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Overexpression of *Arabidopsis ICE1* improves cold tolerance in transgenic *Zoysia japonica*

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Overexpression of *Arabidopsis ICE1* improves cold tolerance in transgenic *Zoysia japonica*

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ABBREVIATIONS

ICE1	Inducer of CBF Expression1
CBF	Inducer of CBF Expression CRT/DRE Binding Factor
COR	Cold-responsive genes
MYB	Myeloblastosis
AP2/ERF	Apetala 2/ethylene response factor
LB	Luria broth
YEB	Yorkshire Electricity Board
MS	Murashige & Skoog
2,4-D	2,4-dichlorophenoxy-acetic acid
BA	6-Benzylaminopurine
PPT	Phosphinothricin acetyltransferase
BAR	Bialaphos
GUS	β-glucuronidase
Ubi M	Ubiquitin promoter
p35S	CaMV 35S promoter
LB	Left border
RB	Right border
T_1	Transgenic 1 generation
T_2	Transgenic 2 generation
T_3	Transgenic 3 generation

ROS	Reactive oxygen species
MDA	Malondialdehyde
SOD	Superoxide dismutase
POD	Peroxidase
CAT	Catalase
APX	Ascorbate peroxidase
SD	Standard deviation
SPSS	Statistical Package for the Social Sciences

ABSTRACT

ICE1 (Inducer of CBF Expression1), is a regulator of cold-induced transcriptome, which plays an important role during cold tolerance signal pathways in *Arabidopsis thaliana*. To enhance the cold tolerance of *Zoysia Japonica*, one of the perennial warm-season turfgrass, the *ICE1* gene was cloned by RT-PCR and constructed into the plant expression vector pIG2 modified from pC3301. The *ICE1* was under the control of ubiquitin promoter and successfully transformed into *Zoysia Japonica* wild type callus and *Arabidopsis thaliana* ecotype Columbia (Col-0) by the *Agrobacterium*-mediated transformation method respectively. After cold treatment at -8℃ for 2 hours and 5 days recovery, The transgenic lines of *Arabidopsis* pIG2-*ICE1*-3, pIG2-*ICE1*-5 showed a higher survival rate 84.5% and 86.9% than the wild type 73.2%. Furthermore, the transgenic lines showed big leaves compared with wild type. RT-PCR and Southern blot analysis of 15 PPT resistant *Zoysia japonica* transgenic lines suggested that *ICE1* had been integrated into *Zoysia japonica* genome successfully. The transgenic plants showed higher *ICE1* expression level compared with wild type by Real time qRT-PCR. Overexpression of *ICE1* both in transgenic *Zoysia Japonica* and *Arabidopsis* showed higher proline content, less MDA content, higher activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT). All of this indicated that overexpression of *ICE1* gene improved cold tolerance of *Zoysia japonica* and *Arabidopsis* at low temperatures.

INTRODUCTION

Zoysiagrass (*Zoysia japonica* Steud. cv. wide leaf) is one of the most popularly cultivated warm-season perennial turfgrass for sports and recreational environments with a well resistance to heat, drought and salinity (Inokuma et al. 1998). Its use is rapidly expanding in the USA and other countries (Toyama et.al. 2003) for the enlargement of urban green areas that provide a better quality of life and improve the mental and physical health (Gusmao et al. 2016). However, zoysiagrass is mainly used in temperate, subtropical and tropical areas due to its tender to cold, so low temperature is one of the limiting factors for *Zoysia japonica* planting in Asia areas and other countries with four distinct seasons.

Low temperature is one of the factors that determines geographical range and cultivation and greatly affects plant productivity (Chinnusamy et al. 2003). Cold stress can be classified as chilling (<20◦C) and freezing (<0◦C) stress. Plants have developed several mechanisms to adapt to the ambient low temperature called cold acclimation. The ability of cold tolerance can be acquired through prior exposure plants to low, non-freezing temperatures (Chinnusamy et al. 2010). Low temperature initiates signaling pathways that control the expression of genes related to cold-tolerance (Miura and Furumoto 2013, Fowler and Thomashow 2002). Cold-responsive genes encode a variety of proteins such as enzymes involved in enhancement of antioxidative mechanisms, transcription factors, lipids, antioxidants, molecular chaperones, antifreeze proteins, and others with a presumed function in tolerance to the dehydration caused by chilling and freezing (Thomashow 1999, Lee et al. 2005, Fowler and Thomashow

2002, Kazuo et al. 2003).

ICE1 (inducer of CBF expression 1), binding to the MYC recognition cis-elements (CANNTG) in the promoter of CBF3/DREB regulon regulates the transcription of *CBF* (Inducer of CBF Expression CRT/DRE Binding Factor) genes during cold acclimation in *Arabidopsis*. Overexpression of *ICE1* improves freezing tolerance in the transgenic plants (Chinnusamy et al. 2010). CBF/DREB (dehydration-responsive element/C-repeat-binding) genes belong to the apetala 2/ethylene response factor (AP2/ERF) family binding to C-repeat CRT/DRE dehydration-responsive elements (G/ACCGAC) promote the transcription of downstream cold-responsive (*COR*) genes, which contributes to low temperature tolerance in *Arabidopsis* (Joaquı´n et al. 1999, Joaqu ń et al. 2011, Zhao & Zhu 2016, Maruyama et al. 2004). *COR* genes encode functional hydrophilic proteins, improving the cell osmoregulation and protecting the plasma membrane of plants under freezing stress (Thomashow 1999). *ICE1-CBF-COR* cold-response pathway is one of the dominant cold signaling mechanisms mediating cold tolerance in *Arabidopsis* and other species (Zhao 2016, Peng et al. 2014, Mohamed et al. 2008, Lee et al., 2005).

Plants can produce reactive oxygen species (ROS) in the normal life activities. The ability of using reactive oxygen species (ROS) is reduced under the low temperature and the excess oxygen is toxic to plants. Too many free radicals will affect the normal life activities of the cell and initiate the membrane lipid peroxidation that cause the damage of membrane and hurts the plants. Plants can clear reactive oxygen species (ROS) through plant antioxidant system including oxidases and antioxidants. The oxidases include peroxidase (POD), superoxide dismutase (SOD), catalase (CAT) and ascorbic acid peroxidase (APX).

Peroxidases (POD) decompose H_2O_2 under low temperature and improve the cold resistance. Superoxide dismutases (SOD) remove active oxygen free radical to improve the oxidation resistance and cold resistance. Malondialdehyde (MDA) is one of the most important products of the membrane lipid peroxidation, which emerged in the case of plants suffer adversity stress or aging. So the MDA content was determined as an indicator of the lipid peroxidation damage to membrane system. Proline is an extremely strong hydrophilic amino acid, which can stabilize the protoplasm condition and metabolic processes in the organization. Under the condition of low temperature, the proline content increased in plant tissue, which can improve the cold resistance of the plants. So the MDA content, proline content, the activity of oxidases like POD, SOD, CAT and APX can be used as indicator to estimate the ability plants cold tolerance.

Arabidopsis ICE1 was successfully cloned and integrated into the lemon genome via *Agrobacterium-transformation*. The transgenic lines improved cold tolerance compared with wild type after low temperature treatment (Huang & Sun 2005). Xiang et al. (2008) cloned *Arabidopsis ICE1* and transformed into Kenjiandao Rice10. Under low temperature, the transgenic plants reduced the rice mortality rate and increased the proline content which suggested that over-expression of *ICE1* improved the tolerance to cold stress in rice. Overexpression of *ICE1* gene in tomato through *Agrobacterium*-mediated transformation increased proline content and the activity of peroxide(POD) and catalase(CAT) in transgenic tomato plants, and the malondialdehyde (MDA) content also lower than non-transgenic plant under low temperature (Juan et al. 2015, Yu et al. 2015). Overexpression of *ICE1* gene in Cucumber showed higher soluble sugars and free proline content, lower malondialdehyde

(MDA) and electrolyte leakage under cold stress (Liu et al. 2010). In this study, we successfully transferred *ICE1* gene into *Zoysia Japonica* callus and *Arabidopsis thaliana* ecotype Columbia (Col-0) by the *Agrobacterium*-mediated transformation method respectively. Our results showed that the *ICE1* we cloned was successfully inserted into the binary vector pIG2 and strong expressed in *Arabidopsis thaliana* even though under the monocotylous maize ubiquitin promoter. Overexpression of *ICE1* both in *Zoysia japonica* and *Arabidopsis* enhanced cold tolerance under low temperature.

MATERIALS AND METHODS

Plant materials and growth condition

Arabidopsis thaliana ecotype Columbia (Col-0) plants were grown in a growth room for *Arabidopsis* with environment of temperature, light, and humidity at 23℃ with normal day condition 16h light and 8h dark (Kang et al. 2011). Leaves grown for three weeks were harvested for RNA extraction. An improved *Zoysia japonica* callus (Song et al. 2010) from the wild type seed of *Zoysia japonica* Steud. cv Duckchang (wide-leaf variety) that was purchased from a seed company (Duckchang Agri-business Co. LTD) was used for transformation. They were provided by Subtropical Horticulture Research Institute, Jeju National University, Korea

Gene cloning of *Arabidopsis thaliana ICE1*

Total RNA was extracted from *Arabidopsis* leaves using Trizlo reagent (Invitrogen). M-MLV reverse transcriptase (Promega, Madison, WI, USA) was used for first-stand cDNA synthesis. The full length of *ICE1* was amplified from cDNA using specific primers *ICE1* forward (5'-CGggatccATGGGTCTTGACGGAAAC-3', with *Bam*HI restriction enzyme digestion site) and *ICE1* reward (5'-TCCcccgggTCAGATCATACCAGCATA-3', with *Sma*I restriction enzyme digestion site). Primers were designed using the ORF sequence of *ICE1* in GenBank (KM593299). The PCR reaction protocol as follows: 94℃ 5 min, 94℃ 30 sec, 60℃ 30 sec, 72℃ 1.5 min, 15℃ 10 min, 30 cycles. The expected PCR fragment was purified by

GeneAll® Expin™ Gel SV, 200p kit (Geneall, LO2001KK, Korea) and cloned into pGEM®-T Easy Vector (Promega, Madison, WI, USA). After overnight ligation, the vector was transformed into *Escherichia coli* TOP10 competent cells and selected on Luria broth (LB) plate containing 100mg/L Ampicillin. Plasmid DNA was extracted from the anti-Amp strains using GeneAll® Exprep™ Plasmid mini, 200p kit (Geneall, M101108H, Korea) and digested with *Bam*HI (Takara, KB3501AA, Japan) and *Sma*I (Takara, K6202AA, Japan). Successfully inserted T-vectors were sent for sequencing.

Binary vector construction and transformation

The correct *ICE1* sequence and pIG2 vector were digested with *Bam*HI and *Sma*I in a 20 μL reaction system respectively: *ICE1* plasmid DNA or pIG2 plasmid DNA 10μL, *Bam*HI 1μL, *Sma*I 1μL, 10X T buffer 1μL, 1% BSA 1μL, ddH_2O 6μL and incubated at 30℃for 3h. All the enzyme reaction solutions were used for electrophoresis. Specific fragments were cut and collected in the 1.5mL tube for purification. After gel purification, the *ICE1* fragment and pIG2 vector were ligated together with T4 DNA Ligase (Takara, K6501DA, Japan) as the following protocol: *ICE1* fragment solution 6μL, pIG2 vector solution 2μL, T4 DNA Ligase 1μL, 10X buffer 1μL and incubated at 4℃ overnight. The constructed vector pIG2-*ICE1* was transformed into *Escherichia coli* TOP10 competent cells and selected on Luria broth (LB) plate containing 100mg/L Kanamycin. Plasmid DNA was extracted from the anti-Kanamycin strains and colony PCR was done to verify pIG2-*ICE1*. Binary vector was introduced into *Agrobacterium tumefaciens* disarmed strain EHA105 (Hood et al. 1993) by the freeze-thaw method (Holster et al. 1978) and grown on YEB plate containing 100 mg/L Kanamycin and

25 mg/L Rifamycin for 48 h.

Culture medium

The basic medium was Murashige & Skoog (MS) medium in *Zoysia japonica* tissue culture and transformation. Callus induction: MS, 4 mg/L Thiamine-HCl, 100 mg/L α-ketoglutaric acid, 2 mg/L 2,4-D, 0.2 mg/L BA; Callus growth: MS, 2 mg/L 2,4-D, 0.4 mg/L Kinetin; *Agrobacterium* culture: YEP, 100 mg/L Kanamycin, 25 mg/L Rifampicin; Infection(liquid): MS, 2 mg/L 2,4-D, 0.4 mg/L Kinetin, 50mg/L Acetosyringone; Co-culture(solid): MS, 2 mg/L 2,4-D, 0.4 mg/L Kinetin, 50mg/L Acetosyringone; Callus selection I: MS, 2 mg/L 2,4-D, 0.4 mg/L Kinetin, 250mg/L Cefotaxime, 1mg/L PPT; Callus selection II: MS+2 mg/L 2,4-D, 0.4 mg/L Kinetin, 250mg/L Cefotaxime+5mg/L PPT; Shoot induction and selection I: MS, 1 mg/L BA, 3mg/L PPT, 250mg/L Cefotaxime; Shoot induction, elongation and selection II: MS, 1 mg/L BA, 1mg/L PPT, 250mg/L Cefotaxime; Root induction and selection : MS, 1mg/L PPT, 250mg/L Cefotaxime.

Plant transformation

For zoysiagrass, transformation was performed according to Agrobacterium mediated transformation method reported by Toyama et al. (2003) using the callus induced from the seed of wide-leaf type of zoysiagrass. After the infection of *A. tumefaciens* EHA105 carrying pIG2-*ICE1* for one day, the callus was co-cultured for three days under dark condition at 25 ℃. The transformed cells from the co-cultured callus were selected was selected in the culture medium with 5 mg/L phosphinothricin (PPT) and then the PPT resistant callus were

transferred to the shoot induction medium and cultured under 18h photoperiod of 30 μmol/m2/s illumination.

For *Arabidopsis*, agrobacterium mediated transformation was done using floral dip transformation method reported by Steven J (1998) with some modification. *A. tumefaciens* EHA105 strain carrying pIG2-*ICE1* grow at 28ºC in YEP liquid culture with 100ppm Kanamycin and 25ppm rifamycine for 36h. Spin down Agrobacterium in 50ml tube and suspend to OD_{600} = 0.8 in 5 % Sucrose solution. Silwet L-77 was added to a concentration of 0.05% before dipping. Dip the bud and flower parts of plant in Agrobacterium solution for 2 to 3 seconds gently. Black plastic bags were put on the pot for 3 days.

Transcription analysis of *ICE1* in transgenic *Arabidopsis*

Total RNA of transgenic lines were extracted from leaves using Trizlo reagent (Invitrogen) according to the procedure of the manufacturer. T_1 transgenic generation and wild type were harvested by liquid nitrogen. First-stand cDNA was synthesized by M-MLV reverse transcriptase (Promega, M adison, WI, USA) using 2μg of total RNA. *ICE1*-F1 and *ICE1*-R1 were used for real-time qRT-PCR. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) including fluorescence signal that can be detected by Opticon-mini system (Bio-Rad). The relative transcript accumulation was calculated using $2^{-\Delta ct}$ Method (Livak & Schmittgen 2001).

Segregation of T_2 generation in transgenic *Arabidopsis*

The T_1 generation seeds of 7 transgenic lines and wild type were surface sterilized by 70%

Ethyl Alcohol for 20 min and inoculated on MS medium with 8ppm PPT. After 14 days, the method described by Xiang (2008) was used to calculate the Segregations of T_2 transgenic generations.

Cold resistance assessment of transgenic *Arabidopsis* under low temperature

The freezing tolerance assays were done as (Hu et al. 2013). The T_2 generations of T_2-3, T_2-5, and wild type each half cultivated on the same MS gelrite medium for 10 days and transferred to a cold chamber. Four days cold acclimation at 4℃was first performed and then the cold chamber was adjusted to -8℃ for freezing treatment. After 6h freezing treatment the plates were removed and incubated at normal growth condition, the survival rates of the seeding were scored after 5 days recovery.

For the phenotype analysis, the T_3 generations of T_3-3, T_3-5 were geminated on 1/2 MS with 8ppm PPT and the wild type grown on 1/2 MS was served as control. 2 weeks plants were transferred to the soil. Freezing treatment was done at -8℃ for 6h after 2 weeks recovery. The leaves of transgenic lines and wild type were harvested.

For MDA, lipid peroxidation was estimated by the level of malondialdehyde (MDA) production using a MDA assay kit (Kit No. A003-1, Nanjing JianCheng Bioengineering Institute, China) manufactured depending on the thiobarbituric acid (TBA) method (Draper and Hadley 1990). For proline, Plant leaves were homogenized with a mortar and pestle in aqueous 3% (w/v) sulfosalicylic acid. After the homogenates were centrifuged at 3500rpm for 10min, supernatant was saved and equal volumes of glacial acetic acid and ninhydrin were added. Proline content was measured by a proline assay kit (Kit No. A107, Nanjing

JianCheng Bioengineering Institute, China) manufactured using the method described by Bates et al. (1973). For SOD and POD, About 0.1 g of plant leaves were homogenized in sodium phosphate buffer (50 mM, pH 7.0), and then the supernatant was collected for detection of enzyme activity by centrifugation at 3500 rpm and 4℃ for 10 minutes. Superoxide dimutase (SOD) and peroxidase (POD) activities in the supernatant were measured according to the methods provided by the manufacturer (Kit No. A001-4 for SOD and Kit No. A084-3 for POD, Nanjing JianCheng Bioengineering Institute, China). SOD and POD activity assays were modified from the methods used by Giannopolitis and Ries (1977) and Cakmak and Marschner (1991), respectively.

Detection and transcription analysis of *ICE1* in *Zoysia japonica* transgenic plants

Total RNA were extracted from leaves of transgenic and wild type zoysiagrass plants using Trizol reagent (Invitrogen) according to the procedure of the manufacturer. First-stand cDNAs were synthesized from 2μg of total RNA using M-MLV reverse transcriptase (Promega, M adison, WI, USA). Real time qRT-PCR was performed with the methods presented by Jung et al. (2006) with thermal cycler dice real time system (TAKARA) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The relative transcript accumulation was calculated using $2^{-\Delta ct}$ Method (Livak & Schmittgen 2001). The optimum cycle number for semi-quantitative RT-PCR conditions was established for each primer set and serial dilutions were used to enable linear amplification. The primers used here were listed in Table 1.

Southern blot analysis of transgenic *Zoysia japonica* plants

Genomic DNA was extracted from wild type and transgenic zoysiagrass leaves by CTAB method (Rogers and Bendich 1985). About 30μg DNA was digested by *Eco*RI at 37℃ for 20h, separated on a 1% agarose gel in an electrophoresis apparatus, and transferred to the Hybond-H^+ nylon membrane (GE Healthcare), which was baked at 80℃ for 2h. A digoxigenin-labeled *ICE1* or *BAR* probe was generated by PCR DIG probe synthesis kit (Roche, Cat. No. 11636090910, Germany) according to the manufacture's protocol. The membrane was hybridized in a hybridization solution (5x saline sodium citrate, 50% formamide, 50 mM sodium phosphate, 2% blocking solution, 0.1% N-lauroylsarconine, and 7% SDS) containing the labeled probe at 60℃ for 16h with gently shaking. Afterward, it was washed twice in 0.2x saline sodium citrate, 0.1% SDS for 15 min at 60℃. Signals were detected by chemiluminescence using CDP^{star} (Roche) and visualized by LAS-4000 (Fuji film).

Cold tolerance assay in transgenic zoysiagrass

The cold tolerance assays for zoysiagrass were done as Mayank (2015) described with some modifications. 10cm height transgenic plants were transferred to 4℃ chamber. To assess cold resistance, the leaves of transgenic plants and control plants were collected at 0day, 7days and 14days. MDA content, proline content, the activity of SOD and POD were detected using the methods as *Arabidopsis*. The transcription activities of cold related genes including superoxide dismutase (SOD), catalase (CAT) and ascorbic acid peroxidase (APX) were detected by Real time qRT-PCR after chilling treatment.

Table 1 Primer sequence used in this study

Name	Oligonucleotides(5'-3')	Note
ICE1-F	CGggatccATGGGTCTTGACGGAAAC	For *ICE1* clone
ICE1-R	TCCcccgggTCAGATCATACCAGCATA	
ICE1-F1	AGCCTATGCTTGAAGGTGAT	For real time qRT-PCR
ICE1-R1	AACATTGAGAAGACAACCCT	
BAR-F	AAGTCCAGCTGCCAGAAACCCAC	For southern blotting
BAR-R	GTCTGCACCATCGTCAACCACTA	
Ubi-F	ATACTTGGATGATGGCA	For colony PCR
Abs-R	CAACGCTGAATATTCCAGAAAGG	
18S rRNA-F	ATGATAACTCGACGGATCGC	For *Arabidopsis* inner control
18S rRNA-R	CCTCCAATGGATCCTCGTTA	
ZjACT-F	AAGGCCAACAGGGAGAAAAT	For *Zoysia japonica* inner control
ZjACT-R	GATAGCATGGGGAAGTGCAT	
ZjCAT-F	GTGTTTTTCATTCGGGATGG	For real-time qRT-PCR
ZjCAT-R	CCCTCCATGTGCCTGTAGTT	
ZjAPX-F	AAGACTTGATGGCGAGCTGT	For real-time qRT-PCR
ZjAPX-R	TCGCATTGCTCTAAGCCTCT	
ZjCuZnSOD-F	GCTCGCTGGTTTGAA ATGTT	For real-time qRT-PCR
ZjCuZnSOD-R	GGAAACAGCACTTTTATTTCCTC	
ZjMnSOD-F	CGGATCTTGGGCAATAAAGA	For real-time qRT-PCR
ZjMnSOD-R	CCGAAAAAGTTGGGAATGAA	

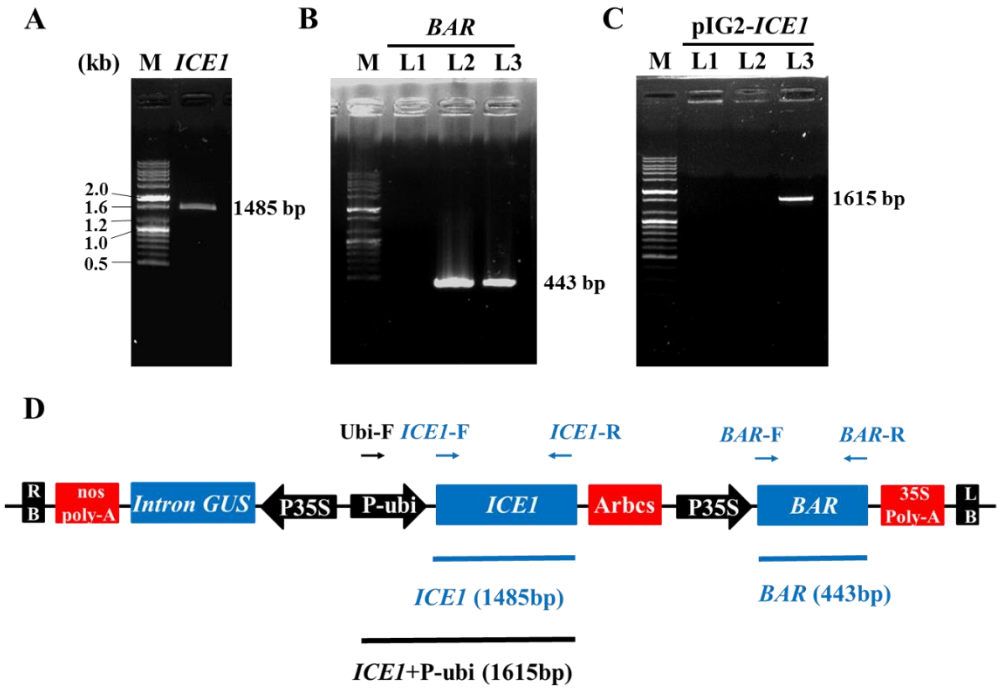

Fig. 1 Isolation of the *ICE1* cDNA clone and identification of DNA fragments in *A. tumefaciens* EHA105 strain harboring binary vector by colony PCR. (A) *ICE1*. (B) *BAR*. (C) A DNA fragment between *Pubi* and *ICE1*. (D) Map of T-DNA region among pIG2-*CE1*. M, 100 bp plus ladder. Lane 1, *A. tumefaciens* EHA105 without binary vector as negative control; Lane 2, pIG2 as empty vector control; Lane 3, pIG2-*ICE1*. *P35S*, CaMV35S promoter, *Pubi*, maize ubiquitin promoter. 35S poly-A, poly A terminator of CaMV 35S. Tnos, Terminator of nopaline synthase gene. LB and RB, left and right border of T-DNA, respectively.

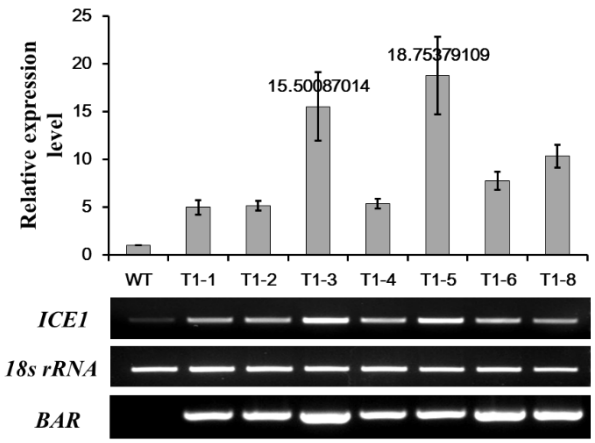

Fig. 2 Transcription analysis of *ICE1* in *Arabidopsis* T_1 generation by real time qRT-PCR. Each sample RNA was extracted from leaves. The relative transcript level was calculated using $2^{-\Delta ct}$ Method. Error bars stand for SD based on three replicated. The transgenic lines were detected by *BAR* gene. *18s rRNA* was used as an internal control.

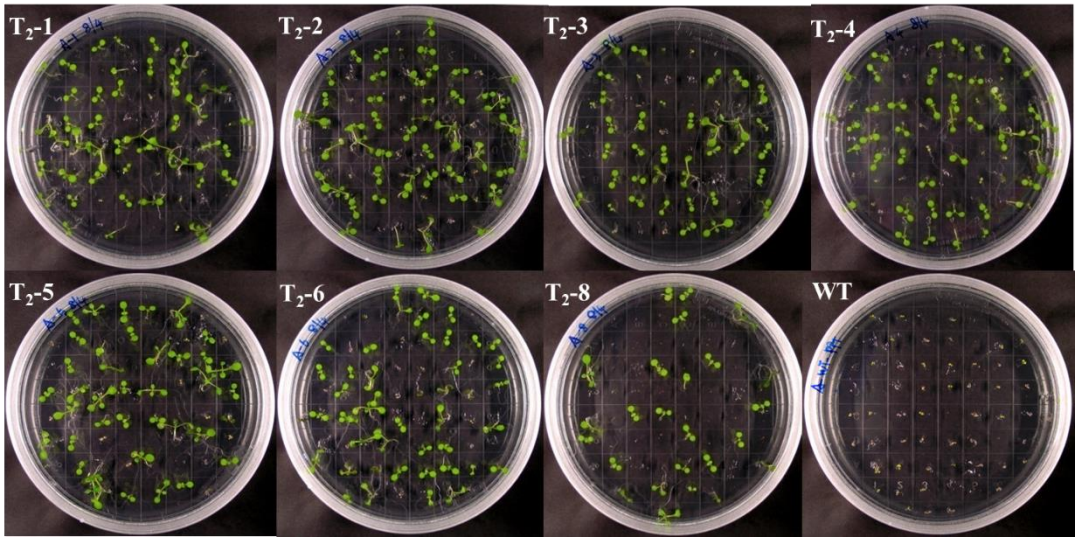

Fig. 3 Germination of *Arabidopsis* T_2 transgenic lines and wild type on 1/2 MS with PPT. T2 generation seeds were surface sterilized by 70% Ethyl Alcohol and inoculated on 1/2 MS medium with 8ppm PPT. After 14 days, The PPT resistant seeds and PPT sensitive seeds in each plat were recorded.

Table 2. Segregation of *Arabdidopsis* T_2 generation for PPT resistance

Plant line	No. T_2 of seed for detection	No.T_2 of PPT resistant seeds	No.T_2 of PPT sensitive seeds	χ^2 (3:1)	Fitness
T_2-1	56	44	12	0.214	H_0
T_2-2	59	45	14	0.006	H_0
T_2-3	50	38	12	0.000	H_0
T_2-4	64	49	15	0.021	H_0
T_2-5	51	38	13	0.007	H_0
T_2-6	62	48	14	0.086	H_0
T_2-8	28	26	2	3.857	H_A
Wild type	52	0	52	-	-

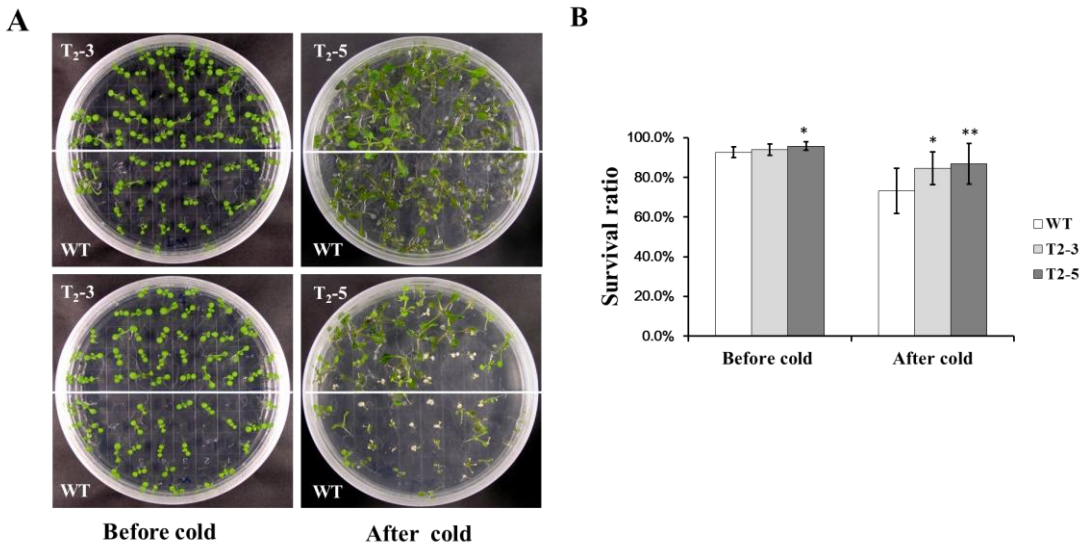

Fig. 4 Overexpression of *ICE1* improved freezing tolerance in *Arabidopsis*. (A) The phenotype of transgenic plants (T_2-3 and T_2-5) and wild type before and after cold treatment. 10 days plants were transferred to 4℃for 4days followed with 6h freezing treatment at -8℃. Pictures were taken after 5 days recovery at 23℃. (B) The survival rate of transgenic and WT plants after recovery. Error bars stand for SD based on three replicated. The data were analyzed using One-Way ANOVA by IBM SPSS Statistics 22.0 (*P <0.05, **P<0.01).

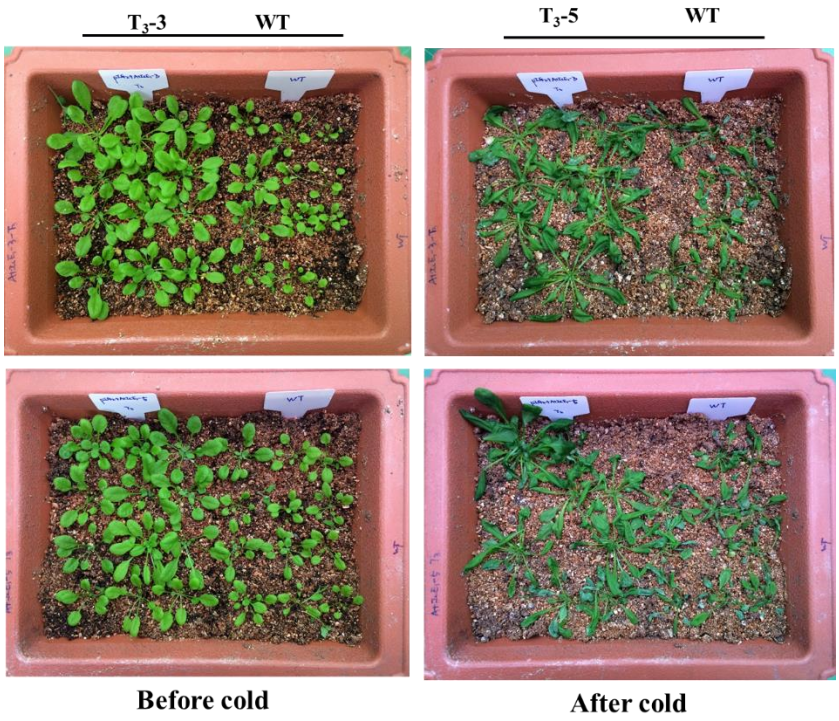

Fig. 5 Phenotype of transgenic *Arabidopsis* and wild type before and after cold treatment. 14 days *Arabidopsis* seedlings grown on PPT selected medium were transferred to soli and cultivated at 23℃ for 2 weeks. freezing treatment was done at -8℃ for 6h. Pictures were taken after 2 days recovery at 23℃.

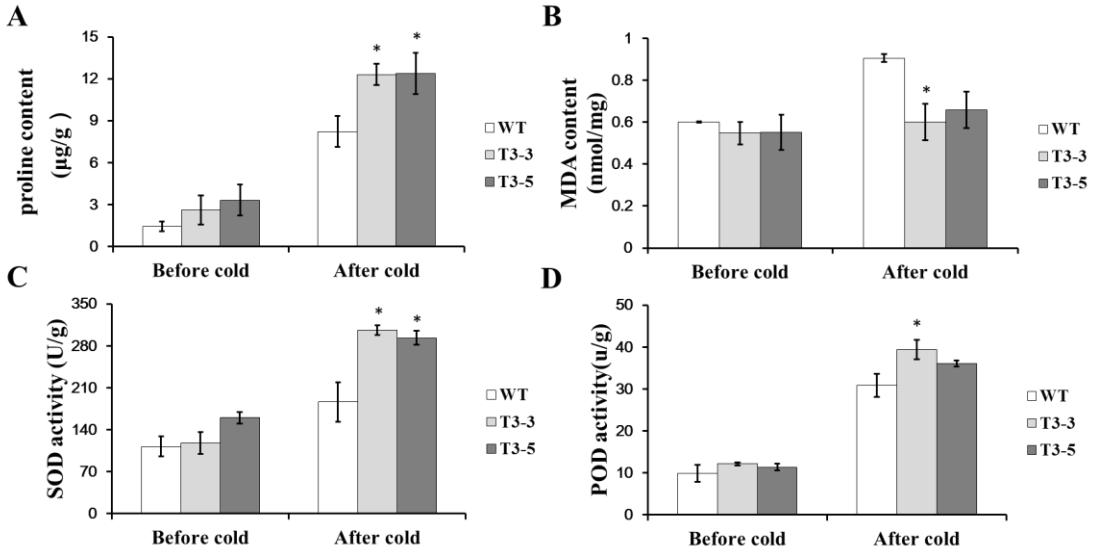

Fig. 6 Overexpression of *ICE1* enhanced cold tolerance in *Arabidopsis.* transgenic and wild type leaves were collected before and after cold treatment at -8℃ for 6h then frozen in liquid nitrogen and stored at -80℃ for analysis. (a) Proline content (b) MDA content (c) SOD activity (d) POD activity. Error bars stand for SD based on three replicated. The data were analyzed using One-Way ANOVA by IBM SPSS Statistics 22.0 (*P <0.05, **P<0.01).

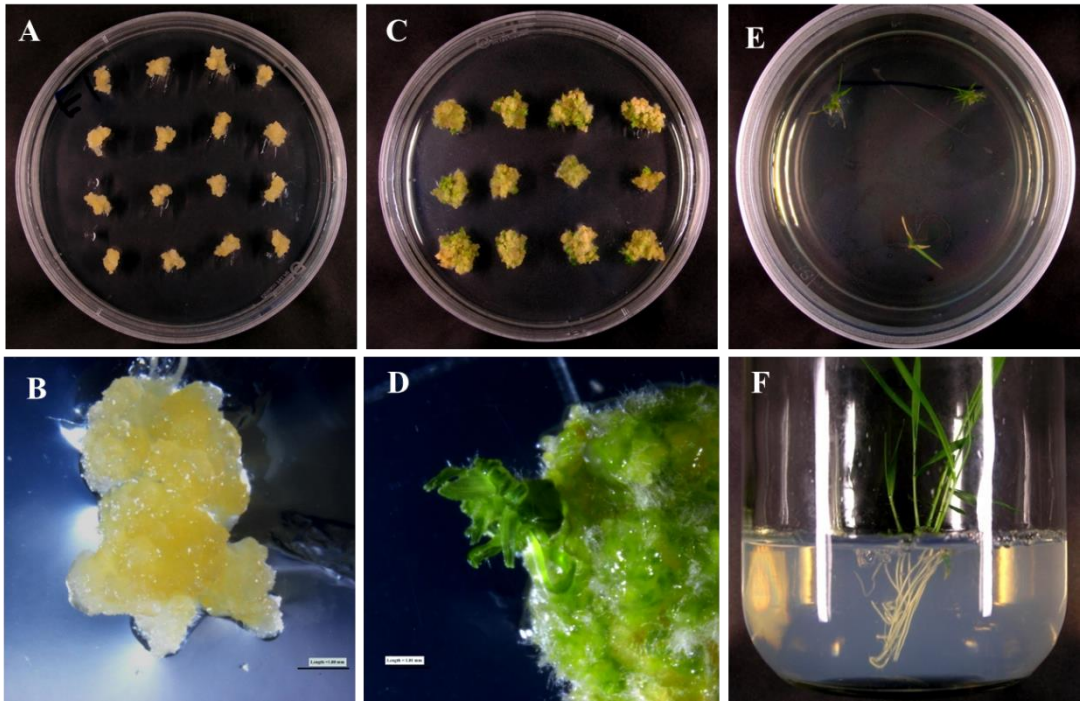

Fig. 7 Generation of the transgenic *Zoysia japonica* plants on PPT selective medium. (A) Callus clumps on PPT selection medium after washing co-cultured callus. (B) One among callus clumps of (A) under stereo microscope. (C) Young shoots being differentiated from callus on PPT-containing shoot induction medium. (D) One among differentiated shoots of (C) under stereo microscope. (E) Putative transformed young shoots transferred into root induction medium on petri dish. (F) Putative transgenic zoysiagrass plant grown in culture bottle. When transgenic plants grew about 3 cm on petri dish, they were taken into culture bottle. Scale bars indicate 1 mm.

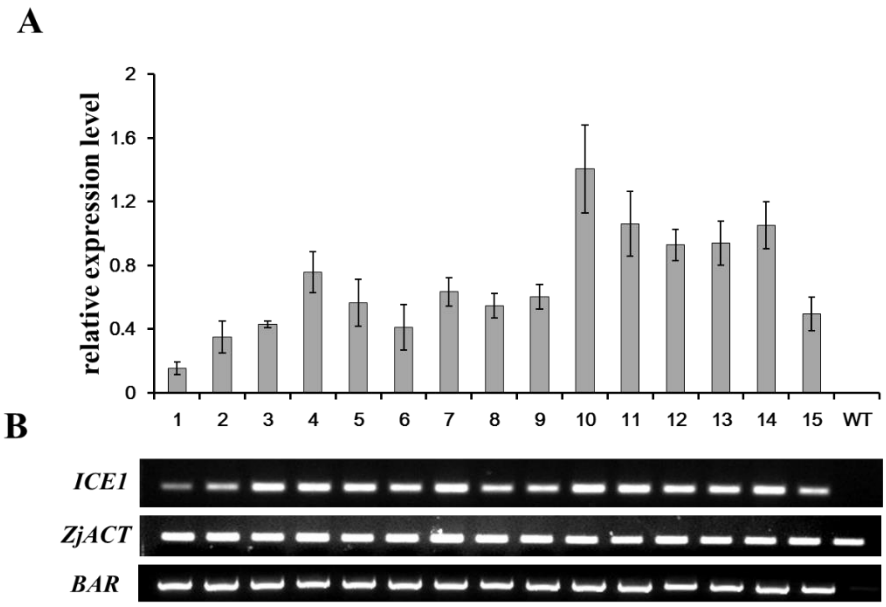

Fig. 8 Transcription level of *ICE1* in T_0 transgenic zoysiagrass. Leaves were harvest and total RNA was extracted for RT-PCR and real time qRT-PCR analysis. (A) Expression pattern of *ICE1* by real time qRT-PCR. Lane1 to 15, zoysiagrass transgenic lines T_0-1 to T_0-15. The relative transcript level was calculated using $2^{-\Delta ct}$ Method. Error bars stand for SD based on three replicated. (B) Electrophoretic analysis of *ICE1* using Ethidium bromide staining of PCR products. *ICE1*-F1 and *ICE1*-R1 were used for RT-PCR of *ICE*. The transgenic lines were detected by *BAR* gene. *ZjACT* was used as the internal control.

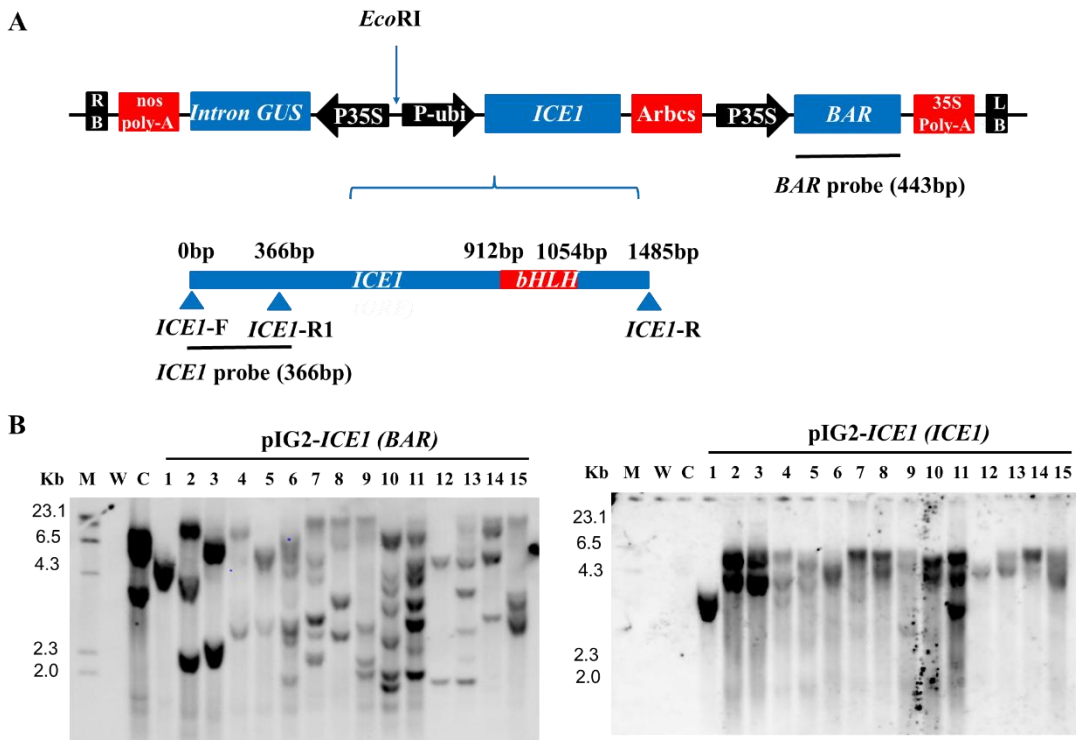

Fig. 9 Southern blot analysis of putative *ICE1* transgenic zoysiagrass. (A) Diagram showing the position and the size of probe in *ICE1* and *BAR* gene. (B) 30㎍ of genomic DNA extracted from leaves of the transgenic plants were hybridized with the *ICE1* and *BAR* probe after digestion by *Eco*RI. The numbers indicate independently transformed plants. Lane1 to 15, transgenic lines T_0-1 to T_0-15; M, lambda *Hind*III molecular marker; W, wild type; C, vector control.

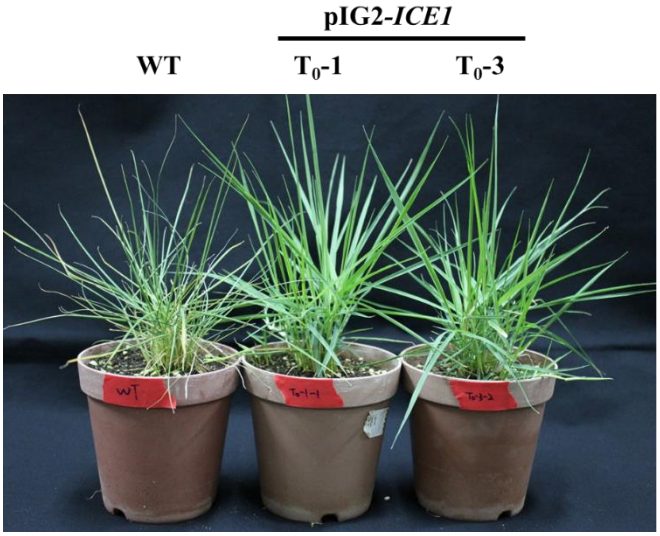

Fig. 10 Phenotype of transgenic *Zoysia japonica* and wild type after cold treatment. Wild type and transgenic zoysiagrass were grown on MS for 4 month and then transferred to outside pots. After two month, transgenic lines and wild type were transferred to 4℃ chamber with 16h light and 8h dark for cold treatment. Pictures were taken after 2 weeks.

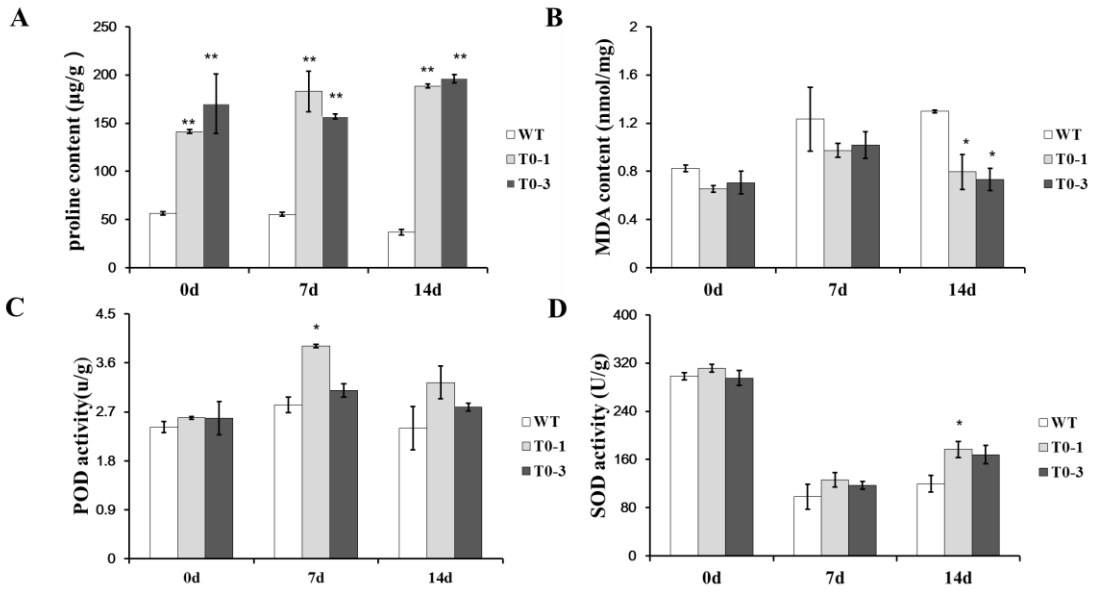

Fig. 11 Overexpression of *ICE1* increases cold tolerance in transgenic *Zoysia japonica.* Wild type and transgenic plants were transferred to 4℃ chamber for cold treatment with 16h light and 8h dark, leaves were collected at 0day, 7days, 14days then frozen in liquid nitrogen and stored at -80℃ for analysis. (A) Proline contents. (B) MDA contents. (C) POD activity. (D) SOD activity. Error bars stand for SD based on three replicated. The data were analyzed using One-Way ANOVA by IBM SPSS Statistics 22.0 (*P <0.05, **P<0.01).

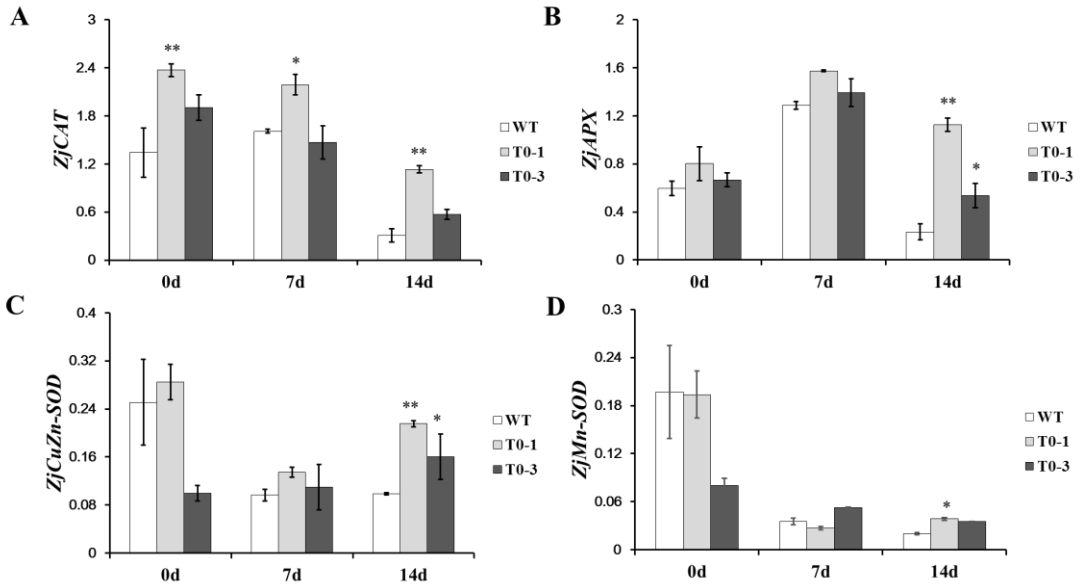

Fig. 12 Transcription analysis of stress related enzymes in transgenic *Zoysia japonica* by real-time qRT-PCR. Wild type and transgenic plants were transferred to 4℃ chamber for cold treatment with 16h light and 8h dark, leaves were collected at 0day, 7days, 14days then frozen in liquid nitrogen and stored at -80℃ for analysis. The relative transcript level was calculated using $2^{-\Delta ct}$ Method. Error bars stand for SD based on three replicated. The data were analyzed using One-Way ANOVA by IBM SPSS Statistics 22.0 (*P <0.05, **P<0.01).

RESULTS

Isolation of *ICE1* and binary vector construction

Using primers designed by the ORF sequence of *ICE1* (Accession No. KM593299) in GenBank, the *ICE1* cDNA clone was isolated by reverse transcriptase PCR (RT-PCR) from the leaf of *A. thaliana* (Chinnusamy et al. 2010). The 1485 bp length of cDNA from ATG to stop codon was confirmed to match perfectly with *ICE1* of GenBank by DNA sequencing (Macrogen Inc. Seoul, Korea). pIG2 vector which modified from pCAMBIA3301 with *Bam*HI and *Sma*I sites was used as the plant expression vector. The cloned *ICE1* DNA was introduced into the pIG2 vector and controlled by the maize ubiquitin promoter vector that was named pIG2-*ICE1* (Fig. 1D). The pIG2-*ICE1* vector was introduced into *A. tumefaciens* disarmed strain EHA105 for plant transformation. The three DNA fragments including *ICE1*, *BAR*, and the fragment between *Pubi* and *ICE1* in the *A. tumefaciens* strain with pIG2-*ICE1* were amplified by colony PCR method (Sheu et al. 2000) and the 1485bp (Fig. 1A), 443bp (Fig. 1B), and 1615bp (Fig. 1C) of DNA fragments in each expected band position on agarose gel were identified.

Transcription analysis of *ICE1* in transgenic *Arabidopsis* plants

To examine the transcription activity of pIG2-*ICE1* in transgenic lines. The *ICE1* transcription activity in T_1 transgenic plants was detected by real-time qRT-PCR. All the transgenic lines showed higher expression pattern compared with Wild type, especially T_1-3 (15.5 times fold higher than Wild type) and T_1-5 (18.8 times fold higher than Wild type) (Fig.

2).

Segregation of *ICE1* in *Arabidopsis* T_2 transgenic generation

The seeds of 7 T_1 transgenic lines and wild type were inoculated on half MS medium with 8ppm PPT after sterilized. After 14 days cultivation at 23℃， both the transgenic lines and the wild type can germinate on the PPT selected medium, but the transgenic plants can grow normal and the wild type become yellow and dead after germination (Fig. 3H). The successfully germinated and survived seeds were regarded as the positive transgenic seeds. In addition, the seeds of death after germination were regarded as the negative transgenic seeds. The segregations of *ICE1* T_2 transgenic generations were calculated according to the formula: χ^2= (|A-3a|-2)2/3(A+a) (A is the number of PPT resistant seeds, a is the number of PPT sensitive seeds, df=1, a=0.05, c2(0.05, 1)=3.84). The segregation ratio of T_2-8 on PPT selected medium didn't show 3:1 by the χ^2 (3:1) test indicated that the *ICE1* was integrated into the *Arabidopsis* genome with multi-copy (Table 2). Other *ICE1* transgenic lines all showed the 3:1 ratio which suggested that the *ICE1* gene was integrated with single-copy.

Overexpression of *ICE1* in transgenic *Arabidopsis* plants increased freezing tolerance

The T_2-3 and T_2-5 were selected to do the further cold tolerance test. The 10 days T_2-3, T_2-5 and wild-type control cultivated in the same agar plates respectively were transferred to 4℃ cold chamber for cold acclimated for 5 days and then subjected to −8°C for 6 h for freezing treatment (Fig. 4A). After 5 days recovery, the transgenic seedlings T_2-3, T_2-5 showed a higher survival rate 84.5% and 86.9% than the wild type control plants 73.2% (Fig.

4B). The result we got was similar with Chinnusamy (2003) published.

To observe the phenotype of overexpression of *ICE1* in *Arabidopsis*, the 14 days PPT resistant T_3 transgenic seeding was transferred to the soil with 2 weeks recovery. The transgenic lines T_3-3, T_3-5 exhibited larger leaves than the WT (Fig. 5).

Same freezing treatment was done to check the cold stress-related biochemical indicator. After cold treatment at -8℃ for 6h, the leaves of transgenic lines and wild type were harvested. All the transgenic lines showed significant higher proline content than wild type (Fig. 6A). Transgenic lines showed lower MDA contents than the wild type (Fig. 6B). The transgenic line T_3-3 showed significant difference compared with wild type. The T_2-3 and T_3-5 showed significant higher POD and SOD activity than wild type (Fig. 6C, D). All that illustrated that the binary vector we constructed was correct and also indicated that the Ubiquitin promoter that well expressed in monocotyledon plants can initiate the *ICE1* gene transcription in dicotyledonous *Arabidopsis thaliana* as well.

Generation of transgenic *Zoysia japonica* plants carrying *ICE1*

The *ICE1* was transformed into *Zoysia japonica* callus which cultured on Callus growth medium at dark condition (Fig. 7A). After Infection at 25℃, 180rpm with infection medium for one day, the callus were placed on co-culture medium at dark condition for 3 days. Elimination was done to remove the excessive *Agrobacterium*. Then the callus was transformed to callus selection medium. After 2 weeks selection, the calluses were placed on shoot induction with 3ppm PPT. The callus began to differentiate into shoot after 3~4 weeks shoot induction medium (Fig. 7C). When the shoot reached to about 1cm (Fig. 7E), they were

moved to root induction medium. After about 2 weeks, the shoot began to take root (Fig. 7F). PCR of *ICE1* and *BAR* using genomic DNA showed that the *ICE1* gene had been transferred into the *Zoysia japonica* genome (Fig. 8B).

Transcription and southern blot analysis of *ICE1* in T_0 transgenic *Zoysia japonica*

Genomic DNA of transgenic *Zoysia japonica* plants was extracted by CTAB method. PCR of *BAR* and *ICE1*were done to detect the transgenic lines using genomic DNA. 15 transgenic plants with expected band were got on the occasion of the wild type as the negative control. Real-time qPCR was done to analysis the transcription activity of *ICE1* in 15 transgenic lines using the same amount of RNA (Fig. 8A). The transgenic plants showed different expression pattern and the wild type didn't express. In addition, Southern blot of the transgenic plants was done using the *BAR* and *ICE1* as the probe (Fig. 9A). The wild type and the empty vector pIG2 were used as the negative and positive control. The result showed that band was presented in all the transgenic plants and the empty vector pIG2 line, no bands could be detected from the wild type line using *BAR* gene as probe (Fig. 9B). Single band was presented in the T_0-1 transgenic line. Two bands were presented in T_0-3, T_0-4, T_0-5, T_0-12 and others showed multiply copy. For *ICE1* probe, band was only presented in the transgenic plants. Single band was presented in the T_0-1 T_0-6, T_0-7 and T_0-12.

Overexpression of *ICE1* increases cold tolerance in transgenic *Zoysia japonica*

To evaluate the function of *ICE1* in zoysiagrass during cold tolerance, T_0-1, T_0-3 and wild type were subjected to cold treatment at 4 ℃. After 2 weeks cold acclimation, leaves of wild

type become rolled and dry. Furthermore, the old leaves at the bottom of the plant become yellow (Fig. 10). In contrast, T_0-1 and T_0-3 transgenic lines grow well.

Proline and MDA contents were also measured which are indicators of biotic or abiotic stress. For proline content, under normal condition before exposure to low temperature, the transgenic plants already accumulated higher proline content, compared to wild-type (Fig. 11A, 0d). During the treatment of low temperature at 4℃, the transgenic plants maintained the increased proline content at 7^{th} and 14^{th} days (Fig. 11A). For MDA content, under normal condition before low temperature treatment, wild-type plant showed very slightly higher MDA content than the transgenic plants (Fig. 11B, 0d). However, wild-type plant increased MDA content at 7^{th} and 14^{th} days after low temperature treatment.

The activity of two important antioxidant enzymes SOD and POD were assessed under the same cold acclimation condition proline and MDA. Under normal growth conditions (0 day), activities of the two enzymes were similar with the wild type control, but the activity of SOD was prominently higher than POD. For SOD activity, 7 days and 14 days cold acclimation caused decrease of SOD activity both in the transgenic lines and the wild type, but T_0-1 was significantly higher than the WT at 14^{th} days (Fig. 6A, 14d). Activity of POD increased at 7^{th} days and slightly decreased at 14^{th} days (Fig. 6B). T_0-1, T_0-3 and wild type showed same change patterns during exposition to cold, but the wild type significantly lower than T_0-1 at 7^{th} days (Fig. 6B, 7d).

Cold related genes were further tested using real time qRT-PCR to determine cold tolerance in transgenic plants. Both the transgenic plants and the wild type showed firstly decreased and then increased of *ZjCAT* (Fig. 12A). There was no difference between wild

type and T_0-3 during the cold acclimation, but T_0-1 significantly higher than wild type and T_0-3 at 7 days. Expression of the *ZjAPX* increased at 7days and then decreased (Fig. 12B). The expression of *ZjCuZn-SOD* and *ZjMn-SOD* decreased continuously both in transgenic lines and wild type (Fig. 12C, D). In the T_0-3, the expression of both *ZjCuZn-SOD* and *ZjMn-SOD* showed higher than the other two lines at 14day.

DISCUSSION

Zoysia japonica is one of most widely used warm-season perennial grass mainly in warm climate regions (Wei et al. 2015). However, the short green period caused by low temperature is the primary reason for its widely planted. The injury by the reason of chilling and freezing varied between the planted years and genotypes in the field research (Patton et al. 2007). Many researches about molecular responses pathway to cold stress were done on *Arabidopsis* and other plants to illuminate the mechanism of plants response to low temperature (Miura et al. 2013, Heidarvand et al. 2010, Thomashow et al. 1999, Miura et al. 2013, Knight et al. 2012, Hannah et al. 2005). When the plants suffer from abiotic stresses, the functional genes in plants including genes for antioxidant enzymes and osmolyte biosynthesis encoding proteins to protect the plant cells and the regulatory genes such as transcription factors and protein kinases regulate the downstream gene expression to increase the cold tolerance. There are multiple cold response regulatory pathways in *Arabidopsis* through transcriptome-profiling experiments during cold acclimation (Fowler et al. 2002). Kazuko (1994) identified a novel cis-acting, dehydration-responsive element (DRE) that was involved in responsiveness to drought, low temperature, or High-Salt Stress (Feng et al. 2013).

An upstream transcription factor *ICE1* pacifically bind to MYC recognition cis-element in the promoter of CBF/DREB1s regulates the expression of *CBF* genes in the low temperature. *ICE,* CBF/DREB1 and *COR* genes pathway in plants occupied important position in response to low temperatures. (Zhou et al. 2011). The *ICE-CBF/DREB1* pathway was positively regulated by jasmonate that acts as an upstream signal during the *Arabidopsis*

freezing tolerance (Hu et al. 2013). *ICE1–CBF* cold response pathway is conserved in variety plant species (Chinnusamy et al. 2010).

In this study, the 1485bp CDS sequence of *ICE1* was cloned and ligated into the binary vector pIG2. Overexpression of *ICE1* in *Arabidopsis* showed higher survival rate compared with wild type and Chinnusamy (2003) isolated one *ice1* mutant that was sensitive to chilling and freezing treatment than the wild type. All these proved that *ICE1* is a positive regulator during chilling and freezing. Furthermore, the *Arabidopsis* transgenic lines in pots showed big leaves than the wild type that was in contrast to the *Arabidopsis ice1* mutant strain showed smaller phenotype than the wild type (Chinnusamy et al. 2003) and were similar with the over-expression of *SlICE1* in transgenic tobacco increase the size of leaves (Feng et al. 2013). In addition, *ICE1* also involved in the Stomata differentiation events and homozygous mutant showed growth defects with small rosettes, short inflorescences, and reduced fertility (Masahiro et al. 2008). Stomata as an important channel for plant to exchange the carbon dioxide and water vapor with the outside world which play an important role in regulating plant photosynthesis, transpiration and water use in the physiological process of plants (Chaerle et al. 2005). That's maybe the increased stomata number in *Arabidopsis* leaves improved the photosynthesis and the ability adapted to the environment. on the *Zoysia japonica* side, the wild type showed rolled leaves and the transgenic lines showed normal after acclimation treatment at 4℃for 2 weeks. That's maybe the water loss coursed by low temperature. We are not sure whether some relationship between the stomata in the transgenic lines and wild type leaves and this water loss phenomenon. So we need to do more experiments to determine the detailed reasons of this phenomenon.

Overexpression of *ICE1*in rice (XIANG et al. 2005), tomato (Yu et al. 2015), Cucumber (Liu et al. 2010) enhance the cold tolerance and the transgenic lines showed lower malondialdehyde (MDA), higher proline (Pro) contents, and higher peroxidase (POD) and catalase (CAT) activities. Overexpression of *ICE1* downstream *CBFs* also increase cold tolerance in plants. Overexpression of *CBF1* and *CBF2* improved plant freezing tolerance (Gilmour 2004). Overexpression of *CBF3* in *Arabidopsis* also increases the freezing tolerance of cold-acclimated plants with high levels of proline (Pro) and total soluble sugars (Gilmour 2000). The *cbf* triple mutants were sensitive to freezing tolerance compared to the wild type after cold acclimation (Zhao et al. 2016). All this demonstrated that *CBF1, CBF2,* and *CBF3* are essential for acclimation and freezing tolerance in plants. In this study, the *Zoysia japonica* transgenic lines also showed high proline content, lower MDA content, high APX, CAT, SOD enzyme activities than wild type at some cold treatment time point. The *ICE1* may be activated the *ZjCBFs* homologous to *CBFs/DREBs* in *Arabidopsis* that regulated *COR* and other stress related genes in *zoysia japonica*. Of course, more researches should be done to investigate the expression patterns of *ZjDREBs*, *COR* genes in the transgenic *Zoysia japonica* under cold stress.

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