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Cloning and functional analysis of *CIVND1*, a member of the OsNAC7 subfamily in *Chrysanthemum morifolium*

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Abstract

NAC transcription factors play important roles in plant growth and development. In this study, *CIVND1* was cloned from the NAC transcription factor of *Chrysanthemum lavandulifolium*. Through bioinformatics analysis, the gene was found to belong to the OsNAC7 subfamily with an open reading frame (ORF) of 1164 bp and encoding 387 amino acids. Through paraffin sectioning and stress treatment, it was found that this gene could increase the thickness of the secondary cell wall of transgenic *Arabidopsis* stems and lead to the growth of more lateral roots to improve the tolerance of *Arabidopsis* seedlings to salt or low temperature. Moreover, the inflorescence elongation rate and height of wild-type *Arabidopsis* were greater than those of transgenic *Arabidopsis*, but the flowering period was later than that of transgenic *Arabidopsis*. These results indicate that *CIVND1* may resist external stress by regulating root growth and can inhibit inflorescence growth and promote flowering. The purpose of this study was to improve stress resistance and promote the growth and development of *C. morifolium* by regulating the expression of *CIVND1*, which is of great significance for the study of chrysanthemum resistance to abiotic stress and growth and development.

Keywords: NAC, *CIVND1*, Stress, *Chrysanthemum lavandulifolium*

1. Introduction

Plants are often affected by various abiotic and biotic stresses, such as drought, high salinity, low temperature, and pathogens, during growth and development. These stresses usually cause damage to plant cells, which affects plant growth and crop production (Sun 2012). Transcription factors play important roles in plant adaptation to the environment and resistance to stress. *A. thaliana* is one of the earliest plants to be studied for NAC transcription factors, which are mainly related to the regulation of secondary wall synthesis (Abe et al. 1997; Mitsuda et al. 2005). Among them, *NST1*, *NST2* and *SND1* belong to the secondary wall NAC family and act as transcription switches to activate secondary cell wall biosynthesis genes (Zhong and Ye 2014). In recent years, it has been found that NAC transcription factors are expressed in different developmental stages and tissues of plants and are closely related to plant lignin synthesis, growth and development, and the regulation of abiotic and biotic stress (Liu et al. 2000, 2018; Wang and Zhang 2018).

The OsNAC7 subfamily is the most studied subfamily in the NAC family and includes *SND1*, *NST1*, *URP7*, *BRN1/2*, *VND1/2/3/4/5/6/7* (Zhong et al. 2006; Ko et al. 2007; Mitsuda et al. 2007; Soyano et al. 2008; Bennett et al. 2010; Yamaguchi et al. 2010; Fendrych et al. 2014; Zhou et al. 2014; Endo et al. 2015; Zhong and Ye 2015). The main functions focus on regulating the formation of secondary cell walls in stems, roots and anthers. They play an important role in lignin regulation, growth and development regulation and stress regulation (Zhou et al. 2014; Fang et al. 2020; Sun et al. 2020; Yang et al. 2020; Yu et al. 2021). Previous studies have shown that *CISND1* in the chrysanthemum brain can lead to tolerance of transgenic tobacco seeds and seedlings to salt and low-temperature environments, reduce the growth of tobacco and lead to early flowering, which results in secondary xylem of transgenic tobacco and secondary xylem cell wall thickening (Aida et al. 1997; Chen et al. 2015; Dong et al. 2018).

VND (*vascular-related NAC domain*) is a group of NAC family transcription factors that encode a specific NAC domain. Seven vascular-related VND genes have been found in *A. thaliana* (He et al. 2019; Duan et al. 2020; Tyrol 2024). To date, a total of 102 NAC family members have been identified in the pepper genome (Huang 2023), 101 in tomato (Zhang 2014), 163 in poplar (Qi 2012), 190 in maize (Zhou 2017), 143 in grape (Sun 2011), and 152 in soybean (Wang 2014). To study VND in *Arabidopsis thaliana*, Zhou and others studied the role of *VND1-VND5* genes in regulating the biosynthesis of the secondary cell wall in *A. thaliana*. *VND1-VND5* were specifically expressed in the vessels, while *VND4* and *VND5* were also expressed in the vessels of the secondary xylem of the root hypocotyl (Zhou et al. 2014). Tan found that *VND1*, *VND2* and *VND3* play special roles in the differentiation of xylem vessel elements in cotyledons (Tan et al. 2018). The

differentiation of xylem vessel elements is highly dependent on *VND1*, *VND2* and *VND3* rather than *VND6* or *VND7*. In addition to *Arabidopsis* and poplar, some studies have investigated *VND* in bananas. Banana *MusaVND1* contains a highly conserved NAC domain and is a homologous gene of *Arabidopsis VND1* (Negi et al. 2018). *MusaVND1* can induce the expression of genes related to lignin and cellulose biosynthesis pathways. The content of lignin and cellulose in transgenic bananas overexpressing *MusaVND1* increased compared to the control (Negi et al. 2015; Tamura 2017; Tan et al. 2018).

Chrysanthemum (*Chrysanthemum lavandulifolium* Ramat) is one of the ten famous flowers in China and is deeply loved by the people (Wu 2016). *Dendranthema lavandulifolium* (Fisch.ex Trautv.) is a *Dendranthema* plant in the Compositae family. It is distributed mainly in Qinghai, Gansu and Xinjiang, China (Liu 2023). It is a near-source species of cultivated chrysanthemum and is considered to be one of the important parental materials for the formation of cultivated chrysanthemum (Chen 2023). However, the current study only conducted a preliminary study of some of these genes, and the specific functions of these genes are not clear.

2. Materials and methods

2.1. Experimental materials

The aseptic seedlings of Chamomile were obtained from the School of Landscape and Forestry of Qingdao Agricultural University and were cultured in 1 / 2MS medium. *Nicotiana benthamiana* seeds were sown on MS mediums and transplanted into a pot with peat substrate when 2-3 leaves had grown. *A. thaliana* was used for genetic transformation of *CIBRN1*. All plant materials were cultured in the artificial climate chamber of Qingdao Agricultural University (Qingdao, China) at a temperature of $\pm 25^{\circ}\text{C}$ and relative humidity of 60 % with light intensity at 2500lx and illumination and darkness periods of 16 hours and 8 hours, respectively.

2.2. Test method

2.2.1. Target gene fragment

Leaves of *C. morifolium* with good growth were selected for total RNA extraction. The total RNA extraction kit used was the FastPure® Universal Plant Total RNA Isolation Kit (Vazyme, China). The reverse transcription kit used was the HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, China). The full-length coding sequence of the target gene was designed according to the chrysanthemum brain genome

data (<http://www.amwayabrc.com>) (Table 1). The full-length coding sequence of the target gene was obtained by high-fidelity DNA polymerase 2 × Phanta Flash Master Mix (Dye Plus) (Vazyme, China).

Table 1. The design of the primer that contains the enzyme tangent site.

Primer name	Sequence (5'→3')	TM/° C	Purpose
<i>CIVND1-F</i>	gcTCTAGAatgatcatggacacagtaga	57.29	Amplification of the full-length <i>VND1</i> sequence with restriction sites.
<i>Cl VND1-R</i>	aaCTGCAGatcaaaaatgcataatcca	54.71	

2.2.2. Bioinformatics analysis

Based on the amino acid sequence of *CIVND1*, nucleotide (amino acid) sequence multiple alignment was performed using MEGA11.0.13, and its sequence was analyzed in detail. DNAMAN version 8.0 (Lynnon Biosoft, Quebec, Canada) was used to align the sequences.

2.2.3. Observation of the subcellular localization signal

The GFP signal was observed by a fluorescence microscope (Leica DM 2500) and a laser confocal microscope (Leica TCS SP5).

2.2.4. Construction of the *super1300* overexpression vector

The design of primers containing restriction sites is shown in Table 1.

According to the ORF region of the *CIVND1* gene and the multiple cloning sites of pSuper1300 : : GFP vector, primers containing PstI endonuclease and KpnI endonuclease restriction sites and without a termination codon were designed. The target fragment containing restriction site was obtained by PCR reaction and agarose gel recovery. The target fragment containing the restriction site and pSuper1300 : : GFP vector were double digested (37 °C, 2h) with endonucleases PstI and KpnI (NEB, Beijing) to obtain the target fragment and linear vector with sticky ends. The digested products were purified by agarose gel electrophoresis, recovered, and ligated with T4 DNA ligase (NEB, Beijing) to form a recombinant plasmid. The recombinant plasmid was transformed into *E. coli* competent DH5α and spread on a solid LB medium containing 50 mg / L kanamycin for resistance screening and identified by colony PCR. The resulting positive plaque was sent to a sequencing company for sequencing and plasmid recovery. The constructed pSuper1300 : : *CIVND1* : : GFP expression vector was transformed into *Agrobacterium tumefaciens* GV3101 (Shanghai, Weidi Biology) by freeze-thawing and spread on the LB solid medium with 3 antibiotics (50mg / L kanamycin, 50mg / L gentamicin, 50mg / L

rifampicin) for resistance screening. The positive plaque was identified by colony PCR and transferred to the liquid LB culture of 3-antibiotics for propagation.

2.2.5. Transformation and screening of *A. thaliana*

The Super1300 : : ClBRN1 : : GFP overexpression vector was transformed into *A. thaliana* by the Agrobacterium-mediated floral dip method, after which the transgenic *A. thaliana* seeds were screened by a resistance screening medium (1 / 2MS + 30g / L sucrose + 6g / L agarose + 50mg / L hygromycin); the homozygous offspring were obtained in the T2 generation.

2.2.6. Stress treatment and phenotypic observation

Transgenic *A. thaliana* was subjected to abiotic stress treatment at the seedling stage, and the tobacco seedlings with the same growth vigor were selected and transferred into the treatment medium. The medium selected was the 1 / 2MS basic medium, with low temperature treatment and control, and 200 nmol / L NaCl was used for the salt treatment. After 3 days of treatment, the root length data were statistically analyzed.

The seedling tobacco was also subjected to stress. Five plants with similar growth vigor were selected from each treatment of Super1300 : : GFP and Super1300 : : *CIVND1* lines for stress treatment. The salt treatment was conducted by applying a 200 mmol · L⁻¹ NaCl solution. Low temperature treatment using a low temperature incubator was performed at a temperature of ± 4 °C with light for 12 h. The two treatments were cultured at ± 25 °C, and a normal water supply was used for the control (CK). After 3 days, the transgenic plants of *A. thaliana* were photographed, and related data were collected.

2.2.7. Observation of the growth and development of transgenic *A. thaliana*

Transgenic *A. thaliana* and the control group were transferred into a 7cmX7cm flowerpot when two true leaves had grown. The same maintenance management was carried out on the experimental group and the control group, and the rosette diameter, plant height and flowering time were measured.

2.2.8. Paraffin sectioning

Samples of the stems of transgenic *Arabidopsis* plants treated with 1cm were taken from above 1/3 of the stem from the soil. The samples were placed in FAA (70 % alcohol : formalin : acetic acid 18 : 1 : 1) for 24h, then soaked in a mixed solution of hydrogen peroxide and glacial acetic acid (1 : 1) for 48h to soften the

material. The samples were dehydrated with ethanol, embedded in paraffin, and divided into 10 µm slices by a microtome. Finally, the samples were stained with safranin green. In the experiment, three biological replicates were set up, the cell wall thickness of 10 cells was randomly measured in the field of view, and statistical analysis was performed.

2.2.9. Data analysis

All treatments mentioned in this study involved at least three independent biological and technical replicates. Data statistics and analysis were performed using Microsoft Excel 2016, GraphPad 8.0.2, and IBM SPSS 27 software.

3. Results

3.1. Bioinformatics analysis of *CIVND1*

The *CIVND1* cDNA was cloned by designing specific primers, and a fragment was amplified and sequenced. The open reading frame (ORF) of *CIVND1* was 1164 bp encoding 387 amino acids. The nucleotide sequence of the *CIVND1* gene was compared with the nucleotide sequence of the chrysanthemum brain CHR0003673-RA, and the two gene sequences were found to be similar (Fig. 1). Conserved domain analysis of the *CIVND1* gene sequence (Fig. 2) found that the gene contains a NAM conserved domain and is a member of the NAC transcription factor family.

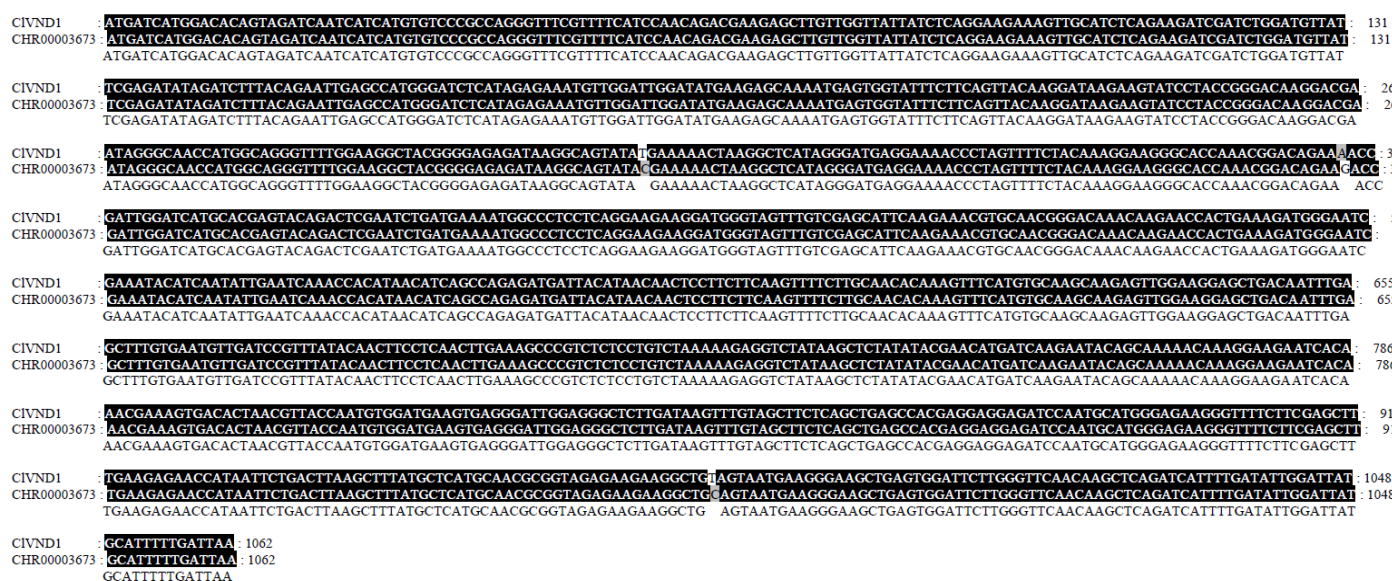


Fig. 1 Cloning and comparative analysis of the *CIVND1* gene in *Chrysanthemum lavandulifolium*

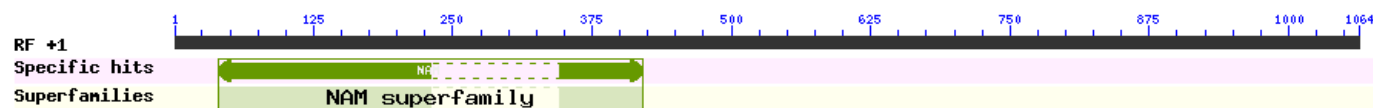


Fig. 2 Conserved domain analysis of the *CIVND1* gene in *C. morifolium*

The amino acid sequence similarity of the gene was compared in NCBI which revealed that the gene was most similar to the NAC gene of Compositae plants, durian, bayberry, poplar and walnut. Phylogenetic tree analysis (Fig. 3) showed that the gene was closely related to the NAC domain protein of *Artemisia annua* and pyrethrum (*Tanacetum cinerariifolium*). In this study, the Ze567 (AB217775) protein was found to be similar to the VND protein in the phylogenetic tree and was upregulated during the formation of xylem vessel molecules in *Zinnia elegans*. The NAC-related protein (PWA76361.1), which is closely related to the *CIVND1* protein, was also named Ze567 in the genome study of *Artemisia annua*.

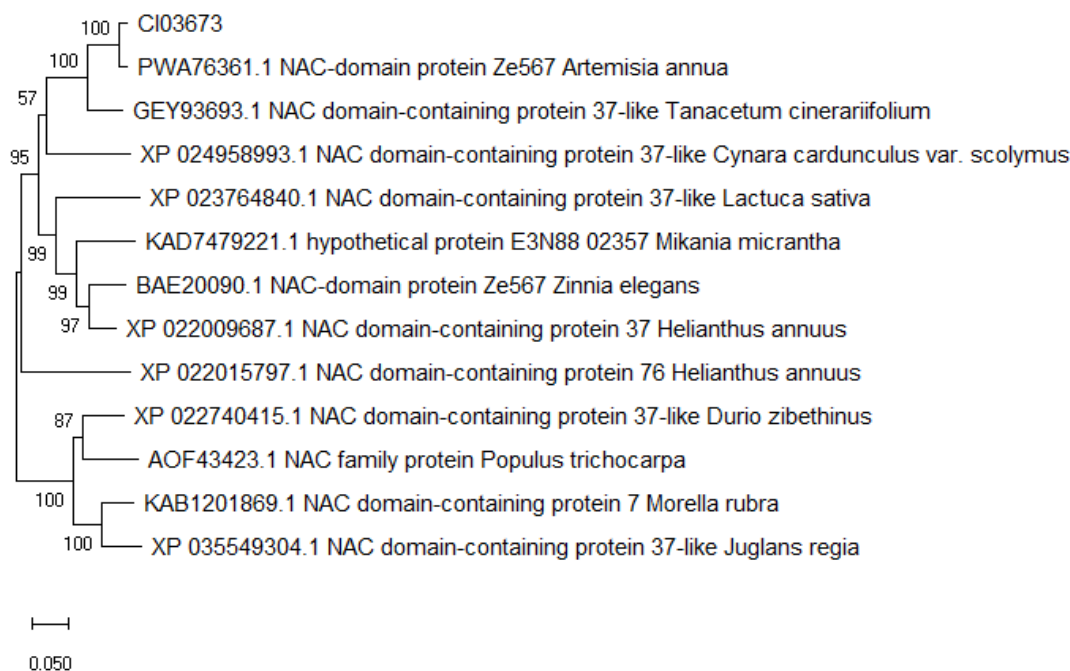


Fig. 3 Evolutionary analysis of the *CIVND1* protein in *Dendranthema lavandulifolium*

Phylogenetic analysis of the *CIVND1* protein with 12 members of the *Arabidopsis* OsNAC7 subfamily (Fig. 4) showed that the gene was closely related to the *AtVND1* and *VND2* proteins, suggesting that *CIVND1* is a VND protein.

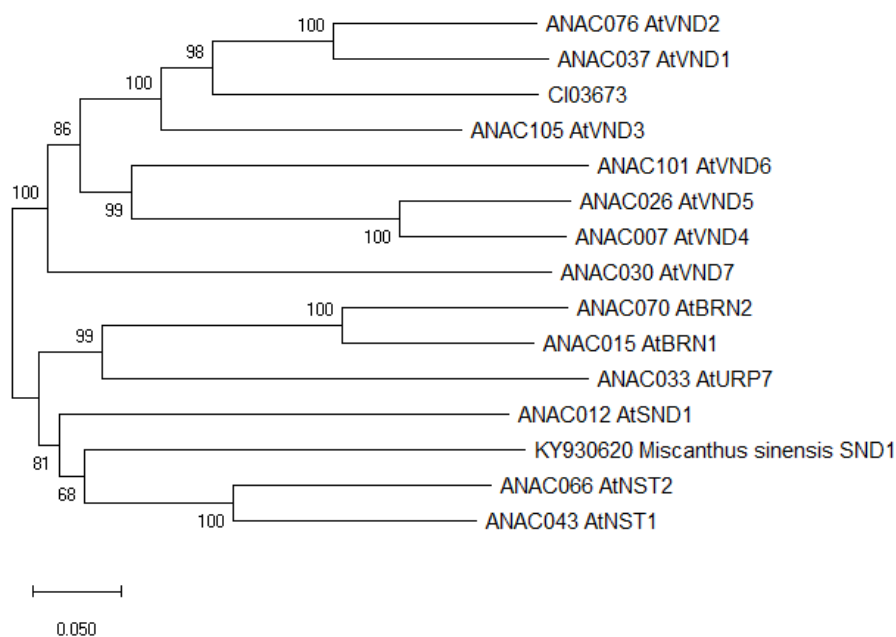


Fig. 4 Homologous alignment of the *CIVND1* protein with OsNAC7 subfamily members in *A. thaliana*

3.2. Subcellular localization of tobacco

In this study, the expression location of the *CIVND1* gene was examined in transgenic tobacco. The fluorescence signal in the leaves of the transgenic plants was observed, and it was found that the pSuper1300 empty transgenic tobacco could recognize the fluorescence signal in the nucleus and cell membrane of the leaves. Fluorescence signals were detected in the nucleus and cell membrane of Super35S::*CIVND1*::GFP transgenic tobacco leaves (Fig. 5). These results indicate that *CIVND1* is localized in the nucleus and cell membrane.

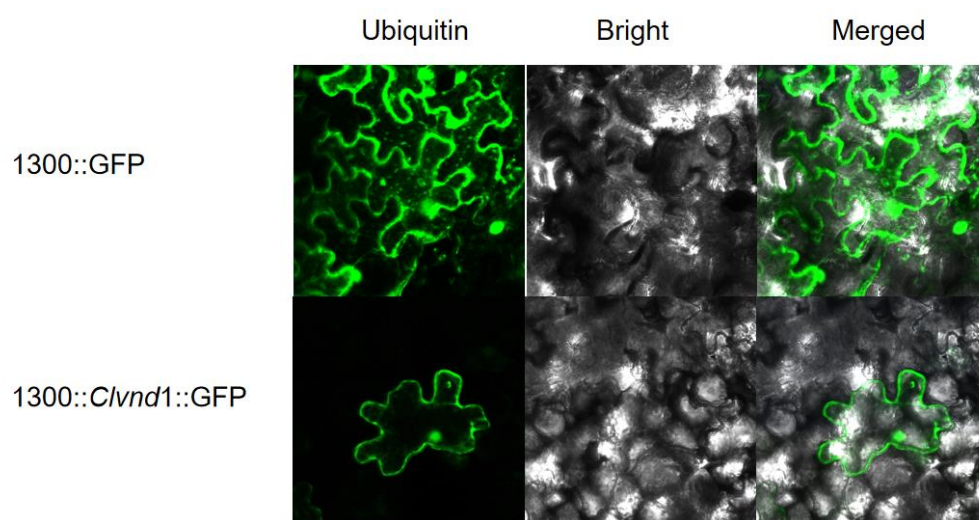


Fig. 5 Subcellular localization of *CIVND1* in tobacco

3.3. Screening of transgenic *A. thaliana*

Transgenic *Arabidopsis* seeds were screened by an *Arabidopsis* resistance screening medium; homozygous offspring were obtained when screened to the T2 generation.

3.4. Response of transgenic *A. thaliana* to NaCl and 4 °C low-temperature treatments

After 3 days of observation (Fig. 6), it was found that the root and upper part of the root of the transgenic *A. thaliana* with the *CIVND1* gene grew significantly in comparison to the control group at 4 °C, and the ability to resist low temperature was enhanced . Under the treatment of 200mmol / L NaCl, the transgenic materials began to produce a large number of short lateral roots, and the upper part of the roots of the transgenic *CIVND1* gene grew significantly.

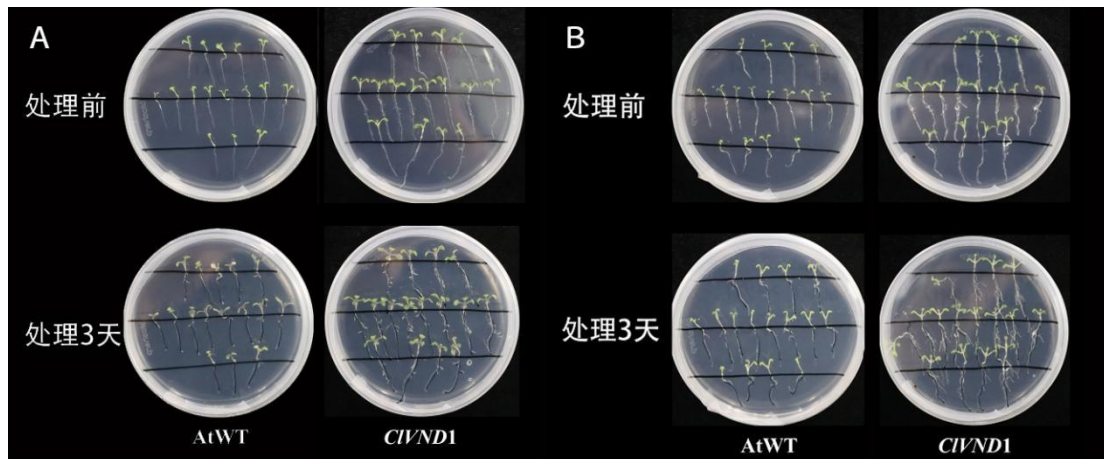


Fig. 6 A. 200mmol / L NaCl treatment for 3 days *Arabidopsis* growth figure; B. Growth of *A. thaliana* treated at 4 °C for 3 days

After observing the significant differences between the experimental group and the control group, the experimental group and the control group were measured and statistically analyzed (Table 2). No significant difference was found in root elongation, lateral root length or survival rate between the experimental group and the control group under treatment, but there was a significant difference in the number of lateral roots. Under the NaCl treatment, the number of lateral roots of transgenic *A. thaliana* with the *CIVND1* gene was greater than that of wild-type *A. thaliana*. Under the 4 °C treatment, the number of lateral roots of transgenic *A. thaliana* with the *CIVND1* gene was also greater than that of wild-type *A. thaliana* (Figs. 7-9).

Table 2. Statistics of the growth status of transgenic *A. thaliana* under stress treatment for 3 days.

Item	200 mmol/L NaCl	4°C
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	Elongation (cm)	Number of lateral root	Average length of lateral root (cm)	Survival rate (%)	Elongation (cm)	Number of lateral root (根)	Average length of lateral root (cm)	Survival rate (%)
<i>CIVNDI</i>	0.1	7	2	1.00	1.6	9	0.6	1.00
	0.2	9	4		0.9	7	0.8	
	0.1	10	3		1	11	0.6	
	0.3	11	2.5		1.5	8	0.5	
	0.2	13	2		1.7	6	0.6	
	0.4	9	4		1.6	7	0.4	
	0.4	9	3		1.1	9	0.6	
	0.25	6	3		1.5	8	0.4	
	0.2	13	4		1.7	6	0.6	
	0.4	6	3		2.1	9	0.8	
	0.3	8	2		1.9	6	0.7	
	0.2	6	3		2	8	0.6	
	0.3	5	4		0.8	11	0.6	
	0.25	10	3		1.2	7	0.7	
	0.2	10	2		0.7	12	0.6	
	0.2	11	3		0.5	9	0.6	
Average value	0.25	8.94	2.97	1.00	1.36	8.31	0.61	1.00
AtWT	0.2	3	0.2	0.75	0.5	4	0.4	1.00
	0.2	2	0.2		1.2	3	0.2	
	0.2	3	0.4		0.4	5	0.1	
	0.2	2	0.2		1.3	4	0.5	
	0.2	3	0.6		1.3	4	0.2	
	0.2	3	0.3		1.2	2	0.3	
	0.2	5	0.4		0.5	5	0.1	
	0.2	3	0.2		0.8	4	0.2	
	0.2	5	0.3		1.6	4	0.1	
	0.2	4	0.4		1	4	0.3	
	0.2	5	0.4		1.3	4	0.4	
	0.2	3	0.2		1.1	3	0.2	
	0.2	2	0.2		0.6	2	0.4	
	0.3	3	0.4		0.9	2	0.2	
	0.2	4	0.3		0.6	4	0.3	
	0.3	2	0.2		0.6	4	0.2	
average value	0.2	3.25	0.31	0.75	0.93	3.63	0.26	1.00

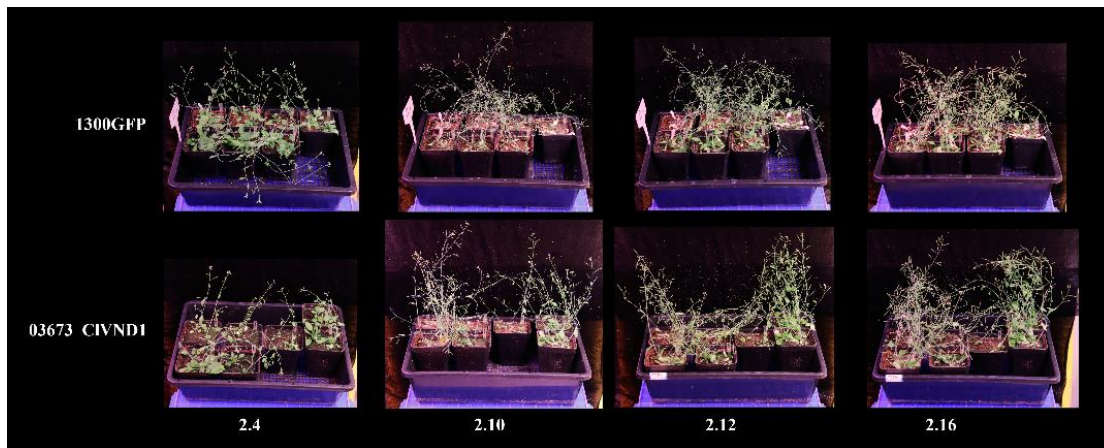


Fig. 7 Untreated *A. thaliana*

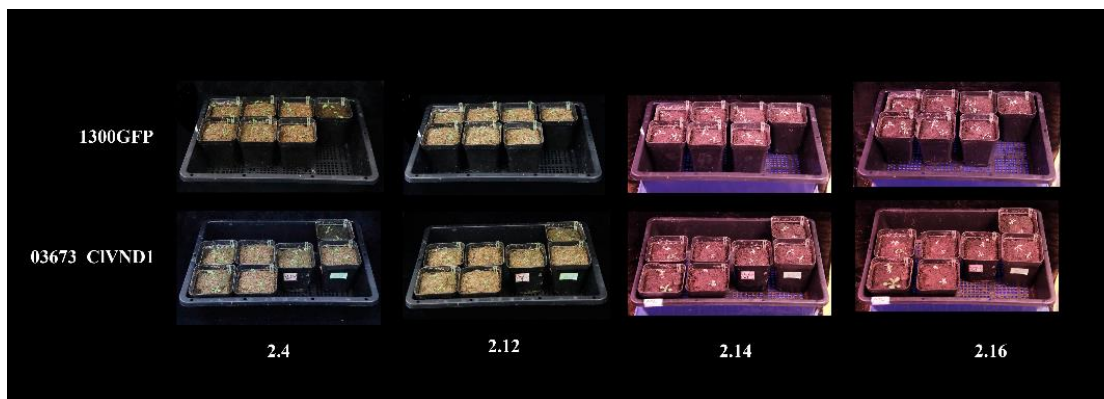


Fig. 8 4 °C low-temperature treatment of *A. thaliana*

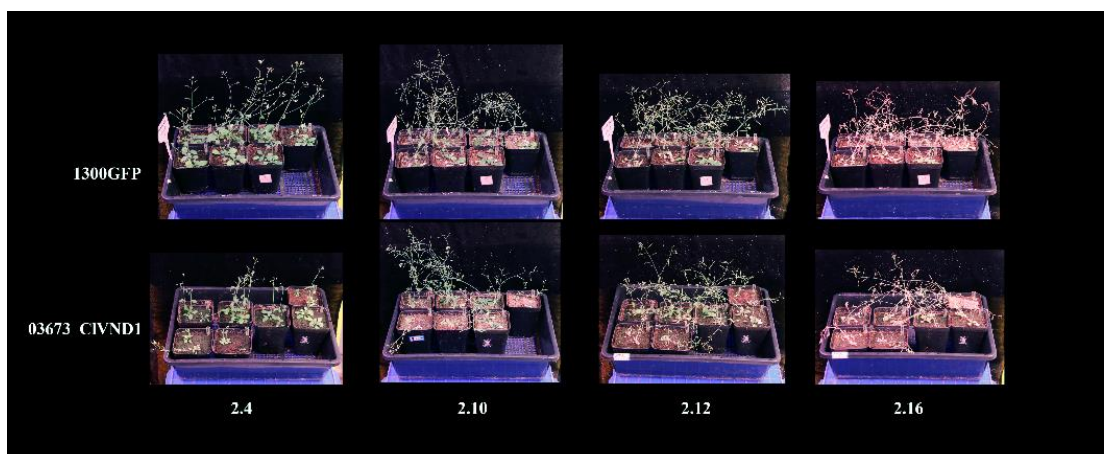


Fig. 9 200mmol / L NaCl treatment of *A. thaliana*

3.5. Observation of the growth and development of transgenic *A. thaliana*

Transgenic *A. thaliana* and the control group were transferred into a 7cmX7cm flowerpot when two true leaves had grown. The statistical results of the rosette diameter were analyzed (Fig. 10). The rosette leaf diameter of the control group was larger than that of the transgenic *Arabidopsis* at the early stage of growth indicating that the vegetative growth of the transgenic *Arabidopsis* was inhibited. The statistical results of *Arabidopsis* height were analyzed (Fig. 11). The inflorescence elongation rate and height of wild-type *Arabidopsis* were greater than those of transgenic *Arabidopsis* indicating that the inflorescence growth of transgenic *Arabidopsis* was also inhibited. However, although the growth and development of transgenic *A. thaliana* were inhibited, its flowering period was earlier than that of the control group. Wild-type *Arabidopsis* plants began to grow inflorescences 9-12 days after transplanting and began to bloom on the 12th day. Transgenic *A. thaliana* with the *CIVND1* gene began to grow inflorescences 7-12 days after transplanting and began to bloom on the 10th day.

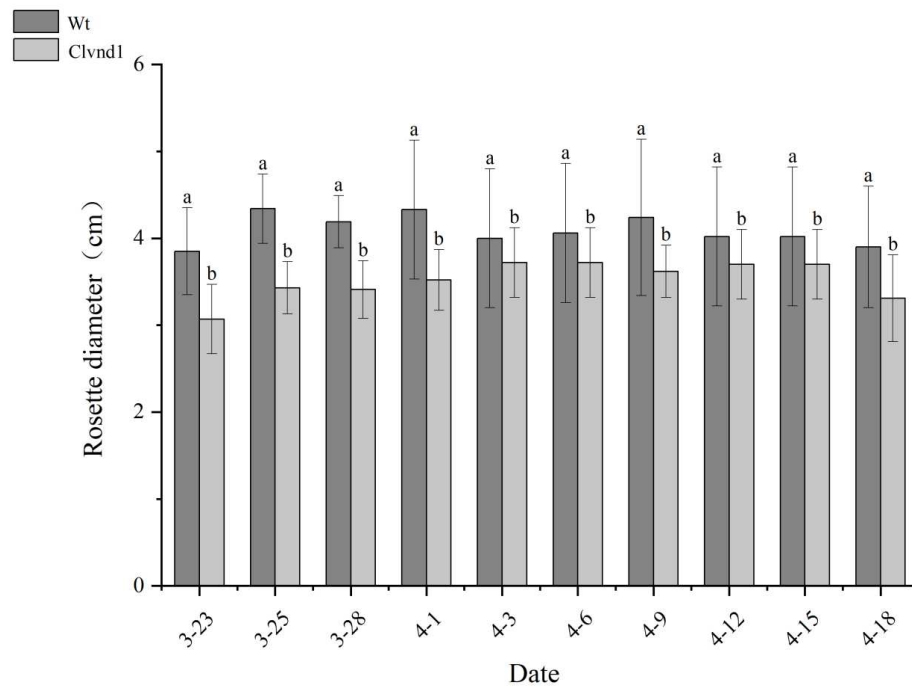


Fig. 10 Transgenic *A. thaliana* rosette diameter statistics

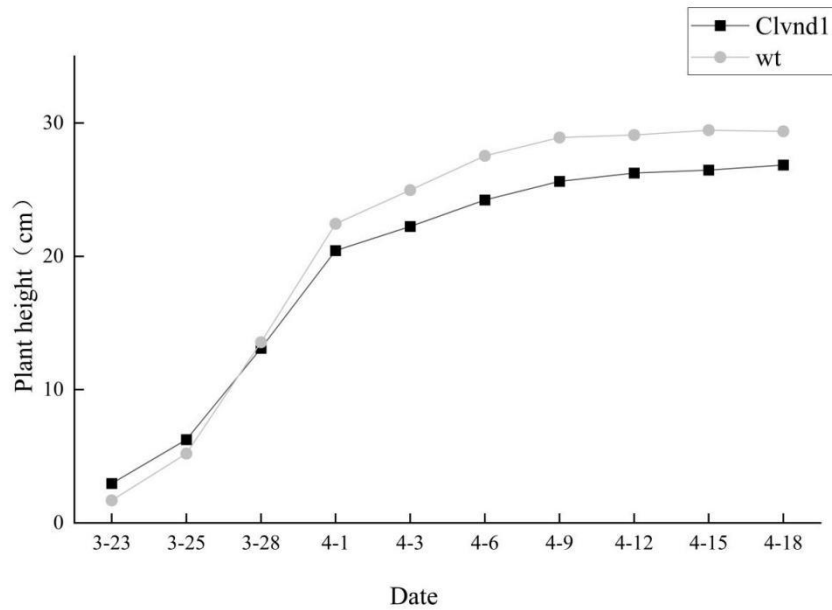


Fig. 11 Growth height statistics of transgenic *A. thaliana*

3.6. Observation of the growth and development of transgenic *A. thaliana*

The 1300 :: *CIVND1* :: GFP and 1300 :: GFP transgenic *A. thaliana* plants shown in Fig. 12 were observed in paraffin sections with samples taken at 3-4cm above the root. It was found that the secondary cell wall of 1300 :: *CIVND1* :: GFP transgenic *Arabidopsis* was larger than that of 1300 :: GFP transgenic *Arabidopsis* (Fig. 12). The paraffin sections of transgenic *A. thaliana* and control *A. thaliana* were amplified and observed (Fig. 12), and the secondary cell wall was measured. The 10 cell walls of 1300 :: GFP transgenic tobacco were measured (Fig. 12), and the average thickness was 4.850 μ m. The 10 cell walls of 1300 :: *CIVND1* :: GFP transgenic *Arabidopsis* were measured, and the average thickness was 11.631 μ m. The thickness of the secondary cell wall in the stems of transgenic *A. thaliana* with the *CIVND1* gene was significantly greater than that of the control group.

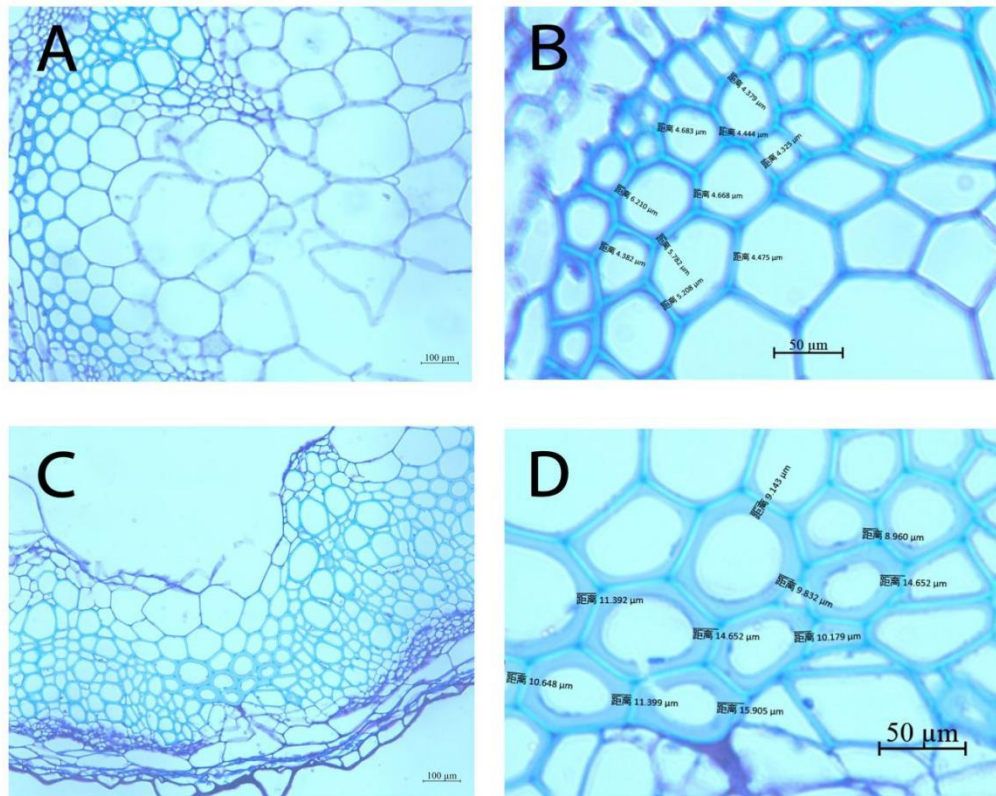


Fig. 12 Stem anatomical structure of 1300: GFP- and *CIVND1*-transgenic *A. thaliana*

4. Discussion

The growth, development, reproduction and production of plants are strongly affected by the combined harmful effects of high salt, drought and other constraints (Wang et al. 2017). Many transcription factors, such as DREB (Liu et al. 1998; Yamaguchi-Shinozaki and Shinozaki 2001, 2007), MYB (Abe et al. 1997), bZIP (Uno et al. 2000), and zinc finger structure (Mukhopadhyay et al. 2004), play important roles in improving plant stress tolerance. Only a better understanding of the mechanisms behind plant tolerance to environmental stress can aid in identifying strategies to improve plant tolerance to abiotic stress (Morran et al. 2011).

The subcellular localization of tobacco and other methods have been used to systematically study the function of the *CIVND1* gene in growth and response to abiotic stress. This study focused on *CIVND1* in the OsNAC7 subfamily. It was cloned, and its conserved domain and phylogenetic relationship were analyzed by bioinformatics methods. The transgenic *A. thaliana* was subjected to low temperature and salt stress treatments, and the paraffin sections of transgenic *Arabidopsis* stems were prepared. In *Arabidopsis*, VND1-7 was found to be related to the induction of xylem cells in vitro. Overexpression of VND6 and VND7 ectopically induced the formation of tubular cells, while the inhibition of VND6 and VND7 expression inhibited the formation of

primary and secondary xylem in roots (Kubo et al. 2005). Similarly, VND1-5 is also specifically expressed in ductal cells, and the overexpression of these genes leads to the ectopic deposition of secondary walls. VND1-VND5, VND6 and VND7 are involved in regulating the thickening of the secondary cell wall of the duct (Soyano et al. 2008; Yamaguchi et al. 2010; Zhou et al. 2014; Endo et al. 2015). The stem segments of transgenic *A. thaliana* were used to make paraffin sections to observe the thickness of the secondary cell wall. The results showed that *CIVND1* could increase the thickness of the secondary cell wall of transgenic *A. thaliana*. Studies have shown that the xylem fiber cell wall of *BpVND1* overexpression (OE) transgenic birch is thicker than that of wild-type birch (Li 2020) which is consistent with the results of this study and indicates that *CIVND1* can promote the synthesis of secondary cell walls. The lateral root growth of transgenic *Arabidopsis* with *CIVND1* under stress conditions was greater than that of wild-type *Arabidopsis* indicating that the *CIVND1* gene plays an important role in the regulation of root growth under stress conditions. When observing the growth and development of transgenic *A. thaliana*, it was found that *CIVND1* inhibited the growth of *A. thaliana*. The rosette leaves were smaller and plant height decreased; however, the flowering time of transgenic *A. thaliana* was 3 days earlier.

The study of the *CIVND1* gene showed that *CIVND1* could increase the lateral root growth of transgenic *Arabidopsis* seedlings under salt and low temperature treatment as well as lead to early flowering and a decrease in growth potential. These findings confirm the potential of the application of NAC transcription factors in plant growth, development breeding and resistance breeding.

5. Conclusions

In summary, *CIVND1* was cloned from *C. lavandulifolium* and identified as a gene of the OSNAC7 subfamily which encodes a nuclear protein and a cell membrane protein. This study found that the *CIVND1* gene can regulate the growth and development of *A. thaliana* as well as improve its stress tolerance, shown by the enhanced tolerance of salt and low temperature, and increase the thickness of the secondary woody cell wall of its stem.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

“All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Wenting He, Yanxi Li , Chendi Mei , Yueyue Liu and Xuebin Song . The first draft of the manuscript was written by Wenting He and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.”

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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