

# Cloning and Functional Analysis of Expansin *TaEXPA9* Homologs in Winter Wheat in Frigid Regions

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## Research Article

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# Abstract

## Background

Low temperature is an important factor that influences the ability of winter wheat to safely overwinter. Excessive low temperatures restrict the regrowth of winter wheat, thus decreasing agricultural output. Non-enzymatic expansins, which are related to plant growth, have been reported to respond to drought, salinity, and low temperature stress. We obtained an expansin gene, *TaEXPA9*, that is induced by low temperature from a transcriptome analysis of ‘Dongnong winter wheat no. 2’—a winter wheat with high cold hardiness—but the expression pattern and function of this gene were unknown. We therefore analyzed the expression patterns of *TaEXPA9-A/B/D* in D2 in response to different abiotic stresses and exogenous phytohormone treatments in different organs. The entire length of *TaEXPA9-A/B/D* was obtained, and green fluorescent labeling was used for subcellular localization analysis of *TaEXPA9-A/B/D* on onion epidermis. The 35S::*TaEXPA9-A/B/D* expression vector was constructed, and an overexpression transgenic *Arabidopsis thaliana* line was obtained to examine the effects of the homologs of this expansin on plant growth and low temperature stress resistance.

## Results

The results showed that *TaEXPA9-A/B/D* transcription significantly increased at 4°C low temperature stress, its expression level was higher in the roots, and *TaEXPA9-A/B/D* was localized to the cell wall. The roots were well-developed in the overexpression *A. thaliana*, and the growth-related markers and setting rate were better than in the wild-type. Recovery was stronger in the overexpression plants after frost stress. At 4°C low temperature stress, the antioxidant enzyme activity and osmoregulatory substance content in the *TaEXPA9-A/B/D*-overexpressing *A. thaliana* plants were significantly higher than in the wild-type plants, and the degree of membrane lipid peroxidation was lower.

## Conclusions

In summary, *TaEXPA9-A/B/D* participates in the low-temperature stress response and may increase the scavenging of reactive oxygen species caused by low temperature stress through the protective enzyme system. Additionally, *TaEXPA9-A/B/D* can increase the levels of small molecular organic substances to resist osmotic stress caused by low temperature.

## 1. Introduction

Low temperature is one of the factors that impede plant growth and development rates. At the same time, low temperature will inhibit the geographical distribution and agricultural output of plants[1, 2]. If vernalization or low temperature acclimatization is not carried out for a period of time before the sowing of winter wheat, the sensitivity of winter wheat towards temperature will significantly increase, and a frigid winter and cold spell in spring will result in the winter wheat being unable to survive[3], thereby decreasing the quality and even output during winter wheat harvest. ‘Dongnong winter wheat no. 2’

possesses high cold hardiness and is rich in genetic resources[4, 5]. Hence, it has important significance in the molecular selective breeding of cold-resistant plants. Mining of superior cold resistance genes in this variety is important in molecular selective breeding to increase the cold hardiness of winter wheat. Under cold stress, the expression of more than 75% of genes on chromosome 21 of wheat will be induced in a common cascade[6]. Similarly, Monroy et al. detected 450 low temperature stress-related genes, and their expression level changes varied in different winter wheat cultivars and with seasonal temperature changes[7]. Therefore, the development of molecular selective breeding methods and the application of related cold-resistance genes can increase the resistance of crops that are sensitive to low temperature, thus increasing agricultural output and preventing economic losses caused by low temperature.

Expansins are cell wall-loosening proteins that are pH-dependent[8]. In 1989, Cosgrove first discovered expansins in a study on cucumber hypocotyl extension[9]. Expansins are non-enzymatic proteins that usually consist of 250–275 amino acids and have a molecular weight of 25–30 kDa[10]. The expansin gene family is divided into four subfamilies. The sequences of expansin family members are highly conserved, with an amino acid sequence homology of 20–25%[11]. Many studies have showed that expansins can generate turgor pressure to cause cell wall loosening and extension. At the same time, changes in cell wall structure promote cell expansion and growth, and this is present in various plant growth and development processes[12, 13], including endosperm development[14], seed germination [15, 16], hypocotyl growth[17], stem elongation[18], pollen tube elongation[19], receptacle growth and maturation[20], vegetative growth of storage root[21], and fruit maturation and softening[22, 23].

Cell walls are the first barrier in plants to encounter environmental stress, and changes in cell wall composition and structure caused by expansins help plants respond to abiotic stresses such as drought[24], salinity[25], high temperature[26] and environmental pollutant[27]. For example, *TaEXPA2*-overexpressing plants can maintain a good moisture content and stronger antioxidant capacity under drought conditions, and their drought resistance is significantly increased[12, 28]. Narayan et al. proved that *EaEXPA1* is a potential target gene that increases drought resistance in sugar cane[29]. Under salinity stress, the expression levels of *NtEXPA4* and some salinity stress-response genes increased to enhance salinity resistance in plants[30]. *PpEXP1* overexpression in tobacco plants decreases biological damage under heat stress, while antioxidant enzymes and seed germination rates are increased[31]. The expression of the expansin *AsEXP1* in creeping bentgrass is induced by high temperature, and its expression in the leaves is significantly upregulated under high temperature stress[32]. In rice, *OsEXPA5*, *OsEXPA7*, and *OsEXPA10* can change pathogen susceptibility in plants and the response to pathogen stress[33]. The *TaEXPB8* gene can increase antioxidant defense by stimulating the secondary metabolism of phenols, having positive effects on cell wall strengthening at the same time. This enables the plant to respond to cadmium toxicity[34]. In addition, expansins play crucial roles in plants under low temperature stress. For example, Zheng et al. analyzed fiber proteins in two cotton cultivars with different temperature sensitivities under low temperature treatment. Their results showed that the expansin content was significantly upregulated in the low temperature-tolerant cotton cultivar[35]. The transcript level of the *CpEXP1* gene in zucchini significantly increases under cold stress, and hence it is speculated that this gene plays a critical role in plant resistance towards cold stress[36]. This result is consistent with

that of Bauerfeind et al., who found that expansins are counterbalancing agents that resist the growth inhibition caused by low temperature stress and are not promoting agents that promote plant growth[16].

Expansins are associated with the morphogenesis of winter wheat root systems and tillers, and there are significant differences in expansin transcript levels in winter wheat cultivars with different cold resistance abilities. The transcript levels of expansins in 'Dongnong' winter wheat are higher than in the cold-sensitive 'Zhongguochun' cultivar[37]. Based on these findings, our group found that the overexpression of *TaEXPB7-B* in *Arabidopsis thaliana* significantly promoted plant growth and tolerance towards low temperature stress[4]. In addition, the *TaEXPA8-B/D* gene could actively respond to 4°C low temperature stress and was highly expressed in the roots. This shows that expansins are important in increasing cold hardiness in winter wheat in frigid regions[5]. In a study on expansins in D2 winter wheat, we found that *TaEXPA9* expression was induced by low temperature stress. However, the expression pattern and function of this gene have not been reported. Therefore, the expression pattern, sequence characteristics, subcellular localization, and gene function in response to low temperature stress of expansins *TaEXPA9-A/B/D* in winter wheat were evaluated in this study. These findings are important for enriching research on expansins and low temperature stress and for providing superior quality low-temperature-resistance genes for molecular selective breeding in the agriculture industry.

## 2. Methods

### 2.1. Plant materials

D2 seeds of the same size and filled with kernels were selected and obtained from the Wheat Selective Breeding Laboratory, College of Agriculture, Northeast Agricultural University. The seeds were soaked in 10% sodium hypochlorite (NaClO) for 8 min. After sterilization, sterile water was used to wash the seeds 8–10 times. The seeds were removed and placed in sterile water for immersion for 12 h. Following that, the seeds were placed in culture dishes that contained two layers of filter paper. Water was sprayed to wet the filter papers. The culture dishes were transferred to the plant culture room. Plants were grown under 25°C for 12 h (daytime) and 18°C for 12 h (nighttime) with 4000 lx during the daytime. Subsequent experiments were carried out after the seedlings had grown to the two-leaf stage.

### 2.2. Cloning and sequence analysis of the *TaEXPA9* gene

The gene file and protein data of the 32nd generation wheat genome were downloaded from the wheat genome website[37]. The full-length sequences of the *TaEXPA9-A/B/D* genes were obtained from the database. Table 1 shows the primers specific for the three sequences that were designed. PCR was used to amplify the *TaEXPA9-A/B/D* gene sequences. The pEASY-T3 Cloning Kit was used for ligating the PCR product with the T3 cloning vector. The ligation product was transformed into *Escherichia coli* and transferred to Comate Bioscience Co., Ltd for sequencing. The full-length *TaEXPA9-A/B/D* sequences were analyzed using the method from Peng and Feng[4, 5].

Table 1  
Primers used in this research.

Name	Primer sequences (5'-3')
<i>TaEXPA9-A</i> (qRT-PCR)	FW: ATGGCCGCCGCAATGC RV: CGCACCTCTCCCCCTCGTT
<i>TaEXPA9-B</i> (qRT-PCR)	FW: ATGGCCGCCGCACTGC RV: CACCTCTGCCCTCGTTGAAC
<i>TaEXPA9-D</i> (qRT-PCR)	FW: ATGGCCGCCGCAATGC RV: GCGCTGCCCTCGTTGAA
<i>ACTIN</i> (Wheat)	FW: TCCAATCTATGAGGGATACACGC RV: TCTTCATTAGATTATCCGTGAGGTC
<i>ACTIN</i> ( <i>Arabidopsis</i> )	FW: CCACATGCTATTCTGCGTTTGGACC RV: CATCCCTTACGATTTACGCTCTGC
<i>TaEXPA9-A</i> (clone the CDS)	FW: ATGGCCGCCGCAATGC RV: CTAGACCCTGAAGTTGTTGCCCTCG
<i>TaEXPA9-B</i> (clone the CDS)	FW: ATGGCCGCCGCACTGC RV: TCAGACCCTGAAGTTCTTGCCCTC
<i>TaEXPA9-D</i> (clone the CDS)	FW: ATGGCCGCCGCAATGC RV: CTAGACCCTGAAGTTCTTGCCCTCG
<i>TaEXPA9-A</i> (addition of <i>Bgl</i> II)	FW: TGACCATGGTAGATCTGATGGCCGCCGCAATGCCACCG RV: CCTTTACTAGTCAGATCGACCCTGAAGTTGTTGCCCTCG
<i>TaEXPA9-B</i> (addition of <i>Bgl</i> II)	FW: TGACCATGGTAGATCTGATGGCCGCCGCACTGCCACCG RV: CCTTTACTAGTCAGATCGACCCTGAAGTTCTTGCCCTC
<i>TaEXPA9-D</i> (addition of <i>Bgl</i> II)	FW: TGACCATGGTAGATCTGATGGCCGCCGCAATGCCACCG RV: CCTTTACTAGTCAGATCGACCCTGAAGTTGTTGCCCTCG

**Suppy Figure. 1 Electrophoretic gel diagram and sequence contrast of *TaEXPA9-A/B/D*. (A)** Electrophoresis gel showing the PCR-amplified *TaEXPA9* gene. Lane 1 show the *TaEXPA9-A* gene; Lane 2 show the *TaEXPA9-B* gene; and Lane 3 shows the *TaEXPA9-D* gene. **(B)** Sequence analysis of *TaEXPA9-A/B/D*. (a) Alignment of the CDS regions of *TaEXPA9-A/B/D*. (b) Alignment of *TaEXPA9-A/B/D* proteins. (c) Alignment of the DPPB\_1 domain of *TaEXPA9-A/B/D*. (d) Alignment of the Pollen\_allerg\_1 domain of *TaEXPA9-A/B/D*.

Name	Primer sequences (5'-3')
<i>TaEXPA9-A</i> (addition of <i>Xba</i> I and <i>Bam</i> HI)	FW:GAGAACACGGGGGACTCTAGAATGGCCGCCGCACTGCCACCG RV: CCGGGGATCCTCTAGACTAGACCCTGAAGTTGTTGCCCTCG
<i>TaEXPA9-B</i> (addition of <i>Xba</i> I and <i>Bam</i> HI)	FW:GAGAACACGGGGGACTCTAGAATGGCCGCCGCAATGCCACCG RV: CCGGGGATCCTCTAGATCAGACCCTGAAGTTCTTGCCCTCG
<i>TaEXPA9-D</i> (addition of <i>Xba</i> I and <i>Bam</i> HI)	FW:GAGAACACGGGGGACTCTAGAATGGCCGCCGCAATGCCACCG RV: CCGGGGATCCTCTAGACTAGACCCTGAAGTTCTTGCCCTCG
<p><b>Suppy Figure. 1 Electrophoretic gel diagram and sequence contrast of <i>TaEXPA9-A/B/D</i>. (A)</b> Electrophoresis gel showing the PCR-amplified <i>TaEXPA9</i> gene. Lane 1 show the <i>TaEXPA9-A</i> gene; Lane 2 show the <i>TaEXPA9-B</i> gene; and Lane 3 shows the <i>TaEXPA9-D</i> gene. <b>(B)</b> Sequence analysis of <i>TaEXPA9-A/B/D</i>. (a) Alignment of the CDS regions of <i>TaEXPA9-A/B/D</i>. (b) Alignment of <i>TaEXPA9-A/B/D</i> proteins. (c) Alignment of the DPPB_1 domain of <i>TaEXPA9-A/B/D</i>. (d) Alignment of the Pollen_allerg_1 domain of <i>TaEXPA9-A/B/D</i>.</p>	

## 2.3. Quantitative RT-PCR analysis of *TaEXPA9* gene

### 2.3.1. Processing of plant materials

Some seedlings at the two-leaf stage were used for drought stress, 4°C low temperature stress, and hormone treatment, with a control group set up. The D2 seedlings from the control group were grown normally at 25°C. To simulate a drought environment in the drought stress group, 20% polyethylene glycol (PEG) 6000 solution was used to soak the roots of the seedlings. In the 4°C low temperature stress group, seedlings were cultured at 4°C and treated with 0.1 µM indole-acetic acid (IAA), 2 µM abscisic acid (ABA), 10 µM salicylic acid (SA), and 10 µM methyl jasmonate (MeJA) following the method of Han[38]. Triplicate samples were collected from each group at 0, 3, 6, 12, and 24 h. The sample weight was 0.1 g. Root, stem, and leaf samples were collected from seedlings at the two-leaf stage. Flowers at the flowering stage and fruits at the fruiting stage were collected. Triplicate samples were collected for each organ, and the sample weight was 0.1 g. The aforementioned samples were stored in a -80°C freezer.

### 2.3.2. RNA extraction and cDNA synthesis

The Trans Zol Up Plus RNA Kit was used to extract total RNA from entire wheat seedlings, and a NanoDrop 2.0 spectrophotometer was used to measure the RNA concentration[39]. The RNA samples with an OD260/280 >1.8, concentration >100 ng/µL, and good integrity were selected for subsequent experiments. The Trans Script® One-step gDNA Removal and cDNA Synthesis Super Mix kit was used for reverse transcription of the extracted RNA into cDNA.

### 2.3.3. Real-time fluorescence quantitative PCR

The TransStart® Top Green qPCR SuperMix was used for quantitative real-time polymerase chain reaction (qRT-PCR). The wheat  $\beta$ -actin gene was used as the internal reference gene. Table 1 shows the independently designed gene-specific primers used for qRT-PCR. The qRT-PCR program was set as denaturation at 40 cycles of 94°C for 5 s, annealing at 62°C for 30 s, and extension at 95°C for 15 s. The  $2^{-\Delta\Delta CT}$  method was used to calculate the expression levels of the genes.

### 2.4. Subcellular localization of *TaEXPA9*

A 20-bp homology box containing the BglII restriction enzyme cleavage site was designed using the primers for *TaEXPA9-A*, *TaEXPA9-B*, and *TaEXPA9-D* (Table 1). The stop codons of the three genes were removed at the downstream region. Single enzyme cleavage was carried out on the pCambia1302 vector and the *TaEXPA9-A*, *TaEXPA9-B*, and *TaEXPA9-D* genes. Homologous recombination was used to obtain the *TaEXPA9-A*-eGFP, *TaEXPA9-B*-eGFP, and *TaEXPA9-D*-eGFP fusion proteins. The 35S::eGFP, 35S::*TaEXPA9-A*-eGFP, 35S::*TaEXPA9-B*-eGFP, and 35S::*TaEXPA9-D*-eGFP vectors were transformed into *Agrobacterium tumefaciens* GV3101 using the freeze-thaw method. An onion epidermis was placed downwards at the side nearest to the mesophyll in Murashige & Skoog (MS) culture medium. The culture temperature was 28°C and transformation was carried out for 24 h away from light. The subcellular localization experiment was performed according to the method of Chen[28]. A confocal microscope was used to observe the transformed onion cells.

### 2.5. Acquisition of *TaEXPA9*-overexpressing *A. thaliana*

Twenty-bp homology box primers containing upstream XbaI and downstream BamHI restriction enzyme sites were designed for the *TaEXPA9-A*, *TaEXPA9-B*, and *TaEXPA9-D* genes (Table 1). Double digestion was separately carried out on the PBI121 vector and *TaEXPA9-A*, *TaEXPA9-B*, and *TaEXPA9-D* genes that contained restriction enzyme sites. Homologous recombination was used to obtain the 35S::PBI121-*TaEXPA9-A*, 35S::PBI121-*TaEXPA9-B*, and 35S::PBI121-*TaEXPA9-D* recombinant vectors. The freeze-thaw method was employed to transform the three recombinant vectors into *A. tumefaciens* GV3101. Inflorescence infiltration[40] was used to transform *A. thaliana* with *A. tumefaciens* containing 35S::PBI121-*TaEXPA9-A*, 35S::PBI121-*TaEXPA9-B*, and 35S::PBI121-*TaEXPA9-D*. After transformation and pod formation in *A. thaliana*, the seeds were grown on ½ MS solid culture medium containing 50 µg/mL kanamycin to screen for T1 plants overexpressing *TaEXPA9-A*, *TaEXPA9-B*, and *TaEXPA9-D* (OE-A/B/D). The method of selecting overexpressing plants was the same as the aforementioned method in which qRT-PCR was used to select homozygous plants[41]. The T3 transgenic plants and wild-type plants were used for further experiments.

### 2.6. Effects of overexpression of *TaEXPA9* on growth of *A. thaliana*

Seeds from wild-type *A. thaliana* and the three types of overexpressing *A. thaliana* plants were sterilized in 10% NaClO for 10 min. Sterile water was used to rinse the seeds 8–10 times, and the seeds were sown

on ½ MS solid culture medium. The culture medium was placed in a 4°C incubator for vernalization for 24 h before the dishes were placed in the plant culture room. On day 4 of culture in the culture medium, the hypocotyl length and number of lateral roots were observed and measured in the plants. On day 8 of culture, the number of root hairs and axial root length were observed and measured in the plants. Seedlings were grown until the 4-leaf stage (10 d) before they were transplanted into soil and grown in the plant culture room. When the plants reached the seed setting stage (100 d of growth), the number of leaves, rosette diameter, and plant height were measured. Finally, watering was no longer carried out for the four types of plants. After 10 d, when the pods completely dried and cracked open, the seed yield and number of pods per plant were measured.

## **2.7. Effects of overexpression of *TaEXPA9* on growth of *A. thaliana***

The aforementioned method was used to grow *A. thaliana* seeds. When the wild-type (WT) and the three types of overexpression plants were grown until 40 d, all plants were divided into two groups, namely the control group and the low temperature treatment group. Plants in the control group were incubated at room temperature (25°C), and plants in the experimental group were incubated in a 4°C incubator. Whole plants was performed at 0, 3, 6, 12, and 24 h. All samples were stored at -80°C and used for the measurement of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activity. The levels of malondialdehyde (MDA), soluble protein, soluble sugar, and proline were measured. These seven physiological markers were measured using assay kits from Suzhou Comin Biotechnology Co. Ltd.

## **2.8. Observation of the *A. thaliana* phenotype under frost stress**

Fifty WT and each of the three overexpression plants were grown, resulting in a total of 200 plants. At 40 d, all plants were incubated in a -20°C incubator for 30 min before being transferred to the plant culture room (25°C) for recovery after frost stress. The survival rate was measured after 8 d.

## **2.9. Statistical analysis**

Excel 2007 (Microsoft Corp., Redmond, WA, USA) was used for sorting and analysis of qRT-PCR, growth and physiological indicators. Average function in Excel2007 was used to average the three repetitions of each group of samples, and stdev.P was used to calculate the standard deviation. The difference significance was analyzed using one-way ANOVA in GraphPad Prism5. Draw a bar chart in Excel2007 based on the above information. In these graphs, error bars represent standard deviation. In histograms of qRT-PCR and physiological indicators, different English lowercase letters represent significant differences between different treatments. Significant differences in histograms of growth indicators were expressed as "\*" ( $P < 0.05$ ) or "\*\*\*" ( $P < 0.01$ ). Chi-square test and visual analysis were performed for survival and mortality data of transgenic and wild-type *Arabidopsis thaliana* using ggstatsplot package in R.Studio.

## 3. Results

### 3.1. *TaEXPA9-A/B/D* expression under abiotic stress and hormone treatments

*TaEXPA9-A/B/D* expression levels showed varying degrees of downregulation after PEG treatment, and these differences were significant compared with 0 h ( $P < 0.05$ ). *TaEXPA9-D* expression decreased to 8.7% after 3 h of treatment. After 12 h of treatment, the *TaEXPA9-A/B/D* expression levels increased compared with 6 h, of which the *TaEXPA9-A/B* expression levels were significantly increased. After 24 h of treatment, *TaEXPA9-A/B/D* genes exhibited almost no expression (Figures 1A, D, and G). Under 4°C low temperature treatment, *TaEXPA9-A* gene expression gradually increased in the first 12 h, and the differences between the various time points were significant. *TaEXPA9-A* expression was downregulated at 24 h, though the difference compared with 0 h was not significant. *TaEXPA9-B/D* expression levels were upregulated at various time points after treatment compared with 0 h, and these differences were significant. *TaEXPA9-A/B* expression levels peaked at 12 h of treatment. Compared with 0 h, the expression levels of *TaEXPA9-A* and *TaEXPA9-B* were 163.6% and 136.7%, respectively (Figures 1A, D, and G).

Under MeJA treatment, the *TaEXPA9-B/D* expression levels gradually decreased as treatment duration increased, and the differences between the various treatment time points were significant ( $P < 0.05$ ). The *TaEXPA9-A* gene was hardly expressed following MeJA treatment. Under the IAA, SA, and ABA treatments, *TaEXPA9-A/B/D* expression was almost completely inhibited (Figures 1B–C, E–F, H–I). In summary, *TaEXPA9-A/B/D* can be upregulated in response to 4°C low temperature stress.

### 3.2. *TaEXPA9-A/B/D* expression in different tissues and different growth stages

Quantitative RT-PCR was used to measure the expression of *TaEXPA9-A/B/D* genes in different tissues at different developmental stages. The expression levels of these three genes were the highest in the roots. The expression levels of *TaEXPA9-B/D* were the lowest in the stems, and the expression level of *TaEXPA9-A* was the lowest in the fruits. Hence, it can be deduced that these three genes may participate in stress responses in plants through similar signaling mechanisms (Figure 2).

### 3.3. Cloning and sequence analysis of *TaEXPA9-A/B/D* genes

In this study, the coding sequences (CDSs) of *TaEXPA9-A/B/D* in D2 seedlings at the two-leaf stage were cloned. The PCR amplification results showed that the length of the target genes was around 800 bp (Figure S1-A), which was consistent with the number of bases in the gene sequence. The CDS lengths of *TaEXPA9-A* and *TaEXPA9-B/D* were 807 and 801 bp, respectively. The similarity of the nucleotide sequences of these three genes was 97.23% (Figure S1-B-(a)). *TaEXPA9-A/B/D* contains three exons and two introns (Supply Figure S3-A). *TaEXPA9-A* encodes for 267 amino acids, and *TaEXPA9-B/D* encodes

for 265 amino acids. The amino acid sequence similarity between the three genes was 96.14% (Figure S1-B-(b)). From this, it was deduced that the three sequences obtained by cloning were homologous genes. Protein structure prediction based on SMART showed that the *TaEXPA9-A/B/D* proteins contained DPDD\_1 and Pollen\_allerg\_1 domains (Figure S1-B-(c/d)) and could induce cell wall loosening[9].

Table S1 shows the basic information of the *TaEXPA9-A/B/D* proteins. Their molecular weights ranged from 28.5–28.9 kDa and their isoelectric points (pIs) ranged from 9.40–9.61. These proteins are basic. The global average hydrophobicity (GRAVY) of these proteins was negative, showing that they are hydrophilic proteins. The instability indices (IIs) of these proteins were greater than 40, showing that these proteins are highly stable. The aliphatic indices (AIs) reflects the stability of the protein[42]. The aliphatic indices (AIs) of these proteins were greater than 60, showing that these proteins can adapt to the environment. In *TaEXPA9-A/B/D* proteins, alanine accounted for most of the amino acids (11–13%), while lysine contributed the lowest proportion (0.7–1.1%) (Table S2). The signal peptides in *TaEXPA9-A* and *TaEXPA9-B/D* are in positions 29–30 and 27–28, respectively (Figure S2-A). The three proteins are hydrophilic secretory proteins with transmembrane structures (Figures S2-B, C) and are located outside the cell membrane (Figure S2-D). The constructed three-dimensional structures of these proteins clearly show the catalytic domain (green region), cellulose binding domain (yellow and red regions), and the signal peptide (dark blue region). There are slight differences in the signal peptide domain (1-1/2) between the *TaEXPA9-A/D* and *TaEXPA9-B* proteins and in the catalytic domain (2-1/2/3). These regions are labeled with a red rectangle in the figure (Figure S3-B). Comparison of EXPA9 proteins from eight plant species, which includes *TaEXPA9-B/D* proteins, and an EXPLA9 protein from one plant species (Figure S3-C) showed that all EXPA9s contain a common conserved sequence. This sequence contains three disulfide bonds formed by eight cysteine residues, a C-terminal cellulose binding domain consisting of six tryptophan residues, and one HFD (His-Phe-Asp) sequence in the center. MEGA7 was used to construct a phylogenetic tree. The results showed that *TaEXPA9-A/B/D* had the closest phylogenetic relationship with *HvEXPA9* (Figure S3-D).

### 3.4. Subcellular localization of *TaEXPA9-A/B/D*

The CELLO v5.2 online prediction software predicted that the *TaEXPA9-A/B/D* proteins were located extracellularly (Figure S2-D). In order to validate the prediction results, we constructed 35S::eGFP, 35S::TaEXPA9-A-eGFP, 35S::TaEXPA9-B-eGFP, and 35S::TaEXPA9-D-eGFP vectors and transformed onion epidermal cells (Figure 3A). Fluorescence microscopy clearly showed that these three proteins were located on the cell wall (Figure 3B).

### 3.5. Phenotypic analysis of *TaEXPA9-A/B/D*-overexpressing *A. thaliana* plants

In order to validate the function of *TaEXPA9-A/B/D*, we separately constructed three 35S: *TaEXPA9-A/B/D* vectors, which were used for *A. thaliana* transformation (Figure 4A). Quantitative RT-PCR was used to measure *TaEXPA9-A/B/D* expression in *A. thaliana* (Figure 4B). Following that, overexpression vectors were transformed into *A. thaliana*. Resistance screening was used to identify overexpression plants, and

the screening results are shown in Figure 4C. The WT and overexpression plants were cultured on vertical plates for 4 days before the hypocotyl length and number of lateral roots were measured. The mean hypocotyl length of the OE-A/D was 7.38 times that of the WT plants, while the mean hypocotyl length of OE-B was 6.75 times that of WT plants (Figures 4D and 5A). Microscopy was used to count the number of lateral roots at identical sites in the WT and overexpression *A. thaliana* roots. The results are shown in Figures 4E, 4F, and 5B. Compared with the WT plants, OE-A/B had 9–12 more lateral roots on average, and this difference was significant ( $P < 0.05$ ), and the OE-D had 6.3 more lateral roots on average, but this difference was not significant.

The number of root hairs and axial roots in *A. thaliana* was measured at 8 d. The results (Figure 4G and 5C) showed that the mean number of root hairs in the OE-A and OE-B/D were 1.49 and 1.69–1.75 times that of the WT, respectively, and these results were significant. The mean axial root length in the three overexpression *A. thaliana* strains was 0.30–0.48 cm longer than in the WT (Figures 4H and 5D), and these differences were significant. From this, it can be seen that the root system of the overexpression plants was more developed than the WT plants, and the overexpression plants possessed greater advantages than the WT plants under the same growth conditions. On 30 d of *A. thaliana* growth, the number of leaves, rosette diameter, and plant height of OE-A/B/D were significantly higher than in the WT (Figures 4I–K and 5E–G). The *A. thaliana* plants were grown until the fruiting stage. Figures 4L and 5H–I show that the number of pods and seed yield in the overexpression plants were significantly higher than in the WT plants. This confirmed that *TaEXPA9-A/B/D* promotes the growth of transgenic *A. thaliana*.

### **3.6. Phenotypic observation of *TaEXPA9-A/B/D*-overexpressing *A. thaliana* plants in response to cold stress**

The D2 transcriptome analysis results showed that the expression of *TaEXPA9-A/B/D* was regulated by low temperature [37], and comparison and observation of the cold stress treatment on the overexpression plants and WT plants intuitively demonstrated the functions of these genes. Compared with the before treatment (Figure 6A), cold stress for 15 min caused leaf softening, wilting, and color deepening in the four types of plants (Figure 6B). After culturing in the plant culture room for 7 d, the growth of the plant leaves gradually recovered (Figure 6C). Phenotypic observation showed that the degree of recovery of OE-A/B/D were greater than the WT plants, but some plants died due to lower resistance. Recording and analysis of plant survival rates indicated that the survival rates of the OE-A/B/D were more than 70% (Table 3), being higher than in the WT. The difference in survival rate between groups was significantly associated with overexpression of *TaEXPA9* ( $\chi^2 = 8.71$ ,  $P = 0.033$ ). Therefore, *TaEXPA9-A/B/D* had positive promoting effects on cold hardiness in transgenic *A. thaliana*.

### **3.7. Measurement of physiological markers in *TaEXPA9-A/B/D*-overexpressing *A. thaliana* plants under low temperature stress**

The activities of antioxidant enzymes, SOD, POD, and CAT in the overexpression plants and WT plants at normal growth conditions and under 4°C treatment were maintained at high levels, and MDA level was low. The SOD activity of the OE-A/B fluctuated with the duration of stress, while the variation trend in SOD activity in the OE-D was stable. Under low temperature treatment for 3 h, SOD activity was 469.0 U/g, 443.6 U/g, and 317.6 U/g, respectively. At 12 h, SOD activity was 316.6 U/g, 483.9 U/g, and 314.3 U/g, respectively (Figure 7A). At low temperature treatment for 6 h, POD activity in the WT plant was at its peak of 224.36 U/g, which was 74.2%, 88.2%, and 54.7% that of the OE-A/B/D, respectively. At 24 h, POD activity in OE-B/D reached their peaks, which was 486.3% and 440.5% of WT plants, respectively (Figure 7B). At 24 h, the CAT content in the three overexpression plants peaked, being 1.60, 1.34, and 1.31 times that of the WT plants, respectively (Figure 7C).

The MDA content in OE-A/B/D at 24 h of treatment was 17.85, 10.71, and 11.82 nmol/g, respectively (Figure 7D), which was significantly lower than in the WT ( $P < 0.05$ ). Comparison of various time points under normal growth conditions and low temperature treatment showed that the soluble protein, soluble sugar, and proline contents in the overexpression plants were all higher than in the WT plants. After 12 h of low temperature treatment, the soluble protein content in the overexpression plants peaked. By contrast, the soluble protein content in the WT plants was at its trough of 0.95 mg/g. The soluble protein content in the three overexpression plants was 426.3%, 362.1%, and 381.1% that in the WT plants, respectively (Figure 7E). As treatment duration increased before 12 h, the soluble sugar content in the WT plants first increased before decreasing, while the soluble sugar content in the overexpression plants gradually increased. After 24 h of treatment, the soluble sugar content in the overexpression plants decreased, but was still 220.3%, 242.5%, and 192.2% that of the WT plants, respectively (Figure 7F). At 6h, the proline content was high in the WT and overexpression plants, which was 11.02 µg/g in WT plants. The proline content in the OE-A/B/D were 227.7%, 279.9%, and 324.8% that of the WT plants, respectively (Figure 7G).

## 4. Discussion

### 4.1. *TaEXPA9-A/B/D* expression in response to abiotic stress and hormone treatment

A large volume of experimental data shows that expansins participate in the response to abiotic stress. Geilfus et al. employed qRT-PCT to examine the effects of salinity stress on the abundance of mRNA in expansin subfamily members in maize cultivars with different salt resistance abilities. The results showed that β-expansin transcript abundance did not decrease in the salt-resistant cultivars, but significantly decreased in the sensitive cultivars. This shows that β-expansin plays an important role in salt resistance in maize[43]. Silva et al. analyzed the transcriptomes of drought-resistant and sensitive sugar cane after 24 h of drought stress and performed qRT-PCR validation. They found that *EXPB8*, which is associated with root elongation, responded to drought stress[44].

The expression of expansins is also regulated by hormones. Our study found that *TaEXPA3-D2*, *TaEXPB7-B* and *TaEXLA2-B* were upregulated in response to ABA, SA and MeJA. However, even though the cis-acting elements that respond to ABA, SA, GA, and MeJA were present in this study, *TaEXPA9-A/B/D* expression was still downregulated when treated with these four hormones. When D2 experiences low temperature stress, the upregulation of *TaEXPA9-A/B/D* may not be mediated by these four hormones.

## **4.2. Organ expression difference and subcellular localization of *TaEXPA9-A/B/D***

Expansins are so named because they regulate cell wall loosening, thereby resulting in cell division and growth. Rice *EXPA10*[45], *OsEXPB2*[46], and *OsEXPB3*[47]; soybean *GmEXPB2*[48]; barley *HvEXPB7*[49], and most plant expansins are localized to the cell wall. In this study, the subcellular localization results showed that *TaEXPA9-A/B/D* mainly localized to the cell wall, which supports this view.

The expansins are expressed differently in different organs [50]. Rice *OsEXPB5* and barley *HvEXPB1* are specifically expressed in the roots[51], and wheat *TaEXPA6* and *TaEXPB8* are expressed in various organs, though their expression levels are the highest in the roots[5, 52]. The results of this study are consistent with previous studies: *TaEXPA9-A/B/D* mRNA abundance was the highest in the roots. Therefore, it can be deduced that these three genes mainly promote root growth and development. *TaEXPA9-A* expression in the stems and *TaEXPA9-B/D* expression in the leaves were second to that of the roots. This also indicates that these three genes may have important roles in organs with intensive growth.

## **4.3. Overexpression of *TaEXPA9-A/B/D* promotes the growth of transgenic *A. thaliana***

Comparison of morphological observations of transgenic and WT *A. thaliana* validated the hypothesis that *TaEXPA9-A/B/D* promotes plant organ growth. Transgenic *A. thaliana* may absorb sufficient moisture and inorganic nutrients from soil through its well-developed root system. The larger leaf area and higher number of leaves ensure that transgenic plants have higher photosynthetic capacity[53]. Plants may accumulate organic matter to promote rapid and healthy growth and produce more kernels in adverse environments. Statistical analysis of the number of inflorescences and pods also satisfied this hypothesis. Therefore, *TaEXPA9-A/B/D* can be considered as a candidate gene for molecular selective breeding to improve crop yield.

## **4.4. *TaEXPA9-A/B/D* enhances low temperature tolerance in transgenic plants**

Plants will generate reactive oxygen species (ROS) under low temperature stress, and the excessive accumulation of ROS will cause membrane degeneration and lipid peroxidation and will result in the production of MDA. In severe cases, this disrupts cell structure and cellular metabolism[1]. The activation of the protective enzymes system (SOD, POD, and CAT) that scavenges ROS protects plants from

oxidative stress damage[54]. It has been reported that the *TaEXPA2*-overexpressing *A. thaliana* can upregulation of *TaMnSOD*, *TaAPX*, *TaCAT*, *TaPOD* and other ROS-scavenging enzyme related genes through reactive oxygen species removal process by antioxidant system, which can maintain low ROS level in *TaEXPA2*-overexpressing *A. thaliana*[12]. In this study, *TaEXPA9-A/B/D* not only increased POD, SOD, and CAT activity and decreased MDA accumulation, but also increased proline, soluble sugar, and soluble protein levels. Proline not only decreases ROS levels to within the normal range but can also decrease intracellular water potential, such as via other soluble small organic molecules. This helps to maintain the osmotic balance in cells, causing cells to be turgid, and increasing plant tolerance to osmotic stress caused by adverse conditions[55]. The conclusions from previous studies provided a rich theoretical basis for this study. Zhang et al. examined the effects of *AstEXPA1* in abiotic stress and found that *AstEXPA1*-overexpressing plants had higher soluble sugar and proline levels than WT[56]. Similar results were obtained in *TaEXPB23*-overexpressing *A. thaliana*[57, 58] and *PttEXPA8*-overexpressing tobacco[59, 60]. Therefore, in addition to increasing antioxidant enzyme activity, the increased levels of small organic molecules may be another important role of *TaEXPA9-A/B/D* in the survival of wheat under low temperature conditions. Although the mechanisms by which expansins regulate antioxidant enzyme activity and increase osmoregulatory levels are still unknown, it is understood from available information that *TaEXPA9-A/B/D* is a promising candidate gene that can be used for the selective breeding of crops with strong resistance towards low temperature and other abiotic stresses.

## 5. Conclusion

The expression of the expansin gene *TaEXPA9* isolated from D2 wheat was induced by low temperature stress, and the protein encoded by this gene localized to the cell wall. *TaEXPA9* overexpression significantly promoted the growth of transgenic *A. thaliana* while simultaneously increasing antioxidant and osmoregulation capacities under low temperature stress. This increased the low temperature tolerance in the plants.

## Declarations

**Author Contributions:** Conceptualization, B.H., and F.L., methodology, X.F., software, J.W., validation, X.F., formal analysis, J.W., investigation, Z.Z., resources, Y.X., data curation, L.L., writing—original draft preparation, Z.Z., writing—review and editing, Z.Z., visualization, L.L., supervision, F.H., project administration, F.L., funding acquisition, F.L. All authors have read and agreed to the published version of the manuscript.”

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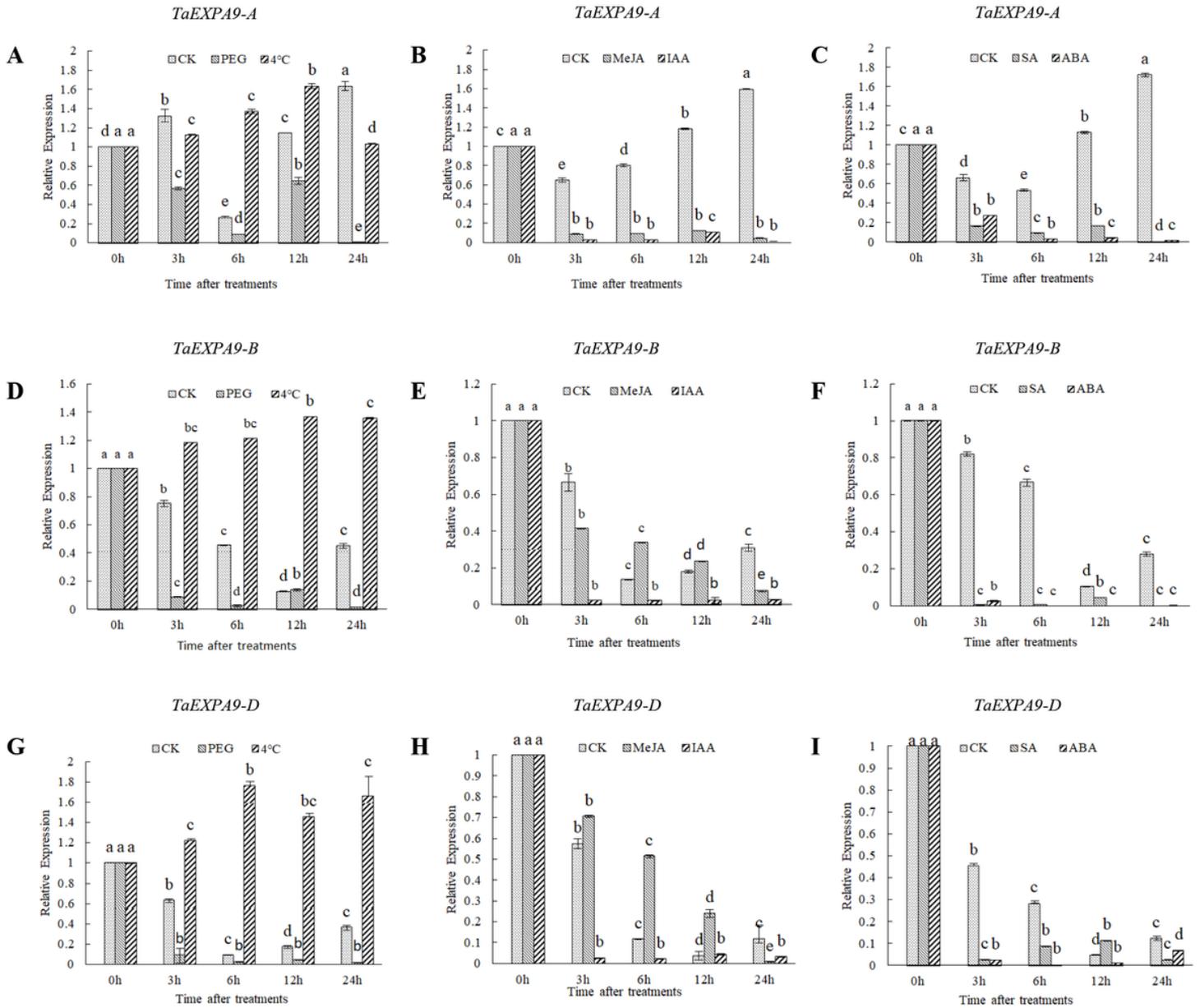
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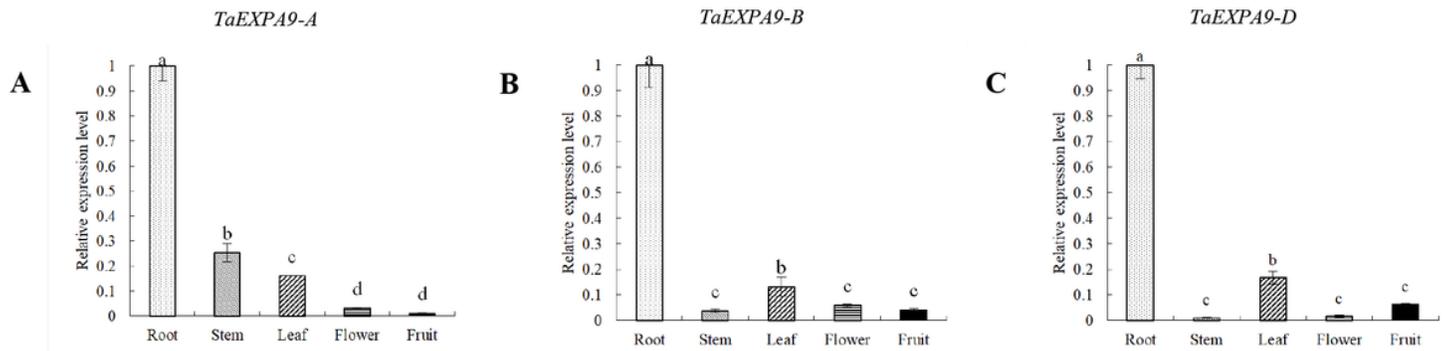
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# Figures



**Figure 1**

TaEXPA9-A/B/D expression under abiotic stress and hormone treatments. (A, D, G). The responses of TaEXPA9-A/B/D to low-temperature stress and drought stress. (B-C, E-F, H-I) The responses of TaEXPA9-A/B/D to plant hormone treatments separately. (B, E, H) The treatments of MeJA and IAA. (C, F, I) The treatments of SA and ABA. The letter “a, b, c, d” indicates the significant difference of gene expression at each time under the same stress treatment.



**Figure 2**

qRT-PCR analysis of the relative expression levels of TaEXPA9-A/B/D in different tissues of D2.(A) TaEXPA9-A, (B) TaEXPA9-B, (C) TaEXPA9-D. The letter “a, b, c, d ” indicates the significant difference in gene expression in the five organs.

**Figure 3**

The subcellular localisation of TaEXPA9-A/B/D.(A) Structure of the expression vector used for subcellular localisation of TaEXPA9-A/B/D. (B) The transient expression of 35S::eGFP and 35S::TaEXPA9-A-eGFP, 35S::TaEXPA9-B-eGFP, 35S::TaEXPA9-D-eGFP in onion epidermal cells. Scale bars, 200 µm. The cells were observed under a confocal microscope after culture on MS medium at 28 °C for 2 days. Onion cell plasmolysis was induced by administering a 30 % sucrose solution for 20 mins prior to observation.

**Figure 4**

Identification and phenotypic observation results of Arabidopsis overexpressing TaEXPA9-A, B and D. WT, OE-A, OE-B and OE-D represent wild-type and overexpressed TaEXPA9-A /B/D Arabidopsis thaliana, respectively. (A) Structure of the expression vector. (B) The expression level of TaEXPA9-A,B and D in transgenic lines as determined by RT-PCR.(C) Screening of transgenic plants resistant. Scale bars, 1 cm. (D) Observation on hypocotyl of Arabidopsis. Scale bars, 1 cm. (E, F) Observation on lateral root of Arabidopsis. Magnification, 10×10 and 10×40. Scale bars, 20 µm. (G) Observation on root hair length of Arabidopsis. Scale bars, 20 µm. (H) Observation on root length of Arabidopsis. Magnification, 10×10. Scale bars, 1 cm. (I, J) Observation on leaf number and rosette diameter of Arabidopsis. Scale bars, 1 cm. (K) Arabidopsis plants height. Scale bars, 5 cm. (L) Arabidopsis seed yield (per plant). Scale bars, 0.5 cm.

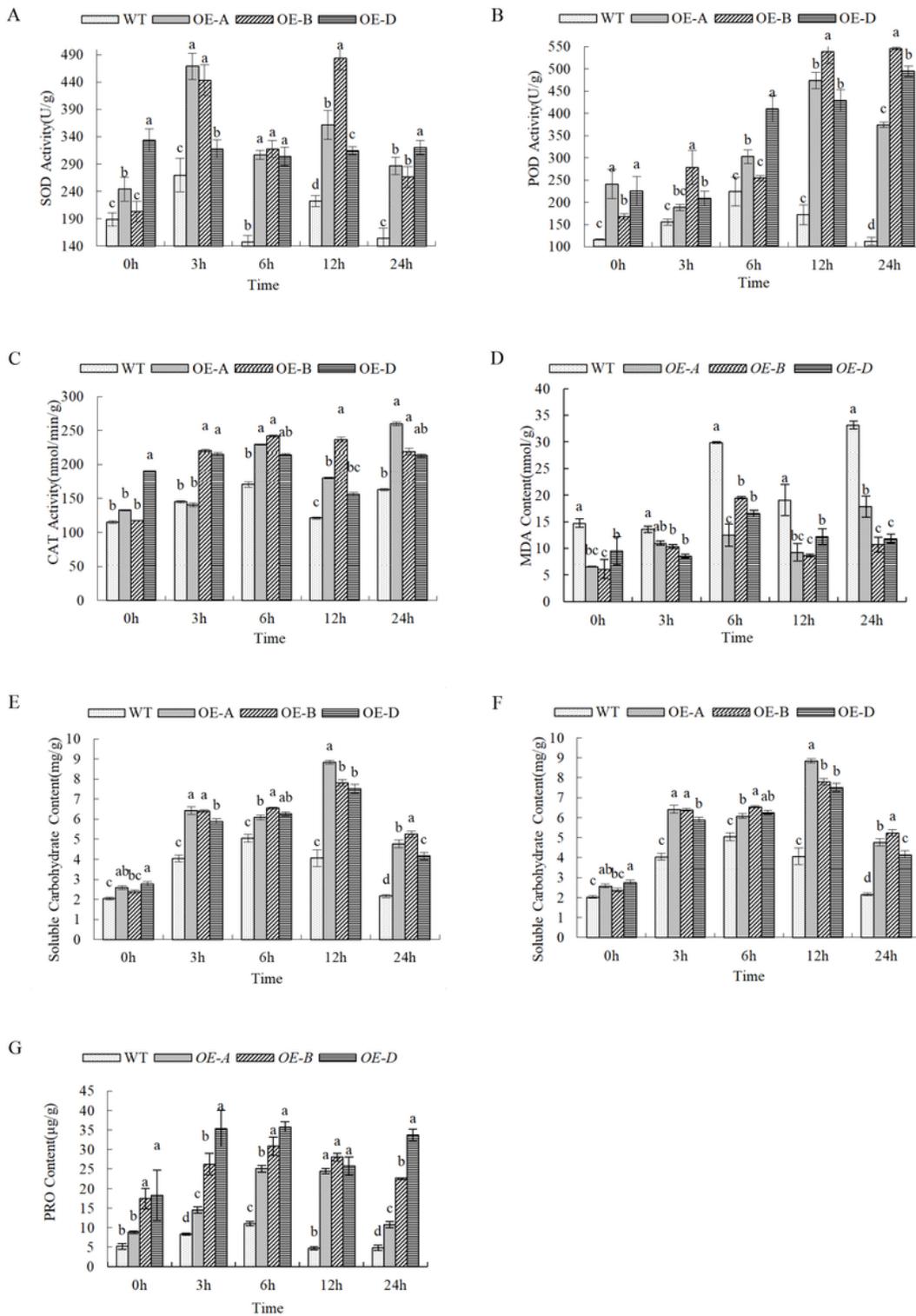
**Figure 5**

Quantitative results of Arabidopsis phenotypic observation (n = 10). (A) Hypocotyl length. (B) Number of lateral roots. (C) Root length. (D) Number of roots hairs. (E) Number of leafs. (F) Rosette of diameter. (G)

Plant height. (H) Number of pods per plant. (I) Weight of seeds per plant. “\*” indicates significant differences between WT and overexpressed plant, “\*” indicates “P < 0.05”, “\*\*” indicates “P < 0.01”.

## Figure 6

Phenotypes and survival rate of the transgenic Arabidopsis overexpressing TaEXPA9-A/B/D and WT plants grown in soil after low-temperature treatment at the rosette leaf stage. (A) Room temperature treatments. (B) -20°C for 15 mins. (C) 7 days of recovery at room temperature. Scale bars, 2 cm. (D) Survival status of wild-type and transgenic and Arabidopsis thaliana plants on the seventh day after exposure to -20 °C (n = 50). The chi-square test was used to obtain the P value for comparison between groups. When P-value was less than 0.05, there was a significant correlation between variables and factors .



**Figure 7**

TaEXPA9-A, B and D enhances low-temperature tolerance in Arabidopsis. Determination of antioxidant capacity and osmotic adjustment substance contents in Arabidopsis under 4 °C treatment (n = 8). (A) SOD activity. (B) POD activity. (C) CAT activity. (D) MDA content. (E) Soluble protein content. (F) Soluble sugar content. (G) Proline content. The letter "a, b, c, d" indicates the significant difference of each component in the same type of plant.

## Supplementary Files

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