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Cloning and functional analysis of expansin *TaEXPA9* orthologs in winter wheat in frigid regions

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Abstract

Long-term low temperatures restrict the regrowth of winter wheat (*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, *TaEXPA9*. It is located in winter wheat cv. Dongnong with high cold hardiness. We analyzed the expression patterns of *TaEXPA9-A/B/D* in this cultivar and conducted a subcellular localization analysis of *TaEXPA9-A/B/D* in the onion epidermis. Transgenic *Arabidopsis thaliana* line with *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 *EXPA9-A/B/D* expression significantly increased at 4 °C, it was higher in the roots than in shoots, and *EXPA9-A/B/D* was localized in the cell wall. The roots were well-developed in the transgenic *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 *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. In summary, *TaEXPA9* orthologous genes participate in the low-temperature stress response, and they might be of great importance in molecular breeding.

Keywords: *Arabidopsis thaliana*, expansins, expression analysis, functional validation, *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 *et al.* 2003, Mishra *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 *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 *et al.* 2019, Peng *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 *et al.* 2009). Similarly, Monroy *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-

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Abbreviations: ABA - abscisic acid; CAT - catalase; D2 - Dongnong winter wheat No. 2; IAA - indoleacetic acid; MDA - malondialdehyde; MeJA - methyl jasmonate; POD - peroxidase; ROS - reactive oxygen species; SA - salicylic acid; SOD - superoxide dismutase.
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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), and fruit maturation and softening (Valenzuela-Riffo *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, *TaEXPA2*-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 *EaEXPA1* is a potential target gene that increases drought resistance in sugar cane. Under salinity stress, the expressions of *NtEXPA4* 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, *OsEXPA5*, *OsEXPA7*, and *OsEXPA10* 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 *TaEXPA8-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 (Peng *et al.* 2019). In a study on expansins in cv. Dongnong, 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 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 *TaEXPA9-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 following 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 ℃ 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 sampled. Triplicate samples were collected for each organ, and the sample mass was 0.1 g. The aforementioned samples were stored in a -80 °C freezer.

Quantitative RT-PCR analysis of *TaEXPA9* 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 (Rodríguez *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 *TaEXPA9-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 $2^{-\Delta\Delta CT}$ 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 *TaEXPA9* gene: The GenBank login number of *TaEXPA9* on the *NCBI* (https://www.ncbi.nlm.nih.gov/) website is No. MK292062, the GeneBank login numbers of *TaEXPA9-A/B/D* genes are not currently recorded. Sequences of the *TaEXPA9-A/B/D* genes were deposited in the wheat genome website (TGACv1,ftp://ftp.ensemblgenomes.org/pub/plants/release-32/fasta/Triticum_aestivum) and can be accessed by their Gene IDs. The Gene ID of *TaEXPA9-A* is TRIAE_CS42_5AL_TGACv1_374454_AA1200520; the Gene ID of *TaEXPA9-B* is TRIAE_CS42_5BL_TGACv1_406965_AA1351930; the Gene ID of *TaEXPA9-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 *TaEXPA9-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 TaEXPA9-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 TaEXPA9-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 TaEXPA9-A/B/D proteins were predicted by *TMHMM Server v. 2.0* (http://www.cbs.dtu.dk/services/TMHMM/)(Krogh *et al.* 2001). The hydropathicity analysis of TaEXPA9-A/B/D proteins was predicted by the *ExPASy-Prot Scale* (https://www.expasy.org/) (Ison *et al.* 2013). The subcellular localization analysis of TaEXPA9-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 TaEXPA9-A/B/D proteins was predicted by *NetPhos* (http://www.cbs.dtu.dk/services/NetPhos/) (Blom *et al.* 1999). Three-dimensional (3D) models of TaEXPA9-A/B/D were predicted using *SWISS-MODEL* (https://swissmodel.expasy.org) (Waterhouse *et al.* 2018). EXPA9 sequences of different species were downloaded from *NCBI* and used to construct phylogenetic trees with TAEXPA9-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 1 500 bootstrap replicates (Tamura *et al.* 2013). The genetic information for plant species is as follows: *HvEXPA9* (*Hordeum vulgare*, GenBank: KAE8814867.1); *SgEXPA9* (*Stylosanthes guianensis*, GenBank: MN540941.1); *CmEXPA9* (*Cucumis melo*, GenBank: QDL52551.1); *AtEXPA9* (*Arabidopsis thaliana*, Gene ID: 831736); *BrEXPLA9* (*Brassica rapa*, Gene ID: 103850441); *AsEXPA9* (*Apostasia shenzhenica*, GenBank: PKA45854.1); *MpEXPA9* (*Mucuna pruriens*, GenBank: RDX86523.1); *NtEXPA9* (*Nymphaea thermarum*, GenBank: KAF3779135.1).

Subcellular localization of *TaEXPA9*: A 20-bp homology box containing the Bg1II restriction enzyme cleavage site was designed using the primers for *TaEXPA9-A*, *TaEXPA9-B*, and *TaEXPA9-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 *TaEXPA9-A*, *TaEXPA9-B*, and *TaEXPA9-D* genes (Fig. 1*A*). Homologous recombination was used to obtain the TaEXPA9-A-eGFP, TaEXPA9-B-eGFP, and TaEXPA9-D-eGFP fusion proteins. The

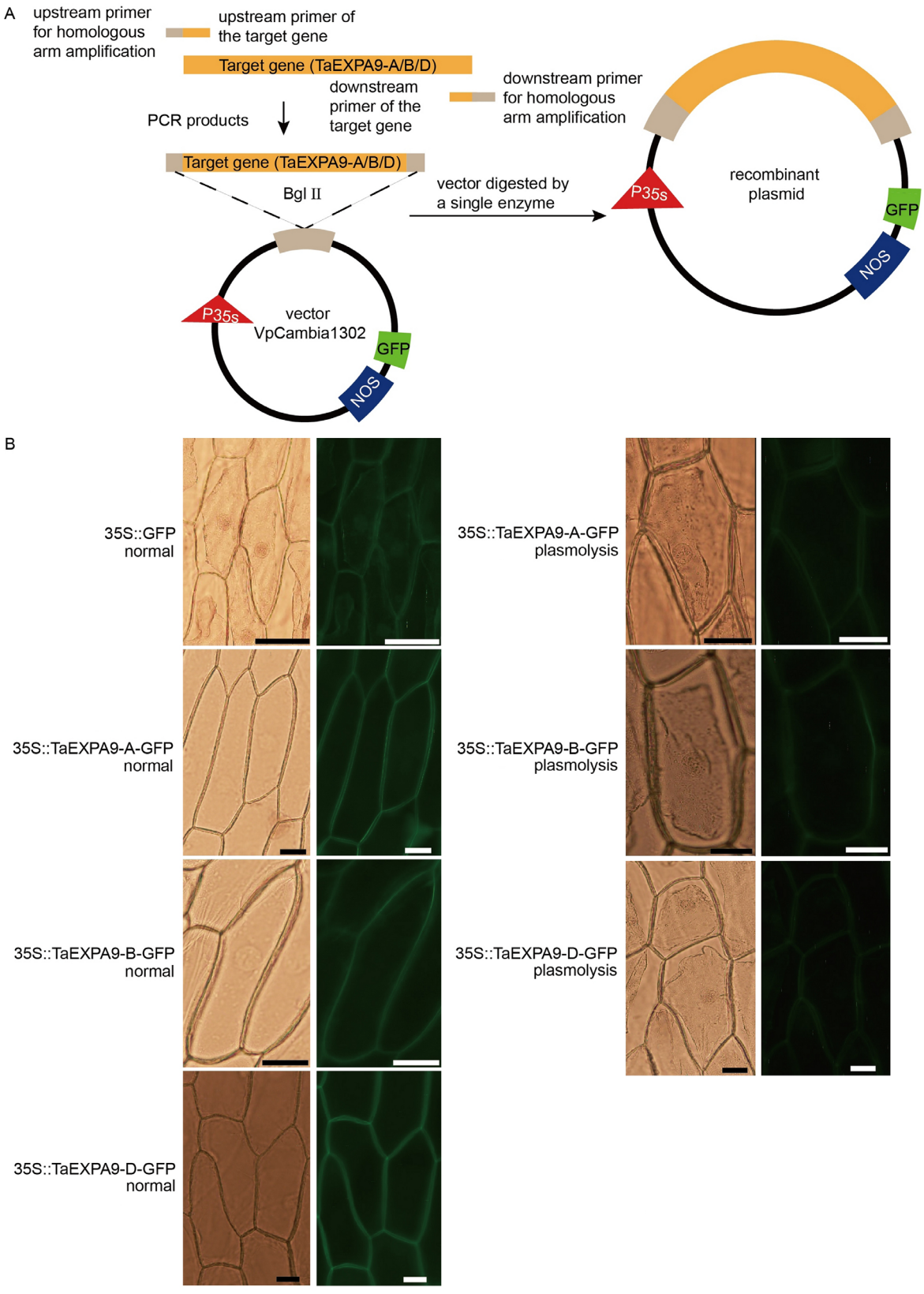

Fig. 1. The subcellular localization of *TaEXPA9-A/B/D*. *A* - Structure of the expression vector used for subcellular localization of *TaEXPA9-A/B/D*. *B* - The transient expression of 35S::eGFP and 35S::TaEXPA9-A-eGFP, 35S::TaEXPA9-B-eGFP, 35S::TaEXPA9-D-eGFP in onion epidermal cells. *Scale bars* 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.

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 (Feng *et al.* 2019). An onion epidermis was placed downwards at the side nearest to the mesophyll in the MS culture medium. The subcellular localization experiment was performed according to the method of Chen (Chen *et al.* 2016). The culture temperature was 28 °C and the transformation was carried out for 24 h in the dark. A confocal microscope was used to observe the transformed onion cells.

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. Double digestion was separately carried out on the PBI121 vector and *TaEXPA9-A*, *TaEXPA9-B*, and *TaEXPA9-D* genes that contained restriction enzyme sites (Fig. 4*A* Suppl.). 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) (Feng *et al.* 2019). Inflorescence infiltration was used to transform *A. thaliana* with *A. tumefaciens* containing 35S::PBI121-TaEXPA9-A, 35S::PBI121-TaEXPA9-B, and 35S::PBI121-TaEXPA9-D (Yu *et al.* 2017). After transformation and pod formation in *A. thaliana*, the seeds were grown on ½ MS solid culture medium containing 50 μg cm^{-3} kanamycin to screen for T1 plants overexpressing *TaEXPA9-A*, *TaEXPA9-B*, and *TaEXPA9-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. *A. thaliana* overexpressing *TaEXPA9-A*, *TaEXPA9-B*, and *TaEXPA9-D* were denoted as OE-A, OE-B and OE-D, respectively.

Effects of overexpression of *TaEXPA9* on the growth of *A. thaliana*: Overexpressed *A. thaliana* and wild-type *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 *A. thaliana* plants of each different type were collected to determine their total yield.

Effects of overexpression of *TaEXPA9* on antioxidant enzymes and osmotic regulatory substances of *A. thaliana*: The aforementioned method was used to grow *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 *et al.* 2019). These seven physiological markers were measured using assay kits from *Suzhou Comin Biotechnology Co*. (Suzhou, China).

Observation of the *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 *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 *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 *A. thaliana* using the *ggstatsplot* package in *R.Studio*.

Results

TaEXPA9-A/B/D expressions 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* expressions increased compared with 6 h, further the *TaEXPA9-A/B* expressions were significantly increased. However, after 24 h of treatment, *TaEXPA9-A/B/D* genes exhibited almost no expression (Fig. 2*A*,*D*,*G*). Under low temperature (4 °C) 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* expressions were upregulated at various time points after treatment compared with 0 h, and these differences were significant. *TaEXPA9-A/B* expressions peaked at 12 h of treatment. Compared with 0 h, the expressions of *TaEXPA9-A*

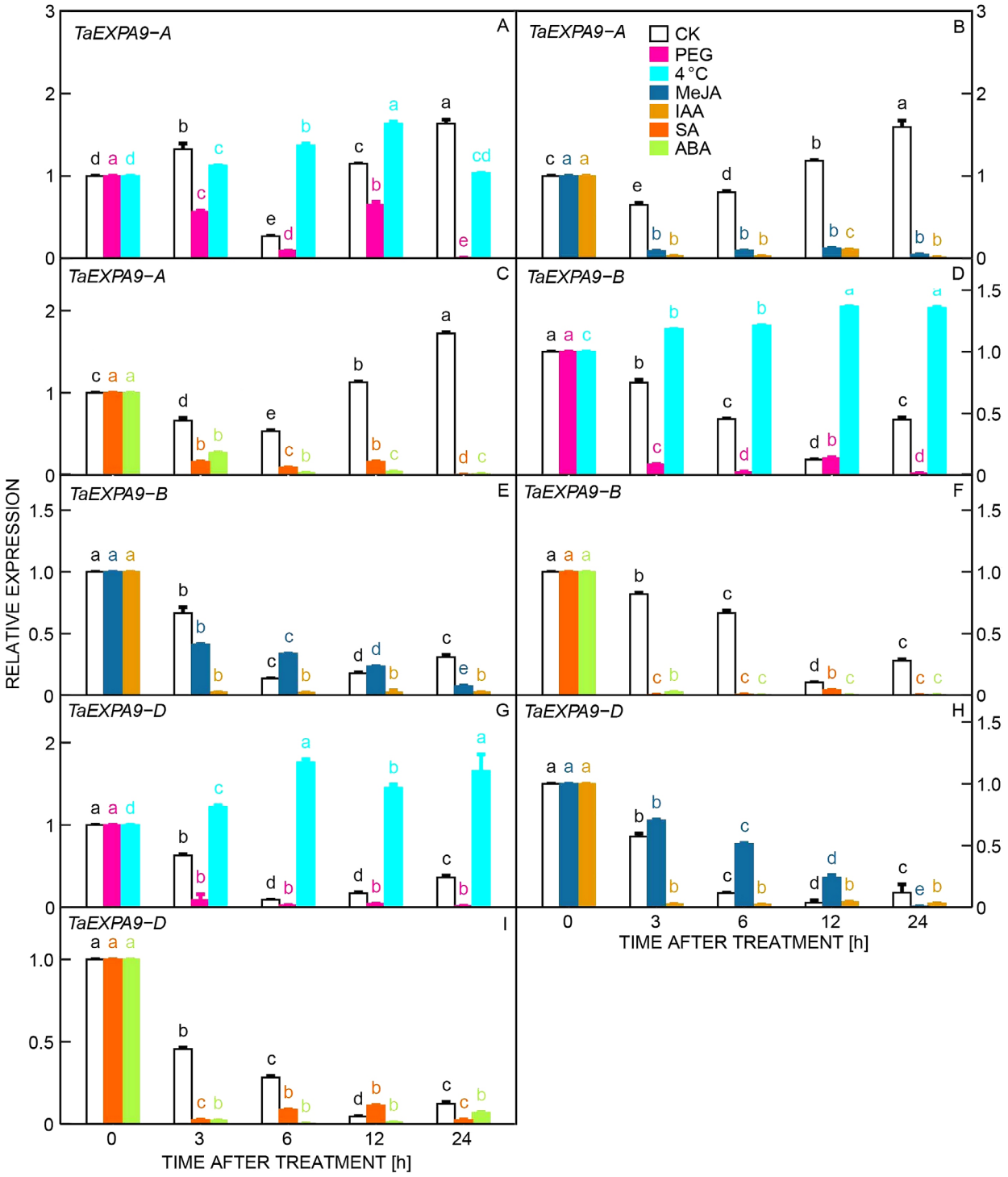

Fig. 2. *TaEXPA9-A/B/D* expression under abiotic stress and hormone treatments. The responses of *TaEXPA9-A/B/D* to low-temperature stress and drought stress (*A* - *TaEXPA9-A*, *D* - *TaEXPA9-B*, *G* - *TaEXPA9-D*). The responses of *TaEXPA9-A/B/D* to plant hormone treatments separately. The treatments of MeJA and IAA (*B* - *TaEXPA9-A*, *E* - *TaEXPA9-B*, *H* - *TaEXPA9-D*). The treatments of SA and ABA (*C* - *TaEXPA9-A*, *F* - *TaEXPA9-B*, *I* - *TaEXPA9-D*). Different lowercase letters indicated that expansin gene expressions were significantly different among different time points under the same treatment ($P < 0.05$).

and *TaEXPA9-B* were 163.6 and 136.7 %, respectively (Fig. 2*A*,*D*,*G*).

Under MeJA treatment, the *TaEXPA9-B/D* expressions 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

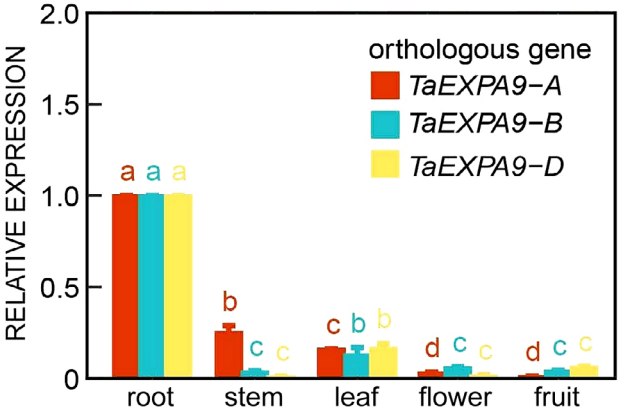

Fig. 3. RT-qPCR analysis of the relative expressions of *TaEXPA9-A/B/D* in different organs of winter wheat cv. Dongong. Means ± SDs. Different lowercase letters indicated significant difference among different organs ($P < 0.05$).

was almost completely inhibited (Fig. 2*B,C,E,F,H,I*). In summary, *TaEXPA9-A/B/D* can be upregulated only in response to low temperature stress.

RT-qPCR was used to measure the expression of *TaEXPA9-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 *TaEXPA9-B/D* were the lowest in the stems, and the expression 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 (Fig. 3).

In this study, the coding sequences (CDSs) of *TaEXPA9-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. 1*A* Suppl.), 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 % (Fig. 1*B* Suppl.). *TaEXPA9-A/B/D* contains three exons and two introns (Fig. 3*A* Suppl.). *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 % (Fig. 1*C* 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 *TaEXPA9-A/B/D* proteins contained DPDD_1 and Pollen_allerg_1 domains (Fig. 1*D-E* Suppl.) and could induce cell wall loosening (Cosgrove 2015).

Table 2 Suppl. shows the basic information of the *TaEXPA9-A/B/D* proteins. Their molecular masses 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) reflect the stability of the protein (Ikai 1980). 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 3 Suppl.). The signal peptides in *TaEXPA9-A* and *TaEXPA9-B/D* are in positions 29 - 30 and 27 - 28, respectively (Fig. 2*A* Suppl.). The three proteins are hydrophilic secretory proteins with transmembrane structures (Fig. 2*B,C* Suppl.) and are located outside the cell membrane (Fig. 2*D* Suppl.). The constructed three-dimensional structures of these proteins clearly show the catalytic domain (green region), cellulose binding domain (yellow and reg 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 Fig. 3*B* Suppl. Comparison of EXPA9 proteins from nine plant species, which includes TaEXPA9-A/B/D proteins, and an EXPLA9 protein from one plant species (Fig. 3*C* Suppl.) 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* only had the closest phylogenetic relationship with each (Fig. 3*D* Suppl.).

The *CELLO v. 2.5* online software predicted that the *TaEXPA9-A/B/D* proteins were located extracellularly (Fig.2*D* Suppl.). In order to validate the results, we constructed 35S::eGFP, 35S::TaEXPA9-A-eGFP, 35S::TaEXPA9-B-eGFP, and 35S::TaEXPA9-D-eGFP vectors and transformed them into onion epidermal cells (Fig. 1*A*). Fluorescence microscopy clearly showed that these three proteins were located in the cell wall (Fig. 1*B*).

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 (Fig. 4*A* Suppl.). The RT-qPCR was used to measure *TaEXPA9-A/B/D* expression in *A. thaliana* (Fig. 4*B* 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. 4*C* Suppl. The WT and transgenic plants were cultured on vertical plates for 4 d before the hypocotyl length (Fig. 4*A*) 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. 4*D* Suppl. and Fig. 4*A*.). 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. 4*E*, 4*F, 4G* Suppl and Fig.4. 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.

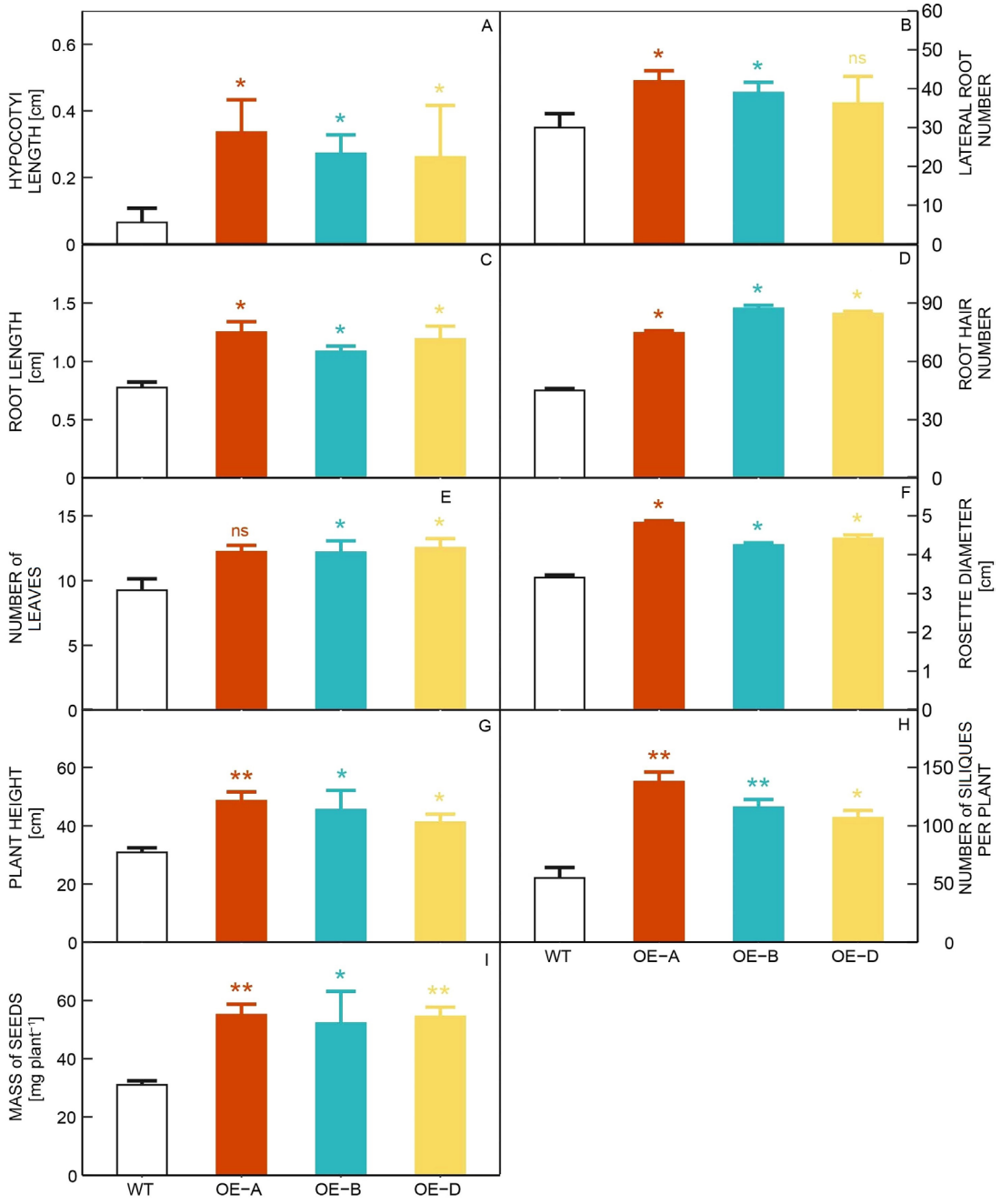

Fig. 4. Quantitative results of *A. thaliana* phenotypic observation. *A* - Hypocotyl length. *B* - Number of lateral roots. *C* - Root length. *D* - Number of roots hairs. *E* - Number of leaves. *F* - Rosette diameter. *G* - Plant height. *H* - Number of pods per plant. *I* - Mass of seeds per plant. Means ± SEs, $n = 10$, *, ** - indicates significant differences between WT and transgenic plants at $P < 0.05$ or $P < 0.01$, respectively.

The number of root hairs and axial roots in *A. thaliana* was measured at 8 d. The results (Fig. 4*G* Suppl. and Fig. 4*C*) 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. 4*I-K* Suppl. and Fig. 4*E–G*). The *A. thaliana* plants were grown until the fruiting stage. Fig. 4*L* Suppl. and Fig. 4*H-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 *TaEXPA9-A/B/D* promoted the growth of transgenic *A. thaliana*.

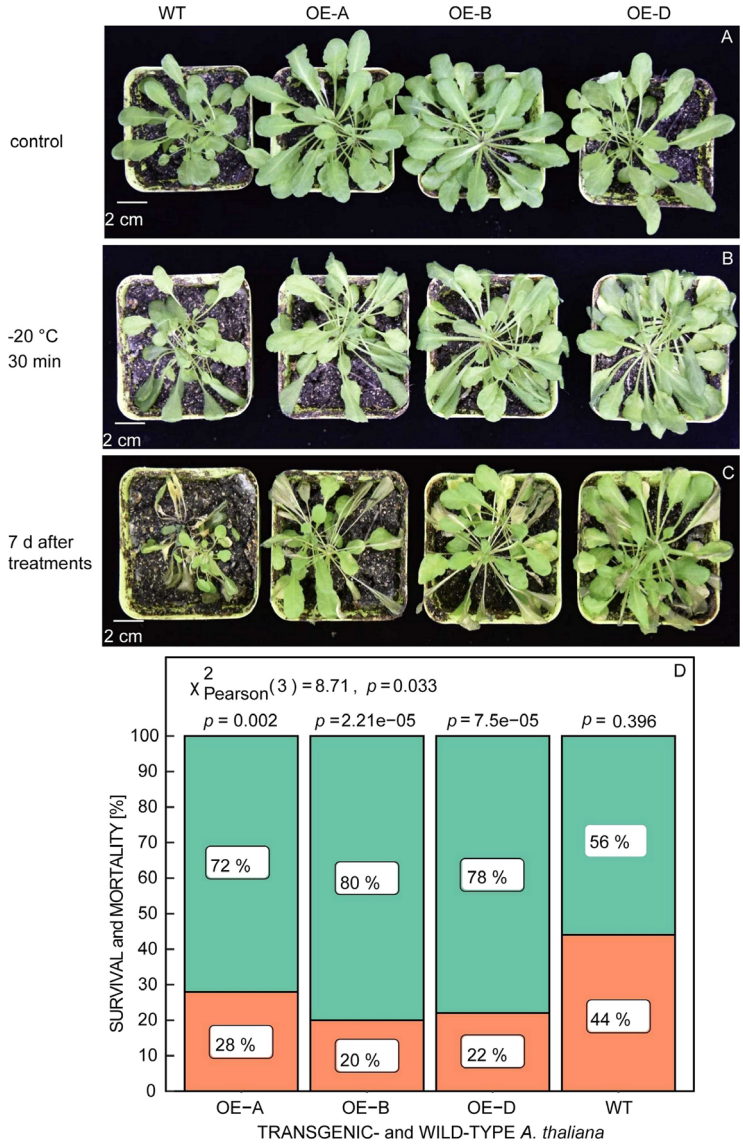

Fig. 5. Phenotypes and survival rate of the transgenic *A. thaliana* overexpressing *TaEXPA9-A/B/D* and WT plants grown in soil after low-temperature treatment at the rosette leaf stage. *A* - Room temperature treatments. *B* - -20 °C for 15 min. *C* - 7 d recovery at room temperature. *Scale bars* 2 cm. *D* - Survival status of wild-type and transgenic and *A. 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 paralogue variables and factors.

The results of D2 transcriptome analysis showed that the expression of *TaEXPA9-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. 5*A*), cold stress for 30 min caused leaf softening, wilting, and colour deepening in the all types of plants (Fig. 5*B*). After culturing in the plant culture room for 7 d, the growth of the plant leaves gradually recovered (Fig. 5*C*). 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. 5*D*) higher than of the WT. The difference in survival rate between groups was significantly associated with overexpression of *TaEXPA9*. Therefore, *TaEXPA9-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. 6*A*). At low temperature treatment

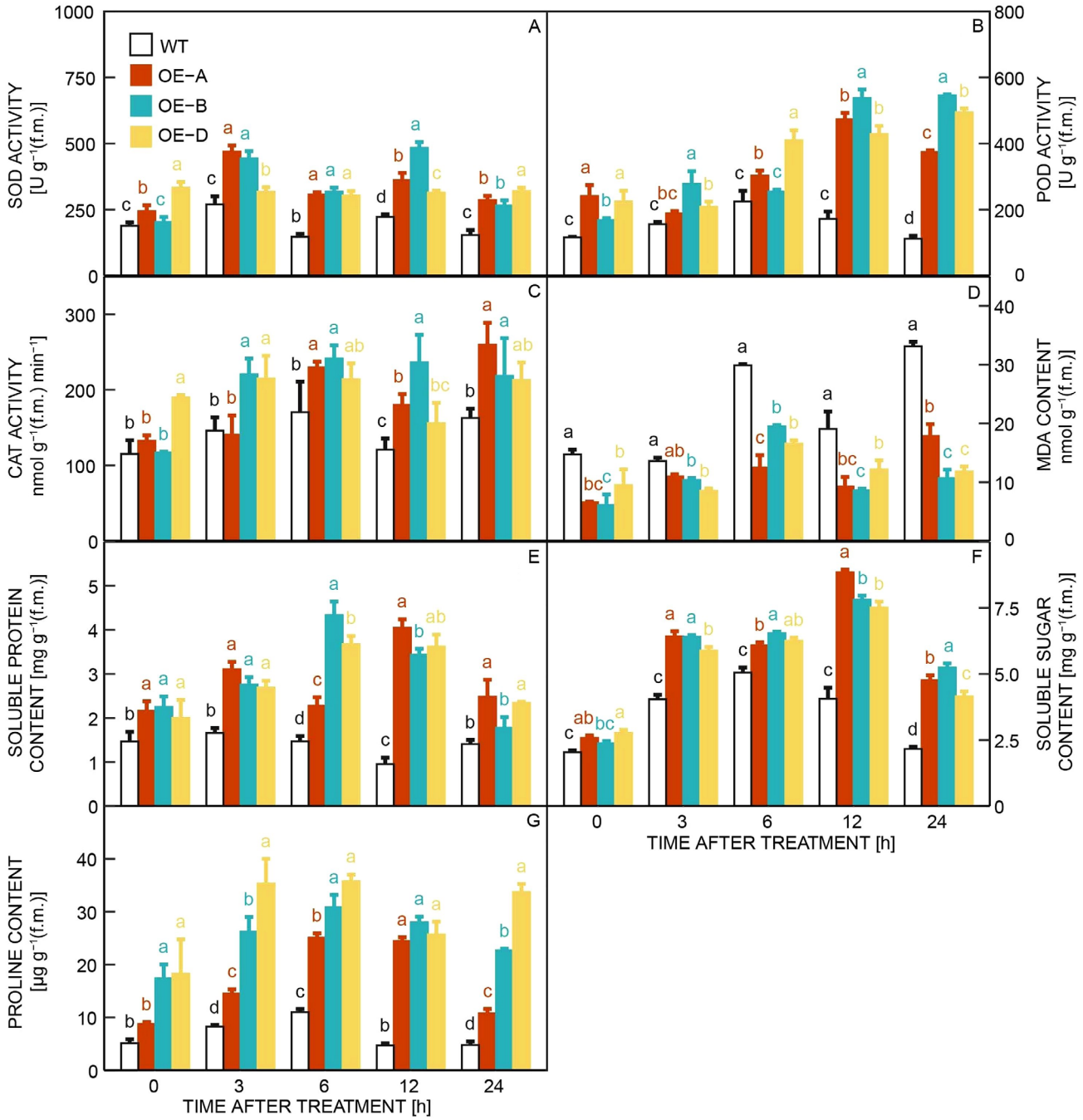

Fig. 6. *TaEXPA9-A*, *B* and *D* enhances low-temperature tolerance in *A. thaliana*. Determination of antioxidant capacity and osmotic adjustment substance contents in *A. thaliana* under 4 °C treatment ($n = 8$). *A* - SOD activity. *B* - POD activity. *C* - CAT activity. *D* - MDA content. *E* - Soluble protein content. *F* - Soluble sugar content. *G* - Proline content. Different lowercase letters indicate that there are significant differences in enzyme activity and substance content of *A. thaliana* between different time points after the same treatment ($P < 0.05$).

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. 6*B*). 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. 6*C*).

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. 6*D*), 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. 6*E*). 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. 6*F*). 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. 6*G*).

Discussion

A large volume of experimental data shows that expansins participate in the response to abiotic stress. Geilfus *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 (Geilfus *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 *TaEXPA9* in winter wheat could respond to low temperature stress of 4 °C (Fig. 2*A*, *D* and *G*). This result was similar to previous results. However, the *TaEXPA9-A/B/D* gene of winter wheat had no significant response to drought stress (Fig. 2*A*,*D*,*G*). This indicates that the same stress tolerance gene has different responses to different stresses (Li *et al.* 2017, Cao *et al.* 2019, Malik *et al.* 2020). Zhang *et al.* (2019) applied RT-qPCR to study the tobacco overexpressing *PttEXPA8* in response to heat, drought, cadmium, cold, and salt stress, and the results showed that tobacco has the largest response to heat stress.

It has been widely reported that plant hormones such as ABA, SA, and MeJA can improve the ability of plants to cope with abiotic stress (Colebrook *et al.* 2014, Lievens *et al.* 2017). Huang *et al.* (2017) studied the contribution of *Cynodon dactylon* resistance at low temperature of 4 °C with or without ABA addition as treatment. They found that exogenous ABA could reduce the content of H_2O_2 and MDA in plants in response to low temperature (Huang *et al.* 2017). The expression of expansins is also regulated by hormones. Previous studies found that *TaEXPA3-D2*, *TaEXPB7-B*, and *TaEXLA2-B* are upregulated in response to ABA, SA, and MeJA (Feng *et al.* 2019). However, *TaEXPA9-A/B/D* expression was still downregulated when treated with these four hormones in this study. When winter wheat experiences low temperature stress, the upregulation of *TaEXPA9-A/B/D* may not be mediated by these four hormones (Fig. 2*B*,*C*,*E*,*F*,*H*,*I*). The results of this experiment were partially supported by previous studies. Liu *et al.* (2019) studied changes in transcriptions of *AnEXPA1* and *AnEXPA2* genes in *Ammopiptanthus nanus* under cold stress, drought stress, and hormone treatment. Their results showed that *AnEXPA1* was only induced by low temperature, while *AnEXPA2* could be induced by hormones. Since plant hormones play an important role in regulating cold tolerance in plants, our study excluded the possibility that this group of orthologous genes were regulated by IAA, ABA, SA, and MeJA. We speculate that the expression of expansin gene may be regulated by other plant hormones under low temperature stress.

Expansins are so named because they regulate cell wall loosening, thereby resulting in cell division and growth. Rice *EXPA10* (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 *TaEXPA9-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 *TaEXPA6* and *TaEXPB8* 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: *TaEXPA9-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. *TaEXPA9-A* expression in the stems (0.25 ± 0.04) and *TaEXPA9-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 *TaEXPA9-A/B/D* promotes plant root growth. Under the same growth conditions, overexpressed plants had more developed roots than WT plants (Fig. 4*E-G* Suppl. and Fig. 4*A-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 *AtEXPA17* and *RhEXPA4* both promoted lateral root formation. Lee *et al.* (2013) showed that *GbEXPATR* overexpression promoted root hair development in *A. thaliana* plants. The high *TaEXPA9-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. 4*I*, *K* Suppl. and Fig. 4*E*, *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 *TaEXPA9-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, *TaEXPA9-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 *TaEXPA2*-overexpressing *A. thaliana* can upregulate *TaMnSOD*, *TaAPX*, *TaCAT*, *TaPOD*, and other ROS-scavenging enzyme related genes which can maintain low ROS content in *TaEXPA2*-overexpressing *A. thaliana* (Yang *et al.* 2020). 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 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 *AstEXPA1* in abiotic stress and found that *AstEXPA1*-overexpressing plants had higher soluble sugar and proline levels than WT (Hao *et al.* 2017). Similar results were obtained in *TaEXPB23*-overexpressing *A. thaliana* (Han *et al.* 2012, Li *et al.* 2014) and *PttEXPA8*-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 *TaEXPA9-A/B/D* in the survival of wheat under low temperature conditions. It is understood from available information that *TaEXPA9-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). *TaEXPA9-A*, *TaEXPA9-B*, and *TaEXPA9-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 *TaEXPA9-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. *TaEXPA9-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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