Cloning and functional analysis of expansin \textit{TaEXPA9} orthologs in winter wheat in frigid regions

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Abstract

Long-term low temperatures restrict the regrowth of winter wheat (\textit{Triticum aestivum} L.), 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 3 gene, \textit{TaEXPA9}. It is located in winter wheat cv. Dongnong with high cold hardiness. We analyzed the expression patterns of \textit{TaEXPA9-A/B/D} in this cultivar and conducted a subcellular localization analysis of \textit{TaEXPA9-A/B/D} in the onion epidermis. Transgenic \textit{Arabidopsis thaliana} line with \textit{EXPA9-A/B/D} overexpression was obtained to examine the effects of the orthologous genes of these expansins on plant growth and low-temperature stress resistance. The results showed that \textit{EXPA9-A/B/D} expression significantly increased at 4 °C, it was higher in the roots than in shoots, and \textit{EXPA9-A/B/D} was localized in the cell wall. The roots were well-developed in the transgenic \textit{A. thaliana}, and the growth-related markers and setting rate were better than in the wild-type. Recovery was stronger in the transgenic plants after freezing stress. At low-temperature stress, the antioxidant enzyme activities and content of osmoregulatory substances in the \textit{TaEXPA9-A/B/D}-overexpressing \textit{A. thaliana} plants were significantly higher than in the wild-type plants, and the degree of membrane lipid peroxidation was lower. In summary, \textit{TaEXPA9} orthologous genes participate in the low-temperature stress response, and they might be of great importance in molecular breeding.

Keywords: \textit{Arabidopsis thaliana}, expansins, expression analysis, functional validation, \textit{TaEXPA9-A/B/D}, winter wheat.

Introduction

Low temperature is one of the factors that impede plant growth and development rates. At the same time, low temperature inhibits the geographical distribution and agricultural output of plants (Yu \textit{et al.} 2003, Mishra \textit{et al.} 2019). If vernalization or low-temperature acclimatization is not carried out for a period of time before the sowing, 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 (Gusta \textit{et al.} 1987), thereby decreasing the quality and even output during winter wheat harvest. Winter wheat cv. Dongnong No. 2 possesses high cold hardiness and is rich in genetic resources (Feng \textit{et al.} 2019, Peng \textit{et al.} 2019). Hence, it is important for the breeding of cold-resistant plants. Mining of superior cold resistance genes in this cultivar is important in molecular selective breeding. Under cold stress, the expression of more than 75 % of genes on chromosome 21 of wheat will be induced in a common cascade (Sun \textit{et al.} 2009). Similarly, Monroy \textit{et al.} (2007) detected 450 low-temperature stress-related genes, and their expression varied in different winter wheat cultivars and with seasonal temperature changes. 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 (Ding et al. 2016). Cosgrove (2015) described expansins during cucumber hypocotyl extension. Expansins are non-enzymatic proteins that usually consist of 250 - 275 amino acids and have a molecular mass of 25 - 30 kDa (McQueen-Mason et al. 1995). The expansins gene family is divided into four subfamilies. The sequences of expansins family members are highly conserved, with an amino acid sequence homology of 20 - 25 % (Sampedro et al. 2005). Many studies have shown that expansins can generate pressure potential 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 (Bashline et al. 2014, Yang et al. 2020), including endosperm development (Borges et al. 2015), seed germination (Bauerfeind et al. 2015, Xu et al. 2020), hypocotyl growth (Boron et al. 2015), stem elongation (Ohtaka et al. 2020), pollen tube elongation (Liu et al. 2021), receptacle growth and maturation (Cui et al. 2021), vegetative growth of storage root (Dong et al. 2020), fruit maturation and softening (Valenzuela-Rillo et al. 2020a,b).

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 (Jin et al. 2020), salinity (Jadamba et al. 2020), high temperature (Flaishman et al. 2015), and environmental pollutant (Ren et al. 2018). For example, TaEXP2-overexpressing plants can maintain good moisture content and stronger antioxidant capacity under drought conditions, and their drought resistance is significantly increased (Chen et al. 2016, Yang et al. 2020). Narayan et al. (2021) proved that EaxEXP1 is a potential target gene that increases drought resistance in sugar cane. Under salinity stress, the expressions of NtEXP44 and some salinity stress-response genes increased to enhance salinity resistance in tobacco plants (Kuluev et al. 2016). PpEXP1 overexpression in tobacco plants decreases biological damage under heat stress, while antioxidant enzymes and seed germination rates are increased (Xu et al. 2014). 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 (Zhou et al. 2011). In rice, OsEXP45, OsEXP47, and OsEXP10 can change pathogen susceptibility in plants and the response to pathogen stress (Kong et al. 2010). The TaEXPB8 gene can increase antioxidant defence 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 (Gomez Mansur et al. 2021). In addition, expansins play crucial roles in plants under low-temperature stress. For example, Zheng et al. (2012) analyzed fiber proteins in two cotton cultivars with different temperature sensitivities under low-temperature treatment. Their results showed that the expansins content was significantly upregulated in the low-temperature-tolerant cotton cultivar. The transcription 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 (Brummell et al. 2004). This result is consistent with that of Bauerfeind et al. (2015), who found that expansins can counterbalance the growth inhibition caused by low-temperature stress but they do not promote plant growth.

Expansins are associated with the morphogenesis of winter wheat root systems and tillers, and there are significant differences in expansins transcription in winter wheat cultivars with different cold resistance abilities. The transcriptions of expansins in wheat cv. Dongnong are higher than in the cold-sensitive cv. Zhongguochun (Zhang et al. 2018). 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 (Feng et al. 2019). In addition, the TaEXP48-B/D gene could actively respond to low-temperature stress of 4 °C and it is highly expressed in roots. This shows that expansins are important in increasing cold hardiness in winter wheat in frigid regions (Feng et al. 2019). In a study on expansins in cv. Dongnong, we found that TaEXP49 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 TaEXP49-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 low-temperature-resistance genes for molecular selective breeding.

Materials and methods

Plants and treatments: Winter wheat (Triticum aestivum L.) cv. Dongnong 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 % (m/v) sodium hypochlorite 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 imbibition 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 a 12-h photoperiod, day/night temperatures of 25/18 °C, an irradiance of 50 µmol m−2 s−1, and air humidity of 50 %. Subsequent experiments were carried out after the seedlings had grown to the two-leaf stage.

Arabidopsis thaliana L. cv. Columbia was provided by the Department of Plant Resources and Molecular Biology, Northeast Agricultural University. Seeds from wild-type A. thaliana and TaEXP49-A/B/D overexpressing 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 Murashige and Skoog (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.

Parts of winter wheat seeds were used in abiotic stress
and hormone treatment experiments done according to (Han et al. 2015). In brief, the trial set up the six treatments and a control group: 1) seedlings of the control group were grown normally at 25 °C; 2) to simulate drought stress, 20 % (m/v) polyethylene glycol (PEG) 6000 solution was used to soak the roots of the seedlings and part of the seedlings were placed at 4 °C for low-temperature treatment; 3) 0.1 μm indole-acetic acid (IAA), 2 μm abscisic acid (ABA), 10 μM salicylic acid (SA), and 10 μM methyl jasmonate (MeJA) was used to treat seedlings, respectively, and 3 wheat samples were randomly selected and mixed into one sample. Triplicate samples were collected from each group at 0, 3, 6, 12, and 24 h. The sample fresh mass was 0.1 g.

For expression analysis in different organs, roots, stems, and leaves of seedlings at the two-leaf stage, flowers at the flowering stage, and siliques in the fruiting stage were selected and mixed into each sample. The triplicate samples were collected for each organ, and the sample fresh mass was 0.1 g. The aforementioned samples were stored in a -80 °C freezer.

**Quantitative RT-PCR analysis of TaEXP A9 gene:** The Trans Zol Up Plus RNA kit (Transgene Biotech Co., Beijing, China) was used to extract total RNA from entire wheat seedlings, and a NanoDrop 2.0 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) was used to measure the RNA concentration (Rodriguez et al. 2020). RNA samples with an absorbance at 260/280 nm ≥ 1.8 and a concentration of ≥ 100 ng mm⁻³ were selected for subsequent experiments (Peng et al. 2019). The Trans Script® One-step gDNA Removal and cDNA Synthesis Super Mix kit (Transgene Biotech Co.) were used for reverse transcription of the extracted RNA into cDNA. The TransStart® Top Green qPCR SuperMix (Transgene Biotech Co.) was used for quantitative real-time polymerase chain reaction (RT-qPCR). The wheat β-actin gene (GenBank: AB181991) was used as the internal reference gene (Ma et al. 2015). Gene-specific primers for RT-qPCR were independently designed based on nucleotide polymorphisms in the cDNA sequences of TaEXP A9-A/B/D (Table 1 Suppl.). The RT-qPCR program was set as 40 cycles of denaturation at 94 °C for 5 s, annealing at 62 °C for 30 s, and extension at 72 °C for 15 s. The 2^−ΔΔCT method was used to calculate the expressions (Livak et al. 2001). Three wheat samples were randomly selected and mixed into one sample. All samples were run in triplicate.

**Cloning and sequence analysis of the TaEXP A9 gene:** The GenBank login number of TaEXP A9 on the NCBI (https://www.ncbi.nlm.nih.gov/) website is No. MK292062, the GenBank login numbers of TaEXP A9-A/B/D are not currently recorded. The sequences of the TaEXP A9-A/B/D genes were deposited in the wheat genome website (TGACv1.ftp://ftpensemblgenomes.org/pub/plants/release-32/fasta/Triticum_aestivum) and can be accessed by their Gene IDs. The Gene ID of TaEXP A9-A is TRIAE_CS42_5AL_TGACv1_374454_AA1200520; the Gene ID of TaEXP A9-B is TRIAE_CS42_5BL_TGACv1_406965_AA1351930; the Gene ID of TaEXP A9-D is TRIAE_CS42_5DL_TGACv1_435145_AA1446640. Table 1 Suppl. shows the primers specific for the three sequences that were designed. PCR was used to amplify the TaEXP A9-A/B/D gene sequences. The PCR program was set as 30 cycles of denaturation at 94 °C for 45 s, annealing at 65 °C for 45 s, and extension at 72 °C for 45 s. The pEASY-T3 Cloning kit (Transgene Biotech Co.) 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. (Jilin, China) for sequencing. SMART website (http://smart.embl-heidelberg.de/) (Letunic et al. 2017) was used to predict TaEXP A9-A/B/D protein domains, and the prediction results were compared and analyzed by DNAMAN software (Wang et al. 2003). Bioinformatics analysis of the sequence was performed as follows: The signal peptide sites of TaEXP A9-A/B/D proteins were predicted by SignalP v. 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen 2017). The transmembrane construction analysis of TaEXP A9-A/B/D proteins were predicted by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al. 2001). The hydrophyticy analysis of TaEXP A9-A/B/D proteins was predicted by the ExPASy-Prot Scale (https://www.expasy.org/) (Iskon et al. 2013). The subcellular localization analysis of TaEXP A9-A/B/D proteins was predicted by CELLO v. 2.5 (http://cello.life.nctu.edu.tw/cgi/main.cgi) (Yu et al. 2004). The phosphorylation sites analysis of TaEXP A9-A/B/D proteins was predicted by NetPhos (http://www.cbs.dtu.dk/services/NetPhos/) (Blom et al. 1999). Three-dimensional (3D) models of TaEXP A9-A/B/D proteins were predicted using SWISS-MODEL (https://swissmodel.expasy.org) (Waterhouse et al. 2018). EXP A9 sequences of different species were downloaded from NCBI and used to construct phylogenetic trees with TAEXP A9-A/B/D proteins by MEGA7 software. Multiple sequence alignments of expansin proteins were analyzed by the MUSCLE program of MEGA7. Neighbor-joining (NJ) tree was constructed with 1,000 bootstrap replicates (Tamura et al. 2013). The genetic information for plant species is as follows: HvEXP A9 (Hordeum vulgare, GenBank: KAE8814867.1); SgEXP A9 (Stylosanthes guianensis, GenBank: MN540941.1); CmEXP A9 (Cucumis melo, GenBank: QDL52551.1); AeEXP A9 (Arabidopsis thaliana, Gene ID: 831736); BrEXPL A9 (Brassica rapa, Gene ID: 103850441); AsEXP A9 (Apostasia shenzhenica, GenBank: P3A45854.1); MpEXP A9 (Mucuna pruriens, GenBank: RDX86523.1); NiEXP A9 (Nymphaea thermarum, GenBank: KAF3779135.1).

**Subcellular localization of TaEXP A9:** A 20-bp homology box containing the BglIII restriction enzyme cleavage site was designed using the primers for TaEXP A9-A, TaEXP A9-B, and TaEXP A9-D (Table 1 Suppl.). 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 TaEXP A9-A, TaEXP A9-B, and TaEXP A9-D genes (Fig. 1A). Homologous recombination was used to obtain the TaEXP A9-A-eGFP, TaEXP A9-B-eGFP, and TaEXP A9-D-eGFP fusion proteins. The

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**ZHAO et al.**

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274
Fig. 1. The subcellular localization of TaEXP49-A/B/D. A - Structure of the expression vector used for subcellular localization of TaEXP49-A/B/D. B - The transient expression of 35S::GFP and 35S::TaEXP49-A::GFP, 35S::TaEXP49-B::GFP, 35S::TaEXP49-D::GFP in onion epidermal cells. Scale bars 100 μm. The cells were observed under a confocal microscope after culture on MS medium at 28 °C for 2 d. Onion cell plasmolysis was induced by administering a 30 % sucrose solution for 20 min prior to observation.
Acquisition of \textit{TaEXP49}-overexpressing \textit{A. thaliana}: Twenty-bp homology box primers containing upstream XbaI and downstream BamHI restriction enzyme sites were designed for the \textit{TaEXP49-A}, \textit{TaEXP49-B}, and \textit{TaEXP49-D} genes. Double digestion was separately carried out on the PBI121 vector and \textit{TaEXP49-A}, \textit{TaEXP49-B}, and \textit{TaEXP49-D} genes that contained restriction enzyme sites (Fig. 4A Suppl.). Homologous recombination was used to obtain the 35S::PBI121-TaEXP49-A, 35S::PBI121-TaEXP49-B, and 35S::PBI121-TaEXP49-D recombinant vectors. The freeze-thaw method was employed to transform the three recombinant vectors into \textit{A. tumefaciens} (GV3101) (Feng \textit{et al.} 2019). Inflorescence infiltration was used to transform \textit{A. thaliana} with \textit{A. tumefaciens} containing 35S::PBI121-TaEXP49-A, 35S::PBI121-TaEXP49-B, and 35S::PBI121-TaEXP49-D (Yu \textit{et al.} 2017). After transformation and pod formation in \textit{A. thaliana}, the seeds were grown on ½ MS solid culture medium containing 50 μg cm⁻³ kanamycin to screen for T1 plants overexpressing \textit{TaEXP49-A}, \textit{TaEXP49-B}, and \textit{TaEXP49-D}. Quantitative RT-PCR was used to confirm transgenic plants. The reaction procedure is the same as above. The T3 transgenic plants and wild-type plants were used for further experiments. \textit{A. thaliana} overexpressing \textit{TaEXP49-A}, \textit{TaEXP49-B}, and \textit{TaEXP49-D} were denoted as OE-A, OE-B and OE-D, respectively.

Effects of overexpression of \textit{TaEXP49} on the growth of \textit{A. thaliana}: Overexpressed \textit{A. thaliana} and wild-type \textit{A. thaliana} were cultured in the same way as mentioned above. On day 4 of the culture, the hypocotyl length and number of lateral roots were observed and measured. On day 8 of the culture, the number of root hairs and axial root length were observed and measured. 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 siliques per plant were measured. Seeds from 21 \textit{A. thaliana} plants of each different type were collected to determine their total yield.

Effects of overexpression of \textit{TaEXP49} on antioxidant enzymes and osmotic regulatory substances of \textit{A. thaliana}: The aforementioned method was used to grow \textit{A. thaliana} seeds. When the wild-type (WT) and the three types of transgenic plants were grown until 40 d, the plants were divided into two groups, namely the control group and the low-temperature treated 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 were sampled 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) activities. The content of malondialdehyde (MDA), soluble protein, soluble sugar, and proline was measured (Feng \textit{et al.} 2019). These seven physiological markers were measured using assay kits from Suzhou Comin Biotechnology Co. (Suzhou, China).

Observation of the \textit{A. thaliana} phenotype under freezing stress: Fifty WT and each of the three overexpressing 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 freezing stress. The survival rate was measured after 8 d.

Statistical analysis: Average function in the basic package of \textit{R} language was used to average the three repetitions of each group of samples, and SD was used to calculate the standard deviation. The difference significance was analyzed using one-way ANOVA in GraphPad Prism5 to draw a bar chart in \textit{R} package ggplot based on the above information. In these graphs, error bars represent standard deviation. In histograms of RT-qPCR 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 \textit{A. thaliana} using the ggstatsplot package in \textit{R.Studio}.

Results

\textit{TaEXP49-A/B/D} expressions showed varying degrees of downregulation after PEG treatment, and these differences were significant compared with 0 h (\(P < 0.05\)). \textit{TaEXP49-D} expression decreased to 8.7 % after 3 h of treatment. After 12 h of treatment, the \textit{TaEXP49-A/B/D} expressions increased compared with 6 h, further the \textit{TaEXP49-A/B} expressions were significantly increased. However, after 24 h of treatment, \textit{TaEXP49-A/B/D} genes exhibited almost no expression (Fig. 2A,D,G). Under low temperature (4 °C) treatment, \textit{TaEXP49-A} gene expression gradually increased in the first 12 h, and the differences between the various time points were significant. \textit{TaEXP49-A} expression was downregulated at 24 h, though the difference compared with 0 h was not significant. \textit{TaEXP49-B/D} expressions were upregulated at various time points after treatment compared with 0 h, and these differences were significant. \textit{TaEXP49-A/B} expressions peaked at 12 h of treatment. Compared with 0 h, the expressions of \textit{TaEXP49-A/B}
and TaEXP A9-B were 163.6 and 136.7 %, respectively (Fig. 2A, D, G).

Under MeJA treatment, the TaEXP A9-A/B/D expressions gradually decreased as treatment duration increased, and the differences between the various treatment time points were significant \( (P < 0.05) \). The TaEXP A9-A gene was hardly expressed following MeJA treatment. Under the IAA, SA, and ABA treatments, TaEXP A9-A/B/D expression
Suppl.). The similarity of the nucleotide sequences of these three genes and bases in the gene sequence. The CDS lengths of (Fig. 1 Suppl.), which was consistent with the number of that the length of the target genes was around 800 bp were determined. The PCR amplification results showed TaEXP A9-A/B/D (Fig. 3). responses in plants through similar signaling mechanisms TaEXP A9-A/B/D were the highest in the roots. The expressions of developmental stages. The expressions of these three genes TaEXP A9-A/B/D contain 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 TaEXP A9-A/B/D only had the closest phylogenetic relationship with each (Fig. 3D Suppl.).

RT-qPCR was used to measure the expression of TaEXP A9-A/B/D genes in different organs at different developmental stages. The expressions of these three genes were the highest in the roots. The expressions of TaEXP A9-B/D were the lowest in the stems, and the expression of TaEXP A9-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 (Fig. 3).

In this study, the coding sequences (CDSs) of TaEXP A9-A/B/D in wheat seedlings at the two-leaf stage were determined. The PCR amplification results showed that the length of the target genes was around 800 bp (Fig. 1A Suppl.), which was consistent with the number of bases in the gene sequence. The CDS lengths of TaEXP A9-A and TaEXP A9-B/D were 807 and 801 bp, respectively. The similarity of the nucleotide sequences of these three genes was 97.23% (Fig. 1B Suppl.). TaEXP A9-A/B/D contains three exons and two introns (Fig. 3A Suppl.). TaEXP A9-A encodes for 267 amino acids, and TaEXP A9-B/D encodes for 265 amino acids. The amino acid sequence similarity between the three genes was 96.14% (Fig. 1C Suppl.). From this, it was deduced that the three sequences obtained by cloning were orthologous genes. Protein structure prediction based on SMART showed that the TaEXP A9-A/B/D proteins contained DPDD_1 and Pollen_allerg_1 domains (Fig. 1D-E Suppl.) and could induce cell wall loosening (Cosgrove 2015).

The CELLO v. 2.5 online software predicted that the TaEXP A9-A/B/D proteins were located extracellularly (Fig. 2D Suppl.). In order to validate the results, we constructed 35S::eGFP, 35S::TaEXP A9-A-eGFP, 35S::TaEXP A9-B-eGFP, and 35S::TaEXP A9-D-eGFP vectors and transformed them into onion epidermal cells (Fig. 1A). Fluorescence microscopy clearly showed that these three proteins were located in the cell wall (Fig. 1B).

In order to validate the function of TaEXP A9-A/B/D, we separately constructed three 35S: TaEXP A9-A/B/D vectors, which were used for A. thaliana transformation (Fig. 4A Suppl.). The RT-qPCR was used to measure TaEXP A9-A/B/D expression in A. thaliana (Fig. 4B Suppl.). Following that, these vectors were transformed into A. thaliana. Resistance screening was used to identify overexpressing (OE) plants, and the screening results are shown in Fig. 4C Suppl. The WT and transgenic plants were cultured on vertical plates for 4 d before the hypocotyl length (Fig. 4D) 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 (Fig. 4D Suppl. and Fig. 4E). Microscopy was used to count the number of lateral roots at identical sites in the WT and transgenic A. thaliana roots. The results are shown in Fig. 4E, 4F, 4G Suppl and Fig. 4H. 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 (Fig. 4G Suppl. and Fig. 4C) 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 higher than that of the WT, respectively, and these results were significant. The mean axial root length in the three OE *A. thaliana* strains was 0.30 - 0.48 cm higher than in the WT (Fig. 4), and these differences were significant. From this, it can be seen that the root system of the OE plants was better developed than of the WT plants, and so the OE plants possessed 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 (Fig. 4L-K Suppl. and Fig. 4E–G). The *A. thaliana* plants were grown until the fruiting stage. Fig. 4L Suppl. and Fig. 4H-I show that the number of siliques and seed yield in the OE plants were significantly higher than in the WT plants. This confirmed that *TaEXP A9-A/B/D* promoted the growth of transgenic *A. thaliana*.
The results of D2 transcriptome analysis showed that the expression of TaEXP A9-A/B/D was regulated by low temperature, and comparison and observation of the freezing stress treatment on the transgenic plants and WT plants intuitively demonstrated the functions of these genes. Compared with the state before treatment (Fig. 5A), cold stress for 30 min caused leaf softening, wilting, and colour deepening in the all types of plants (Fig. 5B). After culturing in the plant culture room for 7 d, the growth of the plant leaves gradually recovered (Fig. 5C). Phenotypic observation showed that the degree of recovery of OE-A/B/D were greater than of 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 % (Fig. 5D) higher than of the WT. The difference in survival rate between groups was significantly associated with overexpression of TaEXP9A. Therefore, TaEXP A9-A/B/D had positive promoting effects on cold hardiness in transgenic A. thaliana.

The activities of antioxidant enzymes (SOD, POD, and CAT) in the transgenic and WT plants at normal growth conditions were high and MDA content was low. The SOD activity of the OE-A/B plants fluctuated during low temperature stress, while the SOD activity in the OE-D was stable. Under low temperature treatment for 3 h, SOD activity was 469.0, 443.6, and 317.6 U g⁻¹, respectively. At 12 h, SOD activity was 316.6, 483.9, and 314.3 U g⁻¹, respectively (Fig. 6A). At low temperature treatment...
for 6 h, POD activity in the WT plant was at its peak of 224.36 U g⁻¹, which was 74.2, 88.2, and 54.7 % of the OE-A/B/D, respectively. At 24 h, POD activity in OE-B/D reached their peaks, which was 386.3 % and 340.5 % more than WT plants, respectively (Fig. 6B). At 24 h, the CAT activity in the three overexpressing plants peaked, being 1.60, 1.34, and 1.31 times higher than that of the WT plants, respectively (Fig. 6C).

The MDA content in OE-A/B/D at treatment for 24 h was 17.85, 10.71, and 11.82 nmol g⁻¹, respectively (Fig. 6D), 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...
content in the overexpressing plants were all higher than in the WT plants. After 12 h of low temperature treatment, the soluble protein content in the transgenic plants peaked. By contrast, the soluble protein content in the WT plants was at its trough of 0.95 mg g⁻¹. The soluble protein content in the three overexpressing plants was 426.3, 362.1, and 381.1 % that in the WT plants, respectively (Fig. 6E). As treatment duration increased up to 12 h, the soluble sugar content in the WT plants first increased before decreasing, while the soluble sugar content in the transgenic plants gradually increased. After 24 h of treatment, the soluble sugar content in the overexpressing plants decreased, but was still 220.3, 242.5, and 192.2 % of that of the WT plants, respectively (Fig. 6F). At 6 h, the proline content was high in the WT and transgenic plants, which was 11.02 μg g⁻¹ in WT plants. The proline content in the OE-A/B/D were 227.7, 279.9, and 324.8 % of that of the WT plants, respectively (Fig. 6G).

**Discussion**

A large volume of experimental data shows that expansins participate in the response to abiotic stress. Geilfuss et al. (2011) employed RT-qPCR to examine the effects of salinity stress on the abundance of mRNA in expansins subfamily members in maize cultivars with different salt resistance abilities. The results showed that β-expansin transcript abundance do not decrease in the salt-resistant cultivars, but significantly decreases in the sensitive cultivars. This shows that β-expansin plays an important role in salt resistance in maize (Geilfuss et al. 2011). Da Silva et al. (2017) analyzed the transcriptomes of drought-resistant and sensitive sugar cane cultivars after 24 h of drought stress and performed RT-qPCR validation. They found that EXPB8, which is associated with root elongation, responded to drought stress. In our study, it was found that the orthologous gene of TaEXP49 in winter wheat could respond to low temperature stress of 4 °C (Fig. 2A, D and G). This result was similar to previous results. However, the TaEXP49-A/B/D gene of winter wheat had no significant response to drought stress (Fig. 2A,D,G). This indicates that the same stress tolerance gene has been upregulated in A. thaliana (Cho et al. 1997), OsEXPB2 (Che et al. 2016), and OsEXPB3 (Lee et al. 2005); soybean GmEXPB2 (Guo et al. 2011); barley HvEXPB7 (He et al. 2015), and most plant expansins are localized to the cell wall. In this study, the subcellular localization results showed that TaEXP49-A/B/D mainly localized to the cell wall, which supports this view. The cell wall is also highly correlated with the response to various abiotic stresses (Cosgrove 1997). Therefore, in this experiment, orthologous genes may respond to low temperature stress through high expression in the cell wall, thus enabling winter wheat to overwinter safely.

The expansins are expressed differently in different organs (Reidy et al. 2001). Rice OsEXPB5 and barley HvEXPB1 are specifically expressed in the roots (Won et al. 2010), and wheat TaEXP46 and TaEXP8 are expressed in various organs, though their expressions are the highest in the roots (Lizana et al. 2010, Peng et al. 2019). The results of this study are consistent with previous studies: TaEXP49-A/B/D mRNA abundance was the highest in the roots (Fig. 3). Therefore, it can be deduced that these three genes mainly promote root growth and development. TaEXP49-A expression in the stems (0.25 ± 0.04) and TaEXP49-B/D expression in the leaves (0.13 ± 0.04; 0.17 ± 0.03) were second to that of the roots (Fig. 3). This also indicates that these three genes may have important roles in organs with intensive growth.

Comparison of morphological observations of transgenic and WT A. thaliana validated the hypothesis that TaEXP49-A/B/D promotes plant root growth. Under the same growth conditions, overexpressed plants had more developed roots than WT plants (Fig. 4E-G Suppl. and Fig. 4A-D). The results of this experiment are consistent with those of many researchers who have studied expansins. Cho et al. (2002) and Lü et al. (2013) found that overexpression of AEXP4A1 and R EXPA4 both promoted lateral root formation. Lee et al. (2013) showed that GhEXP4ATR overexpression promoted root hair development in A. thaliana plants. The high TaEXP49-A/B/D transcription in roots (Fig. 3) of winter
wheat seems to be the reason for the exuberant growth of transgenic *A. thaliana*. In this experiment, in addition to the more developed root system, the development of stem and leaf of the overexpressing plants were better than those of the WT (Fig. 4f, K Suppl. and Fig. 4E, G). Although the studies of Ma et al. (2013), Zhang et al. (2014), Kuluev et al. (2013), and Marowa et al. (2016) can also support our results, they cannot explain the reason why TaEXP A9-A/B/D increases *A. thaliana* yield. From this, we make a hypothesis. 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 (Wu et al. 2018). Plants may accumulate organic matter to promote rapid and healthy growth and produce more kernels in adverse environments. Therefore, TaEXP A9-A/B/D can be considered as a candidate gene for molecular selective breeding to improve crop yield.

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 (Mishra et al. 2019). The activation of the protective enzymes (SOD, POD, and CAT) that scavenges ROS protects plants from oxidative stress damage (Kazemi-Shahandashti et al. 2018). It has been reported that the TaEXP A2-overexpressing *A. thaliana* can upregulate TaMnSOD, TaAPX, TaCAT, TaPOD, and other ROS-scavenging enzyme related genes which can maintain low ROS content in TaEXP A2-overexpressing *A. thaliana* (Yang et al. 2020). In this study, TaEXP A9-A/B/D not only increased POD, SOD, and CAT activity and decreased MDA accumulation, but also increased proline, soluble sugar, and soluble protein content (Fig. 6). Proline not only decreases ROS content within the normal range but can also decrease intracellular water potential 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 (Hosseini et al. 2016). The conclusions from previous studies provided a rich theoretical basis for this study. Zhang et al. (2019) examined the effects of AstEXP A1 in abiogen stress and found that AstEXP A1-overexpressing plants had higher soluble sugar and proline levels than WT (Hao et al. 2017). Similar results were obtained in TaEXP B23-overexpressing *A. thaliana* (Han et al. 2012, Li et al. 2014) and PttEXP A5-overexpressing tobacco (Liu et al. 2016, Zhang et al. 2019). Therefore, in addition to increasing antioxidant enzyme activity, the increased levels of small organic molecules may be another important role of TaEXP A9-A/B/D in the survival of wheat under low temperature conditions. It is understood from available information that TaEXP A9-A/B/D are promising candidate genes that can be used for the selective breeding of crops with strong resistance towards low temperature and other abiotic stresses.

A large number of studies have reported that polyploid plants have better ability to resist environmental stress (Lewis 1980, Otto et al. 2000, Chao et al. 2013, Coate et al. 2013). In *Brachypodium distachyon* and *Brachypodium stacei* the drought resistance of polyploid plants was compared with two diploid plants and polyploid plants had stronger adaptability to drought stress. It was mainly reflected in the increase of endogenous hormones related to resistance and the improvement of osmotic stress resistance ability (Martínez et al. 2018). TaEXP A9-A, TaEXP A9-B, and TaEXP A9-D were transformed into *A. thaliana*, which improved systemically induced freezing resistance of *A. thaliana*. These results proved that the three winter wheat orthologous genes all had the stress resistance function and they are worth studying. Even in winter wheat under low temperature stress, the expression did not increase to more than two times. Studies have shown that the expression of multiple homologous genes can facilitate biological function (Carlson et al. 2017). Under natural low temperature conditions, orthologous genes in winter wheat may be highly expressed in the same time, responding to low temperature stress in a synergistic manner.

**Conclusion**

The expression of the expansin genes TaEXP A9-A/B/D isolated from winter wheat was induced by low temperature stress, and the protein encoded by this gene localized to the cell wall. TaEXP A9-A/B/D 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.

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