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Expression pattern of *FmNAC11* transcription factor in *Fraxinus mandshurica* and its function in improving cold resistance

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Abstract

Background: NAC domain proteins contain one of the largest families of plant specific TFs. NAC (Nam, no apical meristem; ATAF1/2, Arabidopsis transcription activation factor; CUC2, cup-shaped cotyledon) is an acronym for the names of three genes originally described as containing domains, namely NAM (no apical meristem), ataf1/2 (Arabidopsis transcription activator) and CUC2 (cupular cotyledon) are plant specific transcription factors. As a regulator of gene expression, transcription factors (TF) play an important role in plant development and response to hormone signals. In order to clarify the function of *Fraxinus mandshurica NAC11* gene under biological and abiotic stress,

Results: The cloned *FmNAC11* gene of *Fraxinus mandshurica* was 891bp, encoding 296 amino acids. Subcellular localization showed that *FmNAC11* was located in the nucleus and was indeed a transcription factor with transcriptional activation activity. *FmNAC11* can respond to cold, NaCl stress, and IAA, GA, ABA hormone signal induction. The promoter length of the cloned *FmNAC11* gene was 2340bp. By analyzing cis-acting elements, it was found that the promoter of *FmNAC11* gene responded to biological and abiotic stresses, participated in a variety of biological pathways, and regulated biological processes. Four transgenic lines overexpressing *FmNAC11* were obtained by Agrobacterium mediated transformation of WT tissue culture seedlings of *Benthamia tobacco*. The transgenic plants grew rapidly after transplantation. Under the condition of low temperature culture at -5 °C for 24 hours, the wilting and yellowing degree of transgenic plants is significantly less than that of ordinary tobacco, and the transgenic tobacco overexpressing *FmNAC11* performs better in membrane system, osmotic system and ROS scavenging system than ordinary tobacco, indicating that *FmNAC11* gene has the function of improving plant cold resistance.

Conclusion: The gene expression of transgenic tobacco under cold treatment shows that *NAC11* transcription factor has a regulatory relationship with growth related *IAA* and *AUX1* genes, cold resistance related *ice*, *DREB* and *CBF1*, *ROS*

clearance related cat and *SOD* genes, which promotes the rapid growth, cold resistance and ROS clearance ability of transgenic tobacco.

Background

Fraxinus mandshurica belongs to the genus *Fraxinus* of *Oleaceae*. It is a deciduous tree. It is one of the unique *Fraxinus* Species in Northeast China. It grows well and has strong cold resistance. It has good dry type and excellent material. It has an extremely wide range of application scenarios [1-4].

Cold resistance of plants refers to some physiological, biochemical and phenotypic changes when plants are in a low temperature environment for a long time. The low temperature environment seriously affects the growth of trees. The impact on trees mainly includes roots and buds, which directly affects the yield of trees [5-7]. Under cold stress, the accumulation of reactive oxygen species in the membrane system of plant cells increases. The large accumulation of reactive oxygen species is harmful to plants. Peroxidase (POD) and superoxide enzyme (SOD) can remove reactive oxygen species. At the same time, the accumulation of reactive oxygen species leads to the excessive oxidation of membrane lipids and the production of a large amount of malondialdehyde (MDA), which will bind to membrane proteins, the membrane protein is denatured, the permeability of the membrane is increased, and the relative conductivity is increased. Plants also increase the content of soluble sugar and soluble protein to resist cold stress. Under cold stress, many genes encoding proteins with cold resistance function are induced to be highly expressed. Research shows that many CORS genes are obviously induced by cold stress [8-10].

Many *NAC* domain genes have been identified from a range of plant species, including *Arabidopsis*, rice, tobacco, poplar and soybean. The structure of the DNA binding *NAC* domain of *Arabidopsis ANAC* (abscisic acid responsive *NAC*) has been determined by X-ray crystallography at 1.9 Å resolution (protein database codes 1ut4 and 1ut7). This is the first structure identified for members of the *NAC* family of plant

specific transcription regulators. *NAC* protein family contains a variety of plant proteins. Its N-terminal *NAC* domain is conservative and can bind DNA and other proteins. Therefore, it can be identified by the existence of this feature, accompanied by a variety of C-terminal domains. *NAC* domain is not a classical helix turn helix motif; On the contrary, it is a new transcription factor fold, which consists of some twisted B sheets surrounded by some helical elements. *NAC* proteins are involved in transcriptional control in a variety of plant processes. Functions include participating in the development of shoot tip meristem and flower organs, as well as the formation of lateral roots. In addition, *NAC* protein is also related to stress response [11-21].00

At present, more and more studies have shown that *NAC* transcription factors play an important role in different kinds of plants and can resist abiotic stress. However, at present, the research of *NaC11* gene is mainly focused on herbal plants such as *Arabidopsis*, and there is less research on woody plants. The analysis of the expression profile of grape *vnac1* shows that the expression of *NaC11* gene is closely related to the later stage of flower and leaf development, indicating its role in plant senescence. In addition, cold water, wound and defense related hormones such as salicylic acid, methyl jasmonate, ethylene and abscisic acid can induce *vnac1* expression in grape leaves. *Arabidopsis* plants overexpressing *vnac1* showed tolerance to osmotic pressure, salt and cold stress. It shows that *vnac1* is an important regulatory component of plant signal defense cascade. The expression of *amnac1* and *amnac2* genes of *Ilex mongolica* were significantly up-regulated under low temperature and drought stress.

The study of *NaC1* gene also includes: (1) the interaction between auxin inducible *NaC1* protein involved in lateral root formation and ring domain protein *sinat5* weakens auxin signal through ubiquitination and *NaC11* degradation, and participates in the pathway of plant abscisic acid control. (2) There was a significant correlation between the expression level of *zmnac1* and lateral root density in maize. Compared with the wild type, transgenic *Arabidopsis* overexpressing *zmnac1* increased lateral roots. These findings suggest that *zmnac1* plays an important role in lateral root development. (3) *Arabidopsis* *NAC* gene and many crop *NAC* genes are

associated with aging, suggesting that NAC gene is an important regulator in the process of aging. (4) NaC11 is expressed in Arabidopsis root tips, cotyledons and leaves, and the expression level of NaC11 changes in various tissues with the process of plant growth, and its role is different in each period.(5) the accumulation of RhNAC100 transcripts in NAC domain transcription factor genes in Rosa is regulated by ethylene through tiny RNA164 dependent post transcriptional regulation. Overexpression of rhnac100 in Arabidopsis inhibited the expansion of petal cells and significantly reduced petal size. [22-35]。 There is no research on *NaC11* gene in *Fraxinus mandshurica*.

Fraxinus mandshurica is a tree with flowers before leaves. Growing in the north, it needs to deal with extreme conditions such as late spring cold to ensure its own growth and development. We found that *FmNAC11* gene can promote plant growth and improve stress resistance. We cloned the full length of *FmNAC11* gene from *Fraxinus mandshurica* and found that the expression increased under cold stress and exogenous hormone treatment. Overexpression of *FmNAC11* gene in tobacco can improve the cold tolerance of tobacco and accelerate the growth of tobacco. It provides a good molecular basis for obtaining better new varieties of *Fraxinus mandshurica* in the future.

Result

Cloning and sequence analysis of *FmNAC11* gene

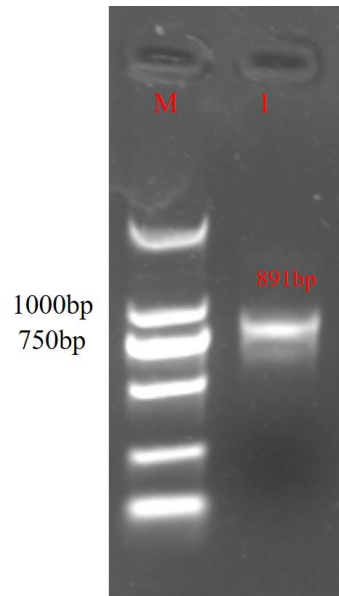


Fig. 1 cloning of *FmNAC11* gene

The gene sequence was screened from the *Fraxinus mandshurica* transcriptome database and named *FmNAC11*. The *FmNAC11* gene was successfully amplified by PCR using the cDNA extracted from the leaves of *Fraxinus mandshurica* as a template through software analysis and primer design (Fig 1). Through NCBI comparison and analysis, it is determined that the total length of the coding region of *FmNAC11* gene is 891bp, encoding 296 amino acids. (Fig 2)

```

1  ATGAGTAGTTTGAGCATGATAGAGGCAAAGCTGCCGCCGGGGTTT
   M S S L S M I E A K L P P G F
46 AGGTTTCAGCCAAGAGATGAAGAATTGATATGTGATTACTTGATG
   R F Q P R D E E L I C D Y L M
91 AAATGGATAATCGGCTGTGGCCACCAGAACCACCTCTGTTAATT
   K W I I G C G H Q N H P L L I
136 GAAGTTGATCTCAACAAGTCCGAGCCTTGGGACGTTCCCAAATCA
   E V D L N K C E P W D V P K S
181 GCATGTGTAGGAGTAAGGAGTGGTATTTTACAGCCAACGCGAT
   A C V G G K E W Y F Y S Q R D
226 CGTAAATATGCAACAGGACTAAGAACAATCGAGCGACACTAACG
   R K Y A T G L R T N R A T L T
271 GGTATTGGAAGGCCACCGGAAAAGATAGACCGATTCTTCGTAAG
   G Y W K A T G K D R P I L R K
316 GGAATCCTAATGGGAATGAGAAAAACATTGGTATTTTACCAGGGC
   G I L M G M R K T L V F Y Q G
361 AGGGCTCCTAAAGGAACAAAACTGATTGGGTCATGCATGAATTT
   R A P K G T K T D W V M H E F
406 CGCCTTGAAGGAGCCTTTGGCTCTCCTAAACCATCATCTGTCAAG
   R L E G A F G S P K P S S V K
451 GAGGATTGGGTTTTATGCAGATTGTTCTACAAAAGGGAGAAGCT
   E D W V L C R L F Y K K G E A
496 GAAGCCAAACCAGAAACAGGAAGCAGCCACGAAAAAGAAATCACC
   E A K P E T G S S H E K E I T
541 ACTTCTCCTTCTTCTTCTCCTCCATTAATGAAACCCTGTCTCACC
   T S P S S L P P L M K P C L T
586 TTTGACCCATATCAGGCCACAACAGATGAGTATAATCATGAGCAA
   F D P Y Q A T T D E Y N H E Q
631 GTGCCCTGCTTCTCCATTTTGAACAATAATTTCTCACAATCACA
   V P C F S I S N N N F S Q I T
676 GAATCATCCAACGAATTCATGCCACATTTGGAGGGTTGCCTGCA
   E S S N E F M P T F G G L P A
721 GATCATAATCTTGAATGTGTTTCATCATCATCAAACTCTGATCAG
   D H N L G M C S S S S N S D Q
766 ACGGTGATCAAAAGCTCTAATCTATCATCTTACCAAAATGGACGAA
   T V I K A L I Y H L T K M D E
811 AGCAATTGCTCCCAAGTTTGGGGATGGAAGTTCAGACAGTTAT
   S N C S P S F G D G S S D S Y
856 CTGTCTGAACTGGGATTGCAGTGAACCATTTATTGA
   L S E L G L Q W N H Y *

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Fig. 2 *FmNAC11* gene and its coding amino acid sequence

***FmNAC11* conserved domain analysis**

The conserved domain of *FmNAC11* gene coding protein of *Fraxinus mandshurica* was predicted by NCBI database CD search

(<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) . The results showed that the FmNAC11 gene coding protein had a NAC domain and belonged to Nam superfamily .Blast in plant transcription factor database (plantTFDB) (http://planttfdb.gao-lab.org/blast_result.php)showed that FmNAC11 had high homology with Arabidopsis NAC No. 1 gene(Fig. 3).

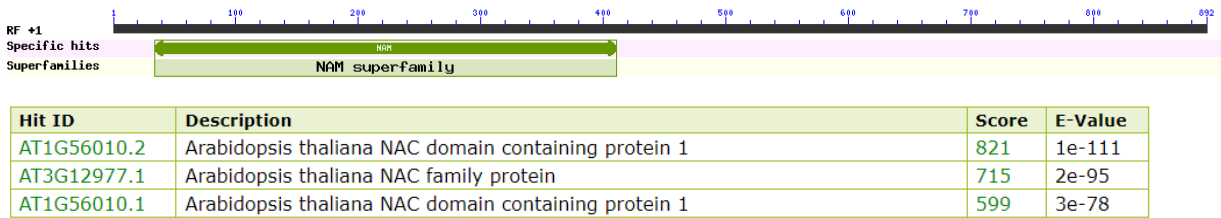


Fig. 3 conserved domain of *FmNAC11* gene

Prediction of the tertiary structure of *FmNAC11* protein

After Swiss model homology modeling, the results of Fraxinus mandshurica NAC11 protein are as follows(Fig 4): the NaC1 domain coverage of FmNAC11 protein is 51.88%(<https://swissmodel.expasy.org/interactive/56MxmS/models/>),and the three-dimensional structure similarity with NAC11 protein in Oryza sativa L is the highest(Fig 5). (<https://swissmodel.expasy.org/templates/3ulx.1>)

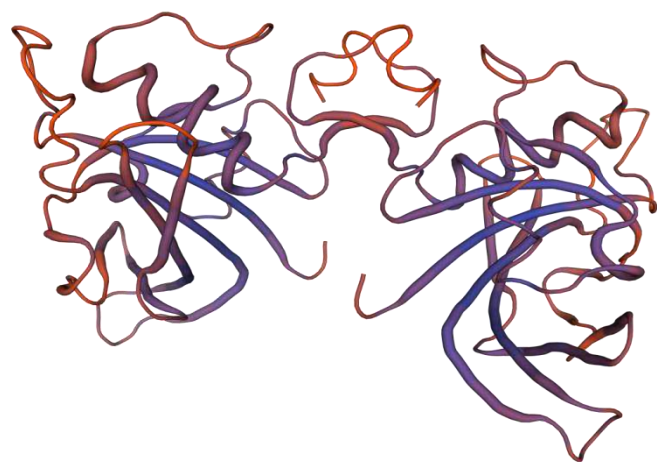


Fig. 4 tertiary structure of *FmNAC11* protein

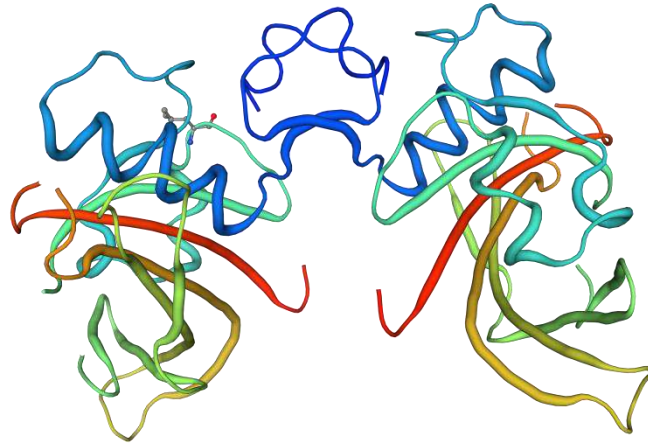


Fig. 5 Tertiary structure of *NAC11* conserved domain in response to *Oryza sativa* L stress

Amino acid sequence alignment and phylogenetic tree construction of *FmNAC11*

To analyze the phylogenetic relationships between *FmNAC11* and the homologous sequences of *NAC11*s in other plants, a phylogenetic tree was constructed using MEGA 5.0 software. *FmNAC11* and other gene sequences were used.

FmNAC11 was most closely related to Oleaceae europaea, *SiNAC11* (*Sesamum indicum*, XP_011095676.1), *CaNAC11* (*Coffea arabica*, XP_027061236.1), *NtNAC11* (*Nicotiana tomentosiformis*, XP_009605814.1), *CsNAC11* (*Camellia sinensis*, XP_028081260.1), (*Beta vulgaris* subsp. *Vulgaris*, XP_010666181.1), as these species were grouped into a clade (Fig.6).

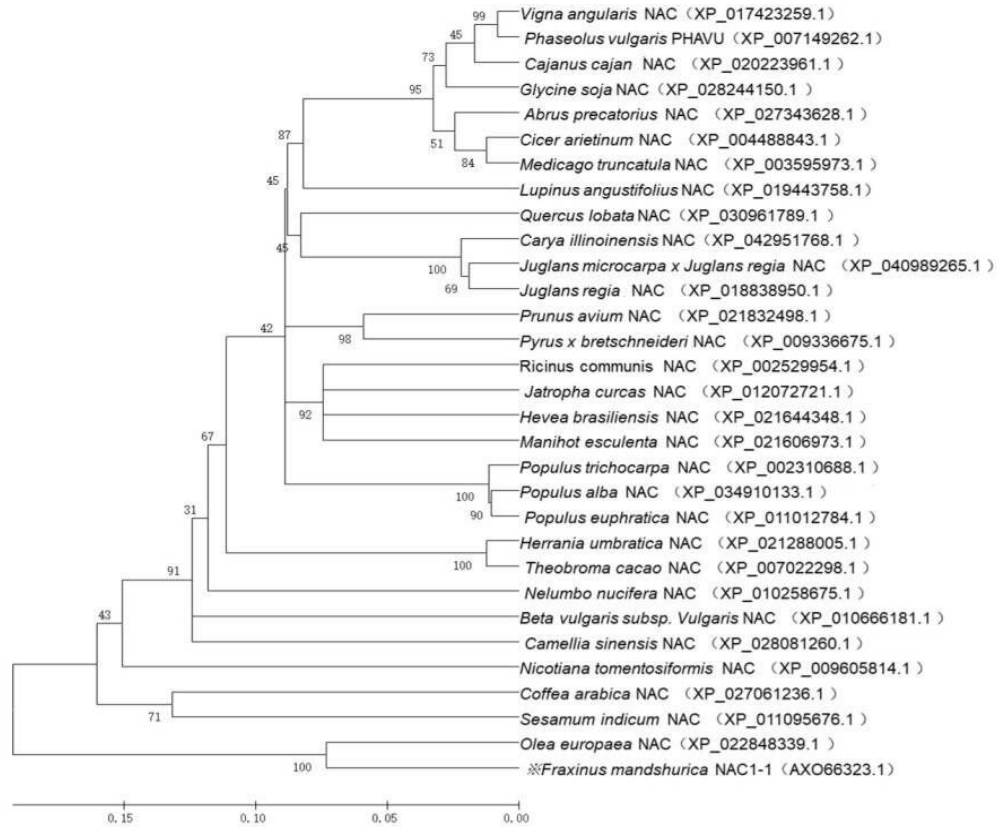


Fig. 6 evolution tree of *FmNAC11* system

Subcellular localization of *FmNAC11* protein

Take the onion epidermal tissue co cultured in MS medium and press it for GFP fluorescence observation (Fig 7). Since there is no localization signal of GFP protein in the empty carrier, the fluorescence signal of empty body located in onion epidermal cell membrane and nucleus can be clearly observed under the microscope. Similarly, the onion nucleus is stained with DAPI, and the nucleus is blue as the positive control, The fluorescence signal of *FmNAC11* GFP fusion protein can only be observed in the nucleus of onion epidermis, indicating that *FmNAC11* is located in the nucleus. The results showed that *FmNAC11* was located in the nucleus and was indeed a transcription factor with transcriptional activation activity.

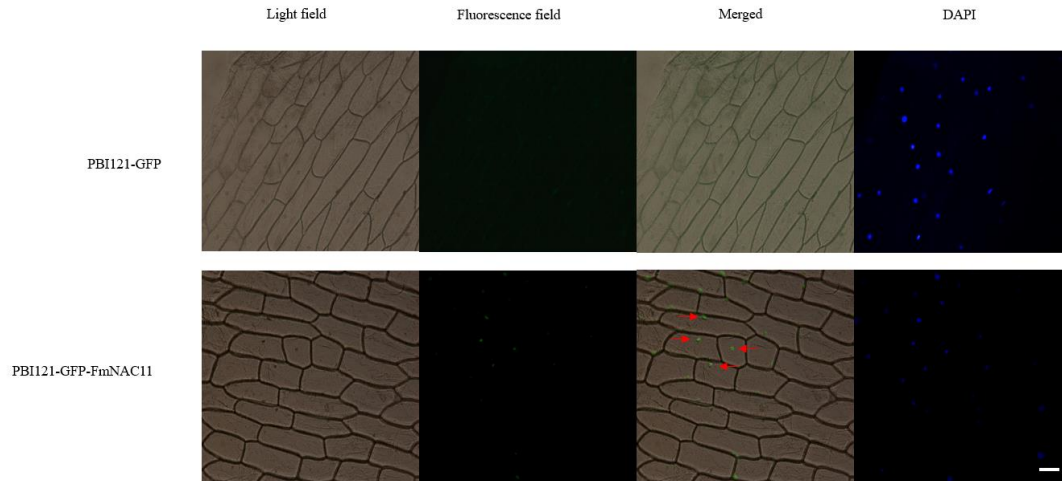


Fig. 7 Subcellular localization of *FmNAC11* protein

Expression pattern of *FmNAC1* in *Fraxinus mandshurica*

Expression pattern of *FmNAC1-1* in *Fraxinus mandshurica* under abiotic stress

The expression of *nac1-1* gene fluctuated with the time of biological stress.

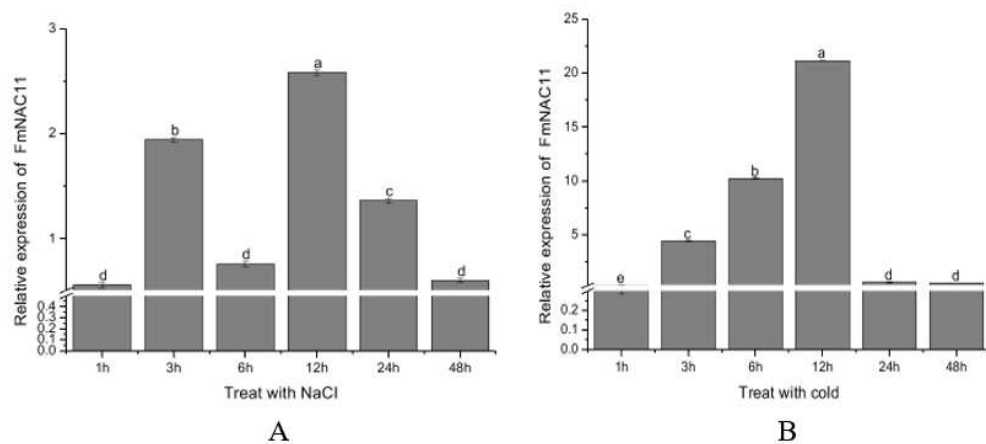


Fig. 8 expression of *FmNAC11* gene under NaCl and cold stress

Salt treatment (Fig. 8A): the expression of *FmNAC11* gene was up-regulated at 3h, 12h and 24h, down regulated at 1h, 6h and 48h, and the expression was the highest at 12h, which was 2.58 times that of the control group. The expression of *FmNAC11* gene in leaves generally showed a trend of first increasing and then decreasing, and then increasing and decreasing, that is, twice increasing and decreasing to "m" type.

Cold treatment (Fig. 8B): the gene expression increased at 3h, 6h and 12h,

decreased at 1H, 24h and 48h, and was the highest at 12h, which was 21.16 times that of the control group. The expression of *FmNAC11* gene in leaves increased first and then decreased. It indicates that *FmNAC11* can respond to cold and NaCl treatment and maintain up-regulated expression.

Expression pattern of *Fraxinus mandshurica* *FmNAC11* gene induced by hormone

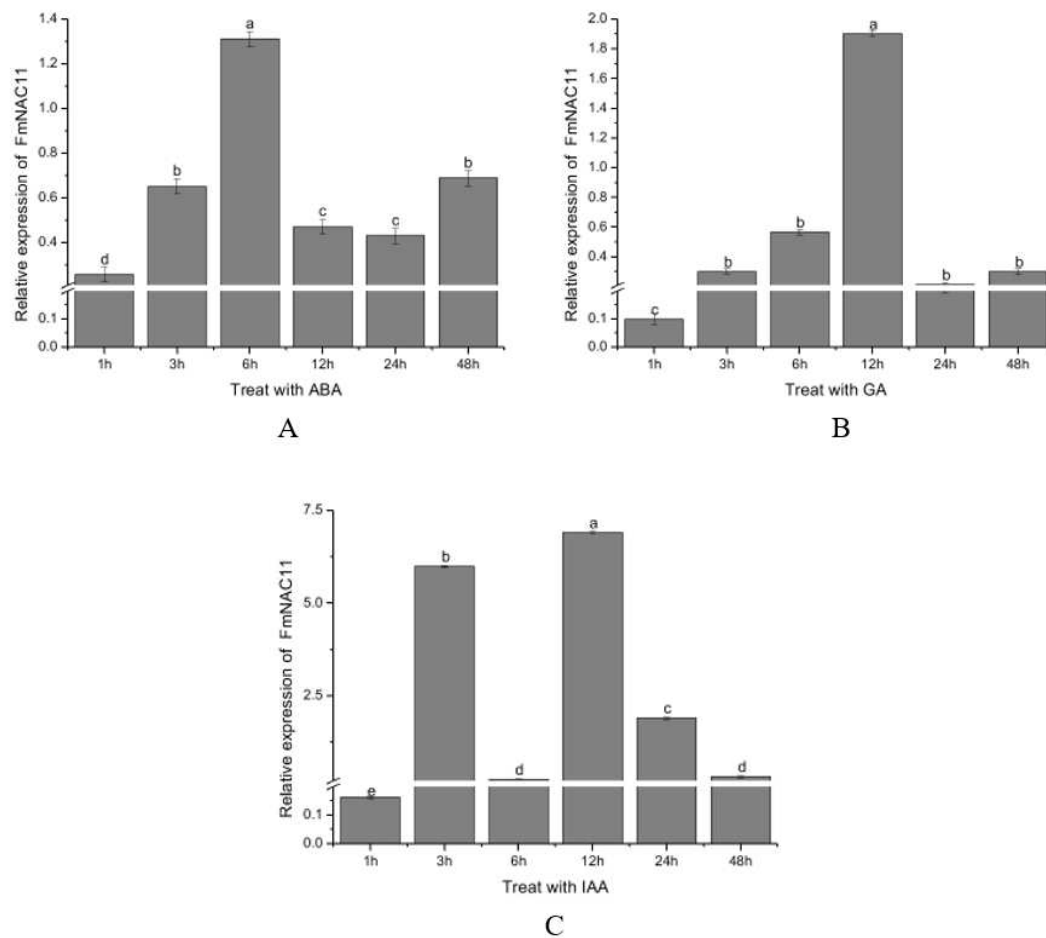


Fig. 9 expression of *FmNAC11* gene induced by IAA, GA and ABA hormone signals

IAA (indoleacetic acid) treatment (Fig. 9C): the expression of *FmNAC11* gene was up-regulated at 3h, 12h, 24h and 48h, down regulated at 1h, 6h and 48h, and the expression was the highest at 12h, which was 6.91 times that of the control group. GA (gibberellin) treatment (Fig. 9B): expression of *fmnac11* gene: it was up-regulated when the treatment time was 12h, and down regulated when the treatment time was 1h, 3h, 6h, 24h and 48h. The expression amount was the highest when the treatment time was 12h, which was 1.9 times that of the control group. ABA (abscisic acid)

treatment (Fig. 9A): the gene expression increased at 6h, decreased at 1h, 3h, 12h, 24h and 48h, and was the highest at 6h, which was 1.31 times that of the control group. It indicates that Fm*NAC11* can respond to the induction of IAA, GA and ABA hormone signals.

Cloning of FmNAC11 promoter and prediction of cis acting elements

The Fm*NAC11* promoter was cloned and 2340 bp was obtained(Figure 10).

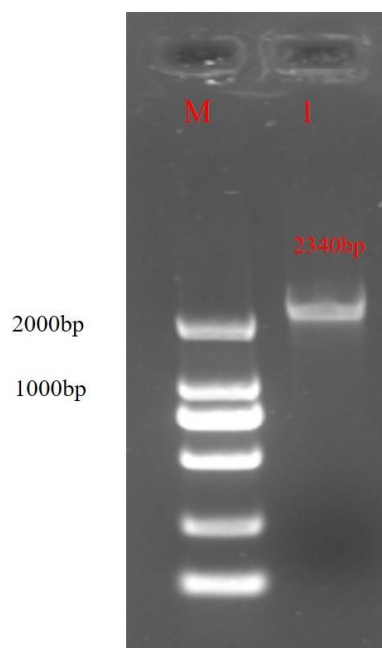


Fig. 10 cloning of Fm*NAC11* promoter

The sequence of *FmNAC11* promoter is as follows (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>):

[illegible]

The cis acting elements of *FmNAC11* promoter were analyzed by plantcare software. The results are shown in Table 1

Table 1 Analysis of cis acting elements of *FmNAC11* promoter

Component name	Element sequence	function	quantity
ARE	AAACCA	Cis acting regulatory elements necessary for anaerobic induction	2
RY-element	CATGCATG	Cis acting regulatory elements involved in seed specific regulation	2
O2-site	GATGACATGG		
CAAT-box	CAAAT	Co cis acting elements of promoter and enhancer regions	8
TATA-box	TACAAAA	Around - 30 core promoter elements around transcription promoter	2
TATA-box	TATA		
GARE-motif	TCTGTTG	Gibberellin reaction element	1
MRE	AACCTAA	Myb binding sites are involved in light response	1
Box 4	ATTAAT	Part of the conserved DNA module involved in the light response	1
TCT-motif	TCTTAC	Part of the light response element	2
TCCC-motif	TCTCCCT		

The cis acting elements in the promoter play a key role in the transcription and activity of the promoter. The plant DNA cis acting regulatory element database was analyzed online by plantcare, and the promoter sequence of *FmNAC11* gene was bioinformatics analyzed to predict its elements and functions. The promoter sequences of *Fraxinus mandshurica FmNAC11* gene can be divided into six categories: transcriptional regulatory elements, photosensitive regulatory elements, stress response elements, hormone response elements, plant tissue-specific elements and specific binding site response elements (Table 1). Among them, the *fmnac11* promoter (2340bp) contains two cis acting regulatory elements are essential for anaerobic induction, two cis acting regulatory elements RY-elements involved in specific regulation, eight common cis acting elements CAAT-box in promoter and enhancer regions, and two core promoter elements TATA box around – 30bp of the transcription promoter. These two transcription regulatory elements are core promoter elements for

eukaryotes to regulate transcription initiation. It is widely distributed in the promoter sequence. At the same time, it also has a gibberellin response element GARE motif, a MYB binding site participating in the light response element MRE, a part of a conserved DNA module participating in the light response, and two light response elements TCT-motif and TCCC-motif. These specific binding site corresponding elements indicate that the promoter of *fmnac11* gene responds to biological and abiotic stresses, participates in a variety of biological pathways, and regulates biological processes.

Acquisition of transgenic tobacco overexpressing *FmNAC11*

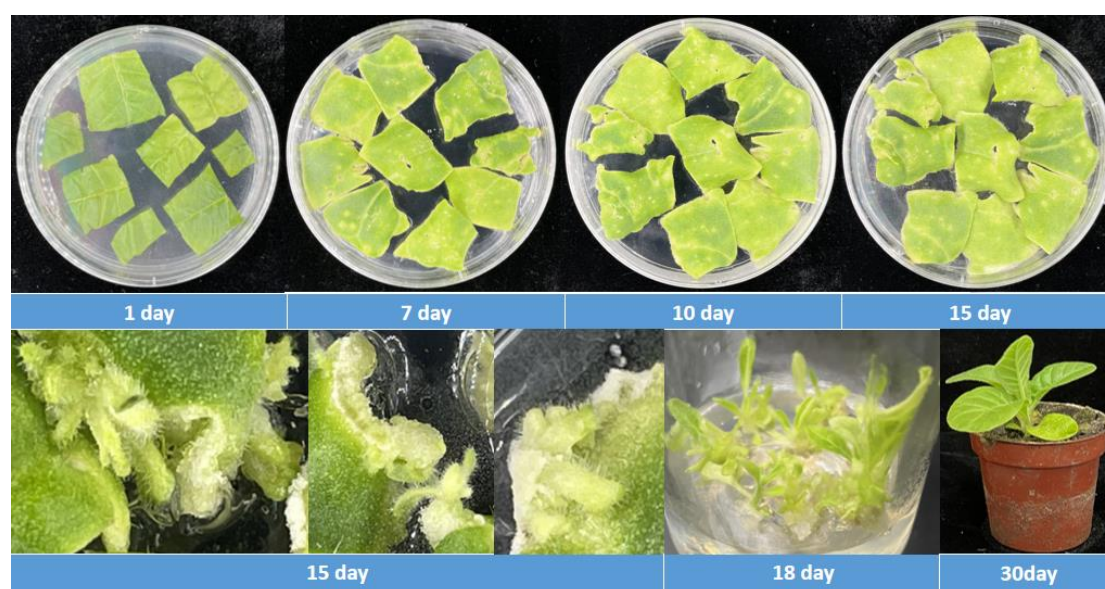


Fig. 11 acquisition of transgenic tobacco overexpressing *FmNAC11*

After three days of pre culture, the cut of the material just began to expand and infect. After three days of co culture in the dark, take photos. It was found that the whole slice expanded a lot, and the material continued to expand at the same time of bacteria removal. After 15 days, it was almost full of plates. At this time, small buds grew on the callus at the cut. The buds were cut off and transferred into rooting screening medium (Fig. 11).

Identification of transgenic lines overexpressing *FmNAC11*

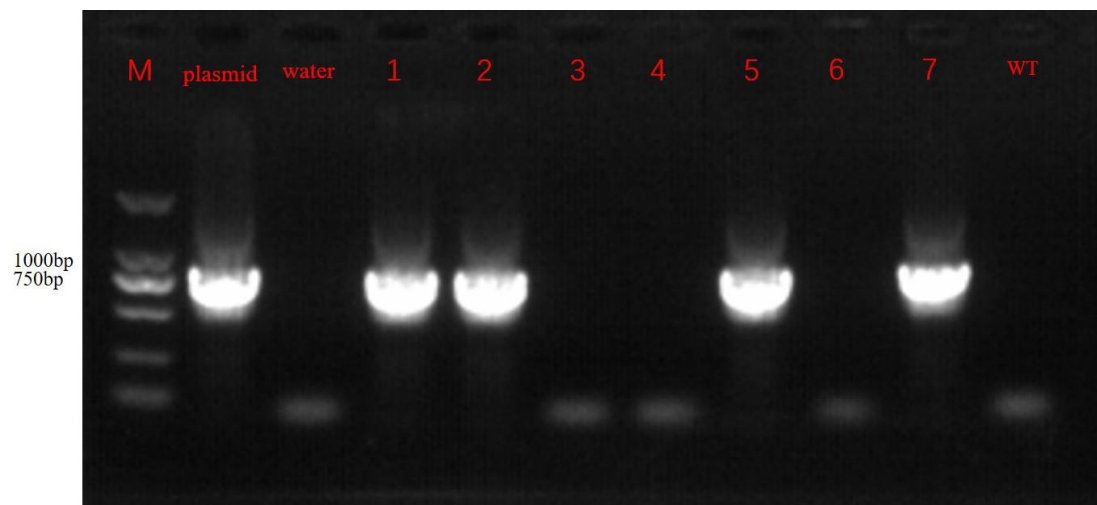


Fig. 12 verification of transgenic tobacco strip overexpressing *FmNAC11*

The successfully constructed overexpression vector was transformed into wt tissue culture seedlings of Benxi tobacco through agrobacterium mediated transformation. The overexpression transgenic tobacco plants were screened through kanamycin resistance to obtain well-growing resistant seedlings. A large number of transgenic tobacco materials were obtained through tissue culture such as differentiation and rooting. The genomic DNA of each strain of WT and transgenic tobacco was extracted respectively and verified by PCR amplification with specific primers. Four transgenic strains were identified (Fig. 12), and the amplified specific bands are consistent with the positive plasmid, the wild-type has no amplified bands.

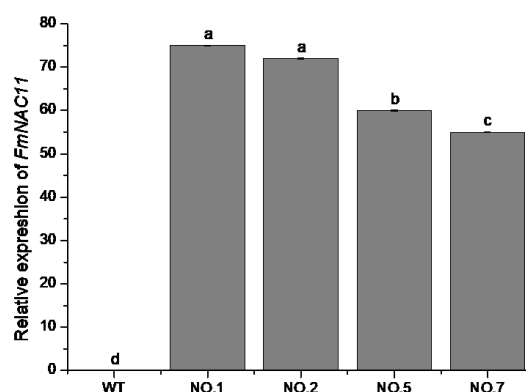


Fig. 13 *FmNAC11* expression of *FmNAC11* transgenic tobacco gene

Four transgenic tobacco lines were analyzed by real-time fluorescence quantitative PCR. Here, when detecting transgenic heterologous overexpression

tobacco, tobacco ntaction gene was used instead of *Fraxinus mandshurica* fntu as internal reference gene (Fig. 13). The quantitative PCR results showed that *FmNAC11* gene was expressed in four strains to varying degrees. The heterologous overexpression level of strain 1 was the highest, 74.99. We selected No.1 with the highest expression for follow-up study.

Growth phenotype analysis of transgenic tobacco plants overexpressing *FmNAC11*



A

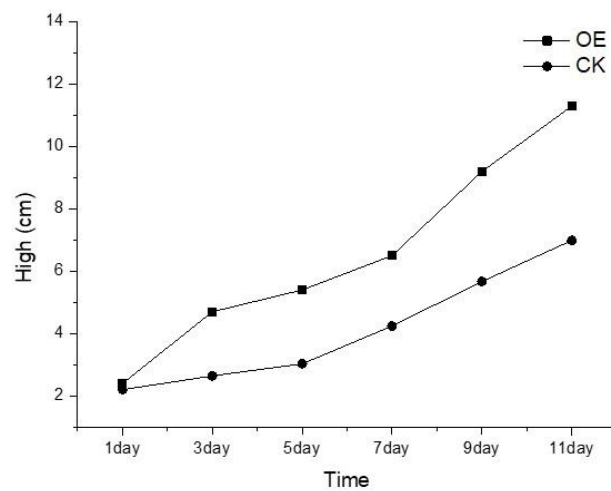


Fig. 14 changes in plant height of *FmNAC11* transgenic tobacco after transplanting

The white arrow indicates the overexpression of transgenic tobacco, and the other three pots are wild-type control at the same period. The plant height of

transgenic tobacco after 11 days of transplantation was measured (Fig. 14A). *FmNAC11* overexpression transgenic tobacco showed a rapid growth phenotype, and the transgenic plant height was 11.3cm, 1.61 times that of WT plant(Fig. 14B). The results showed that overexpression of *FmNAC11* gene accelerated the growth of tobacco plants.

Cold resistance analysis of *FmNAC11* overexpression tobacco plants

Phenotypic observation of transgenic tobacco under low temperature stress

The transgenic and wild-type tobacco seedlings with good growth were selected and placed in a low-temperature incubator at - 5 °C for 24 hours to observe the stress of the plants.



Fig. 15 phenotypic changes of *FmNAC11* transgenic tobacco gene under cold stress

The white arrow indicates the overexpression of transgenic tobacco, and the other three pots are wild-type control at the same time (Fig. 15). That under the condition of low temperature treatment, the situation of overexpression lines is basically the same as that before treatment, while the leaves of wild-type tobacco (WT) are obviously wilting, the main stem begins to yellowing, the main stem softens, and the branches have withered. The results showed that *FmNAC11* gene had strong cold resistance function.

Analysis of physiological indexes of transgenic tobacco under low temperature

stress

Changes of membrane system (MDA, relative conductivity) of transgenic tobacco under low temperature stress

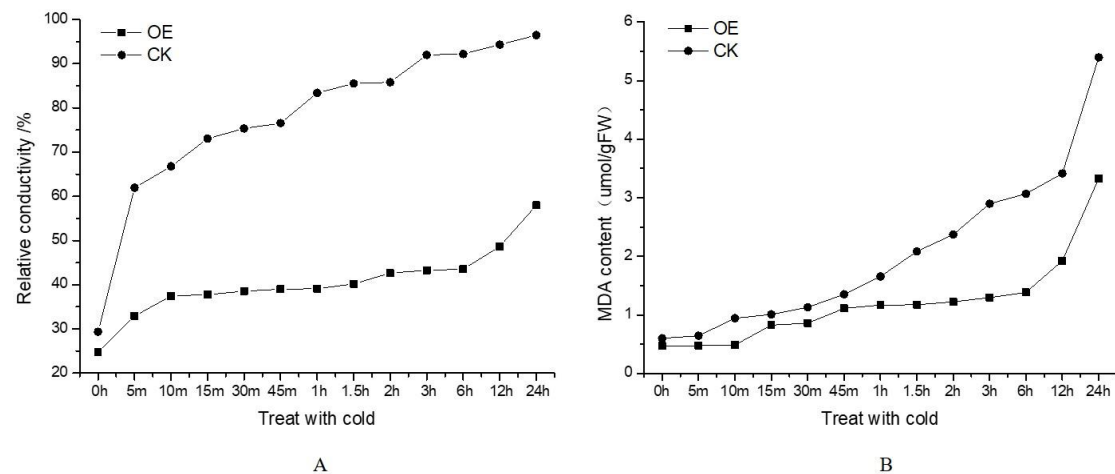


Fig. 16 relative conductivity changes of transgenic tobacco under cold stress

Under cold stress, the MDA content (Fig. 16B) and relative conductivity of the membrane system (Fig. 16A) of *FmNAC11* transgenic tobacco were always lower than that of ordinary tobacco, and showed an upward trend, reaching the highest point in 24 hours, which were lower than 39.9% and 38.4% of ordinary tobacco respectively. It shows that *FmNAC11* transgenic tobacco can resist cold stress and maintain the state of continuous membrane system for a long time, which enhances the tolerance of transgenic tobacco to cold stress.

Changes of osmotic system (soluble sugar and soluble protein) of transgenic tobacco under low temperature stress

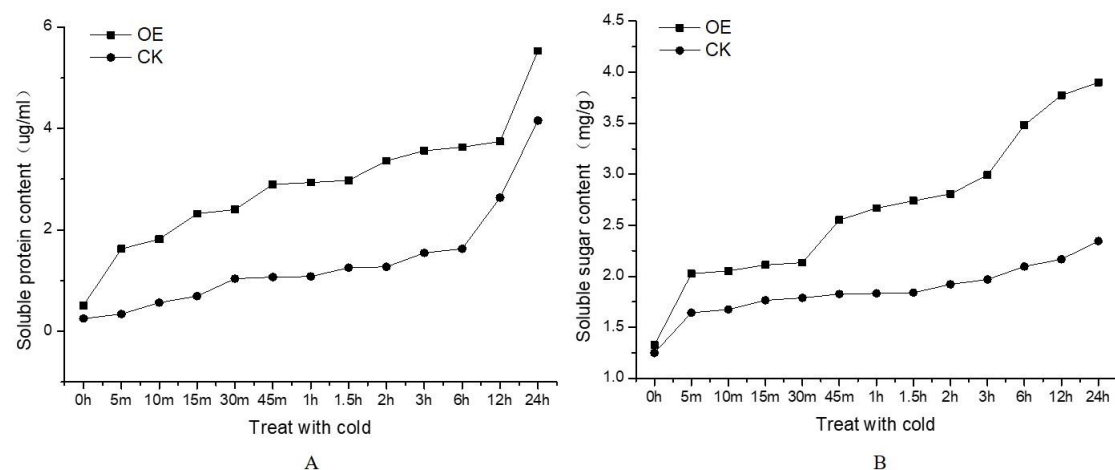


Fig. 17 Changes of soluble protein and soluble sugar content in *FmNAC11* transgenic tobacco under cold stress

Under cold stress, the content of soluble protein (Fig. 17A) and soluble sugar (Fig. 17B) in the osmotic system of *FmNAC11* transgenic tobacco was always higher than that of ordinary tobacco, and showed an upward trend, reaching the highest point in 24 hours, which were higher than 34.1% and 65.5% of ordinary tobacco respectively. It shows that *FmNAC11* transgenic tobacco can increase the solute of osmotic system, reduce the freezing point and enhance the tolerance of transgenic tobacco to cold stress.

Changes of reactive oxygen species scavenging system (POD, SOD) in transgenic tobacco under low temperature stress

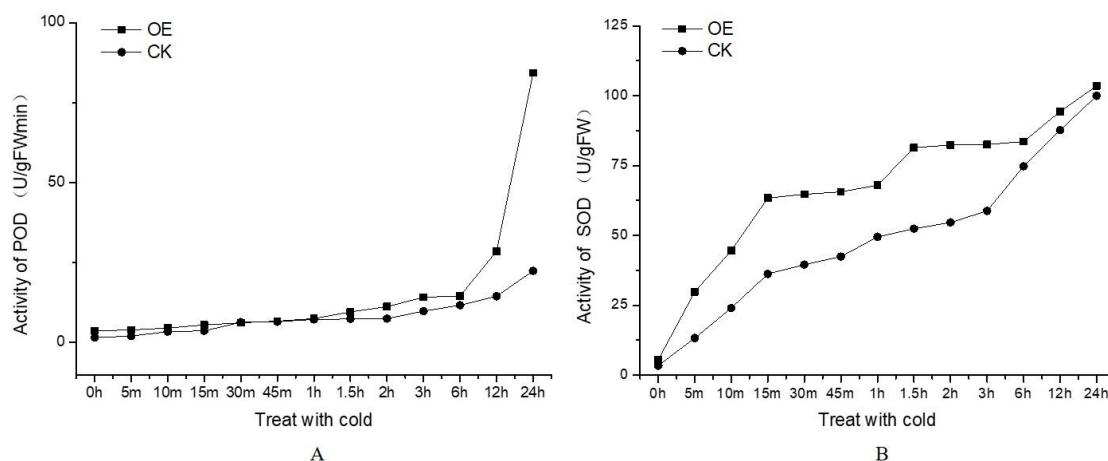


Fig. 18 Changes of POD and SOD activities of *FmNAC11* transgenic tobacco under cold stress

Under cold stress, the activities of POD (Fig. 18A) and SOD (Fig. 18A) in the ROS scavenging system of transgenic tobacco were always higher than those of ordinary tobacco, and showed an upward trend, reaching the highest point in 24 hours, which were higher than 275.7% and 3.6% of ordinary tobacco respectively. It shows that *FmNAC11* transgenic tobacco can resist cold stress and remove accumulated ROS, which enhances the tolerance of transgenic tobacco to cold stress.

Gene expression of transgenic tobacco

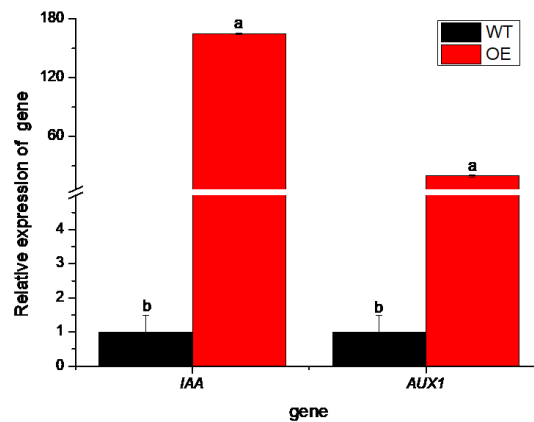
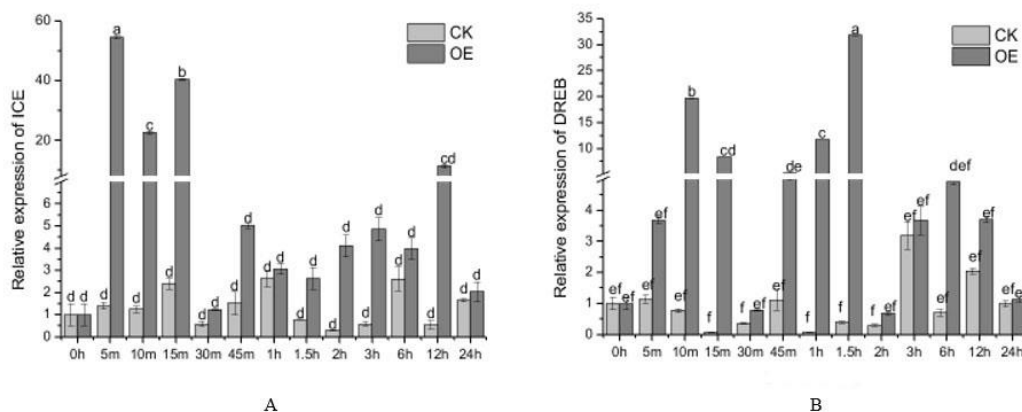
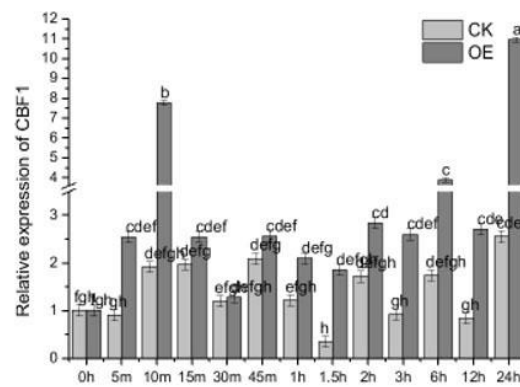


Fig.19 growth related gene expression of *FmNAC11* transgenic tobacco

The expression levels of *IAA* and *AUX1* genes related to the growth of *FmNAC11* transgenic tobacco were higher than that of ordinary tobacco, which were 164.8 times and 19.8 times higher than that of ordinary tobacco, respectively (Fig.19). It shows that there is a regulatory relationship between *FmNAC11* transcription factor and growth related *IAA* and *AUX1* genes, which increases the expression of *IAA* and *AUX1* and promotes the rapid growth of transgenic tobacco.





C

Fig. 20 expression changes of cold resistance related genes in *FmNAC11* transgenic tobacco under cold stress

Under cold stress, the expression of ice, *DREB* and *CBF1* genes related to cold resistance of transgenic tobacco *FmNAC11* was higher than that of ordinary tobacco, and showed a trend of first increasing, then decreasing and then increasing. The expression of *ICE* (Fig. 20A) reached the peak after 5 minutes of cold treatment, 54.61 times higher than that of ordinary tobacco, and the expression was the lowest after 30 minutes of cold treatment. It is 1.21 times that of ordinary tobacco; The expression of *DREB* (Fig. 20B) reached a small peak at 10 minutes of cold treatment, which was 19.65 times that of ordinary tobacco. At 1.5 hours of cold treatment, the expression was the highest, which was 31.82 times that of ordinary tobacco; The expression of *CBF1* (Fig. 20C) reached a small peak at 10 minutes of cold treatment, which was 7.79 times that of ordinary tobacco, and the expression was the highest at 24 hours of cold treatment, which was 10.94 times that of ordinary tobacco; It shows that the transgenic tobacco of *FmNAC11* can quickly respond to the cold signal, maintain the high expression state for a long time, and regulate the expression of related genes. It shows that *FmNAC11* transcription factor has a regulatory relationship with ice, *DREB* and *CBF1* genes related to cold resistance, which increases ice, *DREB* and *CBF1* genes and enhances the tolerance of transgenic tobacco to cold stress.

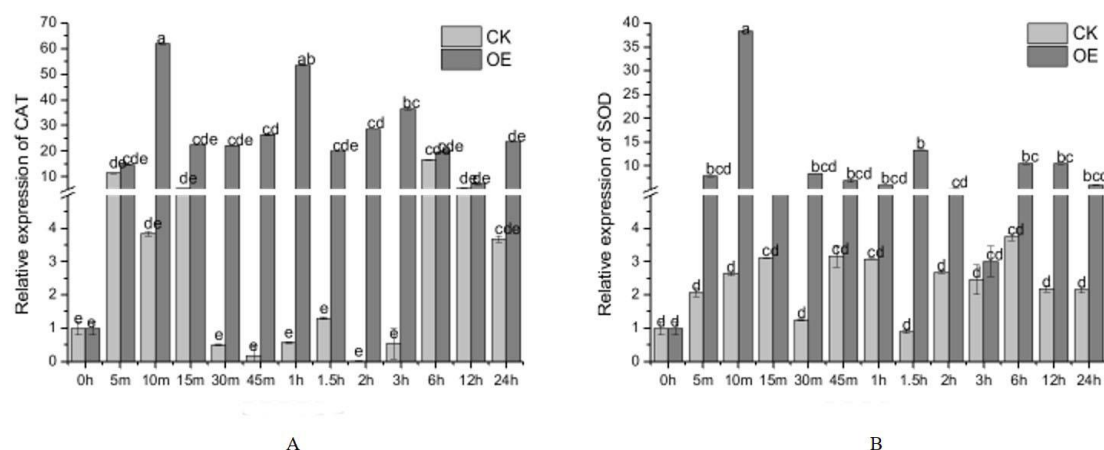


Fig. 21 changes of antioxidant enzyme gene expression in *FmNAC11* transgenic tobacco under cold stress

Under cold stress, the expression of *CAT* (Fig. 21) and *SOD* genes (Fig. 21B) related to ROS clearance in transgenic tobacco was higher than that in ordinary tobacco, and showed a trend of first rising, then falling and then rising. They all reached the highest point after 10 minutes of treatment, which were 23.65 times and 6.04 times higher than that in ordinary tobacco, respectively. It shows that *FmNAC11* transgenic tobacco can quickly respond to the cold signal, maintain high expression for a long time, and regulate the expression of ROS clearance related genes. It shows that *FmNAC11* transcription factor has a regulatory relationship with *cat* and *SOD* genes related to ROS clearance, which increases the expression of *cat* and *SOD* genes, enhances the ability of transgenic tobacco to scavenge reactive oxygen species, and increases the tolerance of transgenic tobacco to cold stress.

Discussion

NAC transcription factors are unique to plants. If the key *NAC* transcription factors in plants are introduced or improved, the purpose of improving wood quality and yield can be achieved. At the same time, it can also enhance the resistance of plants to cold, high salt and drought stress, and improve the comprehensive quality of trees. In this study, *FmNAC11* was cloned by molecular biology related experiments, and the *Fraxinus mandshurica* materials treated with different kinds of exogenous

hormones (IAA, ABA, GA) and abiotic stress (4 °C, NaCl) were detected by fluorescence quantitative PCR. The related bioinformatics analysis of *Fraxinus mandshurica* *NaC11* was carried out to explore the expression of *Fraxinus mandshurica* *NaC11* Structural characteristics to predict the related properties of *FmNAC11* protein. The molecular mechanism of *NaC11* in stress resistance of *Fraxinus mandshurica* was preliminarily revealed, which laid a good theoretical foundation for scientific utilization in the future.

We cloned the *FmNAC11* gene, 891bp, encoding 296 amino acids. The *FmNAC11* protein is a amphoteric protein, with 21 hydrophilic regions and 16 hydrophobic regions. There is no signal peptide and transmembrane domain. The secondary structure is mixed, with a NAC domain, belonging to the Nam superfamily. The tertiary structure model of *FmNAC11* protein was constructed. The coverage of *FmNAC11* protein and *OsNAC11* domain reached 51.88%. The phylogenetic tree of *FmNAC11* was constructed, which showed that the amino acid sequence of *Fraxinus mandshurica* *NAC11* had the highest homology with *Olea europaea* (xp_022848339.1), and the homology was 85.14%. The subcellular localization of *FmNAC11* protein showed that *FmNAC11* was indeed a transcription factor and had transcriptional activation activity.

Under NaCl, cold stress and IAA, GA and ABA hormone signal induction, the expression of *FmNAC11* in *Fraxinus mandshurica* increased, and the expression pattern increased first and then decreased, indicating that *FmNAC11* can respond to cold, NaCl stress and IAA, GA and ABA hormone signal induction. It may play a key role in both stress and growth of *Fraxinus mandshurica*.

The promoter was cloned with a length of 2340 bp. The promoter sequence of *FmNAC11* gene was bioinformatics analyzed by plantcare online analysis, and its elements and functions were predicted. It contained 19 cis acting elements, including an gibberellin response element and a myb binding site involved in light response. It shows that the promoter of *FmNAC11* gene responds to biological and abiotic stress, participates in a variety of biological pathways and regulates biological processes.

The plant height of transgenic tobacco was measured within 11 days after transplanting. The final height of transgenic tobacco was 11.3cm, which was 1.61 times that of wt. the results showed that overexpression of *NAC11* gene accelerated the growth of tobacco plants. Under the condition of low temperature culture at 5 °C for 24 hours, the leaves of wild-type tobacco (WT) obviously wilted and began to yellowing, the main stem softened and the branches withered; The transgenic lines overexpressing *FmNAC11* grew significantly better than the wild-type, and still maintained a healthy state. The stems and leaves were thick and the leaves were green. The results showed that *FmNAC11* gene had strong cold resistance function. Transgenic tobacco grows more vigorously and the plant is stronger at the same time, which enhances the ability to resist the changes of the external environment. *NAC11* gene can promote the growth of roots, so as to enhance the ability of plants to absorb nutrients, so as to make plants grow rapidly and improve cold resistance [45].00

The relative conductivity and malondialdehyde content under low temperature stress can be used to evaluate the degree of membrane damage after low temperature injury. In this study, under cold stress, the MDA content and relative conductivity of the membrane system of *FmNAC11* transgenic tobacco were always lower than that of ordinary tobacco. Even if the cold treatment time of over expressing *FmNAC11* transgenic plants lasted for 24 hours, the MDA content and relative conductivity were also lower than that of the female parent control. With the increase of cold treatment time, the MDA content gradually increased. It indicates that the cell damage is serious. However, it is worth noting that the malondialdehyde content of all samples increased slowly 12 hours before cold treatment. Megha et al. Found that the malondialdehyde content of rape increased with the increase of cold stress time. During low temperature stress, the content of malondialdehyde also fluctuated in chickpea. Low temperature stress can cause plant oxidative stress response through excessive production of reactive oxygen species, and excessive accumulation of reactive oxygen species can cause lipid peroxidation and lead to the accumulation of malondialdehyde content. Under low temperature stress, ROS scavenging enzymes play a major role in ROS scavenging. In this study, the relatively gentle increase of

malondialdehyde content before 12 hours of cold treatment may be due to the increase of catalase and superoxide dismutase activities. When the cold treatment time reaches 24 hours, although the activities of catalase and superoxide dismutase are very high, the accumulation of malondialdehyde content cannot be reduced due to the serious damage to cells caused by long-term low-temperature stress [36-40].⁰⁰

Under cold stress, the soluble protein content, soluble sugar content, pod and SOD of ROS scavenging system of *FmNAC11* transgenic tobacco are always higher than those of ordinary tobacco. Plants improve high and low temperature tolerance through the changes of Osmoregulation Substances (such as the accumulation of soluble sugar and soluble protein). Soluble sugar in plants plays an important role in cold resistance. The accumulation of soluble sugar content under low temperature plays a key role in preventing low temperature injury [41-43]. In this study, the contents of soluble sugar and soluble protein increased with the increase of cold treatment time. The expression of soluble namc11 protein in transgenic plants can increase the content of soluble sugar. The transgenic of *FmNAC11* enhanced the tolerance of transgenic tobacco to cold stress.⁰⁰

The gene expression of transgenic tobacco under cold treatment was as follows: *FmNAC11* had regulatory relationship with growth related IAA and AUX1 genes, cold resistance related ice, *DREB* and *CBF1*, ROS clearance related cat and SOD genes, which promoted the rapid growth, cold resistance and ROS clearance ability of transgenic tobacco.

Many nac-if are reported to be induced by cold stress. And participate in the response of plants to cold stress. *CaNAC2* can improve the sensitivity of pepper seedlings to low temperature; *GMNA20* has a positive regulating effect on the cold tolerance of transgenic *Arabidopsis* plants; *NaC11* transcription factor of banana (*Musa aeveninata*) can enhance the cold tolerance of banana; *SNAC2* of *Oryza sativa* L and pbenac1 of pear give cold tolerance to rice and pear respectively; *NaC11* in tomato is induced by low temperature, which can improve the cold resistance of transgenic plants; *TaNAC2*, *TaNAC47* and *TaNAC67* in wheat can enhance the cold tolerance of transgenic plants. Among the NAC-TFs related to cold stress, several

regulate plant cold resistance through CBF-COR signal cascade. For example, *GmNAC20* mediates stress tolerance by regulating the expression of *cor* gene, *DREB1A / CBF3* and *DREB1C / CBF2*. *GINAC20* can directly bind to the promoters of *DREB1A / CBF3* and *DREB1C / CBF2*, resulting in increased *CBF3* transcription and decreased *CBF2* transcription. In banana, *mannac1* is the target gene of *maice1*. *Mannac1* protein interacts with *macbf1* to regulate the cold tolerance of banana fruit. *Pbenac1* protein can improve the transcription level of some stress-related genes by interacting with *pbeDREB1* and *pbeDREB2A*, so as to enhance the race tolerance of transgenic tobacco. These studies show that NAC-TFs plays a role by downstream of *ICE1* and upstream of *CBF*. [44]⁰

conclusion

Transgenic tobacco overexpressing *FmNAC11* performed better in membrane system, osmotic system and ROS scavenging system than ordinary tobacco, indicating that *FmNAC11* gene can improve the cold resistance of plants. *FmNAC11* transcription factor has regulatory relationship with growth related *IAA* and *AUX1* genes, cold resistance related *ICE*, *DREB* and *CBF1*, ROS clearance related *CAT* and *SOD* genes, which promotes the rapid growth, cold resistance and ROS clearance ability of transgenic tobacco, and provides a theoretical basis for improving *Fraxinus mandshurica* germplasm resources and obtaining better *Fraxinus mandshurica* varieties in the future.

Materials and method

Experimental materials

Fraxinus mandshurica seeds were collected from the experimental forest farm of Northeast Forestry University. The seedlings of *Fraxinus mandshurica* seeds 15 days after germination were treated with NaCl, low temperature, IAA, GA and ABA, and sprayed on the leaves. The control group did not do any treatment. Then the culture was continued under normal conditions. Samples were taken at 1 hour, 3 hours, 6 hours, 12 hours, 24 hours and 48 hours after treatment, and repeated three times

each. The samples were stored in the refrigerator at - 80 °C.

Cloning of the full length of *FmNAC11* gene

(1) Clone primer design

Primer primer5 was used for primer design, and the results are shown in table 4-1

Table 4-1 corresponding primers in the coding region of cloned FmNAC1 gene

Primer name	Primer sequence	TM value
FmNAC1-F	5'-AAAATGAGCAGTCTGAGCATGG-3'	59.6
FmNAC1-R	5'-TCAATGGTAATTCCACATTGAAGGC-3'	59.7

(2) PCR system and procedure

The cDNA of *Fraxinus mandshurica* obtained above was used as a template, and the NAC11 gene was amplified with the designed and synthesized upstream and downstream primers. The PCR system is shown in Table 4-2:

Table 4-2 cloning system of FmNAC1 gene

Buffer	TwoμL
Taq	Zero point twoμL
dNTP	One point sixμL
FmNAC1-F	OneμL
FmNAC1-R	OneμL
CDNA template	OneμL
ddH2O	Up to 20μL

PCR program settings:

Pre denaturation: 94 °C, 5 min

30× cycle: $\left\{ \begin{array}{l} \text{Denaturation: 94 °C, 30 s} \\ \text{Annealing: 57 °C, 45 s} \end{array} \right.$

Extension: 72 °C, 30 s

Extension: 72 °C, 10 min

4°C, ∞

Connection and transformation of *FmNAC11* with easy-t5 vector

1. Add the following reagents into the PCR tube, and the reaction system is:

PCR purified product (5ng / ml), 4ml; Easy-t5 carrier (10NG / ml) 1ml.

2. Shake slightly, mix and centrifuge, react at 25 °C for 5 minutes, centrifuge after the reaction, and then place on ice.

3. Connect the easy-t5 carrier according to the instructions, then apply the plate, select a single colony after overnight culture, and then culture until the bacterial solution is turbid.

4. After PCR verification, the positive bacterial solution was sent to the company for sequencing.

Subcellular localization of FmNAC11 protein

For the method of vector construction, see cloning and genetic transformation of bhlh transcription factor gene of *Populus deltoides*

Bioinformatics analysis of *FmNAC11* gene

The following software and websites are used for bioinformatics analysis of *FmNAC11* gene, dnaman: nucleotide analysis; ProtParam: physical and chemical properties of protein; ProtScale: hydrophilic / hydrophobic; SignalP: signal peptide; Gor4: secondary structure; TMpred: transmembrane structure prediction; Proline: multiple sequence alignment; Mega 5.0: construct evolutionary tree.

Stress and hormone signal induction of *Fraxinus mandshurica* seedlings

The tissue cultured seedlings of *Fraxinus mandshurica* were stressed with 4 °C low temperature and NaCl respectively, and induced with ABA, IAA and GA for 1h, 3h, 6h, 12h, 24h and 48h; The control group did not do any treatment, and the time gradient was the same as above. See 3.1.2 for the method

Table 3-4 fluorescence sequence of real-time PCR primers

Primer name	Primer sequence	TM value
QFmNAC1-F	5'-CAAGTGTCTGCTTCTCCATTTTA-3'	62.1
QFmNAC1-R	5'-AGGGTTCGTCGTGTCTCTCGT -3'	61.9

Experimental materials:

Wild type tobacco (WT) (*N. tabacum*) is preserved in the forest bioengineering Laboratory of Northeast Forestry University.

Experimental method:

1) Vector construction, genetic transformation, identification and propagation of transgene: Construction of *FmNAC11* overexpression vector and preparation of *Agrobacterium* by three parent hybridization. Refer to the methods in Liang Nansong's master's thesis for reference;The genetic transformation, transgenic identification and propagation of tobacco refer to the methods in Dr. Zhao Xingtang's thesis.

2) Cold treatment: - 5 °C low temperature incubator for cold treatment;

3) Real time fluorescence quantitative PCR reaction: the quantitative primer sequences of ICE1, DREB, CBF1, cat, SOD and other genes related to cold tolerance in tobacco refer to the primer sequences of related genes in Liu Ying's master's thesis. The gene expression detection method is the same as that in Chapter 3.

Abbreviations

IAA:3-Indoleacetic acid;AUX1:AUXIN RESISTANT
1;DREB:dehydration-responsive element-binding protein;CBF1:C-repeat/DRE
binding factor 1;CAT:Catalase;SOD:Superoxide
dismutase;GA:Gibberellin;ABA:Absciscic
Acid;MDA:Malondialdehyde;SOD:Superoxide dismutase;POD:Peroxidase.

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Authors' contributions

CY conceived the idea of the study and designed the experiments. CY performed most of the experiments. CY, SF, LZ collected and analysed the data. CY, SF involved in writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated and analysed during the current study are available in the National Center for Biotechnology Information repository. And the sequence of FmNAC11 in this study can be accessed at accession numbers: FmNAC11 (MG269829.1,

[https://www.ncbi.nlm.nih.gov/nucleotide/MG269829.1?report=genbank&log\\$=nuclalign&blast_rank=1&RID=HDXDRHRJ013](https://www.ncbi.nlm.nih.gov/nucleotide/MG269829.1?report=genbank&log$=nuclalign&blast_rank=1&RID=HDXDRHRJ013))

In addition, all databases used in this study are open for public and the links are as follows:

PlantTFDB: http://planttfdb.gao-lab.org/blast_result.php

ExPasy: <https://swissmodel.expasy.org/interactive/56MxmS/models/>; <https://swissmodel.expasy.org/templates/3ulx.1>.

Plantcare: <http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>

Declarations

Ethics approval and consent to participate

The experimental materials are obtained in Maoershan Experimental Forest Farm of

Northeast Forestry University, and part of the complete plants were cultured in the greenhouse of Northeast Forestry University. Experimental research and field studies on plants are comply with relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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