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Research article

DlICE1, a stress-responsive gene from *Dimocarpus longan*, enhances cold tolerance in transgenic *Arabidopsis*

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ABSTRACT

ICE1 (*inducer of CBF expression 1*) encodes a typical MYC-like basic helix-loop- helix (bHLH) transcription factor that acts as a pivotal component in the cold signalling pathway. In this study, *DlICE1*, a novel ICE1-like gene, was isolated from the southern subtropical fruit tree longan (*Dimocarpus longan* Lour.). *DlICE1* encodes a nuclear protein with a highly conserved bHLH domain. *DlICE1* expression was slightly upregulated under cold stress. Overexpression of *DlICE1* in *Arabidopsis* conferred enhanced cold tolerance via increased proline content, decreased ion leakage, and reduced malondialdehyde (MDA) and reactive oxygen species (ROS) accumulation. Expression of the ICE1-CBF cold signalling pathway genes, including *AtCBF1/2/3* and cold-responsive genes (*AtRD29A, AtCOR15A, AtCOR47* and *AtKIN1*), was also significantly higher in *DlICE1*-overexpressing lines than in wild-type (WT) plants under cold stress. In conclusion, these findings indicate that *DlICE1* is a member of the bHLH gene family and positively regulates cold tolerance in *D. longan.*

1. Introduction

Cold stress is one of the most adverse environmental stressors, not only influencing plant growth and development by significantly reducing crop productivity but also restricting the geographical distribution of many important crops (Pearce, 2001). Longan (*Dimocarpus longan* Lour.), originating from South China or Southeast Asia, is the most important evergreen fruit tree belonging to the family Sapindaceae. As a delicious drupe fruit and an important source of traditional Chinese medicines, longan is widely cultivated in South Asia and Southeast Asia. (Mei et al., 2014; Lin et al., 2017). However, longan is sensitive to low temperatures. In the subtropics, unpredictable cold extremes and abnormal weather occur occasionally, and cold stress and freezing conditions not only affect fruit productivity and quality but also threaten the survival of longan trees, causing great economic loss in southern China. Furthermore, the mature stage of longan is very short in southern China, and fresh fruit is vulnerable to storage at low temperatures. To prolong the mature stage and sale duration of longan, it is important to extend the planting area to high-latitude and high-altitude regions. Therefore, there is an urgent need to develop cold-tolerant longan cultivars. Several studies have reported the physiological, biochemical and molecular changes associated with the response of longan to cold stress (Chen et al., 2012; Liu et al., 2014). However, due to the high degree of genetic heterozygosity, long juvenile stage and limited understanding of the molecular mechanisms underlying the response of longan to low temperatures, little progress has been made in the breeding of cold-tolerant longan.

Accumulating evidence have revealed that plants have evolved a series of precise physiological, biochemical and molecular mechanisms to adapt to cold environments (Thomashow, 1999; Kaplan et al., 2007). Among these pathways, the C-repeat binding factor (CBF)-dependent pathway is the most important cold signalling pathway in plants. The expression of CBF genes is rapidly induced by cold stress, and the CBFs can then bind to the conserved C-repeat/dehydration response element (CRT/DRE) in the promoters of cold-regulated (COR) genes to activate COR gene expression and enhance cold tolerance in plants (Stockinger et al., 1997; Liu et al., 1998). *ICE1* encodes a typical MYC-like basic helix-loop-helix (bHLH) transcription activator and was the first identified upstream *CBF* regulator (Chinnusamy et al., 2003). ICE1 protein can specifically bind to the MYC cis-element (CANNTG) in the promoter of the *CBF1-3* gene and induce gene expression at low temperatures (Chinnusamy et al., 2003; Ding et al., 2015). ICE1 is a positive regulator

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in the cold signalling pathway. Overexpression of *ICE1* can significantly enhance cold stress tolerance in Arabidopsis. In contrast, in the *ice1* mutant, the expression of *CBFs* and downstream COR genes is significantly inhibited at low temperatures, significantly reducing plant resistance to chilling and freezing stress (Chinnusamy et al., 2003; Lee et al., 2005). *ICE2*, a paralog gene of *ICE1*, is also a transcriptional activator that positively regulates *CBF* expression and cold tolerance in plants (Fursova et al., 2009).

The function of the *ICE1* homologues in the regulation cold response is conserved in many plant species, such as wheat (Badawi et al., 2008), canola (*Brassica napus*) (Zhang et al., 2018), tomato (Feng et al., 2013), banana (Shan et al., 2014), apple (Feng et al., 2012), pear (*Pyrus ussuriensis*) (Huang et al., 2015a) and citrus (Huang et al., 2015b) . Moreover, heterologous expression of the maize, grape (*Vitis amurensis*), rice or rubber (*Hevea brasiliensis*) *ICE1* orthologues in Arabidopsis significantly enhanced the cold tolerance of the plants (Xu et al., 2014; Deng et al., 2017; Lu et al., 2017; Yuan et al., 2017). These studies reveal the conserved function of *ICE1* in the regulation of cold tolerance in plants.

ICE1 is constitutively expressed in roots, leaves and other tissues tested (Chinnusamy et al., 2003), but the expression of this gene is not induced by cold stress in Arabidopsis (Chinnusamy et al., 2003; Miura et al., 2007). Therefore, the functions of the ICE1 protein are mainly regulated by post-translational modifications (PTMs). PTMs participate in the process of cold response by modulating the activity and stability of ICE1. It is widely reported that PTMs in the ICE1 protein mainly include ubiquitination, sumoylation and phosphorylation. HOS1 (high osmotic expression 1), a ubiquitin E3 ligase, directly interacts with and ubiquitinates the ICE1 protein, leading to degradation of the protein via the 26S proteasome pathway (Dong et al., 2006). SIZ1 (SAP and Miz1), a SUMO E3 ligase, was reported to sumoylate the ICE1 protein at Lys393 and simultaneously attenuate the ubiquitination of the protein by HOS1, thereby enhancing the stability of the ICE1 protein (Miura et al., 2007). OST1 (open stomata 1) is a pivotal kinase in the ABA signalling pathway that phosphorylates ICE1 at Ser278 to promote the stability and transcriptional activity of the protein, causing activation of *CBFs* and downstream COR gene expression (Ding et al., 2015). Recent studies revealed that the protein kinases MPK3 and MPK6 phosphorylate ICE1 at Ser94, Thr366 and Ser403, promoting ICE1 protein degradation, thereby negatively regulating CBF gene expression and freezing tolerance in plants (Li et al., 2017; Zhao et al., 2017). A recent study in rice indicated that OsMAPK3 phosphorylates and stabilizes OsICE1, which directly regulates *OsTPP1* expression, thereby promoting trehalose synthesis and positively regulating cold tolerance (Zhang et al., 2017). These findings indicate that PTMs in the ICE1 protein are distinct in different plant species; however, ICE1 is also the central component in the cold response signalling pathway.

To date, the molecular mechanisms and the main components of the cold signalling pathway involved in the response of longan to cold stress remain poorly understood. In this study, the *D. longan ICE1* (*DlICE1*) gene was identified and characterized for its function in the response to cold stress. Overexpression of *DlICE1* in Arabidopsis enhanced cold tolerance by regulating physiological parameters and cold response genes.

2. Materials and methods

2.1. Plant materials and cold treatments

Trees of the major longan (*D. longan* Lour.) cultivar ‘shixia’ were cultivated in the South China Agricultural University Germplasm Resource Nursery, Guangzhou, China. Healthy and uniform mature autumn branches with even pinnately compound leaves were selected and incubated in 4 °C growth chambers for cold treatment. The leaves were collected 0, 1, 3, 6, 12 and 24 h after cold treatment. Branches treated under normal conditions (25 °C) were used as controls. For examination of tissue-specific expression of *DlICE1*, samples of apical buds, young annual stems, young red leaves, mature autumn leaves and petioles were collected in winter from 5-year-old mature trees cultivated in an orchard. All the samples were frozen immediately in liquid nitrogen and stored at −80 °C until further use.

2.2. DlICE1 gene cloning and sequence analysis

Total RNA from all the samples was extracted using the RNAprep Pure Plant Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. RNase-free DNase I was employed to remove DNA contamination. Approximately 800 ng of total RNA per sample was used to synthesize first-strand cDNA using the M-MLV RTase cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer's instructions. The *AtICE1*(AAP14668.1) protein sequence was used as bait to identify potential ICE1-like genes in longan genome database (Lin et al., 2017) and obtained by BLAST analysis in TBtools (Chen et al., 2018). The matching sequence with the lowest E-value was selected as a putative longan ICE1-like gene. The longan ICE1-like gene was amplified using gene-specific primers (Table S1). The cDNA from a sample treated at 4 °C for 6 h was used as a template with KOD-PLUS-NEO *Taq* polymerase (TOYOBO, Japan) following the manufacturer's instructions. The PCR products were sub-cloned into the pMD19-T cloning vector (TaKaRa, Japan) for sequencing.

The amino acid sequence of DlICE1 was determined by the ExPASy Translate tool (https://web.expasy.org/translate/). The molecular weight (Mw) and theoretical isoelectric point (*pI*) were calculated by the online ProtParam tool (http://www.expasy.org/tools/protparam.html). A similar analysis was performed by using NCBI BLAST software (http://www.ncbi.nlm.nih.gov/) (Wang et al., 2018). Multiple sequence alignment was conducted using ClustalX software. A phylogenetic tree was constructed using the neighbour-joining (NJ) method with MEGA 7.0 software, and bootstrap values with 1,000 replications were employed (Kumar et al., 2016).

2.3. Expression analysis of DlICE1 under cold stress

Real-time quantitative PCR (RT-qPCR) was conducted to evaluate the expression levels of *DlICE1* under cold stress. The primer used for RT-qPCR analysis of *DlICE1* were listed in Table S1. Amplification was performed in an ABI 7500 real-time PCR system (Life Technologies Corporation, Beverly, MA, USA). The 20-μL PCR contained 10 μL of 2 × SYBR Green Real MasterMix (SYBR Green, Applied Biosystems), 100 ng of cDNA, and 0.25 μM each primer for amplification of *DlICE1*. The variations in relative expression between samples was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and longan *β-actin* was used as the internal control. The primers used were listed in Table S1. Each sample was measured in triplicate.

2.4. Subcellular localization of DlICE1

The *DlICE1* open reading frame (ORF) without a stop codon was amplified using PCR. The products were cloned into *Hin*dIII and *Bam*HI restriction sites of pGreen-35S-GFP vector (Lee et al., 2012) using the In-FusionTM PCR Cloning Kit (Clontech) to produce a fusion construct with green fluorescence protein (GFP). The primer pairs used for vector construction are listed in Table S1. After verification by sequencing, the 35S: DlICE1-GFP fusion vector and negative control 35S:GFP vector were transferred into *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method, and the *A. tumefaciens* cultures were infiltrated into the abaxial surfaces of *Nicotiana benthamiana* leaves. After incubation for 24–48 h at 25 °C. The fluorescence signal of leaf epidermal cells were detected, then leaf protoplasts were isolated and incubated with 0.1 μg ml^{-1} DAPI for 15 min. The fluorescence signals were detected under the fluorescence microscope (Olympus BX53). Excitation and emission filters Ex480 ± 20/DM510/BA535 ± 25 and Ex360-

370/DM410/BA440 ± 20 were used for GFP and DAPI, respectively.

2.5. Transcriptional activation assay of DlICE1

The full-length coding region of *DlICE1* were amplified using PCR. The product was subcloned into the frame of the sequences encoding the GAL4 DNA-binding domain in *Eco*RI/*Bam*HI sites of pGBKT7 vector. The primer pairs used for Y2H assay are listed in Table S1. After verification by sequencing, the constructed plasmids pGBKT7-DlICE1, positive control pGBKT7-p53 and negative control pGBKT7, were independently transformed into yeast strain Y2HGold by the lithium acetate method (Clontech, PT1172-1). The transformed yeast cells were plated for 3 d on selection media SD/-Trp and SD/-Trp-His-Ade, followed by incubated with 20 $\mu g\,ml^{-1}$ X-α-gal for blue color development, to verify the transactivation of DlICE1 according to their growth status and the activity of α-galactosidase.

2.6. Plasmid construction and plant transformation

The coding region of *DlICE1* was amplified using primers containing the *Xba*I restriction site and then sub-cloned into the pPZP6k90 vector with a modified cauliflower mosaic virus (CaMV) 35S promoter to construct the recombination vector pPZP6k90-DlICE1. The primers used were listed in Table S1. After verification of the sequence, the recombinant plasmid was introduced into A. tumefaciens strain GV3101 by the freeze-thaw method. To produce transgenic plants, the A. tumefaciens strains were then transformed into wild-type (WT) Arabidopsis (Columbia ecotype, Col-0) by the floral dip method (Clough and Bent, 1998). Positive plants were screened on MS medium containing 100 $mg\,L^{-1}$ kanamycin and verified by genomic PCR using gene-specific primers of *DlICE1*. In addition, *DlICE1* expression in the homozygous T_3 transgenic lines was assayed by RT-qPCR. The Arabidopsis actin gene (*AtACTIN2*) was used as an internal control (Ding et al., 2015), and the primers used were listed in Table S1. Three homozygous T_3 transgenic lines were used for further stress tolerance experiments and physiological measurements.

2.7. Assessment of the cold tolerance of the transgenic lines

WT and transgenic lines were planted in plastic pots filled with a 3:1 mixture of soil and vermiculite under a normal photoperiod of 16 h light and 8 h dark at 22 °C. For freezing treatment, three-week-old seedlings were treated at −4 °C for 6 h after cold acclimation (48 h at 4 °C), followed by 4 °C in the dark for 12 h. The plants were transferred to normal conditions for recovery for 6 d, and then, the survival rates were determined. Photos were taken before freezing and after recovery. The rosette leaves were collected for analysis of ion leakage, proline content, malondialdehyde (MDA) content and reactive oxygen species (ROS) accumulation. For chilling treatment, the seedlings were placed at 4 °C for 0, 6, 12 and 24 h, and leaves were collected at different timepoints for expression analysis of cold-responsive genes regulated by DlICE1.

2.8. Measurement of proline content, ion leakage, MDA content and ROS accumulation

The proline content was measured by the sulfosalicylic acid-ninhydrin method as previously described (Shi et al., 2012). The ion leakage assay was performed as previously described (Ding et al., 2015). Five leaves of seedlings treated with cold and freezing treatment were placed in 50-mL tubes with 10 mL of ddH_2O_2 (S0) and shaken at 100 rpm for 30 min, and then, the conductivity (S1) was determined. Conductivity (S2) was detected after the samples were boiled at 100 °C water for 15 min and shaken at 22 °C for 1 h. Ion leakage was calculated by applying formula S1-S0/S2-S0 × 100%. MDA is an indicator of the level of membrane lipid peroxidation caused by damage in transgenic plants. MDA concentrations were estimated using the thiobarbituric acid method as described by Guo and Crawford (2005). Histochemical staining with 1 $mg\,ml^{-1}$ 3,3′ -diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) in 10 mM potassium phosphate buffer (pH 7.8) was used to analyses the in situ accumulation of H_2O_2 and $O_2^{\cdot-}$, as described by Shi et al. (2010). Quantitative measurement of H_2O_2 and $O_2^{\cdot-}$ was performed using specific detection kits (Suzhou Comin Biotechnology, China).

2.9. Expression analysis of cold-responsive genes regulated by DlICE1

Expression of ICE1-CBF cold signalling pathway genes such as *AtCBF1/2/3*, *AtRD29A*, *AtCOR15A*, *AtCOR47* and *AtKIN1* was evaluated by RT-qPCR in *DlICE1* transgenic plants, and WT plants were used as controls. *AtACTIN2* was used as a reference gene. The specific primers used for RT-qPCR were listed in Table S1. The variation in relative expression between samples was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Three duplicates of each experiment were conducted. The data represent the mean ± SD. One-way Duncan's multiple comparison tests were performed to determine the significance of the experimental means at $P < 0.05$.

3. Results

3.1. Cloning and bioinformatics analysis of DlICE1

An increasing number of studies have revealed that ICE1-like genes, as key transcription factors, play importent roles in the cold tolerance of different plants. However, to date, the ICE1-like genes have not been identified in *D. longan*. We isolated a putative longan ICE1-like gene (Dlo_005414.1) in longan genome. The full-length cDNA sequence was amplified, and sequence analysis indicated that the ORF was 1602 bp in length, encoding a predicted polypeptide of 533 amino acids (aa), with an estimated molecular weight of 58.43 kDa and a pI of 5.15. The longan ICE1-like protein exhibited 78% similarity to CtICE1 and 49% similarity to AtICE1, therefore nominated as *DlICE1* (Genbank accession No. MN088827). There is another ICE1-like homologous gene (Dlo_023998.1) in longan, named as *DlICE2* (Genbank accession No. MN217565), the DlICE2 protein exhibited 47.4% similarity to DlICE1 (Fig. S1). The cDNA and deduced amino acid sequences of *DlICE1/2* were listed in the supplemental file. In this study, we mainly discuss the function of *DlICE1* in longan response to low temperatures.

Multiple sequence alignments indicated that DlICE1 protein contained a typical MYC-like bHLH domain, a zipper region (bZIP) (aa 342–389 and 390–419), a serine-rich region (S-rich) in the N-terminus (aa 84–100), and an ACTUUT-ACR-like domain in the C-terminus (aa 462–522), all of which are defining characteristics of bHLH TFs. DlICE1 and other plant ICE1-like proteins were highly conserved at the C-terminus but varied at the N-terminus (Fig. 1A). Phylogenetic analysis was carried out using the sequences of DlICE1 and ICE1-like proteins from other plants. ICE1-like proteins are highly conserved in different plant species and are widely present in dicots and monocots. DlICE1 and DlICE2 is most closely related to CtICE1 of *Citrus trifoliata* and VaICE1/2 of *Vitis amurensis*, respectively. All of them belong to the dicotyledonous branch (Fig. 1B). *CtICE1* and *VaICE1/2* has been reported to act as an important transcription factor that plays a role in cold tolerance (Huang et al., 2015b; Xu et al., 2014). These results suggested that *DlICE1/2* might be involved in the cold stress response of longan.

3.2. Tissue-specific and cold-inducible expression of DlICE1

To examine the tissue-specific expression of *DlICE1*, *DlICE1* expression in apical buds, young leaves, mature leaves, young stems and petioles was examined by RT-qPCR. *DlICE1* was expressed in all the tissues tested, with the highest expression observed in young leaves and the lowest expression observed in young stems (Fig. 1C). We also

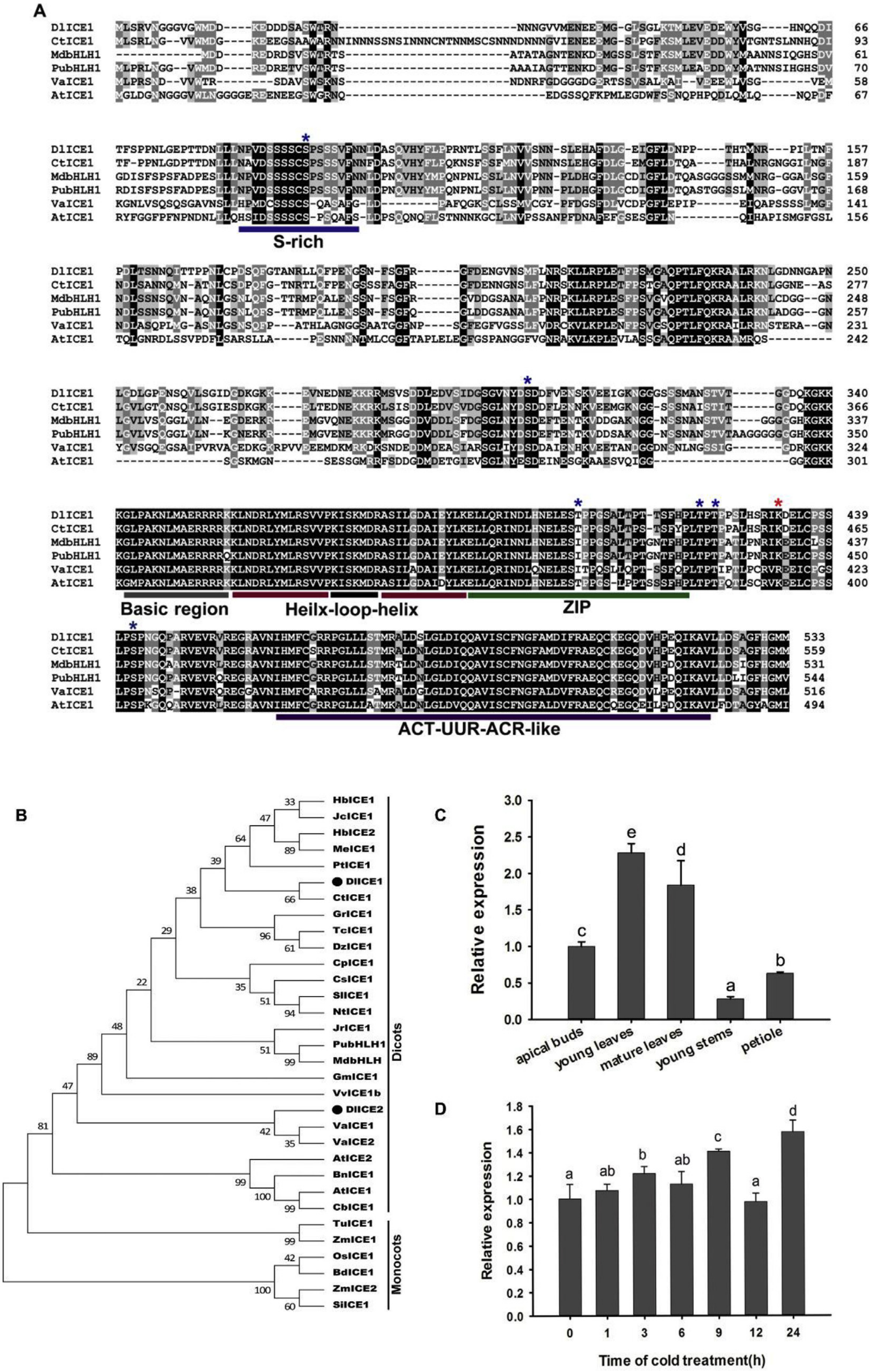

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Fig. 1. Amino acid sequence, phylogenetic and expression analysis of *DlICE1*. (A) Multiple sequence alignment of DlICE1 and ICE1-like proteins from other plant species. The sequences and accession numbers are as follows: CtICE1 [Citrus trifoliata, AIZ78178.1]; MdbHLH1 [Malus domestica, ABS50251.1]; PubHLH1 [Pyrus ussuriensis, AML33329.1]; VaICE1 [Vitis amurensis, AGP04217.1]; AtICE1 [*Arabidopsis thaliana*, AAP14668.1]. Identical and similar amino acid residues are represented by black and grey shadows, respectively. Three predicted conserved regions of ICE-like proteins are labelled: a MYC-like basic helix-loop-helix leucine zipper (bHLH-ZIP), a serine-rich region (S-rich) and an ACT-UUR-ACR-like domain. The sumoylation sites and the phosphorylation sites are indicated by red and blue asterisks, respectively. (B) Phylogenetic analysis of DlICE1/2 [Dimocarpus longan, MN088827/MN217565] and ICE1-like proteins from other plant species. The neighbour-joining method was used to construct the tree. The GenBank accessions of the predicted ICE1-like protein sequences used are as follows: AtICE2 [A. thaliana, NP_172746.2]; BdICE1 [Brachypodium distachyon, XP_003567427.1]; BnICE1 [Brassica napus, XP_013698429.1]; CbICE1 [Capsella bursa-pastoris, AAS79350.1]; CpICE1 [Carica papaya, NP_001306859.1]; CsICE1 [Camellia sinensis, ACT90640.2]; DzICE1 [Durio zibethinus, XP_022730447.1]; GmICE1 [Glycine max, NP_001238560.1]; GrICE1 [Gossypium raimondii, XP_012489049.1]; HbICE1/2 [Hevea brasiliensis, XP_021673395.1/ARK08718.1]; JcICE1 [Jatropha curcas, NP_001306859.1]; JrICE1 [Juglans regia, AHJ79155.1]; MeICE1 [Manihot esculenta, XP_021592727.1]; NtICE1 [Nicotiana tomentosiformis, XP_009625133.1]; OsICE1 [Oryza sativa Japonica Group, BAD88163.1]; PtICE1 [Populus trichocarpa, ABN58427.1]; SiICE1 [Setaria italica, XP_004971151.1]; SlICE1 [Solanum lycopersicum, NP_001274718.1]; TcICE1 [Theobroma cacao, EOY22617.1]; TuICE1 [Triticum urartu, EMS66007.1]; VaICE2 [Vitis. amurensis, AGP04218.1]; VvICE1b [Vitis vinifera, AGQ03811.1]; ZmICE1/2 [*Zea mays*, PWZ26337.1/ACG46593.1]. (C) Differential expression patterns of *DlICE1* in various tissues (apical buds, latex, young leaves, mature leaves, young stems and petiole). (D) Time-course expression patterns of *DlICE1* in response to cold stress. The data represent the means of three biological replicates ± standard deviations (SDs). Different letters (a–d) indicate significant differences by Duncan's multiple comparison tests at $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

examined the time-course expression patterns of *DlICE1* in response to cold stress. The transcription of *DlICE1* was significantly induced at 3 h, followed by a gradual decline from 6 h to 12 h and a distinct increase to the peak value at 24 h after cold treatment (Fig. 1D).

3.3. DlICE1 was localized in the nucleus

Sequence analysis showed that the DlICE1 protein contains a potential nuclear localization signal (NLS), suggesting that this protein is located in the nucleus for transcriptional regulation. To verify this hypothesis, the fusion construct 35S:DlICE1-GFP and the negative control vector 35S:GFP were transiently expressed in the *N. benthamiana* leaf epidermis cells. Fluorescence microscopic imaging showed that the control GFP was uniformly distributed throughout the cell both in the leaf epidermal cells or protoplasts (Fig. 2A; Figs. S1 and S2). However, the 35S: DlICE-GFP was observed exclusively in the nucleus, coincident with DAPI fluorescence signal in protoplasts (Fig. 2A), implying that DlICE1 was localized in the nucleus.

3.4. Transcriptional activation of DlICE1 in yeasts

Transcriptional activity is an important feature of transcription factors. The transcriptional activity of DlICE1 was analyzed using Yeast two-hybrid system. The results showed that all the yeast cells transformed with pGBKT7-DlICE1 and pGBKT7-p53 (positive control) grew well in selection medium SD/-Trp and the SD/-Leu/-Trp/-Ade, and also exhibiting quite strong α-galactosidase activity. However, the yeast cells transformed with the empty constructs pGBKT7(negative control) did not grow on SD/-Leu/-Trp/-Ade medium (Fig. 2B). These results indicate that DlICE1 may potentially act as a transcriptional activator.

3.5. Overexpression of DlICE1 enhanced cold tolerance in Arabidopsis

To investigate the function of *DlICE1* in cold tolerance, we generated transgenic *Arabidopsis thaliana* plants overexpressing *DlICE1*. Three transgenic lines (E1, E2 and E8) showed high expression of *DlICE1* in the eight homozygous lines analysed by RT-qPCR, and these lines were used for further analysis (Fig. 3A). Under normal conditions, no significant difference in morphology was observed between the transgenic lines and WT plants (Fig. 3B). Under freezing treatment (-4 °C for 6 h) after cold acclimation (48 h at 4 °C) and recovery for 6 d in a normal ambient environment, transgenic lines presented less severe injury than the WT (Fig. 3B). The survival rates of the transgenic lines (73.3% for E1, 55% for E2 and 48.3% for E8) were significantly higher than those of the WT (25%) (Fig. 3C). These findings suggested that overexpression of *DlICE1* improved the freezing tolerance of transgenic *Arabidopsis*.

3.6. Overexpression of DlICE1 affects proline content, ion leakage, MDA content and ROS accumulation under cold stress

Physiological indexes such as ion leakage, MDA content, proline content, and ROS accumulation are commonly used to evaluate stress resistance capacity during the response to a variety of abiotic stressors

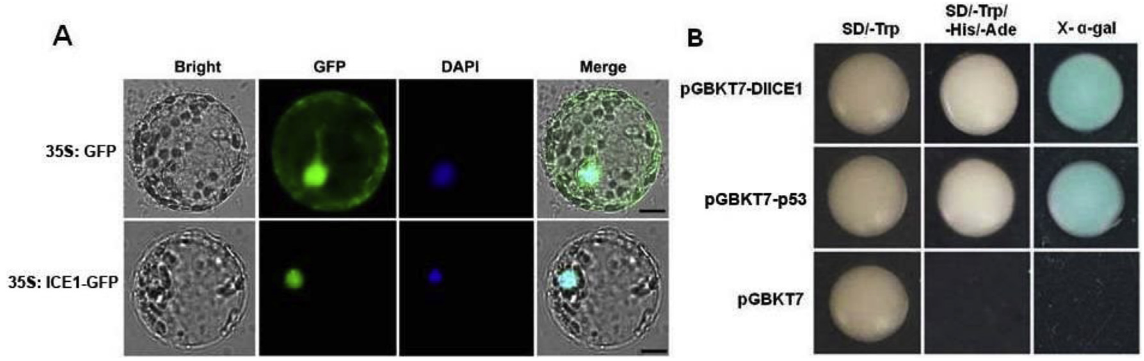

Fig. 2. Subcellular localization and transcriptional activation assay of DlICE1.(A) Subcellular localization of the DlICE1 protein in Nicotiana benthamiana protoplasts. 35S: DlICE1-GFP fusion constructs in Agrobacterium tumefaciens strain GV3101 were transiently transformed into epidermal cells of N. benthamiana leaves. The GFP and DAPI signals were visualized under a fluorescence microscope after 48 h of incubation at 25 °C; 35S: GFP was used as negative control. DAPI was used to stain the nucleus (pseudo-color, blue). Scale bar, 10 μm. (B) Transcriptional activation assay of DlICE1 protein in yeast cells. The yeast strain Y2HGold transformed with fused plasmids pGBKT7-DlICE1, positive control (pGBKT7-p53) and negative control (pGBKT7) cultured on selection medium SD/-Trp, SD/-Trp-His-Ade and SD/-Trp-His-Ade with X-α-gal staining. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

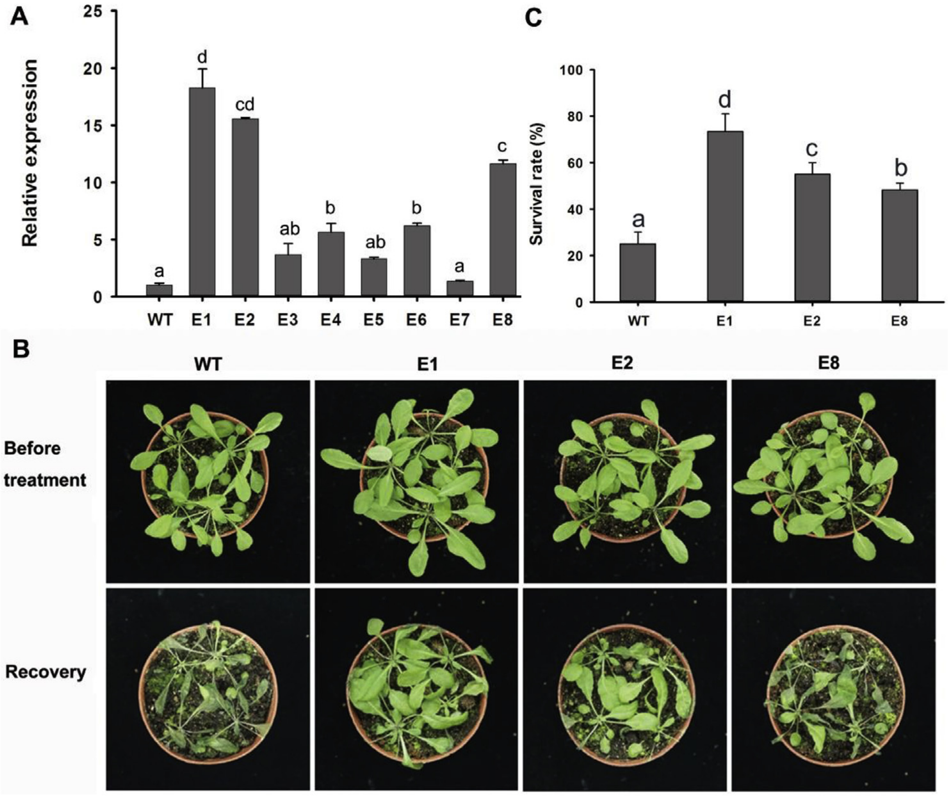

Fig. 3. Overexpression of DlICE1 enhanced cold tolerance in Arabidopsis. (A) Confirmation of *DlICE1* expression in positive transgenic Arabidopsis. mRNA levels in transgenic lines and the WT were detected by RT-qPCR using AtACTIN2 as the internal control. Freezing phenotypes (B) and survival rates (C) of the transgenic lines E1, E2, and E8 and the WT. Three-week-old pot-grown plants were treated at −4 °C for 6 h after cold acclimation (48 h at 4 °C) and recovery for 6 d. 20 seedlings per line for each freezing treatment. The data represent the means of three biological replicates ± standard deviations (SDs). Different letters (a–d) indicate significant differences by Duncan's multiple comparison tests at $P < 0.05$.

(Xu et al., 2014; Lu et al., 2017; Yuan et al., 2017; Zhang et al., 2018). Proline is considered to be an adaptive product, produced by plants in response to various types of environmental stress. Proline promotes plant stress resistance by enhancing cellular osmotic regulation, maintaining normal membrane and protein functions, and inhibiting stress-induced ROS production. Under normal conditions (22 °C), proline accumulation in the *DlICE1*-overexpressing lines was similar to that in the WT plants. However, after cold (4 °C for 48 h) and freezing (−4 °C for 6 h) treatments, distinctly higher proline levels were observed in the transgenic lines than in the WT plants (Fig. 4A).

Ion leakage is an important indicator of cell membrane injury during the plant response to environmental stress. Under normal conditions (22 °C), ion leakage in the transgenic lines was similar to that in the WT plants. However, during cold and freezing treatments, ion leakage levels were significantly higher in the WT plants than in the transgenic lines (Fig. 4B), suggesting the occurrence of relatively severe cell membrane injury in the WT and enhanced cold and freezing tolerance of the *DlICE1*-overexpressing lines.

MDA is also a reliable index of the plant response to stress. Stress-induced ROS production results in the decomposition of polyunsaturated lipids, resulting in the generation of MDA. Upon exposure to cold and freezing conditions, the MDA content was significantly lower in the transgenic lines than in the WT plants (Fig. 4C). In other words, the decrease in MDA levels might reflect a reduction in ROS accumulation in transgenic plants. ROS, including hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and superoxide ($O_2^{\cdot -}$), are produced in plants in response to various environmental stressors (Tyystjarvi, 2013). In this study, quantitative measurement showed that H_2O_2 and $O_2^{\cdot -}$ contents in the transgenic lines were distinctly lower than in the WT (Fig. 4D and E). We further assayed H_2O_2 and $O_2^{\cdot -}$ accumulation in situ by histochemical staining with DAB and NBT. Lighter staining was observed in the transgenic leaves than in the WT leaves under chilling temperatures (Fig. S3). These results indicated that the transgenic lines exhibited relatively low ROS accumulation under cold conditions.

Taken together, these results demonstrated that overexpression of *DlICE1* enhanced cold and freezing tolerance by increasing proline production, reducing ion leakage, and decreasing MDA levels and ROS accumulation in transgenic Arabidopsis plants at low temperatures.

3.7. DlICE1 positively regulates CBFs and cold-responsive gene expression under cold stress

To further elucidate the molecular mechanism underlying the response of *DlICE1*-overexpressing lines to cold stress, we investigated the expression patterns of cold-induced CBF family genes and the downstream cold-responsive genes using qRT-PCR. The expression of cold-induced CBF family genes, including *AtCBF1, AtCBF2 and AtCBF3*, were rapidly induced and peaked at 6 h after cold stress treatment in both the *DlICE1* transgenic lines and WT plants. However, the maximum expression levels of the three CBF genes were significantly higher in all the *DlICE1* transgenic lines than in the WT (Fig. 5A-C). The cold-responsive genes, which contain conserved CRT/DRE motifs in their promoters, are downstream target genes of CBF, such as *AtRD29A, AtCOR15A, AtCOR47* and *AtKIN1*(Stockinger et al., 1997). The expression of these COR genes was gradually induced to a maximum value after exposure to cold stress for 24 h in both the transgenic lines and the WT. Nevertheless, the expression of all these genes was significantly higher in the transgenic lines than in the WT plants (Fig. 5D-G). These results indicated that overexpression of *DlICE1* positively regulates the expression of the CBF genes and downstream COR genes in response to cold stress, thereby contributing to enhanced cold tolerance .

4. Discussion

Longan is a freezing sensitive plant, cold stress has become one of the most important limiting factors affecting the geographical location for cultivation and the fruit productivity and quality. However, little is known about cold response signalling pathway in longan. ICE1 is the first identified and the best-characterized positive regulator of *CBF* genes (Chinnusamy et al., 2003). To date, numerous studies shows that

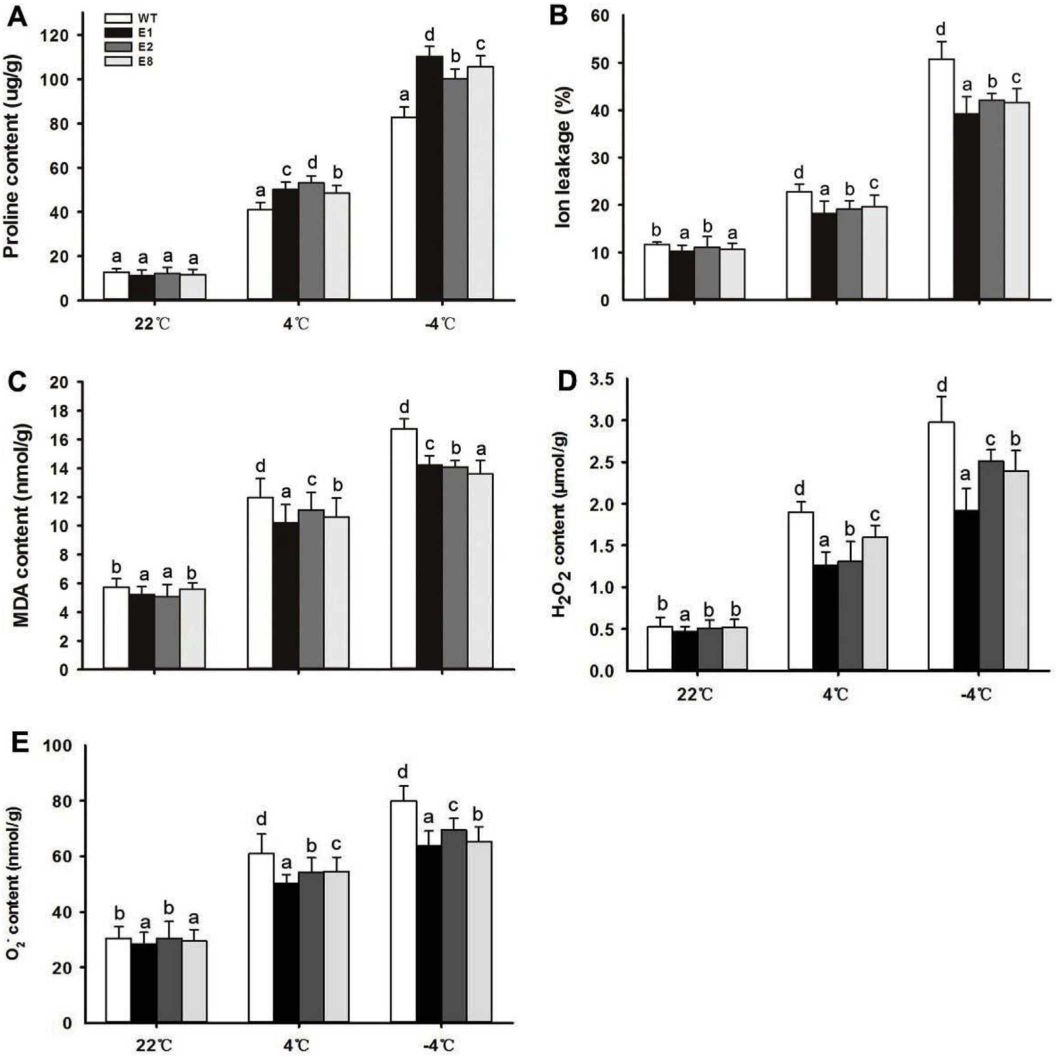

Fig. 4. Changes in physiological parameters in WT and transgenic lines under cold stress. Proline content (A), ion leakage (B), MDA (C), H_2O_2 (D) and $O_2^{\cdot-}$ (E) accumulation were determined in three-week-old plants of WT and the transgenic lines E1, E2 and E8 under cold stress (4 °C for 48 h) and freezing (−4 °C for 6 h) treatments. The data represent the means of three biological replicates ± standard deviations (SDs). Different letters (a–d) indicate significant differences by Duncan's multiple comparison tests at $P < 0.05$.

ICE1 is one of the central components in the cold response pathways of plants (Ding et al., 2019). However, there are few reports about the function of *ICE2* in responses to cold stress, except in *Arabidopsis* (Fursova et al., 2009), *Vitis amurensis* (Xu et al., 2014) and rice (Deng et al., 2017). In this study, we mainly discuss the function of *DlICE1* which would as the start to investigate the molecular mechanisms of longan response to low temperatures. In further study, we will characterize the function of *DlICE2* to help us understand the underlying roles of the ICE1-like genes in longan.

In this study, the *DlICE1* gene was isolated in longan. *DlICE1* encodes a typical bHLH protein which contains a MYC bHLH-ZIP domain. The bHLH-ZIP region is a unique domain of bHLH family members and is responsible for the binding of transcription factors to DNA cis-elements (Chinnusamy et al., 2003). The previous studies demonstrated that bHLH transcription factor play important roles in responses to abiotic stresses, including cold, drought and high salinity stresses (Chinnusamy et al., 2003; Seo et al., 2011; Ji et al., 2014). The sumoylation and the phosphorylation sites are highly conserved with ICE1 in Arobidopisis, suggesting that the activity and stability of DlICE1 may also be regulated by these post-transcriptional regulation (PTMs).

Multiple sequence alignment and phylogenetic analysis indicated that DlICE1 is highly homologous to CtICE1, VaICE1 and MdCIbHLH1. Previous reports have shown that these ICE1 proteins play important role in the cold response (Feng et al., 2012; Xu et al., 2014; Huang et al., 2015b). In addition, subcellular localization and transactivation activity assay confirmed that DlICE1 is a typical transcription factor that primarily functions in the nucleus. These results indicate that DlICE1 is a novel ICE1 homologue that may play an important role in the cold tolerance of longan.

Expression analysis revealed that *DlICE1* was constitutively expressed in all the tissues tested and was expressed at low levels and slightly induced under cold stress in longan. These results further suggest that PTMs may be more important for its function. Furthermore, our results are consistent with previous studies in the tropical species *H. brasiliensis* (Yuan et al., 2017) but differs from the results for *MdCIbHLH1, CtICE1* and *PuICE1*, the expression of which was significantly induced by low temperature (Feng et al., 2012; Huang et al., 2015a, 2015b). These disparities between *DlICE1* and other fruit tree plants might be attributed to the inherent differences among plant species.

In our study, physiological indexes analysis showed that *DlICE1*-overexpressing lines showed improvement of survival rates and cryotolerance via increased proline production, reduced ion leakage, and decreased MDA levels and ROS accumulation. These results showed

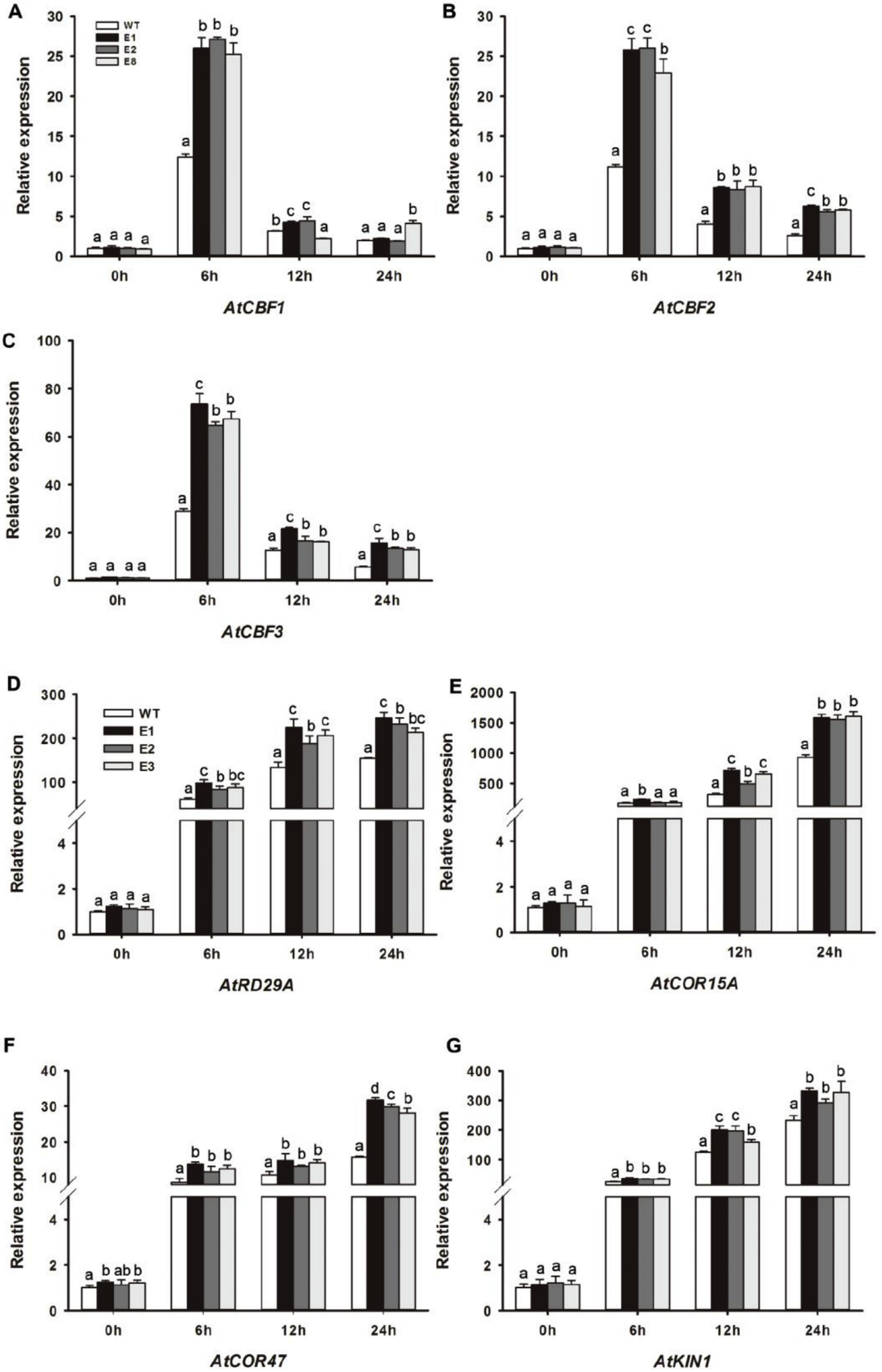

Fig. 5. Expression of cold-responsive genes in WT and the transgenic lines under cold treatment. Three-week-old pot-grown plants of the WT and transgenic lines E1, E2 and E8 were treated at 4 °C for 0, 6, 12 and 24 h. The ICE1-CBF cold signalling pathway genes, including *AtCBF1/2/3* (A-C), *AtRD29A, AtCOR15A, AtCOR47* and *AtKIN1* (D-G), were assayed by RT-qPCR using *AtACTIN2* as the internal control. The data represent the means of three biological replicates ± standard deviations (SDs). Different letters (a–d) indicate significant differences by Duncan's multiple comparison tests at $P < 0.05$.

that *DlICE1* plays a positive regulatory role in the response of plants to cold stress, similar with the previous studies in other plant species. Overexpression of *VaICE1* and *VaICE2* (*V. amurensis*) in Arabidopsis can alter ion leakage and proline and MDA levels to improve cold tolerance (Xu et al., 2014). *ZmmICE1*-overexpressing Arabidopsis plants showed decreased ion leakage, reduced MDA levels and enhanced cold tolerance (Lu et al., 2017). Overexpression of *H. brasiliensis ICE1* in Arabidopsis has been shown to increase plant survival rates under freezing treatments (Yuan et al., 2017). These findings indicate that ICE1 regulates CBF-independent cold signalling pathways in not only temperate plants but also tropical and subtropical crops such as *D. longan.*

CBF transcription factors are downstream regulatory genes of ICE1 homologues. The expression of three members of the CBF family can be rapidly and transiently induced by low temperatures (Gilmour et al., 1998; Liu et al., 1998; Medina et al., 1999). Subsequently, cold-induced CBF transcription factors can bind to the conserved CRT/DRE cis-element (CCGAC) in the promoters of COR genes to activate COR gene expression and enhance cold tolerance in plants (Stockinger et al., 1997; Liu et al., 1998). In this study, the expression of *AtCBF1/2/3* was rapidly induced in both the *DlICE1* transgenic lines and WT plants under cold stress, and the maximum relative expression at 6 h in the transgenic lines was significantly higher than that in the WT. The expression of *AtRD29A, AtCOR15A, AtCOR47* and *AtKIN1,* which have more than one CRT/DRE cis-element in the promoter region, was highly induced by overexpression of *AtICE1* (Chinnusamy et al., 2003). Here, we found that expression of these COR genes were gradually induced by cold stress, and the maximum expression at 24 h was significantly higher in the transgenic lines than in the WT plants. These results indicated that overexpression of *DlICE1* positively regulates the expression of cold-induced CBF genes and downstream cold-responsive genes in response to cold stress, thereby contributing to enhanced cold tolerance.

In conclusion, *DlICE1*, a novel ICE1-like gene, was identified from *D. longan*. DlICE1 was localized in the nucleus. Overexpression of *DlICE1* in Arabidopsis increased the proline content, decreased electrolyte leakage, reduced MDA and ROS accumulation, and significantly induced the expression of cold-induced CBFs and downstream cold-responsive genes, all of which contributed to the enhanced cold tolerance of the plants.

Author contributions

C.M.L. and J.X.F. conceived the project and designed the experiments. X.Y.Y., R.W., Q.L.H., and S.L.L. performed experiments and analysed the data. X.D.M., H.H.J., J.T.Z., and G.B.H. were involved in data analysis. X.Y.Y., J.X.F. and C.M.L. wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.08.007.

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