Genetics and Molecular Research

Pattern of *CsICE1* expression under cold or drought treatment and functional verification through analysis of transgenic *Arabidopsis*

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ABSTRACT. *CsICE1* is thought to be involved in hardiness resistance of tea plants. Using seedling cuttings of biennial Wuniuzao in this study, the pattern of *CsICE1* expression under cold temperature (4°, -5°C), drought [20% polyethylene glycol 6000 (PEG-6000)], and plant hormone [200 mg/L abscisic acid (ABA), 1 mg/L brassinolide (BR)] treatment was studied by real-time quantitative PCR. Additionally, stress resistance, such as the freezing resistance of *CsICE1,* was studied using *Arabidopsis* lines transformed with sense or anti-sense *CsICE1* via *Agrobacterium tumefaciens* infection. Our results showed that *CsICE1* mRNA could be induced under -5°C, PEG, ABA, or BR treatment, although the pattern of expression differed for all treatments. Compared to wild type (WT) and anti-sense *ICE1* transgenic lines, sense lines displayed higher relative germination rates under salt and drought stress. After freezing treatment, the sense transgenic lines over-expressing *CsICE1* showed a higher survival rate, increased levels of proline, and decreased levels of malonaldehyde. Conversely, compared with WT, anti-sense *ICE1* transgenic lines had lower proline levels and higher malonaldehyde levels under freezing conditions. Our study

indicates that *CsICE1* is an important anti-freezing gene and that over-expression of *CsICE1* can improve cold resistance and enhance salt and drought tolerance of transgenic lines.

Key words: *CsICE1*; Expression pattern; Real-time quantitative PCR; Tea plant

INTRODUCTION

Tea plants prefer warm temperatures. A better understanding of the underlying mechanism of plant cold resistance could improve plant cold tolerance and further increase tea yield. Numerous reports have shown that *CBF/DREB* is an optimum anti-cold gene. As a transcriptional activator, *inducer of CBF expression* (*ICE1*) binds to the promoter of *C-repeat/dehydration-responsive element binding factors* (*CBF*) and functions as an activator of *CBF*. Low expression of *ICE1* is maintained under normal temperature conditions. Following its induction at freezing temperatures, the ICE1 protein could improve plant freezing tolerance by binding to the *MYC cis*-element of the *CBF3* promoter and further inducing expression of the downstream anti-cold gene *COR* (Gilmour et al., 1998; Chinnusamy et al., 2003; Xin et al., 2007; Miura and Hasegawa, 2008; Huang et al., 2012; Dong et al., 2013).

CBF/DREB over-expressing transgenic lines waste much energy in maintaining constitutive induction of cold related genes under normal temperature conditions. With a poor growth rate, the plant usually displays a short phenotype (Dubouzet et al., 2003; Liu et al., 2010a; Akhtar et al., 2012). Transgenic lines over-expressing *ICE1* do not exhibit a growth defect. Transcriptome analyses of *ICE1* mutants via gene chip hybridization showed that ICE1 plays a key role in the early response to cold stress by regulating the expression of early cold-responsive genes and those related to abscisic acid (ABA) and auxin (Lee et al., 2005; Jiang et al., 2013; Peng et al., 2013). Huang and Sun (2005) successfully integrated *AtICE1* into the lemon genome via *Agrobacterium tumefaciens* infection, and the detection of cold tolerance in transgenic lines is still ongoing. Xiang et al. (2008) cloned *ICE1*, which was subsequently integrated into Kenjiandao Rice 10, and the transgenic rice showed a lower death rate and had a higher proline content under cold temperature when compared with wild type plants. So far, a variety of plants have been successfully transformed with the *ICE1* gene, such as citrus (Huang, 2005), *Populus suaveolens* (Lin et al., 2007*)*, apple (Zhao, 2007; Feng et al., 2012), *Cymbidium grandiflorum* (Zhang et al., 2008), tobacco (Zheng et al., 2009), cucumber (Liu et al., 2010b), rice (Xiang et al., 2011), and chrysanthemum (Chen et al., 2012). Compared to control plants, all of these transgenic lines displayed improved cold tolerance. *ICE1* has been cloned from *Capsella bursa-pastoris* (Wang et al., 2005), wheat (Badawi et al., 2008), cucumber (Liu et al., 2010b), *Eucalyptus camaldulensis* L. (Lin et al., 2011*)*, lettuce (Xiang et al., 2011), chrysanthemum (Chen et al., 2012), and tea (Wang et al., 2012). The pattern of *CsICE1* expression at normal temperature and at 4°C was investigated, and it was not found to be induced at 4°C. So far, few functional studies investigating *CsICE1* transgenic lines or its expression under drought, ABA, and brassinolide (BR) treatment have been published. Fundamental data and a theoretical basis are needed to further understand the role of *CsICE1* in the regulation of freezing tolerance in tea plants. In this study, we further investigated the expression pattern of *CsICE1* under cold temperature, freezing stress, drought, and plant hormone (ABA and BR) treatment using quantitative real-time (qRT) PCR. Because it is not

yet possible to transform tea plants, we chose *Arabidopsis* as the host plant for *CsICE1* sense and anti-sense transgenic analysis via *A. tumefaciens* infection. A series of functional studies were carried out utilizing these transgenic lines. Our results provide information for further exploration of the function of *CsICE1* in the cold resistance pathway of tea plants.

MATERIAL AND METHODS

Plant materials

Cutting seedlings of biennial tea plant (*C. sinensis* cv. Wuniuzao) were chosen to study the pattern of *CsICE1* expression. Wild type (WT) *Arabidopsis thaliana* (ecotype Columbia).

Primer design and synthesis

RT-PCR primers were designated I-1 and I-2 based on the *CsICE1* sequence obtained from GenBank (GenBank accession No.: JX029153); real-time PCR primers specific for the reference gene *CsGAPDH* were designated GAPDH-F and GAPDH-R based on the coding sequence deposited in NCBI (GenBank accession No.: GE651107). The sense *CsICE1* fragment was amplified using primer pair I-3 (with an *Xba*I cutting site at the 5' terminus) and I-4 (with a *Sac*I cutting site at the 5' terminal). The anti-sense *CsICE1* fragment, which contains the full-length coding region, was amplified using the primer pair I-5 (with a *Sac*I cutting site at the 5' terminus) and I-6 (with an *Xba*I cutting site at the 5' terminal). Primer synthesis and sequencing was conducted by Shanghai Sangon Biotech. Company, Shanghai, China. All primer sequences are listed in Table 1.

Table 1. Primer sequences.

No.	Oligonucleotides (5'-3')	Note
I1	GGAGAACCTTGGGCTAGACAT	For real-time qRT-PCR
I2	TCTATAGGCATGAGAAAGAGCA	For real-time qRT-PCR
GAPDH-F	TTGGCATCGTTGAGGGTCT	For real-time qRT-PCR
GAPDH-R	CAGTGGGAACACGGAAAGC	For real-time qRT-PCR
I3	GCTCTAGAATGGAAGATAGAGGAGAT	For Ti transgenic plants
I4	CGAGCTCCGATTGAAGAAGAAACCTAC	For Ti transgenic plants
I5	CGAGCTCATGGAAGATAGAGGAGAT	For Ti transgenic plants
I6	GCTCTAGACGATTGAAGAAGAAACCTAC	For Ti transgenic plants

Total RNA extraction and reverse transcription

Total RNA was extracted using an EASY spin rapid plant total RNA extraction kit (Yuanping-hao Bio, China). Two leaves from one early bud of the annual biennial Wuniuzao served as the material for RNA extraction. Reverse transcription was performed using ReverTAid™ First Strand cDNA Synthesis Kit (Thermo, Massachusetts, USA). The one-step method of qRT-PCR was used.

Analysis of gene expression pattern

Cuttings of annual tea plant seedlings (*C. sinensis* cv. Wuniuza) were divided into

groups and subjected to different treatments: cold stress at 4°C (kept in the darkness in an incubator), freezing stress at -5°C (kept in the darkness in an incubator), drought (irrigated with 20% polyethylene glycol 6000 (PEG-6000) solution until there was overflow in the bottom), 200 mg/L ABA, and 1 mg/L BR. The leaves were harvested for total RNA extraction at set time points. Maxima SYBR Green qPCR Master Mix (2X) was used for qRT-PCR analysis. The PCR was carried out in a 10-μL system with the following program: 95°C for 10 s, 58°C for 10 s, 72°C for 10 s, for 45 cycles. The relative expression level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method as previously described (Livak and Schmittgen, 2001). *GAPDH* was used as the internal reference gene. A sample taken at 0 h served as the calibration sample.

Screening and molecular verification of transgenic lines

Positive T0 *Arabidopsis* seeds were primarily selected on MS medium containing 50 mg/L kanamycin. The positive candidates were further verified through molecular identification. Genomic DNA for PCR identification was extracted from positive genomic DNA containing corresponding DNA fragments: the primer pair I-3 and I-4 for sense transgenic lines; and the primer pair I-5 and I-6 for anti-sense transgenic lines. Furthermore, total RNA from all of the transgenic and WT lines was extracted and reverse transcribed into cDNA. Identification of transgenic lines at the transcriptional level was conducted using the cDNA obtained above.

Germination capacity of transgenic seeds under osmotic stress

Seeds of transgenic and WT lines (both sense and anti-sense) were seeded on solid 1X MS growth medium containing 150 mM NaCl and 200 mM mannitol (pH 5.4-5.8) for 14 days. The relative germination rate was calculated and images of all transgenic and WT lines were captured under different conditions. The germination rate of seeds grown on solid 1X MS medium was set as the contrast germination rate. Relative germination rate = germination rate of treatment group / contrast germination rate x 100%.

Phenotype observation, survival rate determination, and biochemical indicator testing under freezing stress

Arabidopsis seedlings grown in a culture dish for two weeks were transferred to a -6°C freezer for 6 h, a 4°C light incubator for 12 h, and then to normal conditions to a 22°C light for 16 h, 20°C for 8 h at light incubator for 4 days, successively. Images of all plant materials were obtained. The degree of frostbite was determined based on the integrity and degree of chlorosis of the leaf blade. Additionally, *Arabidopsis* underwent the same treatments following two weeks of potting. The degree of frostbite and the survival rate were determined. Proline (sulfosalicylic acid method) and malondialdehyde contents (thiobarbituric acid method) were analyzed in all samples collected at 0 min and at 6 h after treatment (Wang et al., 2012).

Data analysis

Analysis of variance of single complete randomized factors was done using the DPS software.

RESULTS

Freezing stress induces the expression of *CsICE1*

The effect of cold (4°C) and freezing stress (-5°C) on the expression of *CsICE1* was studied using its expression in tea leaves at room temperature (RT) as the control. The results showed that freezing stress (-5°C) induced the expression of *CsICE1*, which peaked (4.1 fold compared with control) 1 h after treatment and then decreased sharply to initial levels (Figure 1). Cold stress (4°C) had no effect on the expression of *CsICE1* throughout the treatment process.

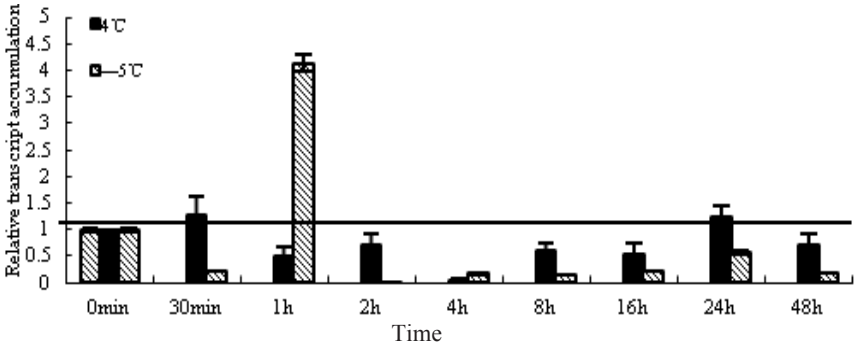

Figure 1. Relative accumulation of *ICE1* transcripts in response to low temperature.

Expression of *CsICE1* under drought conditions and treatment with plant hormones (ABA and BR)

The pattern of *CsICE1* expression following treatment with 20% PEG, 200 mg/L ABA, and 1 mg/L BR were also investigated (Figure 2). Treatment with PEG, ABA, or BR could induce the expression of *CsICE1,* while differences were seen in terms of temporal expression and abundance in response to each condition. Peak expression was observed at 16 h (11.3 fold increase versus control), 8 h (4.3 fold increase), and 4 h (5.9 fold increase) following treatment with 20% PEG, 200 mg/L ABA, and 1 mg/L BR, respectively. Following all treatments, the level of *CsICE1* expression returned to initial levels during the later treatment stage (24 and 48 h).

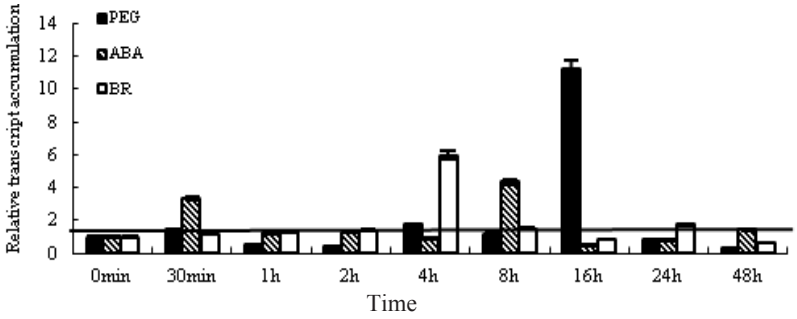

Figure 2. Relative accumulation of *CsICE1* transcripts under PEG, ABA, and BR treatments.

Identification of *Arabidopsis* transgenic lines

In this study, we found that although untransformed seeds could germinate on solid Ka-MS medium, their cotyledons gradually underwent etiolation and death (Figure 3A). Transgenic seedlings could maintain healthy growth on Ka-MS medium (Figure 3B). The positive seedlings were further identified at a molecular level. Four sense and six anti-sense lines were selected through genomic DNA-PCR analysis (Figure 4A). No PCR product was obtained when WT genomic DNA was used as a template. Detection of *CsICE1* mRNA in transgenic lines by RT-PCR (Figure 4B) further confirmed that *CsICE1* was successfully integrated into the *Arabidopsis* genome and could be transcribed into mRNA.

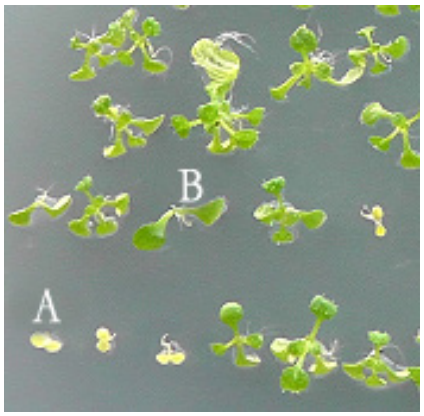

Figure 3. Transgenic *Arabidopsis* plants with PvP5CS1 grown on 1X MS + 0.8% agar + 50 mg/L Kan culture medium plates (**A.** untransformed plants, **B.** transgenic plants).

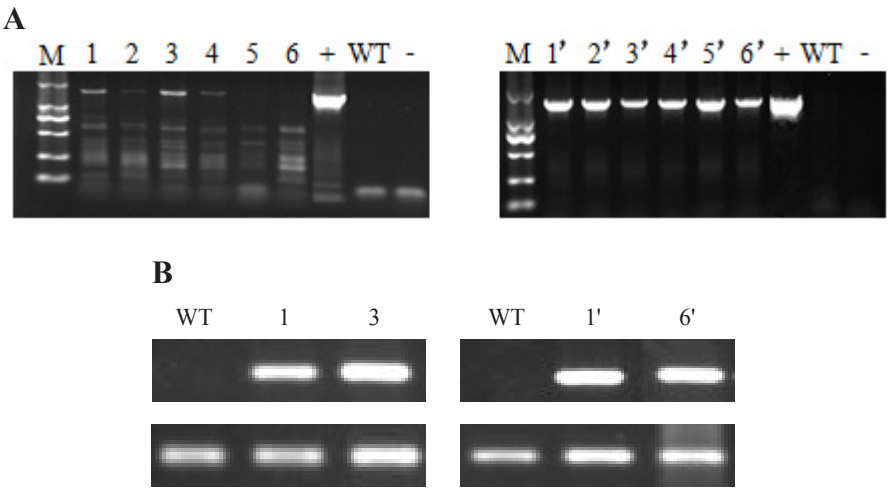

Figure 4. Genomic DNA-PCR (**A**) and RT-PCR (**B**) detection of sense and antisense transgenic *Arabidopsis* plants. +: positive control lane using corresponding plasmid as PCR template; -: negative control lane using H_2O as the PCR template.

Germination rate of *Arabidopsis* transgenic lines under osmotic stress

The germination rate was higher under osmotic stress in sense transgenic lines when compared with that of anti-sense lines and WT plants (Figure 5A), while no significant differences were observed between the germination rate of anti-sense lines and WT plants. The average germination rates of sense lines subjected to 150 mM NaCl and 200 mM mannitol treatment were 2.6- and 1.9-fold those of WT plants, respectively, and 1.8- and 1.9-fold those of anti-sense lines, respectively (Figure 5B).

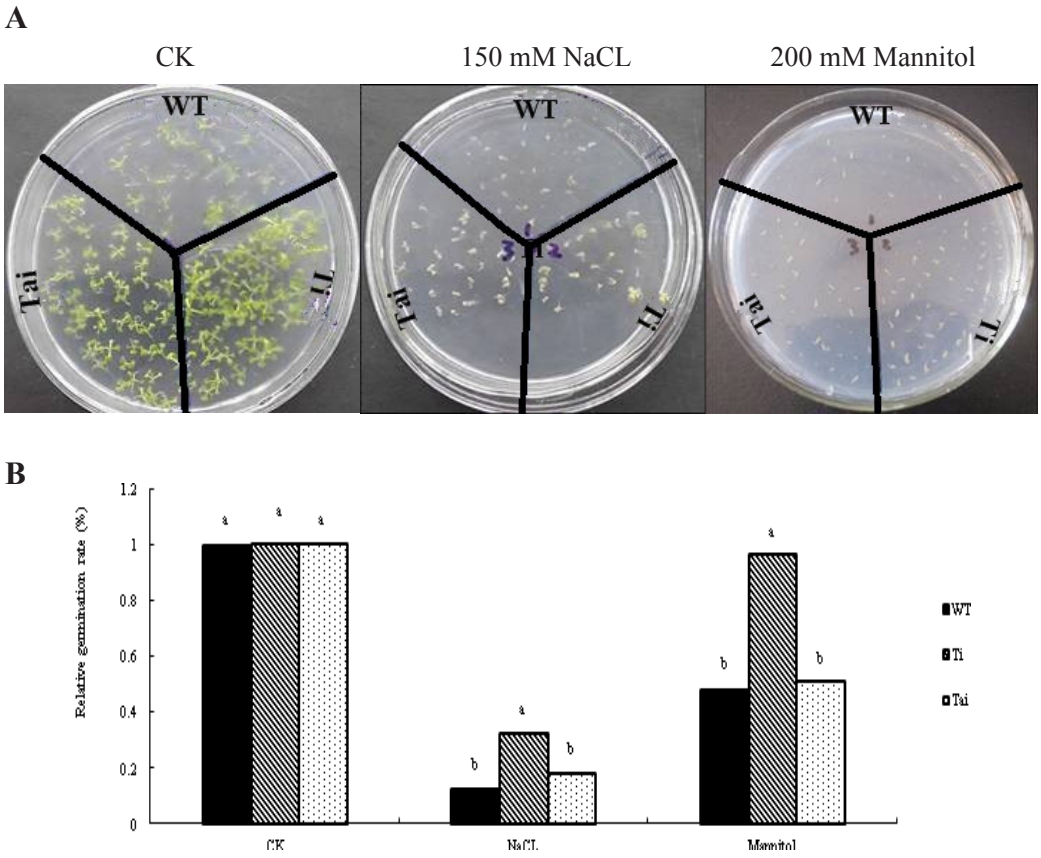

Figure 5. Germination (**A**) and relative germination rate (**B**) of transgenic *Arabidopsis* seeds exposed to different stresses. Ti: sense transgenic plants; Tai: anti-sense transgenic plants. The different lowercase letters on top of the histogram represent a significant difference ($P < 0.05$).

Strong cold tolerance of sense transgenic lines under freezing stress

The degree of frostbite was determined based on the integrity and chlorosis condition of the leave blade. Compared with WT and anti-sense transgenic lines, the leave blades of sense transgenic lines maintained better integrity and were greener and healthier when grown

at -6°C for 6 h (Figure 6A). Sense lines and WT lines displayed survival rates of 98.5 and 88.5% ($P < 0.01$). Anti-sense transgenic lines showed the lowest survival rate of 78.4% and poorest growth status after freezing treatment (Figure 6B).

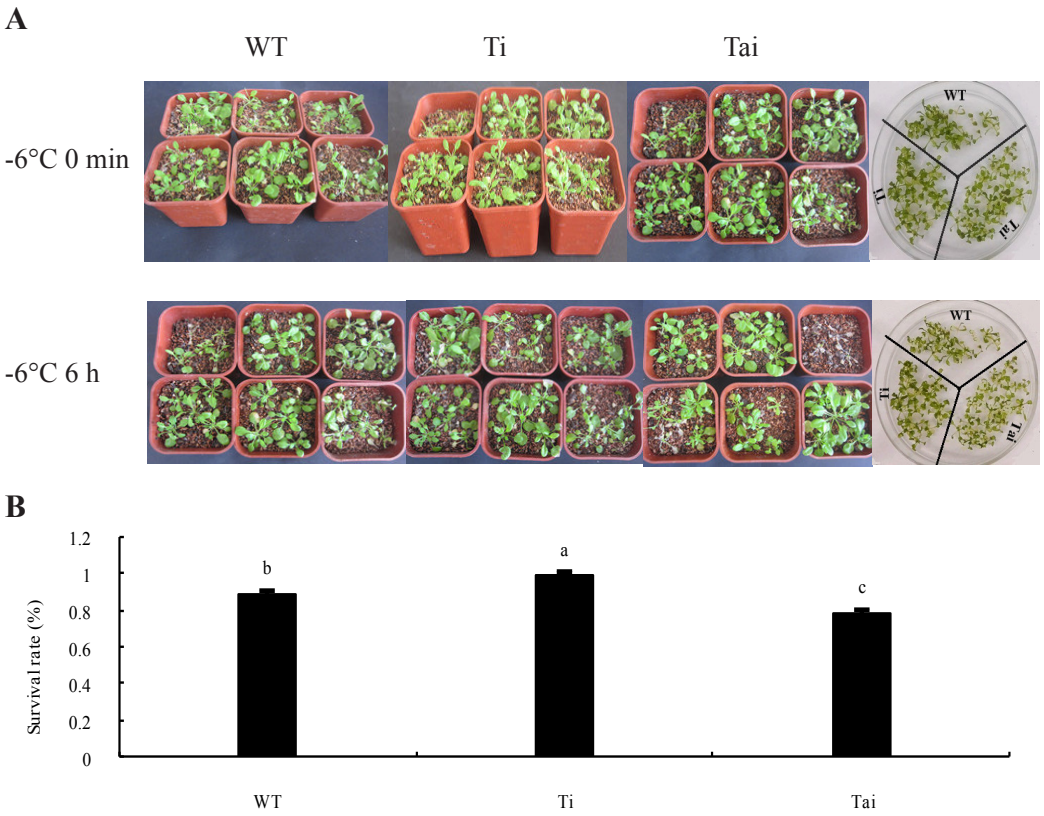

Figure 6. Phenotype identification (**A**) and the survival rate (**B**) of WT and sense and anti-sense transgenic plants under freezing stress. The different lowercase letters on top of the histogram represent a significant difference ($P < 0.05$).

Determination of physiological indicators of transgenic lines under freezing stress

Our results indicate that the transgenic and WT lines contained similar levels of proline and malonaldehyde (Figure 7A and B) before any treatment. After 6 h under -6°C, sense lines showed the highest accumulation of proline (1.25 μg/g) and the lowest accumulation of malonaldehyde (Figure 7). These changes in the levels of physiological indicators indicate that *CsICE1* endowed *Arabidopsis* with strong freezing tolerance, which is consistent with the plant phenotypes observed under freezing stress. These results further demonstrate that *CsICE1* is a positive regulator of the cold tolerance signal pathway in plants. The anti-sense transgenic plants displayed higher malonaldehyde content and lower proline content compared with WT (Figure 7). This indicates that self-protection against freezing stress in plants could be hampered by reverse integration of exogenous *CsICE1* into the genome.

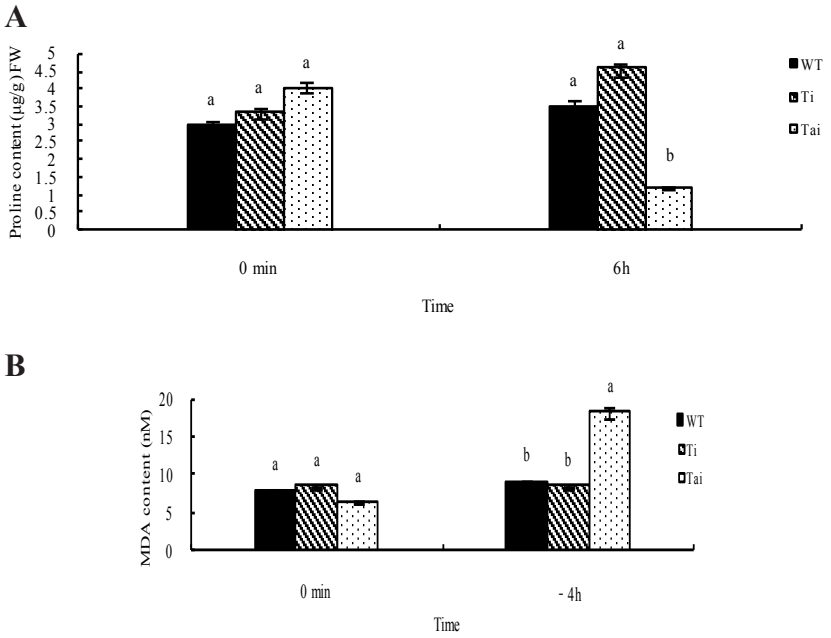

Figure 7. Proline (**A**) and MDA (**B**) contents in WT, sense transgenic, and anti-sense transgenic plants under freezing stress. The different lowercase letters on top of the histogram represent a significant difference ($P < 0.05$).

DISCUSSION

Tea is a healthy drink that is popular worldwide. Tea plants prefer warm climates to cold or freezing temperatures. Tea seedlings encounter numerous low temperature stresses, such as freezing, cold winds, snow, frost, and cold springs. Therefore, it is of great importance to determine the cold response pathway in tea plants. The genes involved in the signaling pathway could be utilized to breed plants that have high cold tolerance. In our study, the pattern of *CsICE1* expression induced under freezing stress is typical of that exhibited by stress responsive genes. Previous studies showed that the level of *EcaICE1* expression was not changed following 4°C treatment for almost 24 h (Lin et al., 2011), while *ICE1* expression in *Raphanus sativus*, lettuce, and cabbage was induced at 4°C, and expression of all three *ICE1s* reached the highest level at 12 h, which could be maintained until 24 h. These data indicate that *ICE1* is possibly involved in the regulatory pathway of cold resistance in plants. However, there are no reports of the pattern of *ICE1* expression under freezing stress. Additionally, our results showed that *Arabidopsis* transformed with sense *CsICE1* was more resistant to freezing stress, which further demonstrates that *CsICE1* is a positive regulator of the freezing response.

Our research also showed that *CsICE1* could be induced by drought or salt treatment. *Arabidopsis* seeds transformed with sense *CsICE1* became more tolerant to osmotic stress. This indicates that *CsICE1* is protective against drought and salt as well as freezing, which is consistent with previous reports on *MdICE1* (Zhao, 2007), *OsbHLH2* (Zhou et al., 2009), and *CdICE1* (Chen et al., 2012).

In order to combat low temperatures, plants undergo a series of physiological, biochemical, and gene expression changes when they encounter cold stress (Thomashow, 1999; Theocharis et al., 2012). We found that *CsICE1* could be induced by exogenous ABA treatment, which indicates that *CsICE1* is possibly involved in the ABA signaling pathway. Other anti-cold genes such as *LEA* and *CBF,* are also induced by ABA treatment (Xiong et al., 2002; Knight et al., 2004; Jeon and Kim, 2013). An ABA responsive element was found in the promoter region of *PkbHLH2*, and *PkbHLH2* can be regulated by the ABA pathway (He et al., 2013). *MbDREB1* enhanced cold resistance in apple through both ABA dependent and independent pathways (Yang et al., 2011). *AtICE1* improved plant cold tolerance via the ABA independent network (Gilmour et al., 1998). More research is needed to determine the underlying mechanisms of *CsICE1* expression in the ABA signaling pathway.

Under adverse environmental conditions, plants synthesize large amounts of hormones, such as ABA, BR, and gibberellic acid (GA). These hormones are important signaling molecules that are involved in protection against stress. Cross-talk occurs between different hormone pathways. *OsGSR1* is induced by exogenous GA, while exogenous BR can repress the induction effect of GA on *OsGSR1* (De Vleesschauwer et al., 2012). Normally, ABA induces the expression of the proline synthesis gene *P5CS1,* while BR represses the effect of ABA on *P5CS1* (Abrahám et al., 2003). In the present study, exogenous BR induced the expression of *CsICE1*, which indicates that BR has a positive effect on the ABA-independent pathway of *ICE1-CBF-COR*.

In conclusion, our study showed that *CsICE1* is an important anti-freeze gene, which has a complicated regulatory pathway. Over-expression of *CsICE1* in *Arabidopsis* not only enhanced cold tolerance, but also promoted resistance to drought and salt. More detailed research is needed to unveil cross-talk between these regulatory pathways. Therefore, *CsICE1* could serve as a candidate molecular marker for use when breeding tea plants with high resistance to freezing.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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