The cold-stress responsive gene DREB1A involved in low-temperature tolerance in Xinjiang wild walnut

Liqun Han1,2, Kai Ma3, Yu Zhao3, Chuang Mei1, Aisajan Mamat2, Jixun Wang2, Ling Qin1,3 and Tianming He1

1 College of Horticulture, Xinjiang Agricultural University, Urumqi, China
2 Institute of Horticultural Crops, Xinjiang Academy of Agricultural Sciences/Key Laboratory of Genome Research and Genetic Improvement of Xinjiang Characteristic Fruits and Vegetables/Xinjiang Fruit Science Experiment Station, Ministry of Agriculture, Urumqi, China
3 College of Plant Science and Technology, Beijing University of Agriculture, Beijing, China

ABSTRACT

Background. Low-temperatures have the potential to be a serious problem for plants and can negatively affect the normal growth and development of walnuts. DREB1/CBF (Dehydration Responsive Element Binding Protein 1/C-repeat Binding Factor), one of the most direct transcription factors in response to low-temperature stress, may improve the resistance of plants to low-temperatures by regulating their functional genes. However, few studies have been conducted in walnut. The Xinjiang wild walnut is a rare wild plant found in China, with a large number of excellent trait genes, and is harder than cultivated walnuts in Xinjiang.

Methods. In this work, we identified all of the DREB1 members from the walnut genome and analyzed their expression levels in different tissues and during low-temperature stress on the Xinjiang wild walnut. The JfDREB1A gene of the Xinjiang wild walnut was cloned and transformed into Arabidopsis thaliana for functional verification.

Results. There were five DREB1 transcription factors in the walnut genome. Among them, the relative expression level of the DREB1A gene was significantly higher than other members in the different tissues (root, stem, leaf) and was immediately un-regulated under low-temperature stress. The overexpression of the JfDREB1A gene increased the survival rates of transgenic Arabidopsis lines, mainly through maintaining the stability of cell membrane, decreasing the electrical conductivity and increasing the activities of antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). Additionally, the expression levels of cold-inducible genes like AtKIN1, AtERD10, AtRD29A, AtCOR15A and AtCOR47, were significantly increased. These results showed that the JfDREB1A gene may play an important role in the response to cold stress of the Xinjiang wild walnut. This study contributes to our understanding of the molecular mechanism of the Xinjiang wild walnut’s response to low-temperature stress and will be beneficial for developing walnut cultivars with improved cold resistance.

Subjects Agricultural Science, Molecular Biology, Plant Science

Keywords Xinjiang wild walnut, JfDREB1A gene, Low-temperature stress, Transgenic Arabidopsis, Cold resistance
INTRODUCTION

Low temperatures are an abiotic stress that affect the growth, development, and distribution of plants, and may even lead to plant death (Colinet, Renault & Roussel, 2017; Shi, Ding & Yang, 2018). As global climate change intensifies, extreme climates and low-temperature disasters will occur more frequently. The study of a plant’s response to low-temperature stress is a topic of great interest in the field of plant research and has important theoretical and practical value for ensuring the plant’s sustainable development (Ding, Shi & Yang, 2019; Gong et al., 2020; Wu, Mao & Zhao, 2021).

Through evolution, plants have gradually formed a complex and efficient response mechanism to adjust their phenotypic structure, physiological characteristics, and molecular mechanism to adapt to low-temperature stress. The most intuitive performance is their phenotypic changes, especially morphological characteristics like leaf wilting, tissue softening and dehydrating, even withering and dying (McCully, Canny & Huang, 2004; Ruelland et al., 2009). The fundamental mechanism of plant cold stress lies in the damage of the membrane system. The cell membrane is the main medium for the exchange between cells and environmental substances, and its stability is an important factor to judge the strength of plant cold stress tolerance (Muramatsu et al., 2015). Physiological indexes can more accurately reflect the response of plants to low-temperature stress, among them relative electric conductivity, superoxide dismutase (SOD) activity, peroxidase (POD) activity, and catalase (CAT) activity are used to identify the response of plants under low-temperature stress (Anwar et al., 2018; Kim & Tai, 2011; Velikova, Yordanov & Edreva, 2000).

Plants can respond to low-temperature stress by regulating gene expressions in the stress signal pathway through transcription factors (Agarwal et al., 2006; Zhu, 2016). DREB are specific transcription factors in the APETALA2/ethylene response factor (AP2/ERF) family, and combine with dehydration responsive element/C-repeat (DRE/CRT) elements for expressing abiotic stresses such as low-temperature, drought and salt (Haake et al., 2002; Magome, Yamaguchi & Hanada, 2004; Shi, Ding & Yang, 2018; Xie et al., 2019; Zhao et al., 2016). DREB transcription factors are divided into six subgroups, A1–A6. DREB1 belongs to the A1 subgroup which contains a plant-specific and highly-conformed AP2 domain consisting of approximately 60 amino acids and two characteristic sequences (PKK/RPAGRTKFRETRHP, DSAWR) (Jaglo et al., 2001; Nakano et al., 2006; Sakuma et al., 2002). DREB1/CFB genes have been identified in rice, tomato, poplar, grape and other plants, and played a key role in the response to low-temperatures (Ito et al., 2006; Maestrini et al., 2009; Xiao et al., 2006; Zhang et al., 2004). Among them DREB1A/CFB3, DREB1B/CFB1 and DREB1C/CFB2 were reported to be sensitive to low-temperature stress (Fowler & Thomashow, 2002; Park et al., 2015; Gilmour, Fowler & Thomashow, 2004; Thomashow, 2010).

Walnut, the genus Juglans of Juglandaceae, is one of the four major nuts in the world. Low-temperatures can seriously affect the normal growth and development of the walnut, resulting in a negative economic impact. Therefore, it is very important to study the response mechanism of the walnut to low-temperature stress. Wild plants are...
important materials from which to obtain resistance genes that have been selected by their exposure to extreme natural environments. Xinjiang wild walnut \((Juglans\ Fallax\ D.)\) is a rare wild plant resource in China and the direct ancestor of cultivated walnuts in Xinjiang. It has formed many unique variations in the long-term evolution process and has great potential for utilization \((Dong\ et\ al.,\ 2012)\). Our team found that there were more cold-resistant varieties in the Xinjiang wild walnut community than cultivated walnuts \((Han\ et\ al.,\ 2019)\). It is important to analyze the molecular mechanisms of the Xinjiang wild walnut in response to low-temperature stress and to detect cold resistance candidate genes. Therefore, we identified all \(DREB1\) members from the walnut genome \((https://www.ncbi.nlm.nih.gov/bioproject/291087)\), analyzed the expression levels of the \(DREB1\) genes in different tissues and low-temperature stress, and identified the key gene, \(DREB1A\), in response to low-temperature stress. The \(JfDREB1A\) gene of the Xinjiang wild walnut was cloned and transformed into Arabidopsis to verify the functional mechanism. This study may provide an important candidate gene for improving the cold-resistance of walnut by transgene technique.

**MATERIALS & METHODS**

**Plant materials**

The experimental materials were the ‘Microoblonga’ type of the Xinjiang wild walnut, which has been shown to have strong cold resistance. We collected the mature seeds stored at 4 °C for three months, the seeds were sowed in nutrition bowls (Peat soil: perlite = 3:1) and were irrigated with water, resulting in 100 seedlings. The roots, stems and leaves were gathered when the seedlings grown to five compound leaves. Meanwhile 35 seedlings with consistent growth were selected for 0 h, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h at 4 °C in the dark in the low temperature artificial climate incubator (DLRX-450B-LED, Jinwen, Shanghai, China). Each sample had three replicates, with five seedlings per replicate. We collected two to five leaves under the parietal lobe and the leaves were frozen immediately in liquid nitrogen and stored at −80 °C before RNA extraction.

The Columbia wild-type Arabidopsis was used as the receptor material. The transgenic and wild-type Arabidopsis were seeded in a MS medium and were cultured at 23 °C for 16/8 h (day/night). The seedlings were divided into two parts: one cultured in medium for 14 days, the other were transferred to the nutrition bowls (Peat soil: vermiculite: perlite = 1:2:0.5) and grew for 21 days which were used for phenotypes, physiological indexes and genes expression analyzing. The sampling method was the same as above.

**Identification of DREB1s transcription factors in walnut**

The walnut whole genome sequence \((PRJNA291087)\) was downloaded from NCBI \((https://www.ncbi.nlm.nih.gov/bioproject/291087)\). The AP2 Hidden Markov Model \((PF00847)\) was downloaded from the Pfam \((http://pfam.xfam.org)\) database. The sequences were screened by HMMER3.0 for those containing AP2 domains from the walnut whole genome. The sequence structures were predicted using the SMART \((http://smart.embl.de/smart/batch.pl)\) and ExPASy-PROSITE \((https://prosite.expasy.org)\), then the partial length and repetitive sequences were removed. All DREB transcription
factors of walnut were identified according to the number and characteristic of their AP2 domains (Nakano et al., 2006).

The Arabidopsis DREB transcription factors family was downloaded from the Arabidopsis genome database (https://www.arabidopsis.org/). We aligned multiple sequences with the DREB transcription factors of walnut and Arabidopsis using Clustal W. A phylogenetic tree was constructed using MEGA 7.0 with the neighbor-joining method, which the execution parameters were bootstrap method 1000, Poisson model, and pairwise deletion.

Real-time quantitative PCR verification
Total RNA was extracted from leaves of Xinjiang wild walnut seedlings using an RNA extraction kit (TIANGEN, Beijing, China), and 1 \( \mu \)g was used to synthesize the cDNA by using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Shanghai, China). Primers were designed with Primer Premier 6.0 software according to the sequence of five DREB1 genes in walnut, and 18S rRNA was used as the reference gene (Table S1). Amplification was performed using an qTOWER \(^{3}\) G fluorescent quantitative PCR instrument (Analytikjena Company, Jena City, Thuringia, Germany) according to the manufacturer’s instructions (SYBR Green qPCR Master Mix kit (Servicebio, Wuhan, China)), which contained 7.5 \( \mu \)L of qPCR mix, 0.75 \( \mu \)L each of forward and reverse primers (2.5 \( \mu \)M), 2.0 \( \mu \)L of cDNA templates and ddH\(_2\)O added to 15 \( \mu \)L. The PCR cycling conditions were: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and the melting curve was 65–95 °C for 0.3 °C/15 s. Each sample had three replicates and the relative expression levels were calculated by the method of \( 2^{-\Delta\Delta Ct} \).

Gene cloning
The total RNA was extracted and reverse transcription was performed as described above. The primers were designed with Primer Premier 6.0 software according to the DREB1A gene sequence in walnut (Table S1). Amplification was performed using a Biometra TAdvanced 96G PCR instrument (Analytikjena Company, Jena City, Thuringia, Germany). The PCR amplification system followed the manufacturer’s instructions (PrimeSTAR GXL Premix (TaKaRa, Dalian, China)) which contained 12.5 \( \mu \)L of PrimeSTAR GXL Premix, 0.5 \( \mu \)L each of forward and reverse primers (10 \( \mu \)M), 1 \( \mu \)L of cDNA templates and ddH\(_2\)O added to 25 \( \mu \)L. The PCR cycling conditions were: 98 °C for 5 min followed by 30 cycles of 98 °C for 10 s, 56 °C for 15 s, 72 °C for 60 s. The amplified products were determined by 1% agarose gel electrophoresis, then purified and recycled using MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (TaKaRa, Dalian, China), connected with pMD19-T vector (TaKaRa, Dalian, China), and transformed by the Escherichia coli DH5 \( \alpha \) competent cell. The full-length cDNA sequence of JfDREB1A gene was obtained by sequencing.

Generation of transgenic Arabidopsis thaliana lines
The open reading frame (ORF) of the JfDREB1A gene was constructed in the pCAMBIA1304 vector (Cambia, Canberra, Australia). The recombinant plasmid was transformed into the Agrobacterium tumefaciens GV3101 strain by freezing and thawing, then transformed into Arabidopsis by soaking (Ni et al., 2013). Eventually, the T3 generation transgenic Arabidopsis lines were obtained through breeding.
Survival rates of seedlings counting
The petri dish and potted seedlings of transgenic and wild-type Arabidopsis with consistent growth were treated at $-7^\circ C$ in the dark for 5 h, 8 h and 6 h, 9 h respectively in the low temperature artificial climate incubator (DLRX-450B-LED, Jinwen, Shanghai, China), then were recovered after 4 d at 23 $^\circ C$ for 16/8 h (day/night). The survival rates of the seedlings were counted.

Relative electric conductivity, SOD, POD and CAT measuring
The potted seedlings of transgenic and wild-type Arabidopsis were treated at 4 $^\circ C$ in the dark for 0 h, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h. The relative electric conductivity was measured using a conductometer (DDS-307A, REX, China). The SOD activity was measured using nitrogen-blue tetrazole photoreduction, POD activity was measured using guaiacol colorimetry, and the CAT activity was measured using ammonium molybdate colorimetry, all the methods were carried out according to the instructions of the kits (COMIN, Suzhou, China).

Functional genes verification
The potted seedlings of transgenic and wild-type Arabidopsis were treated with 4 $^\circ C$ in the dark for 0 h, 8 h. Total RNA extraction, reverse transcription, and qPCR amplification were performed as described above. Primers were designed according to the sequence of cold-inducible genes, and the AtUBQ3 gene (NC_003076.8) was used as an internal reference (Table S1). The relative expression levels were calculated by the method of $2^{-\Delta\Delta Ct}$.

Statistical analysis
The data were analyzed using SPSS 21 software (SPSS, Inc. Chicago, IL, USA) and presented as means ± standard deviation (SD), one-way analysis of variance (ANOVA), and the Duncan’s multiple comparison tests at a significance level of $p < 0.05$.

RESULTS
DREB1s genes in walnut
We found 197 protein sequences containing the AP2 domain from the whole genome database of walnut. A total of 192 protein sequences were identified as being a member of the AP2 family after excluding incomplete and repetitive sequences, with 61 protein sequences of the DREB subfamily (Fig. S1, Tables S2 and S3). Multiple sequence alignment results showed that the DREB subfamily in walnut was divided into six subgroups (A1, A2, A3, A4, A5 and A6). DREB1 belonged to the A1 subgroup with five genes (WALNUT_00028779, WALNUT_00022069, WALNUT_00024569, WALNUT_00004985 and WALNUT_00004984) (Fig. 1, Table S3). These five genes were homologous to DREB1A, DREB1B, DREB1C, DREB1Da and DREB1Db in Arabidopsis, respectively (Table 1).

Expression pattern of DREB1s genes
Tissue-specific expression patterns of the five DREB1 genes were analyzed using Xinjiang wild walnut seedlings. As shown in Fig. 2, DREB1A and DREB1B were highly expressed in
Figure 1  Phylogenetic tree of DREB transcription factor families in walnut and *Arabidopsis thaliana*. Full-length amino acid sequences were aligned using the Clustal W software package. The phylogenetic tree was constructed using the MEGA 7.0 software package (https://www.megasoftware.net/). The phylogenetic trees were derived using the neighbor-joining (NJ) method with bootstrap value of 1,000 replicates. The tree divided the *DREB1* into six subgroups (A1-A6) represented by different colored clades. The *DREB1* genes are annotated with red font in walnut and *Arabidopsis*.

Table 1  Homologous sequences of five *DREB1* genes related *AtDREB1* in walnut.

| NO | Walnut gene | Arabidopsis thaliana | Identity | E-value | Score |
|----|-------------|----------------------|----------|---------|-------|
| 1  | WALNUT_00028779 | DREB1A | AT4G25480 | 55.30 | 4e−62 | 192 |
| 2  | WALNUT_00022069 | DREB1B | AT4G25490 | 55.61 | 1e−63 | 198 |
| 3  | WALNUT_00024569 | DREB1C | AT4G25470 | 52.69 | 5e−51 | 164 |
| 4  | WALNUT_00004985 | DREB1D | AT5G51990 | 39.29 | 2e−41 | 444 |
| 5  | WALNUT_00004984 | DREB1D | AT5G51990 | 38.68 | 7e−44 | 148 |
Figure 2  Expression levels of DREB1 genes in different tissues of Xinjiang wild walnut. (A–C) The expression levels of five DREB1 genes in the leaf, stem, root of Xinjiang wild walnut seedlings, respectively. Five DREB1 genes from the walnut genome sequence (PRJNA291087) were identified through bioinformatics methods. The roots, stems and leaves were sampled for expression analysis after the seedlings of the Xinjiang wild walnut grew to five compound leaves. The error bars indicate the SDs from three biological replicates. The different letters above the bars indicate significant differences at the $P < 0.05$ level according to Duncan’s multiple comparison tests.

the root, stem and leaf, among which DREB1A was higher, while the expression levels of DREB1C and DREB1Db were low in these tissues. The expression levels of DREB1Da were low in the root, but high in both the stem and the leaf.

The expression patterns of DREB1s genes in the seedlings of Xinjiang wild walnut were determined under low-temperature stress (4 °C) to select for the cold responsive member. The results showed that the expression level of the DREB1A gene was up-regulated rapidly 1 h after the low-temperature stress, increased to the highest level at 8 h, then decreased (Fig. 3). While the expression levels of the DREB1B, DREB1Da and DREB1Db genes increased slightly, and fewer folds. The expression levels of the DREB1C gene remained lower during this process with no obvious changes (Fig. 3).

Obtaining the *JfDREB1A* gene from Xinjiang wild walnut

Analysis of the expression levels of the DREB1s genes showed that the DREB1A gene was significantly higher than other members in the different tissues and was immediately un-regulated under low-temperatures. It can be regarded as a key DREB1 gene in response to low-temperature stress in walnut. This gene was cloned from the Xinjiang wild walnut and named *JfDREB1A*, with a coding region for 645 bp (Fig. S2, Table S4), showing the structural characteristics of the DREB1/CBF transcription factor (Fig. S3).
Overexpression of the *JfDREB1A* gene enhanced tolerance of transgenic *Arabidopsis* under low-temperature stress

An overexpression vector was constructed to transform the *JfDREB1A* gene into *Arabidopsis*. The overexpressed T3 generation transgenic homozygous strains were obtained (Fig. S4).

The transgenic and wild-type *Arabidopsis* seedlings were treated at −7 °C. The results showed that there were no significant differences in the phenotype between transgenic and wild-type *Arabidopsis* seedlings under normal growth conditions (23 °C) (Figs. 4B and 4D). After being exposed to the low-temperature stress at −7 °C for 5 h and a recovery period of 4 d, most of the leaves of petri dish seedlings were still green. The survival rates of transgenic and wild-type seedlings were 100% and 92%, respectively. Transgenic seedlings still maintained a high survival rate (85%) when the chilling stress extended to 8 h, while the leaves of wild-type seedlings were severely damaged by freezing with a survival rate of only 5% (Figs. 4A and 4B).

The potted seedlings showed similar results. The survival rate of transgenic seedlings was 91%, while wild-type seedlings was 72% after the low-temperature stress at −7 °C for 9 h (Figs. 4C and 4D). These results indicated that the overexpression of *JfDREB1A* improved the cold resistance of transgenic *Arabidopsis* seedlings.
Overexpression of the JfDREB1A gene caused the physiological change in transgenic Arabidopsis

The relative electrical conductivity, SOD activity, POD activity, and CAT activity of transgenic and wild-type Arabidopsis seedlings were measured at 4 °C to analyze the effects of JfDREB1A gene overexpression on the physiological activities of plant cells. The results showed that the relative electrical conductivities gradually decreased and was lowest at 24 h and that transgenic Arabidopsis were always lower than the wild-type (Fig. 5A). Under low-temperature stress, the SOD activities, POD activities, and CAT activities of transgenic and wild-type Arabidopsis gradually increased, ascending most significantly at 8 h and reaching the highest levels at 24 h. The antioxidative enzyme activities of transgenic Arabidopsis were always higher than wild-type Arabidopsis under low-temperature stress (Figs. 5B–5D).

Overexpression of the JfDREB1A gene increased the transcription level of cold-inducible genes

To further evaluate the involvement of the JfDREB1A gene to the cold stress response, we analyzed the expression levels of six cold-inducible genes which are related to low-temperature stress in Arabidopsis. The results showed that the expression levels of all genes were up-regulated except for ATRAB18. The AtKIN1, AtERD10, AtRD29A, AtCOR15A and AtCOR47 genes were significantly higher than the control (0 h). The expression levels...
Figure 5  Changes in physiological parameters of transgenic and WT Arabidopsis under low temperature stress. (A–D) Relative electric conductivity, SOD, POD, and CAT activities were measured in transgenic and WT Arabidopsis seedlings under low temperature stress. WT, Wild type Arabidopsis; JfDREB1A, Transgenic Arabidopsis lines. The error bars indicate the SDs from three biological replicates. The asterisks above the bars indicate significant differences at the $P < 0.05$ level according to Duncan’s multiple comparison tests.

DISCUSSION

Transcription factors regulate the expression of specific plant genes in response to abiotic stress such as low-temperature. DREB1/CFB can recognize CRT/DRE elements of the functional genes promoter region and plays an important role in the plant’s response to abiotic stress (Cook et al., 2004; Gilmour et al., 1998). The function of the DREB1 gene in the ICE1 (inducer of CBF expression 1)-CBF (C-repeat (CRT)-binding factor)-COR (cold responsive) cold signal pathway of plant had been reported extensively (Ding, Shi & Yang, 2019; Gilmour et al., 1998; Siddiqua & Nassuth, 2011; Stockinger, Gilmour & Thomashow, 1997). However, there are no reports on the function of DREB1 transcription factors in walnut, especially in the Xinjiang wild walnut.

In this study, the DREB members of walnut and Arabidopsis were mapped to draw a phylogenetic tree, and five DREB1 transcription factors of walnut were identified. These
Figure 6  Expression levels of cold-inducible genes in transgenic and WT *Arabidopsis* under low temperature stress. (A) AtKIN1 (NM_121601.2) gene, (B) AtERD10 (NM_180616) gene, (C) AtRAB18 (U75603.1) gene, (D) AtRD29A (D13044.1) gene, (E) AtCOR15A (NC_003071.7), (F) AtCOR47 (NM_101894.4). The potted seedlings of transgenic and WT *Arabidopsis* were treated at 4°C for 0 h (control), 8 h. The leaves were used for expression analysis, and the AtUBQ3 gene was used for internal reference. WT, Wild type *Arabidopsis*; JfDREB1A, Transgenic *Arabidopsis* lines. The error bars indicate the SDs from three biological replicates. The asterisks above the bars indicate significant differences at the \( P < 0.05 \) level according to Duncan’s multiple comparison tests.

Figure 6—full-size DOI: 10.7717/peerj.14021/fig-6

were named DREB1A, DREB1B, DREB1C, DREB1Da, and DREB1Db after being aligned with *Arabidopsis*. The expression levels showed that there were high levels of DREB1A in the root, stem, leaf. It was immediately up-regulated under low-temperature stress, and was found at significantly higher levels than the other four DREB1 homologous genes. DREB1A can be regarded as a key gene involved in the regulation process of walnut to low-temperature stress.

Overexpression is one of the most common methods used to study gene function (Prelich, 2012). Previous studies have shown that the overexpression of the DREB1A gene can activate the expression of cold-inducible genes and promote the cold tolerance of transgenic plants (Gilmour et al., 2000; Hong et al., 2015; Maruyama et al., 2004; Oh et al., 2005; Shah et al., 2014). This study showed that the heterologous overexpression of the JfDREB1A gene improved the tolerance and increased the survival rates of transgenic *Arabidopsis* seedlings under low-temperature stress (Fig. 7).

A series of physiological responses occurred in plants under low-temperature stress. The cell membrane perceived the low-temperature stimulation, which led to the destruction of the cell structure and the loss of corresponding metabolic function (Gautier et al., 2013; Lyons, 1973). The stability of the plant cell membrane system is an important factor to consider when assessing the cold tolerance of plants (Muramatsu et al., 2015). The degree of damage to the cell membrane structure and function can be measured by its
Cold stress model of transgenic seedlings regulated by overexpression of JfDREB1A gene. Solid lines indicate positive regulation, and dotted line indicate negative regulation. The overexpression of the JfDREB1A gene enhanced the survival rates of transgenic seedlings, decreased the relative electrical conductivities and increased SOD activities, POD activities, and CAT activities to maintain the stability of cell membrane, also positive regulated the expression of cold-induced genes. These results indicated that the expression of JfDREB1A gene could enhance the cold resistance of Arabidopsis.
electrical conductivity (Liu et al., 2017). In this study, the relative electrical conductivities of transgenic seedlings were gradually decreased and were always lower than that of wild-type Arabidopsis under low-temperature stress (Fig. 7). The SOD activities, POD activities, and CAT activities of transgenic seedlings gradually increased and were always higher than wild-type Arabidopsis for 0–24 h at 4 °C (Fig. 7). Antioxidant enzymes are important in plants, and may eliminate redundant reactive oxygen species (ROS) to maintain the stability of the membrane system when their activity increases (Green & Fluhr, 1995). Therefore, the overexpression of JfDREB1A in transgenic Arabidopsis can reduce the contents of osmotic regulatory substances and improve the activities of antioxidant enzymes, thereby regulating the tolerance of plants to low-temperature stress.

The DREB1 protein binds to the CRT/DRE elements of the target cryogenic response genes to induce the expression levels of genes which respond to low-temperature (Shi, Ding & Yang, 2018). At present, CRT/DRE elements have been found in the cold stress-inducible genes AtKIN1, AtERD10, AtRAB18, AtRD29A, AtCOR15A, and AtCOR47, which are regulated by the DREB1 protein in Arabidopsis (Aleyanova et al., 2020; Ding, Shi & Yang, 2019; Stockinger, Gilmour & Thomashow, 1997). We determined the expression of the above genes to further understand the activation of the JfDREB1A gene to downstream functional genes in transgenic Arabidopsis. The expression levels of AtKIN1, AtERD10, AtRD29A, AtCOR15A and AtCOR47 were significantly increased under low-temperature stress (Fig. 7). These may be the direct target genes of JfDREB1A, however, further experiments are needed for verification.

CONCLUSIONS

In this work, five DREB1 genes were identified in the walnut. Among them, the relative expression levels of the DREB1A gene were high in the root, stem, and leaf and were immediately up-regulated under low-temperature stress. Its levels were significantly higher than the other four DREB1 homologous genes. The JfDREB1A gene of Xinjiang wild walnut was cloned. The overexpression of the JfDREB1A gene in Arabidopsis was shown to enhance cold resistance in transgenic seedlings and improve the response to low-temperature stress, as well as positively regulate the expression of cold-induced genes. These results indicated that the expression of the JfDREB1A gene may enhance the cold resistance of Arabidopsis, which may play an important role in the response to cold stress of the Xinjiang wild walnut.

ACKNOWLEDGEMENTS

We would like to thank the Xinjiang wild walnut Nature Reserve for providing the seeds.
ADDITIONAL INFORMATION AND DECLARATIONS

Funding
This work was supported by the Project of Renovation Capacity Building for the Young Sci-Tech Talents Sponsored by Xinjiang Academy of Agricultural Sciences (No. xjnkq-2019002) and the National Natural Science Foundation of China (No. 32060665). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures
The following grant information was disclosed by the authors:
Project of Renovation Capacity Building for the Young Sci-Tech Talents Sponsored by Xinjiang Academy of Agricultural Sciences: xjnkq-2019002.
National Natural Science Foundation of China: 32060665.

Competing Interests
The authors declare there are no competing interests.

Author Contributions
• Liqun Han conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
• Kai Ma performed the experiments, prepared figures and/or tables, and approved the final draft.
• Yu Zhao performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
• Chuang Mei analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
• Aisajan Mamat analyzed the data, prepared figures and/or tables, and approved the final draft.
• Jixun Wang conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.
• Ling Qin conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.
• Tianming He conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.

Data Availability
The following information was supplied regarding data availability:
The raw data is available in the Supplemental Files.

Supplemental Information
Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.14021#supplemental-information.
REFERENCES

Agarwal PK, Agarwal P, Reddy MK, Sopory SK. 2006. Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Reports* **25**:1263–1274 DOI 10.1007/s00299-006-0204-8.

Aleynova OA, Kiselev KV, Ogneva ZV, Dubrovina AS. 2020. The grapevine calmodulin-like protein gene CML21 is regulated by alternative splicing and involved in abiotic stress response. *International Journal of Molecular Sciences* **21**(21):7939 DOI 10.3390/ijms21217939.

Anwar A, Bai LQ, Miao L, Liu YM, Li SZ, Yu XC, Li YS. 2018. 24-Epibrassinolide ameliorates endogenous hormone levels to enhance low-temperature stress tolerance in cucumber seedlings. *International Journal of Molecular Sciences* **19**(9):2497 DOI 10.3390/ijms19092497.

Colinet H, Renault D, Roussel D. 2017. Cold acclimation allows *Drosophila* flies to maintain mitochondrial functioning under cold stress. *Insect Molecular Biology and Biochemistry* **80**:52–60 DOI 10.1016/j.ibmb.2016.11.007.

Cook D, Fowler S, Fiehn O, Thomashow MF. 2004. A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **101**:15243–15248 DOI 10.1073/pnas.0406069101.

Ding YL, Shi YT, Yang SH. 2019. Advances and challenges in uncovering cold tolerance regulatory mechanisms in plants. *New Phytologist* **222**:1690–1704 DOI 10.1111/nph.15696.

Dong YZ, Zhu XH, Chen H, Liang FH, Wang ZY. 2012. Investigation and analysis on the wild walnut in Gongliu, Xinjiang. *Journal of Plant Genetic Resources* **13**:386–392 DOI 10.3389/fpls.2016.01866.

Fowler S, Thomashow MF. 2002. *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *The Plant Cell* **14**:1675–1690 DOI 10.1105/tpc.003483.

Gautier J, Passot S, Penicaud C, Guillemin H, Cenard S, Lieben P, Fonseca F. 2013. A low membrane lipid phase transition temperature is associated with a high cryotolerance of *Lactobacillus delbrueckii* subspecies *bulgaricus* CFL1. *Journal of Dairy Science* **96**:5591–5602 DOI 10.3168/jds.2013-6802.

Gilmour SJ, Fowler SG, Thomashow MF. 2004. *Arabidopsis* transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Molecular Biology* **54**:767–781 DOI 10.1023/B:PLAN.0000040902.06881.d4.

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF. 2000. Overexpression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiology* **124**:1854–1865 DOI 10.1104/pp.124.4.1854.
Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF. 1998. Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold induced COR gene expression. The Plant Journal 16:433–442 DOI 10.1046/j.1365-313x.1998.00310.x.

Gong ZZ, Xiong LM, Shi HZ, Yang SH, Herrera-Estrella LR, Xu GH, Chao DY, Li JR, Wang PY, Qin F, Li JJ, Ding YL, Shi YH, Wang Y, Yang YQ, Guo Y, Zhu JK. 2020. Plant abiotic stress response and nutrient use efficiency. Science China Life Sciences 63:635–674 DOI 10.1007/s11427-020-1683-x.

Green R, Fluhr R. 1995. UV-B-induced PR-1 accumulation is mediated by active oxygen species. The Plant Cell 7:203–212 DOI 10.2307/3869996.

Haake V, Cook D, Riechmann JL, Pineda O, Thomashow MF, Zhang JZ. 2002. Transcription factor CBF4 is a regulator of drought adaptation in Arabidopsis. Plant Physiology 130:639–648 DOI 10.1104/pp.006478.

Han LQ, Ma K, Ding JW, Yan P, Mei C, Wang JX. 2019. Physiological response and evaluation of cold resistance of Xinjiang wild walnut under low temperature stress. Journal of Northwest Forestry University 34:98–101 DOI 10.3389/fpls.2016.01866.

Hong B, Tong Z, Ma N, Kasuga M, Yamaguchi-Shinozaki K, Gao JP. 2015. Expression of the Arabidopsis DREB1A gene in transgenic chrysanthemum enhances tolerance to low temperature. The Journal of Horticultural Science and Biotechnology 81:1002–1008 DOI 10.1080/14620316.2006.11512162.

Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. 2006. Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant and Cell Physiology 47:141–153 DOI 10.1093/pcp/pci230.

Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF. 2001. Components of the Arabidopsis C-Repeat/Dehydration-Responsive element binding factor cold-response pathway are conserved in Brassica napus and other plant species. Plant Physiology 127:910–917 DOI 10.1104/pp.010548.

Kim S-I, Tai TH. 2011. Evaluation of seedling cold tolerance in rice cultivars: a comparison of visual ratings and quantitative indicators of physiological changes. Euphytica 178:437–447 DOI 10.1007/s10681-010-0343-4.

Liu R, Liu QG, Fan JX, Gong DY, Wu XB, Han SQ, Lei CY. 2017. Research advances in the cold-resistance physiology biochemistry and molecular mechanism in fruit crops. Molecular Plant Breeding 15:2028–2034 DOI 10.13271/j.mpb.015.002028.

Lyons JM. 1973. Chilling injury in plants. Annual Review of Plant Physiology 24:445–466 DOI 10.1146/annurev.pp.24.060173.002305.

Maestrini P, Cavallini A, Rizzo M, Giordani T, Bernardi R, Durante M, Natali L. 2009. Isolation and expression analysis of low temperature-induced genes in white poplar (Populus alba). Journal of Plant Physiology 166:1544–1556 DOI 10.1016/j.jplph.2009.03.014.
Magome H, Yamaguchi S, Hanada A. 2004. Dwarf and delayed-flowering 1, a novel Arabidopsis, mutant deficient in gibberellin biosynthesis because of overexpression of a putative AP2 transcription factor. *The Plant Journal* **37**:720–729 DOI 10.1111/j.1365-313X.2003.01998.x.

Maruyama K, Sakuma Y, Kasuga M, Ito Y, Seki M, Goda H, Shimada Y, Yoshida S, Shinozaki K, Yamaguchi-Shinozaki K. 2004. Identification of cold-inducible downstream genes of the Arabidopsis DREB1A/CBF3 transcriptional factor using two microarray systems. *The Plant Journal* **38**:982–993 DOI 10.1111/j.1365-313X.2004.02100.x.

McCully ME, Canny MJ, Huang CX. 2004. The management of extracellular ice by petioles of frost-resistant herbaceous plants. *Annals of Botany* **94**:665–674 DOI 10.1093/aob/mch191.

Muramatsu M, Harada Y, Suzuki T, Niino H. 2015. Relationship between transition of fracture mode of carbon fiber-reinforced plastic and glass transition temperature of its resin. *Advanced Composite Materials* **25**:143–158 DOI 10.1080/09243046.2014.986844.

Nakano T, Suzuki K, Fujimura T, Shinshi H. 2006. Genome-wide analysis of the ERF gene family in Arabidopsis and rice. *Plant Physiology* **140**:411–432 DOI 10.1104/pp.105.073783.

Ni ZY, Hu Z, Jiang QY, Zhang H. 2013. GmNFYA3, a target gene of miR169, is a positive regulator of plant tolerance to drought stress. *Plant Molecular Biology* **82**:113–129 DOI 10.1007/s11103-013-0040-5.

Oh SJ, Song SI, Kim YS, Jang HJ, Kim SY, Kim M, Kim YK, Nahm BH, Kim JK. 2005. Arabidopsis CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. *Plant Physiology* **138**:341–351 DOI 10.1104/pp.104.059147.

Park S, Lee CM, Doherty CJ, Gilmour SJ, Kim Y, Thomashow MF. 2015. Regulation of the Arabidopsis CBF regulon by a complex low-temperature regulatory network. *The Plant Journal* **82**:193–207 DOI 10.1111/tpj.12796.

Prelich G. 2012. Gene overexpression: uses, mechanisms, and interpretation. *Genetics* **190**:841–854 DOI 10.1534/genetics.111.136911.

Ruelland E, Vaultier M-N, Zachowski A, Hurry V. 2009. Cold signalling and cold acclimation in plants. *Advances in Botanical Research* **49**:35–150 DOI 10.1016/s0065-2296(08)00602-2.

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K. 2002. DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochemical and Biophysical Research Communications* **290**:998–1009 DOI 10.1006/bbrc.2001.6299.

Shah SH, Ali S, Jan SA, Jalal Ud D, Ali GM. 2014. Piercing and incubation method of in planta transformation producing stable transgenic plants by overexpressing DREB1A gene in tomato (*Solanum lycopersicum* Mill.). *Plant Cell Tissue and Organ Culture* **120**:1139–1157 DOI 10.1007/s11240-014-0670-6.
Shi YT, Ding YL, Yang SH. 2018. Molecular regulation of CBF signaling in cold acclimation. *Trends in Plant Science* 23:623–637 DOI 10.1016/j.tplants.2018.04.002.

Siddiqua M, Nassuth A. 2011. *Vitis CBF1* and *Vitis CBF4* differ in their effect on *Arabidopsis* abiotic stress tolerance, development and gene expression. *Plant, Cell and Environment* 34:1345–1359 DOI 10.1111/j.1365-3040.2011.02334.x.

Stockinger EJ, Gilmour SJ, Thomashow MF. 1997. *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proceedings of the National Academy of Sciences of the United States of America* 94:1035–1040 DOI 10.1073/pnas.94.3.1035.

Thomashow MF. 2010. Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. *Plant Physiology* 154:571–577 DOI 10.1104/pp.110.161794.

Velikova V, Yordanov I, Edreva A. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants Protective role of exogenous polyamines. *Plant Science* 151:59–66 DOI 10.1016/S0168-9452(99)00197-1.

Wu D, Mao DH, Zhao XY. 2021. Advances in molecular mechanism of plant response to low temperature. *Life Science Research* 26:67–75 DOI 10.16605/j.cnki.1007-7847.2020.08.0239.

Xiao HG, Siddiqua M, Braybrook S, Nassuth A. 2006. Three grape CBF/DREB1 genes respond to low temperature, drought and abscisic acid. *Plant, Cell and Environment* 29:1410–1421 DOI 10.1111/j.1365-3040.2006.01524.x.

Xie ZL, Nolan TM, Jiang H, Yin YH. 2019. AP2/ERF transcription factor regulatory networks in hormone and abiotic stress responses in *Arabidopsis*. *Frontiers in Plant Science* 10:228 DOI 10.3389/fpls.2019.00228.

Zhang X, Fowler SG, Cheng HM, Lou YG, Rhee SY, Stockinger EJ, Thomashow MF. 2004. Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant *Arabidopsis*. *The Plant Journal* 39:905–919 DOI 10.1111/j.1365-313X.2004.02176.x.

Zhao CZ, Zhang ZJ, Xie SJ, Si T, Li YY, Zhu JK. 2016. Mutational evidence for the critical role of CBF transcription factors in cold acclimation in *Arabidopsis*. *Plant Physiology* 171:2744–2759 DOI 10.1104/pp.16.00533.

Zhu JK. 2016. Abiotic stress signaling and responses in plants. *Cell* 167:313–324 DOI 10.1016/j.cell.2016.08.029.