups [14]. The C-terminus of NAC protein has highly repetitive simple amino acids, containing a large amount of Ser, Thr, Glu, and some acidic amino acid residues [15]. Some studies have found that the C-terminal domain of NAC protein has the function of transcriptional activation domain in yeast and plant cells [16].

In the NAC gene family, the earliest discovered *Petunia* NAM gene is related to plant morphogenesis. Plants with NAM gene mutations may wither during the seedling stage due to the inability to form normal apical meristem tissue in the stem [14]. The NAC transcription factor family has been found to not only participate in plant growth, development, and morphogenesis, but also participate in various stress resistance responses, especially abiotic stress. The double mutant plants of *Arabidopsis CUC1* and *CUC2* were found to exhibit healing of stamens, sepals, and cotyledons [13]. The *Arabidopsis CUC3* gene is involved in regulating the formation of stem tip meristem and cotyledon edges [17]. Overexpression or inhibition of the *NAC1* gene in *Arabidopsis* leads to an increase and decrease in the number of lateral roots [18]. Overexpression of the soybean NAC gene *GmNAC20* in *Arabidopsis* promotes the generation of lateral roots in plants [19]. *Arabidopsis ANAC019*, *ANAC055*, and *ANAC072* can bind to the promoter sequences of drought response factors. Overexpression of *Arabidopsis* transgenic plants can improve drought resistance [20]. *ATAF1* plays a role in drought resistance by

participating in regulating the expression of related genes in osmotic stress response [21]. *Arabidopsis ANAC096* can help plants survive under dehydration osmotic stress [22]. *TsNAC1* can target an important proton transport protein to improve plant salt tolerance [23].

Previous studies have mainly focused on NAC transcription factors discovered in model plants. In recent years, with the progress of transcriptomics and molecular breeding technology, the NAC family transcription factors in coniferous tree species have gradually been discovered, and research on gene function is also gradually underway. Overexpression of the *PwNAC2* in *Arabidopsis* can enhance the tolerance of plants to stress treatment [24]. The PtaVNS protein in *Pinus taeda* participates in the process of tracheid formation by regulating the transcription induction of genes required for SCW formation and PCD [25]. *LoNAC1* and *LoNAC2* in *L. olgensis* can be induced to express under drought and salt stress [26].

In this study, *LoNAC3* gene in the NAC family of *L. olgensis* was identified through transcriptome data from the laboratory. The gene coding region sequence was cloned and isolated from the cDNA of *L. olgensis*, and the *LoNAC3* promoter sequence was obtained from the gDNA of *L. olgensis*. Through analysis of sequence information and expression ability, as well as research on overexpressing *LoNAC3* tobacco, the potential role of *LoNAC3* gene in plant growth and development was preliminarily elucidated, enriching the NAC family genes of larch and providing a molecular basis for studying the growth and stress resistance mechanisms of larch.

Methods

Plant materials, growth conditions, and treatments

The seedlings of *L. olgensis* (Jixi species) and wild-type tobacco used in the experiment were preserved by the State Key Laboratory of Tree Genetics and Breeding of Northeast Forestry University.

Non-lignified (approximately 60 days), semi-lignified (approximately 120 days) and lignified (180 days) *L. olgensis* were used as experimental material. Roots, stems and needles were separated, wrapped in aluminium foil, labelled, rapidly frozen in liquid nitrogen and stored at −80°C. The 60-day *L. olgensis* material was treated with solutions of PEG6000 (25% w/v), NaCl (250 $mmol.L^{-1}$), $NaHCO_3$ (50 $mmol.L^{-1}$), 50 $mg.L^{-1}$ Gibberellin A3 (GA_3), abscisic acid (ABA), N-(phenylmethyl)-9H-purin-6-amine(6-BA), indole-3-acetic acid(IAA), methyl jasmonate (MeJA), and water (control) at 0h, 48h, 72h, and 84h, respectively. Samples were taken uniformly at 96h (three samples for each treatment and time) [26].

After disinfection, tobacco seeds are inoculated onto 1/2MS solid culture medium to germinate and obtain sterile seedlings. Select individual plants with good growth potential for tissue culture and propagate them as genetic transformation receptor materials [27].

RNA extraction and DNA extraction

Total RNA was extracted using Universal Plant Total RNA Extraction Kit (BIOTEKE, Beijing, China), and cDNA obtained through reverse transcription using total RNA as a template (ReverseScript ^{RT} reagent Kit, TaKaRa). Plant DNA was extracted using Universal Plant DNA Extraction Kit (Omega).

Isolation and sequence analysis of LoNAC3

Design primers based on transcriptome data (forward:5'ATGATTTATCAGTATGGGCCAG3'; reverse:5'TTACTTACGAGTTCCATAAATCTAT3') and amplify PCR using cDNA from *L.olgensis* as a template. Purify the target DNA bands and sequence them to obtain the sequence information of *LoNAC3*. Upload the sequence information of the successfully aligned *LoNAC3* gene to NCBI (GenBank: MW206689).

Perform sequence multiple comparison analysis on the amino acid sequence of *LoNAC3* using BioEdit software. MEGA5.0 software was used to construct the Neighbour-Joining tree, and the structural domains of the sequences were predicted and analysed with the online tool NCBI CD-search. Protparam (https://web.expasy.org/cgi-bin/protparam/protparam) was used to predict and analyse the physicochemical properties of the protein encoded by *LoNAC3* gene. GOR4 (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html) and SwissModel (https://www.swissmodel.expasy.org/) was used to predict the secondary structure and three-dimensional structure of the protein, respectively. Utilizing WOLF PSORT (https://wolfpsort.hgc.jp/) predict the subcellular localization of proteins.

Isolation and Vector construction of LoNAC3 promoter

Primers were designed based on the NCBI *Larix kaempferi* genome (taxid: 54800, *L. kaempferi* GenBank assembly GCA_013171265.2 Nucleotide BLAST): Forword primers (5'-3') GCCCCGTTTGTCGTAG; Reverse primers (5'-3') CATGTCCTGTAAACAGAAAACAGGATTT. Using the DNA of *L. olgensis* as a template, a fragment of approximately 2000 bp before the start codon (ATG) of *LoNAC3* was cloned and insert it into the BamH□ and Nco□ enzyme cleavage sites of the pCAMBIA1301 plant expression vector to obtain the recombinant plasmid.

Activity validation and bioinformatics analysis of LoNAC3 promoter

Transfer the constructed plasmid (*LoNAC3*pro:: GUS) into *Agrobacterium* GV3101.Cultivate *Agrobacterium* in LB containing 50mg/L kan and Rif until OD600 = 0.8 (200rpm, 28 ℃). Soak tobacco seedlings that have grown for a week in *LoNAC3*pro:: GUS *Agrobacterium* (OD600 = 0.5 ~ 0.6) suspended at 1/2MS, shake for 3 to 4 hours (120 rpm, 25 ℃), and then incubate them in dark at 25 ℃ on 1/2MS medium for 2 days. Place them in the prepared GUS staining solution. After incubating at 37 ℃ for 12 hours, decolorize the stained tobacco seedlings in 95% alcohol. Utilizing PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) predict the cis acting element of the promoter.

Analysis of LoNAC3 expression in L. olgensis

Primer Premier 5.0 software designed the qRT-PCR primers: Forword primers (5'-3') GCACGATTGTTATTTTAGCC; Reverse Primers (5'-3') ACACGAGAGTCTTTCGCATTCC, the primers specifically screened by gel electrophoresis detection. *LoTubulin* was selected as a reference gene (Forword primers:5'GCCGTGCTGCTGGATAATGAGG3';Reverse primers:5'TGTCTGGAACTCAGTCACATCAACG3') and amplification was performed automatically according to the Real Master Mix (SYBR Green) kit 20ul system and an ABI 7500 real-time PCR instrument was used. Three replicates were set up in the quantitative PCR instrument and the results analysed by the $2^{-\Delta\Delta Ct}$ method [28].

Vector construction and genetic transformation of LoNAC3 gene

Insert the CDS sequence of the cloned *LoNAC3* into the XbaI and HindIII enzyme cleavage sites of the VB191104 plant expression vector to obtain the recombinant plasmid. After successful sequencing of the connecting products, the plasmid was extracted and transferred to *Agrobacterium* GV3101. Using the *Agrobacterium* mediated leaf disc transformation method, tobacco leaves were infected with *Agrobacterium* GV3101 (VB191104-*LoNAC3*), and the infected transgenic leaves differentiated into resistant buds on a differentiation medium containing hygromycin (25mg/L). After the isolated resistant buds were extracted from a culture medium containing hygromycin, they were transplanted to a culture medium containing hygromycin for rooting. After rooting, tobacco seedlings were transplanted into a nutrient soil substrate for cultivation [27].

Identification and phenotypic observationof overexpressed tobacco

Tobacco leaf DNA was extracted from the seedlings, and PCR identification was performed. Targeted bands were confirmed as positive seedlings. The seeds of the positive seedlings were collected and screened for hygromycin. After 2 generations of resistance screening, harvest T2 generation resistant tobacco seeds, and after sowing T3 generation on 1/2MS medium for 10 days, extract the entire RNA of each strain. Identify the RNA level through RT-qPCR, and select the three strains with the highest relative expression level for the next experiment.

Select the three overexpression strains and wild-type tobacco with the highest relative expression levels and sow them on 1/2MS medium. After normal growth for 7 days, move them to the soil to continue growing. Observe the growth status of the tobacco, record the height and diameter of each strain after stem extraction (45 days), calculate the growth rate, and observe the flowering time.

Stress resistance analysis of overexpressed tobacco

Sow each tobacco strain onto a 1/2MS medium containing 0.1M NaCl (5.844g/L) and 0.2M (36.4344g/L) mannitol, and record the seed germination rate. Sow wild-type seeds and overexpressed seeds on 1/2MS for 3 days. After the white heads of the seeds are exposed, transfer the seeds with the same conditions to 1/2MS medium containing 0.1M NaCl and 0.2M mannitol, place them vertically, and cultivate them in a constant temperature incubator at 25℃. After about 10 days of cultivation, measure the root length.

All strains of tobacco grown for fifteen days were subjected to stress treatment without watering or with water containing 0.2M NaCl. After 15 days of treatment, the peroxidase (POD) activity and malondialdehyde (MDA) content of each strain of tobacco were measured. DAB and NBT were used to analyze in situ ROS accumulations, transgenic gametophytes were incubated in 1 mg/mL DAB solution (pH 3.8) or NBT solution (10 mM phosphate buffer, pH 7.8) for 6–8 h, then boiled ethanol (98%) for 10 min to remove chlorophyll. To further validate the role of *LoNAC3* in abiotic stress, six genes (*NtSOD*, *NtPOD*, *NtCAT*, *NtSOS*, *NtGST*, *NtAPX*) related to ROS clearance, were selected to detect their relative expression levels (Table 1).

Table 1
List of primer sequences used in qRT-PCR

Genes	Forward primers (5'-3')	Reverse primers (5'-3')
NtSOD	AGCTACATGACGCCATTTCC	CCCTGTAAAGCAGCACCTTC
NtPOD	CCTCAGCTTCAAGCATTATGTCCA	ACCTTTGTAGAAGCATCGGTCCAC
NtCAT	AGGTACCGCTCATTCACACC	AAGCAAGCTTTTGACCCAGA
NtSOS	GCGTGCTTATTTCCACCTTTTG	TTTGATGACGGCTCCCCAGT
NtGST	GCTTGGGTTAAGGGATTG	AGACAACTACTTCAGACAGA
NtAPX	GCTGTTCGACGAGTTGTTAACAG	CTCTGGCTGAGTTGTTGTTGG
NtActin	AGGTACCGCTCATTCACACC	AAGCAAGCTTTTGACCCAGA

Results

Isolation and bioinformatics analysis of LoNAC3

Using the cDNA of *L. olgensis* as a template, the PCR amplification product was located correctly with clear bands. The sequence information of *LoNAC3* gene was obtained through gel recovery sequencing and uploaded to NCBI (GenBank: MW206689). The gene has a length of 1185bp and encodes 394 amino acids. NCBI conserved structure analysis revealed the presence of a NAM conserved domain in *LoNAC3*, which is located at the N-terminus (from 12 to 139) of the gene. According to the subfamily classification of Ooka H et al.[12], *LoNAC3* belongs to the OSNAC7 subgroup through evolutionary tree and homology analysis with *Arabidopsis* NAC family proteins (Fig. 1A). Phylogenetic analysis shows that *LoNAC3* shares similar homology with *Pinus taeda*, *Picea sitchensis*, and *Picea wilsonii* (Fig. 1C). Motif analysis results also confirmed that the N-terminus is more conserved and the C-terminus exhibits diversity (Fig. 1B), consistent with the structural characteristics of the NAC family.

According to the physical and chemical properties prediction on the Protparam website, the theoretical molecular weight of *LoNAC3* protein is 45659.20 Da (1Da = $1g.mol^{-1}$), and the predicted isoelectric point is 6.53. The negatively charged residues in the amino acid composition are dominant, and the instability

coefficient is greater than 40. It is an unstable protein with a hydrophobicity of -0.851. The subcellular localization prediction results of WOLF PSORT show that *LoNAC3* is mainly concentrated in the nucleus. GOR4 predicted its secondary structure and found that the *LoNAC3* protein is mainly randomly curled, with 21.07% α-helices, 15.23% extension chains, 63.71% random coils, and lack β-turns. (Fig. 1B), which has some similarity with the identified NAC gene of *L. olgensis* [26]. The SwissMode homologous modeling method was used to predict the tertiary structure of *LoNAC3* (Fig. 1B), and the aligned model was A0A5S9HI04.1.A NAC transcription factor(gene: *PtaVNS5*, organism: *Pinus taeda* (Loblolly pine)), with a sequence identity of 95.41%.

Cloning and cis-acting elements analysis of the LoNAC3 promoter

Using NCBI genome data of *L. kaempferi* as a reference, primers were designed to clone the sequence information of the approximately 2000bp fragment before the *LoNAC3* start codon using the entire gDNA of *L. olgensis* as a template. Select the correctly sequenced *LoNAC3*pro: GUS *Escherichia coli* positive strain plasmids, transfer them into *Agrobacterium* receptive cells using heat shock method, apply plates, select single colonies for expansion culture, and use the bacterial solution as a template for PCR detection. The target bands are clear (Fig. 2B), and the recombinant plasmid has been successfully transformed into *Agrobacterium*. By infecting tobacco, it was found that the *LoNAC3* promoter can initiate GUS expression in tobacco, with promoter activity, which is weaker than the 35S promoter (Fig. 2C).

The prediction results on the PlantCARE website show that in addition to multiple common cis-acting element in promoter and enhancer regions (CAAT box), core promoter element around − 30 of transcription start (TATA box), there are also multiple MYB recognition sites on the *LoNAC3* promoter. Analysis found that there are two cis-acting element involved in defense and stress responsiveness, as well as multiple cis-acting regulatory element involved in light responsiveness (G-Box) on the promoter. In addition, there are also multiple hormone related elements, three cis-acting element involved in the abscisic acid responsiveness, two cis-acting regulatory element involved in the MeJA-responsiveness and one gibberellin-responsive element (Fig. 2A).

Analysis of LoNAC3 gene expression patterns

Through the analysis of qRT-PCR results, it can be seen that *LoNAC3* has the highest relative expression level in the leaves of *L. olgensis* at three time periods (Fig. 2D). Under abiotic stress treatment, the relative expression level of $NaHCO_3$ showed little change, and the expression level was down regulated at 24 and 96 hours; the changes in relative expression levels were relatively small at 12h and 24h under NaCl and PEG treatment, but were more significantly upregulated at 48h and 96h, reaching a tenfold increase (Fig. 2E). Under hormone treatment, the relative expression level of *LoNAC3* induced by different hormones varied. The expression level of *LoNAC3* was lower at several time points under IAA and 6-BA treatment than that of WT; The relative expression level of *LoNAC3* gene under GA_3 treatment reached a maximum of 1.99 times at 12 hours; the relative expression levels of ABA and MeJA under

two hormone treatments showed significant changes, reaching their maximum at 24 hours, reaching 3.60 and 8.16 times, respectively (Fig. 2F).

Obtaining and phenotypic observation of overexpressed tobacco

The transgenic resistant buds obtained through *Agrobacterium* mediated leaf disc transformation grew well on a medium containing hygromycin (25mg.L^{-1}) and were reinduced into seedlings (Fig. 3A-D). Extracting tobacco leaf DNA for PCR identification, there were clear and correctly positioned target bands (Fig. 3F), and the *LoNAC3* gene was successfully integrated into the DNA of resistant tobacco seedlings. Collect the seeds of positive seedlings and screen them for hygromycin. The resistant seedlings grew well but the wild-type was unable to grow (Fig. 3E). After 2 generations of resistance screening, the T2 generation resistant tobacco seeds were harvested, and the T3 generation was seeded on 1/2MS medium. After 10 days, RNA was extracted from the entire tobacco plant of each strain. RNA levels were identified through RT-qPCR, and all positive strains were upregulated (Fig. 3G). Select OE3, OE4, and OE7 with the highest relative expression levels for the next experiment.

Compared to the wild-type, overexpressed tobacco showed a situation of plant dwarfism (Fig. 3H). Throughout the growth process, the plant height of overexpressed tobacco was lower than that of wild-type, and the ground diameter of overexpressed tobacco was also smaller than that of wild-type (Fig. 3I-J). As the tobacco grew, the ground diameter difference gradually decreased. Within 45–90 days, the growth rate of plant height was lower than that of wild-type plants, while the growth rate of ground diameter was higher than wild-type plants, and flowering occurred earlier than wild-type plants.

Analysis of stress resistance of tobacco seeds

The germination rate of tobacco seeds sown on 1/2MS medium was almost 100%, while the germination rate of tobacco seeds sown on 1/2MS medium containing 0.2M NaCl and 0.3M mannitol was almost zero. Observing the germination rate of various tobacco strains sown on a 1/2MS medium containing 0.1M NaCl (5.844g.L^{-1}) and 0.2M mannitol (36.4344g.L^{-1}), it was found that the germination rate of overexpressed *LoNAC3* tobacco was lower than that of wild-type tobacco (Fig. 4E-F), and wild-type tobacco seedlings showed better growth status (Fig. 4A-B). In addition, after stress treatment, the root length of overexpressed tobacco was shorter than that of wild-type tobacco (Fig. 4C-D).

Analysis of stress resistance of tobacco

Drought and salt stress can lead to the accumulation of reactive oxygen species, causing damage to plants. Under normal growth conditions, there was no significant difference in POD activity and MDA content between transgenic strains and wild-type tobacco. DAB and NBT staining showed only slight staining of plant leaves, but after treatment, transgenic strains showed weaker POD activity and lower

MDA content (Fig. 5A). Different strains of tobacco also experienced varying degrees of deepening of staining, while overexpressing tobacco showed significantly deeper staining, indicating that it accumulated more hydrogen peroxide and superoxide ions (Fig. 5B). Similarly, under normal circumstances, the expression level of genes is low and there is no significant difference between different strains. After stress treatment, the expression level of genes increases, and the growth of most genes in transgenic tobacco is lower than that in wild-type tobacco (Fig. 5C).

Discussion

Bioinformatics analysis of LoNAC3

Through bioinformatics analysis, *LoNAC3* is a full-length gene sequence containing a NAM specific domain which located at the N-terminus. In terms of instability coefficient, *LoNAC3* greater than 40 is an unstable protein, and the *LoNAC3* protein may appear in stages. The predicted hydrophobicity of the protein is negative, suggesting that it is a hydrophilic protein. Through sequence alignment of NCBI, it was found that *Picea wilsonii* NAC transcription factor *PwNAC30* with high homology to *LoNAC3*, negatively regulates the abiotic stress tolerance of transgenic *Arabidopsis* [29]. *LoNAC3* belongs to the OSNAC7 subgroup through evolutionary tree and homology analysis with *Arabidopsis* NAC family proteins. *ANAC012* (*NST3*) in the same subgroup negatively regulates the development of *Arabidopsis* wood fibers [30], and *ANAC033* (*SMB*) is involved in the development of Arabidopsis root cap [31]. It is speculated that *LoNAC3* may also be involved in the stress resistance and development processes related to *L. olgensis*. Further experimental analysis is needed for this conclusion.

Expression analysis of LoNAC3 gene and promoter

Through the analysis of qRT-PCR results, it can be seen that *LoNAC3* can detect fluorescence signals in different parts of *L. olgensis* at three stages, indicating that the specific fragments of *LoNAC3* have been successfully amplified in these parts, and *LoNAC3* is expressed at all stages. *LoNAC3* has the highest relative expression level in leaves, but overall, the difference in expression levels among different parts is not significant. Under abiotic stress, the relative expression level of *LoNAC3* indicates that it is more likely to participate in drought and salt stress responses, and is more likely to participate in later regulation. Under several different hormone treatments, *LoNAC3* was induced to varying degrees of expression. The relative expression levels varied significantly under MeJA and ABA hormone treatments, suggesting a greater correlation between *LoNAC3* and these two hormones.

Due to the lack of genomic data information of *L. olgensis*, different from using RACE technology to clone promoter sequence information, this experiment designed primers based on NCBI *L. kaempferi* genomic data, cloned the *LoNAC3* promoter sequence using *L. olgensis* gDNA as a template, and constructed the *LoNAC3*pro:: GUS plasmid. The *LoNAC3* promoter can initiate GUS expression in tobacco and has promoter activity. The analysis of cis-acting elements in the promoter shows that there are three ABA responsive elements and two MeJA responsive elements on the *LoNAC3* promoter.

ABA plays a crucial role in stress response and regulates various developmental processes such as seed maturation and dormancy, organ abscission, and leaf senescence in plants [32, 33]. Methyl jasmonic acid (MeJA) is a member of the jasmonic acid family. JAs not only play an important role in defense responses to biological stress [34], but also participate in a series of plant responses to abiotic stress [35–37]. Both hormones play important roles in various plant activities, and expression analysis results indicate that the *LoNAC3* gene is likely associated with two hormone signals, and the response mechanism needs further research.

Preliminary analysis of LoNAC3 gene function

In order to preliminarily analyze the function of the *LoNAC3* gene, this experiment analyzed *LoNAC3* overexpressed tobacco. It was found that transgenic tobacco exhibited stunted growth and poorer performance under stress, accumulating more MDA content and exhibiting lower POD activity under stress treatment, indicating that the *LoNAC3* gene can participate in plant response to drought and salt stress through the reactive oxygen species pathway.

Conclusion

This experiment cloned a gene with a NAM specific conserved domain named *LoNAC3* from *L. olgensis* and obtained its promoter sequence information. Bioinformatics analysis identified *LoNAC3* as a NAC family gene belonging to the *OSNAC7* subgroup, and speculated that its function is related to the secondary wall. Through the analysis of *LoNAC3* sequence and promoter expression, it can be concluded that *LoNAC3* is involved in the drought and salt stress response processes of *L. olgensis*, and is induced by ABA and MeJA expression. Overexpression of *LoNAC3* leads to stunted tobacco growth and negatively regulates its tolerance to drought and salt stress through the reactive oxygen species pathway.

Declarations

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Not applicable.

Author contributions

Qing Cao and Lei Zhang conceived and designed the study. Qing Cao, Tiantian Zhang and Lu Liu performed the experiments. Daixi Xu, Chen Wang and Qingrong Zhao analyzed the data. Qing Cao and Junfei Hao wrote the paper. Hanguo Zhang and Lei Zhang reviewed and edited the manuscript. All authors read and approved the manuscript.

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Data availability

Data will be made available on request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no known competing financialinterests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

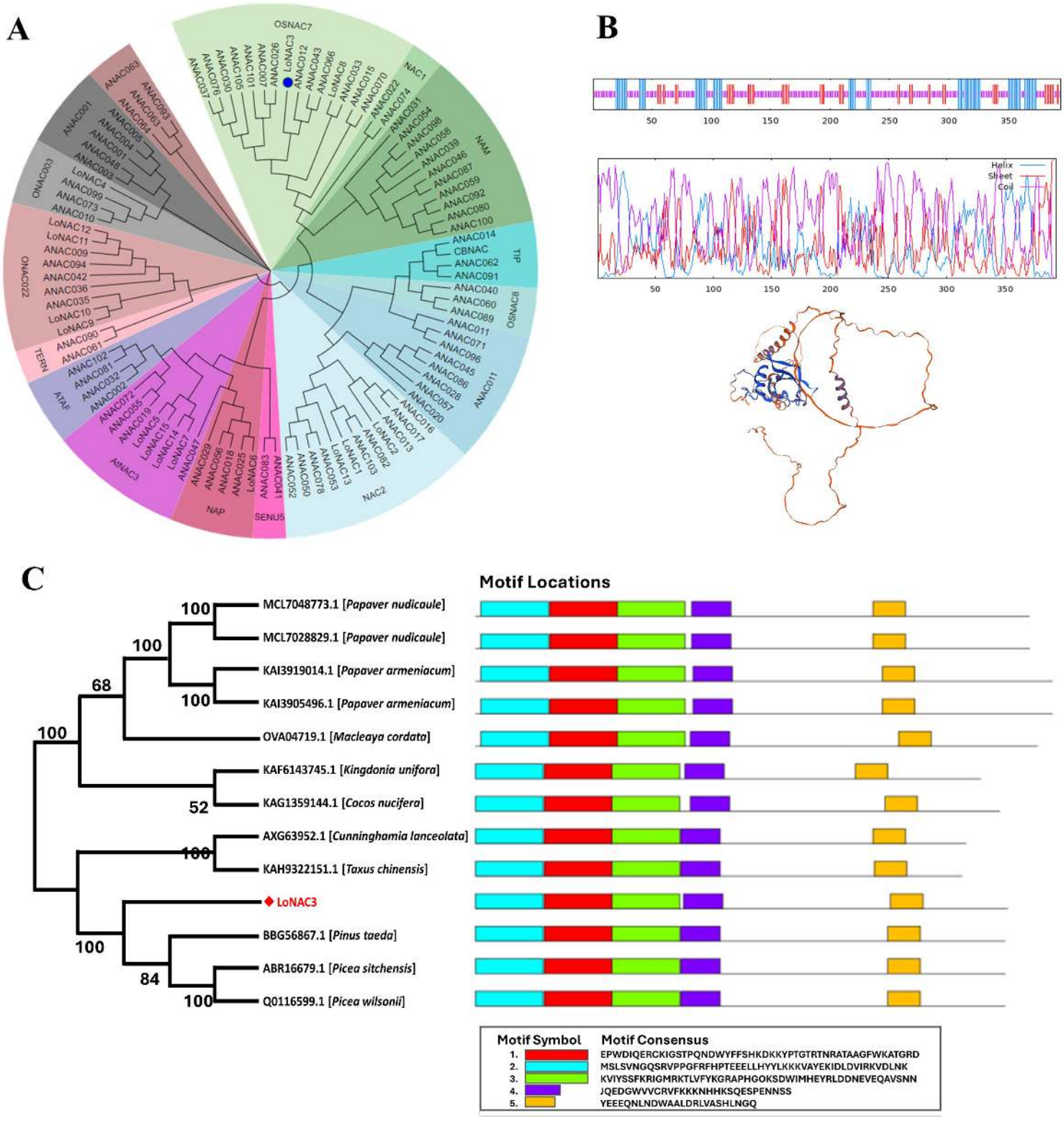

Figure 1

Isolation and bioinformatics analysis of *LoNAC3*. (A)Subgroup classification analysis of *LoNAC3*. **(B)** Secondary structure and tertiary structure prediction of LoNAC3 protein.**(C)** The phylogenetic tree and motif analysis of *LoNAC3*.

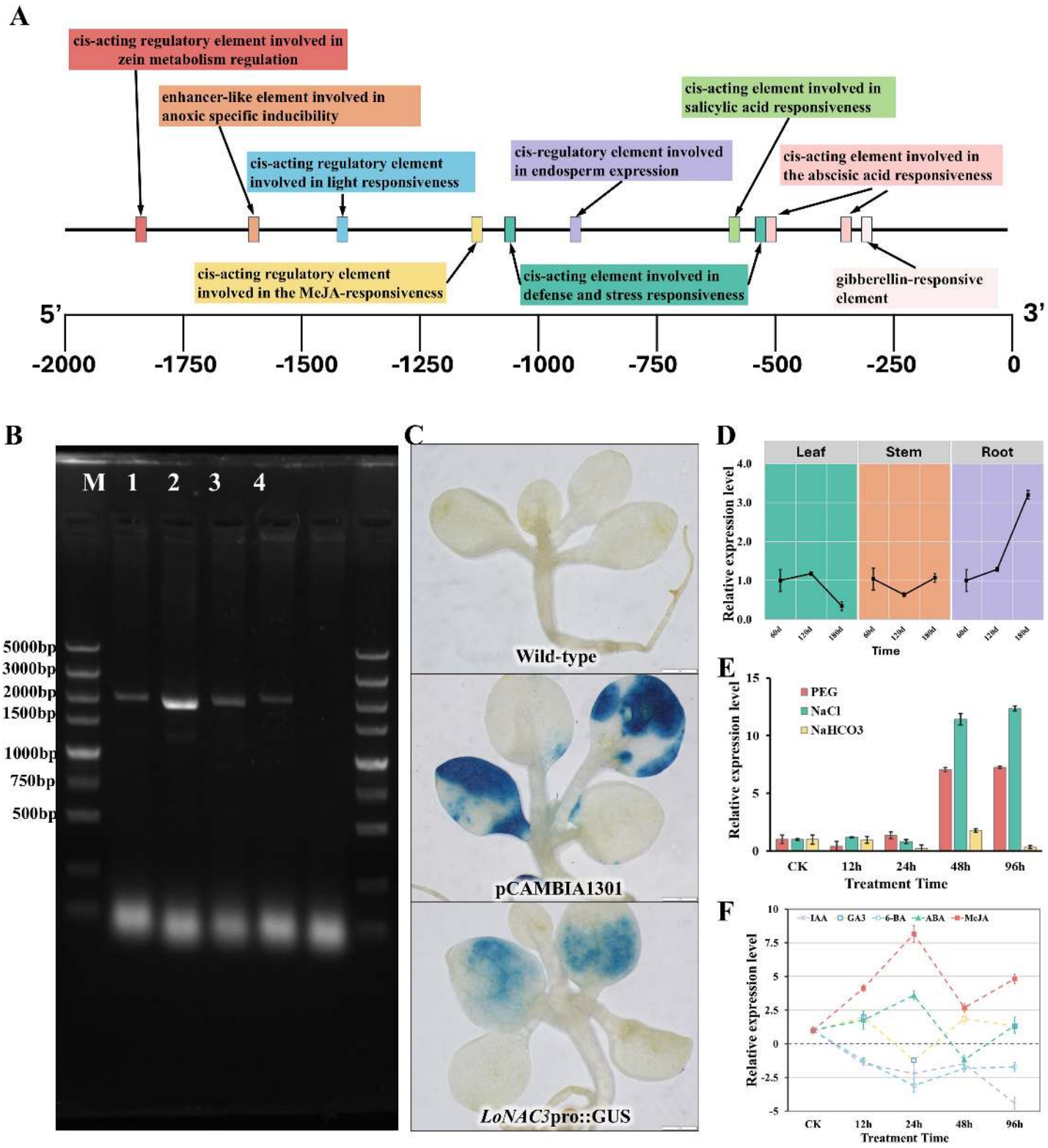

Figure 2

Analysis of *LoNAC3* promoter and gene expression patterns. (A) The cis-acting elements in *LoNAC3* promoter sequence. (**B**) PCR amplified product of *LoNAC3* promoter. (**C**) The results of tobacco GUS staining. **(D)** Expression analysis of *LoNAC3* in different growth stages of *L. olgensis.* (**E**) Expression analysis of *LoNAC3* under different stress treatment. (**F**) Expression analysis of *LoNAC3* under different hormone treatment.

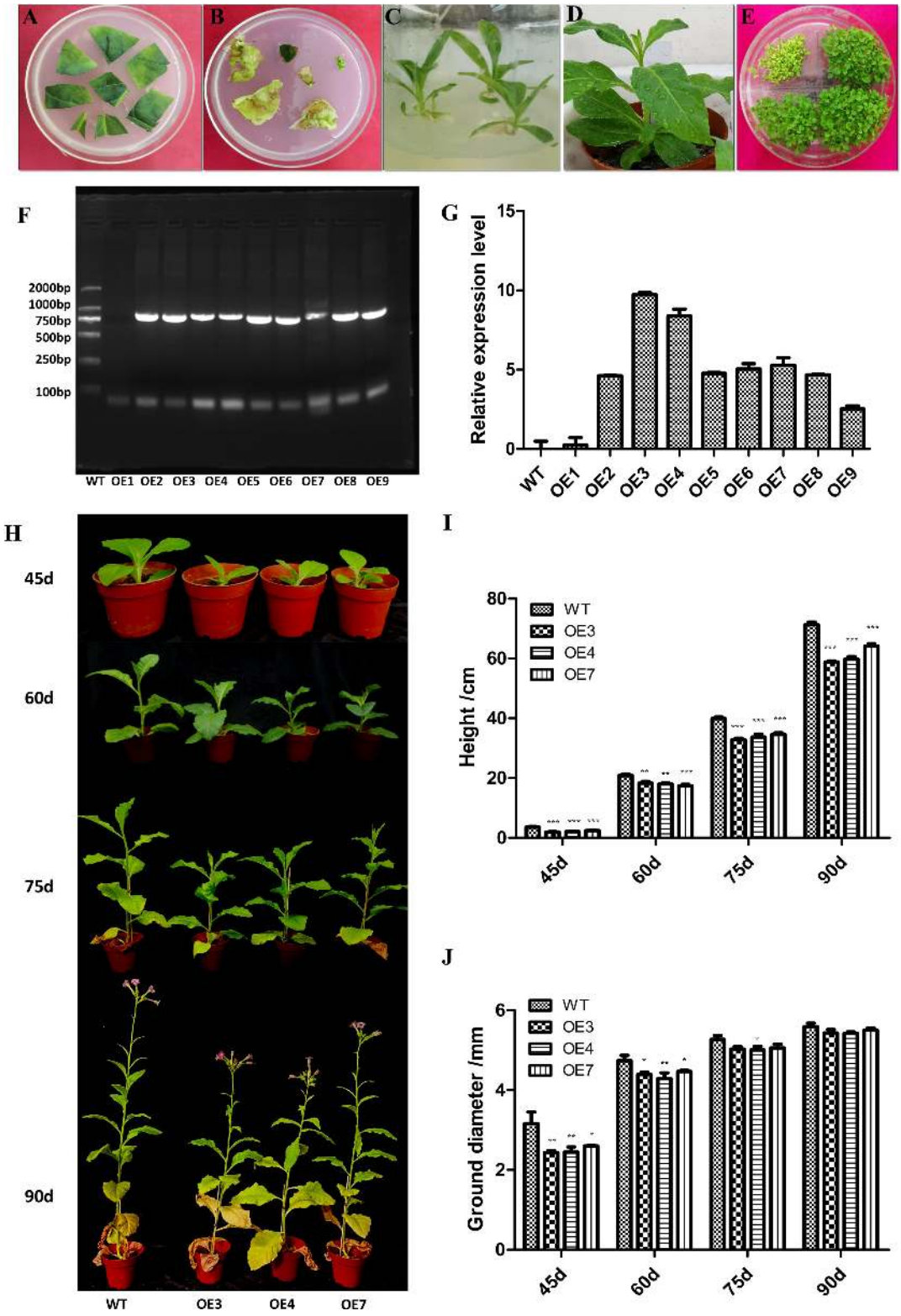

Figure 3

Obtaining and phenotypic observation of overexpressed tobacco. (A) Co-cultivation. **(B)** Induced resistant differentiated buds. **(C)** Rooted tissue culture seedlings. **(D)** Transplanted into soil. **(E)** Selection of transgenic tobacco seed resistance (25mg.L^{-1} Hyg). **(F)** PCR detection of VB191104-*LoNAC3* transgenic tobacco. **(G)** Relative expression levels of VB191104-*LoNAC3* transgenic tobacco. **(H)** Phenotype of tobacco. **(I)** Plant height of tobacco with different asexual strains. **(J)** Ground diameter of

tobacco with different asexual strains. Significance was determined by the least significant difference, asterisks indicate statistically significant differences from WT (*P < 0.05, **P < 0.01, ***P < 0.001).

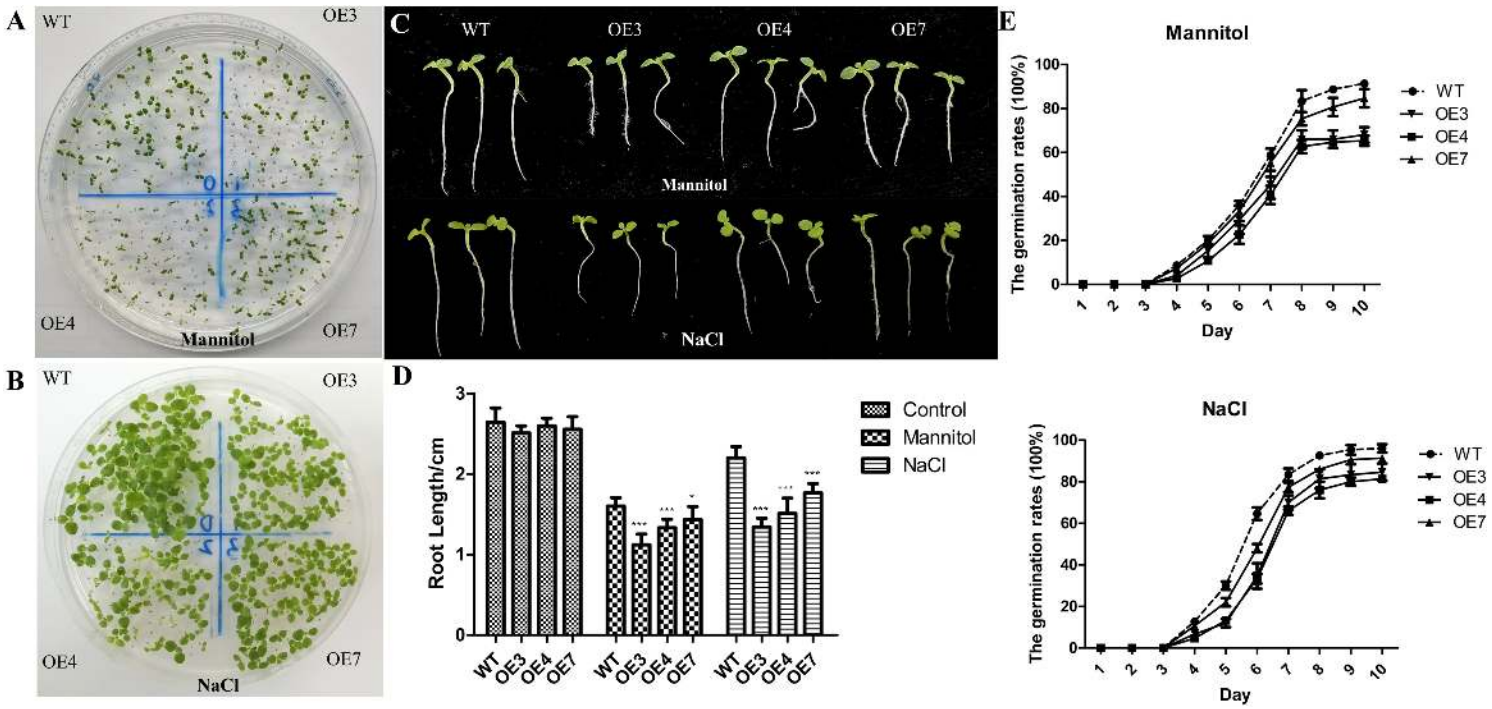

Figure 4

Analysis of stress resistance in genetically modified tobacco seeds. (A) Sowing tobacco seeds in 1/2MS medium containing 0.2M mannitol for 10 days. **(B)** Sowing tobacco seeds in 1/2MS medium containing 0.1M NaCl for 10 days. **(C-D)** Root length of tobacco under stress treatment. Significance was determined by the least significant difference, asterisks indicate statistically significant differences from WT (*P < 0.05, **P < 0.01, ***P < 0.001). **(E)** The germination rate of Tobacco in 1/2MS medium with 0.2M mannitol. **(F)**The germination rate of Tobacco in 1/2MS medium with 0.1M NaCl.

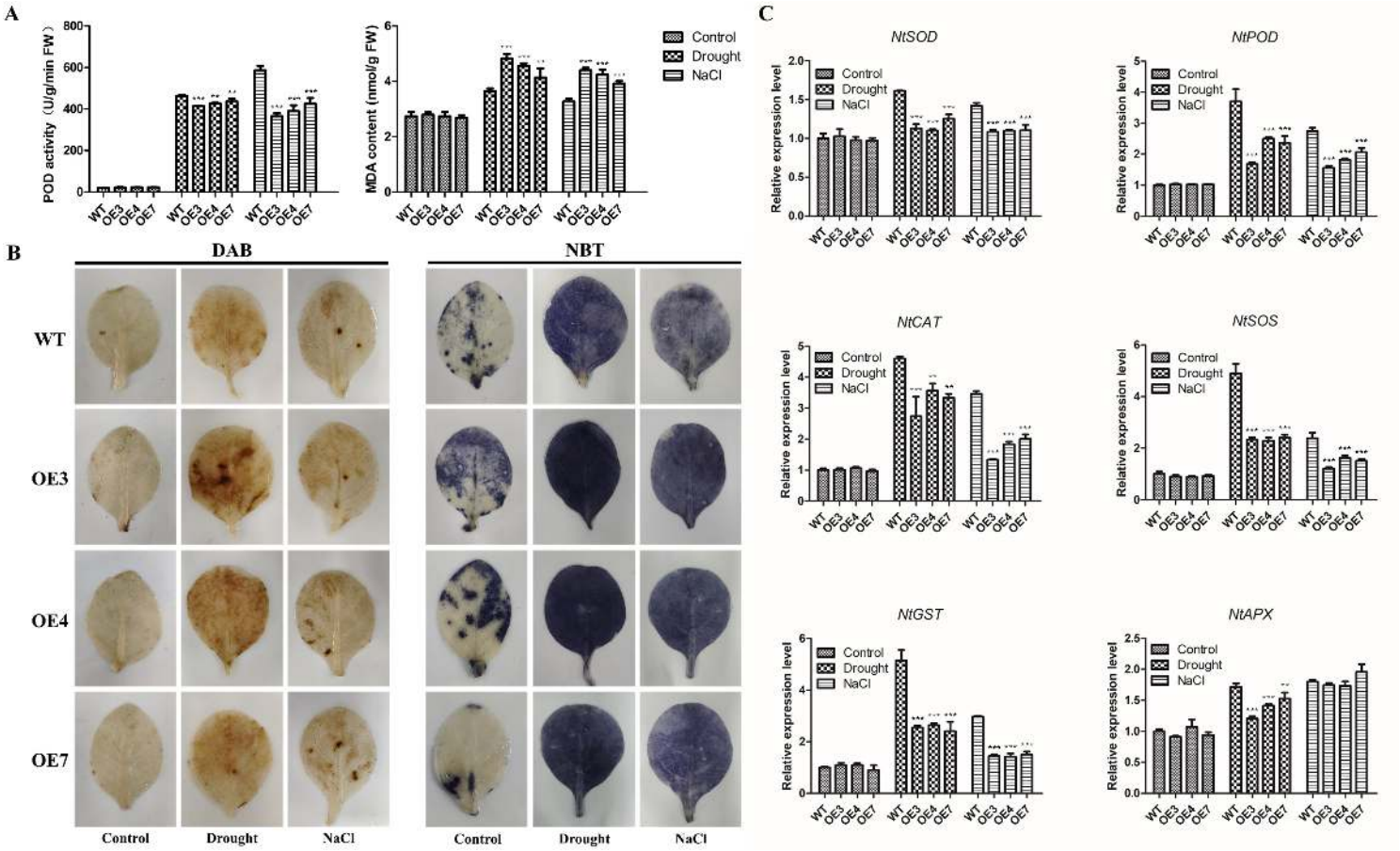

Figure 5

Analysis of stress resistance in tobacco. (A) Determination of POD activity and MDA content in various tobacco strains before and after treatment. **(B)** DAB and NBT staining of tobacco leaves before and after treatment. **(C)** Detection of ROS-related gene expression levels before and after treatment.Significance was determined by the least significant difference, asterisks indicate statistically significant differences from WT (*P < 0.05, **P < 0.01, ***P < 0.001).