



# Cloning and transformation of INDUCER of CBF EXPRESSION1 (*ICE1*) in tomato

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**ABSTRACT.** The tomato plant (*Solanum lycopersicum* Mill.) is sensitive to cold, and low field temperatures can result in shortened growth periods and decreased crop yield. Transcription of CRT/DRE-binding factor (*CBF*) is regulated by INDUCER of CBF EXPRESSION1 (*ICE1*). *CBF* activates many downstream genes that confer cold tolerance on plants. *ICE1* has been used in genetic engineering to improve cold-resistance in several plant species. Here, *ICE1* in a plant expression vector was used to transform a tissue-cultured rhubarb tomato variety using *Agrobacterium tumefaciens*. The transgenic and control plants were compared at 4°C for 0, 24, and 72 h. We measured leaf physiological indicators related to cold resistance, including malondialdehyde (MDA) and proline (Pro) contents, and peroxidase (POD) and catalase (CAT) activities. At 72 h, the MDA content in transgenic plants was significantly lower than in control plants, indicating a lower membrane lipid injury. The Pro contents and the CAT and POD activities in the transgenic plants increased significantly compared with those of the control plants. For Pro, the increase continued over the prolonged stress exposure, while CAT and POD activities reached peak

levels at 24 h. These results are consistent with the roles of Pro, CAT, and POD in defending the integrity of plant cells. Our study not only improves the cold resistance of tomato, but also provides the foundation for further research on the role of *ICE1* as a transcription factor in plant cold resistance.

**Key words:** Transcription factor; *ICE1* gene; Cold tolerance; Tomato

## INTRODUCTION

The tomato plant (*Lycopersicon esculentum* Mill.) is a highly cold-sensitive crop. At temperatures of 10, 8, and 5°C, its growth is respectively affected, incrementally decreased, and then stopped completely. Low temperatures often result in a shorter growth period, growth retardation, plant dwarfism, and decreased yield (Lin et al., 2000). In recent years, studies addressing cold tolerance found that cold-induced genes play important roles in the tomato plant's resistance to low temperatures and cold acclimation (Thomashow, 1999). The transcription factor CRT/DRE-binding factor (*CBF*) binds to the promoter of a low temperature-induced gene containing the CRT/DRE regulatory elements to regulate the gene's expression level when induced by a series of low temperatures, and to improve the plant's cold resistance (Stockinger et al., 1997; Liu et al., 1998). *CBF* has been widely used in genetic engineering to improve cold resistance in crops (Hsieh et al., 2002; Savitch et al., 2005). *CBF* can also regulate the expression levels of multiple functional genes in plants under drought, high salt, and low-temperature stresses. Li et al. (2010) found that *CBF1* is expressed in cucumber plants under low temperature and salt stresses, but not under drought or abscisic acid stress.

The regulation of transcription factors and effector genes, collectively called cold-regulated (*COR*) genes, is necessary for cold tolerance. INDUCER of CBF EXPRESSION1 (*ICE1*) is an upstream transcription factor that controls the transcription of *CBF*, which activates many downstream genes to confer cold tolerance on plants. *ICE1* encodes a bHLH transcription factor similar to the transcriptional activation gene, *MYC*, which is inactive at normal temperatures but specifically binds with *CBF3* promoter sequences at low temperatures, inducing *CBF3* expression. When *CBF3* binds to the DRE sequence in the promoter of a downstream gene, it induces the expression of a series of downstream *COR* genes and other genes that play a role in the plant's adaptation to cold. Up-regulating this pathway, using an *ICE1* transgene, has been shown to improve cold resistance and growth, without producing abnormalities (Chinnusamy et al., 2003). Presently, transgenic *ICE1* plants, including *Arabidopsis* (Chinnusamy et al., 2003), citrus (Huang, 2005), *Populus suaveolens* (Lin et al., 2007), apple (Zhao, 2007), *Cymbidium* (Zhang et al., 2008), rice (Xiang et al., 2008), tobacco (Zheng et al., 2009; Lin et al., 2011), cucumber (Liu et al., 2010), and chrysanthemum (Chen et al., 2012), have improved cold resistance when compared with non-transgenic plants. In the present research, we transformed the tissue-cultured rhubarb tomato variety with *ICE1* in a plant expression vector using *Agrobacterium tumefaciens*. These experiments were designed not only to significantly improve the cold resistance of the tomato plant, but also to provide information to assist further studies on the role of *ICE1* in cold resistance.

## MATERIAL AND METHODS

### Material

The experiments were performed in the research center of the Northeast Agricultural

University and Education Ministry's Key Laboratory of Biology for Vegetables in Cold Regions in Heilongjiang Province in 2007 to 2009.

## **Tomato**

The tomato variety used was a tissue culture-derived rhubarb tomato, provided by Education Ministry's Key Laboratory of Biology for Vegetables in Cold Regions in Heilongjiang Province. The variety was grown in a tissue culture incubator to produce plant materials for transformation.

## **Vector and reagent**

Recombinant plasmid pMD18-*ICE1* was contributed by Associate Professor Yinying Zheng, College of Life Sciences, Shihezi University, and *Agrobacterium* strain EHA105 and plant expression vector pCAMBIA3301 (referred to as p3301 thereafter) were contributed by Associate Professor Wang Yong, Northeast Agricultural University. Restriction endonucleases (*Nco*I and *Bst*EII), T4 DNA ligase, kanamycin (Kan), and carboxybenzyl penicillin (Carb) were purchased from the Takara Company (Takara, Japan). Molecular weight markers Trans2k and Trans15k were purchased from TransGen Biotech Co., Ltd. (Beijing, China).

## **Culture media**

The media used in the experiments included basal medium containing Murashige and Skoog medium (MS) + 2.0 mg·L<sup>-1</sup> 6-benzyladenine (6-BA) + 0.2 mg·L<sup>-1</sup> indoleacetic acid (IAA); co-culture medium containing MS + 2.0 mg·L<sup>-1</sup> 6-BA + 0.2 mg·L<sup>-1</sup> IAA; selective medium containing MS + 2.0 mg·L<sup>-1</sup> 6-BA + 0.2 mg·L<sup>-1</sup> IAA + 20 mg·L<sup>-1</sup> Kan + 300 mg·L<sup>-1</sup> Carb; and rooting medium containing 1/2 MS + 0.5 mg·L<sup>-1</sup> IAA + 10 mg·L<sup>-1</sup> Kan + 300 mg·L<sup>-1</sup> Carb.

## **Methods**

### **Primer design and polymerase chain reaction (PCR)**

Primers were designed based on the *A. thaliana ICE1* mRNA sequence (GenBank accession No. AY195621) as reported by Chinnusamy et al. (2003). The upstream primer P1 (5'-CCATGGGTCTTGACGGAAACAAT-3') and downstream primer P2 (5'-GGTAACCTCAGATCAT ACCAGCATACCCTG-3') were synthesized with *Nco*I and *Bst*EII sites, respectively. PCR conditions were as follows: pre-denaturation at 94°C for 5 min; denaturation at 94°C for 30 s, following by 30 cycles of annealing at 59°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. The PCR reactions contained 1 µL template, 2.5 µL 10X Taq buffer, 2.5 µL 2.5 mmol·L<sup>-1</sup> dNTP, 1 µL each primer, 0.1 µL Taq DNA polymerase, and water to 25 µL. After agarose gel electrophoresis, PCR products were recovered using a DNA Gel Extraction Kit (Harbin Marine Biotechnology Co., Harbin, China).

### **Construction of the expression vector p3301-ICE1**

*ICE1* and p3301 were cut with *Nco*I and *Bst*EII and purified for ligation with T4 DNA ligase. The ligation reactions were transformed into *E. coli* DH5α competent cells using the freeze and thaw

method, and plated out and screened for positive colonies. Plasmid DNA was extracted and sequenced.

### ***Agrobacterium transformation***

Recombinant expression plasmids were transformed into *Agrobacterium* EHA105 by freezing and thawing in liquid nitrogen, and plated on YEB agar medium containing 50 mg·L<sup>-1</sup> Kan. Colonies were picked up after 48 h, and individually grown in liquid medium on a shaker. The plasmid was extracted and used as a template for PCR amplification with the gene-specific primers P1 and P2. The transformed *A. tumefaciens* EHA105 was stored at -80°C for subsequent use.

### ***Tomato transformation***

Under sterile conditions, the tomato cotyledons were cut into squares of 0.5 cm x 0.5 cm, and then placed on the pre-culture medium for two days. *A. tumefaciens* EHA105 containing the recombinant plasmid p3301-*ICE1* was grown with shaking to an OD600 of between 0.4 and 0.6, and used for infecting the pre-cultured cotyledon tissue. The infection lasted 8 to 10 min and excess bacterial liquid was blot-dried. The infected explants were placed on the co-culture medium for 36 h, and then on the selective medium, which was changed every 14 d. When the cotyledon calli differentiated into small buds, the buds were cut off and placed on the rooting medium containing Kan to regenerate resistant seedlings.

### ***Detection of transgene and analysis of transgene expression***

The genomic DNA of regenerated tomatoes was used as template in PCR reactions to screen and identify positive plants. Non-transgenic rhubarb tomatoes were used as a negative control. For quantitative reverse transcriptase PCR (qRT-PCR), a housekeeping gene was used as an internal control. Using the Trizol method, total RNA from PCR positive-plants was extracted (after exposure to dark conditions in an incubator at 4°C for 12 h) and qRT-PCR was performed. The transformed and control plants were grown at 4°C for 0, 24, and 72 h, and harvested to measure the leaf physiological indicators related to cold resistance, such as malondialdehyde (MDA) and proline (Pro) contents, and peroxidase (POD) and catalase (CAT) activities. The experiments were conducted in triplicate and the data were averaged. The ninhydrin colorimetric method was used to measure Pro content (Zou, 1998), the thiobarbituric (TBA) method was used to measure the MDA content (Zhang et al., 1989), the guaiacol method was used to measure POD activity (Cakmak and Marschner, 1992), and the hydrogen peroxide decomposition volume method was used to measure CAT activity (Zhao et al., 1998).

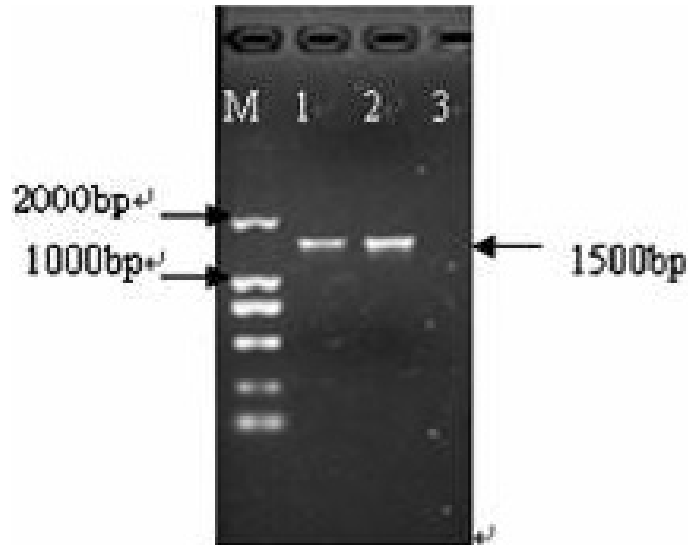
### ***Data analysis***

Microsoft Excel software was used for the statistical analysis.

## **RESULTS**

### ***PCR amplification of ICE1***

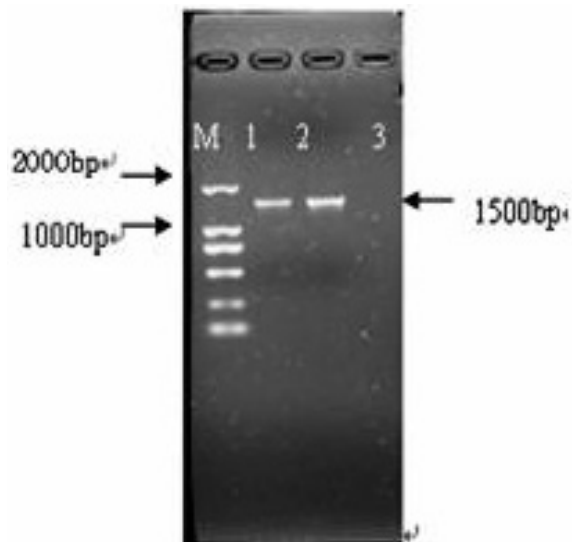
With the plasmid pMD18-*ICE1* as the template, and P1 and P2 as the primers, the cDNA coding region of *ICE1* was amplified, producing a band of anticipated size (1500 bp, Figure 1).



**Figure 1.** Electrophoresis of the PCR products of pMD18-ICE1. *Lanes 1 and 2*, pMD18-ICE1; *Lane 3*, water; *M*, DNA marker Trans2k.

### Construction of p3301-ICE1

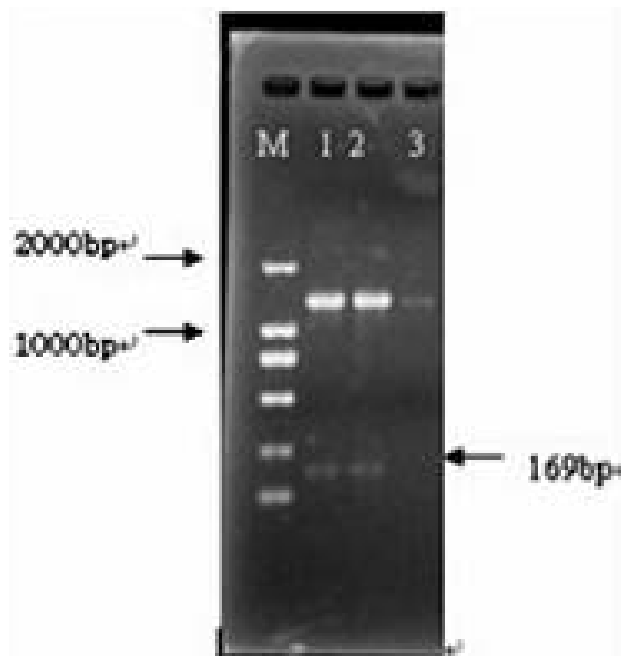
The recombinant plasmid p3301-ICE1 was verified by an *Nco*I and *Bst*EII double digestion and produced expected PCR bands at 1500 bp (Figure 2-1, 2-2). The *Bgl*II (located at 169 bp inside *ICE1*) digestion produced a band of approximately 169 bp (Figure 2-3), indicating that the expression vector p3301-ICE1 obtained contained *ICE1*.



**Figure 2-1.** Electrophoresis of PCR products of p3301-ICE1. *Lane 1*, pMD18-ICE1 amplification products; *Lane 2*, p3301 amplification products; *Lane 3*, water; *M*, DNA marker Trans2k.



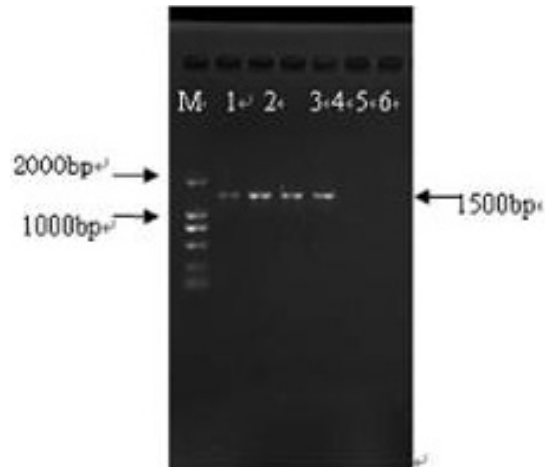
**Figure 2-2.** Enzyme-digested products of plant expression vector p3301-ICE1. *Lanes 1 and 2*, p3301-ICE1; *Lane 3*, P3301; *Lane 4*, pMD18-ICE1 amplification products; M, DNA marker Trans15k.



**Figure 2-3.** BglII-digested electrophoresis pattern. *Lane 1*, pMD18-ICE1 digested by BglII; *Lane 2*, p3301-ICE1 digested by BglII; *Lane 3*, pMD18-ICE1 amplification products; M, DNA marker Trans2k.

### Transformation of p3301-*ICE1* into *Agrobacterium* EHA105

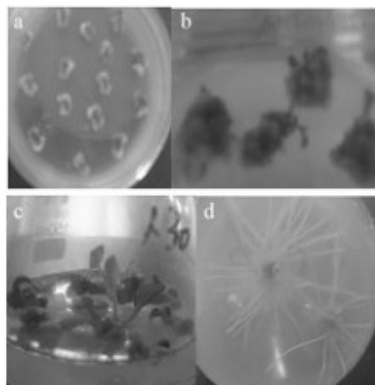
When *ICE1*-specific primers P1 and P2 were used to amplify the DNA from *Agrobacterium* EHA105 transformation with p3301-*ICE1*, an amplicon of expected 1500 bp was obtained, indicating the strain was successful (Figure 3).



**Figure 3.** PCR product of *Agrobacterium* EHA105 in individual colonies transformed with p3301-*ICE1*. *Lane 1*, p3301-*ICE1*; *Lanes 2-4*, *Agrobacterium* EHA105 (p3301-*ICE1*) colonies; *Lane 5*, water; *Lane 6*, non-transformed *Agrobacterium* EHA105; M, DNA marker Trans2k.

### Transgenic production

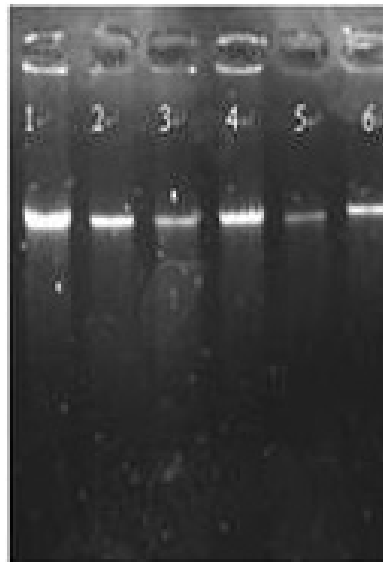
Transformed tomato cotyledons were placed on the selection medium and approximately 14 days later calli began to appear (Figure 4a), and they differentiated into bud clusters approximately 40 days later (Figure 4b). When the buds grew to approximately 2 cm long (Figure 4c), they were cut off and transferred to rooting medium. Approximately 10 days later, the tomato plants began to root (Figure 4d). Finally, five normally growing tomato plants transformed with *ICE1* were produced.



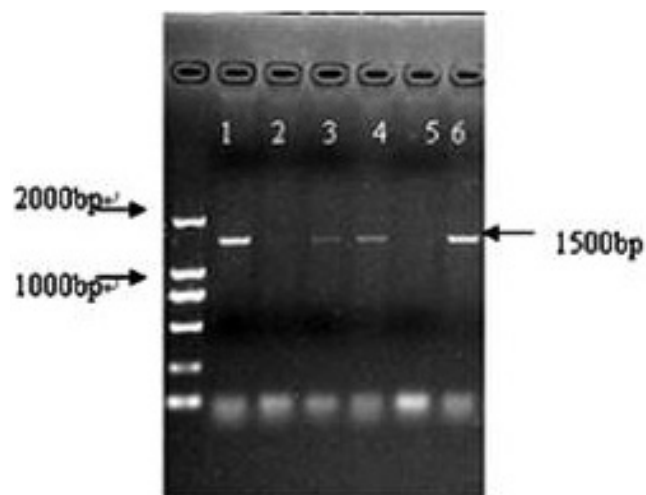
**Figure 4.** Transgenic tomato plants containing *ICE1* after *Agrobacterium tumefaciens*-mediated transformation. a, calli; b, differentiation of callus and bud clusters; c, growth of bud clusters; d, rooting.

### Molecular analysis of transgenic tomato

The genomic DNA was extracted from Kan-resistant plants using the CTAB method (Figure 5-1) and used for a PCR analysis with the *ICE1*-specific primers P1 and P2. The results revealed expected bands (1500 bp) in three tomato plants and the positive control (Figure 5-2). These three PCR-positive plants were subjected to a 4°C low temperature treatment for 12 h, and an RT-PCR analysis revealed that the three plants had intact *ICE1* RNA (Figure 5-3) and the amplicons were 1500 bp long (Figure 5-4).

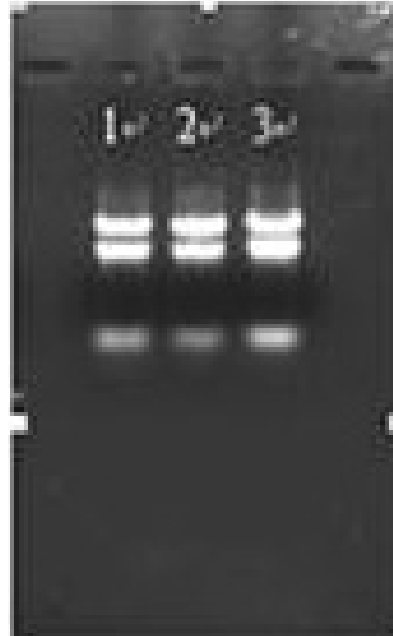


**Figure 5-1.** Electrophoresis of *ICE1* transgenic tomato DNA. Lanes 1-5, transgenic tomato; Lane 6, non-transformed tomato.

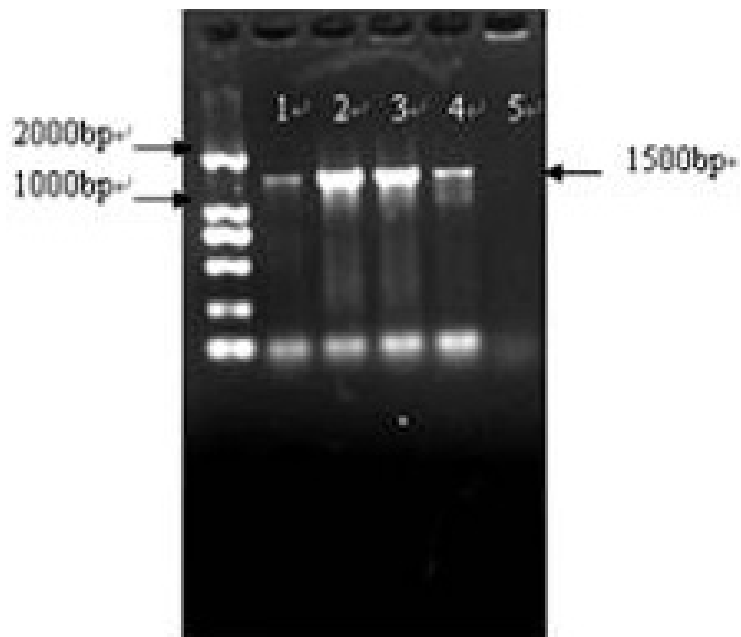


**Figure 5-2.** PCR detection of *ICE1* transgene in tomato plants. Lanes 1-5, transgenic tomato; Lane 6, p3301-*ICE1*; M, DNA marker Trans2k.





**Figure 5-3.** Electrophoresis of tomato RNA. *Lanes 1-3*, transgenic tomato.



**Figure 5-4.** RT-PCR detection of ICE1 transgene in tomato plants. *Lanes 1-3*, transgenic tomato; *Lane 4*, positive control; *Lane 5*, negative control; M, DNA marker Trans2k.

## Cold resistance of transgenic plants

The MDA content in the transgenic plants showed a declining trend with increasing exposure to low-temperature from 0 h to 72 h, although the increase was slight from 0 h to 24 h. This was also true for the control plants. At 72 h, the content of MDA in transgenic plants was significantly lower than that of the control plants (Figure 6-1). The Pro contents of transgenic plants and control plants increased under low-temperature stress. Compared with control plants, the Pro content in the transgenic plants increased with increasing stress duration (Figure 6-2). The changes in CAT and POD activity followed similar trends in the transgenic and control plants, increasing first and then decreasing over the low-temperature stress period. Under low-temperature stress, the CAT and POD activity levels in the transgenic plants increased significantly compared with those in the control plants. The CAT and POD activity levels were at their highest at 24 h (Figure 6-3, 6-4).

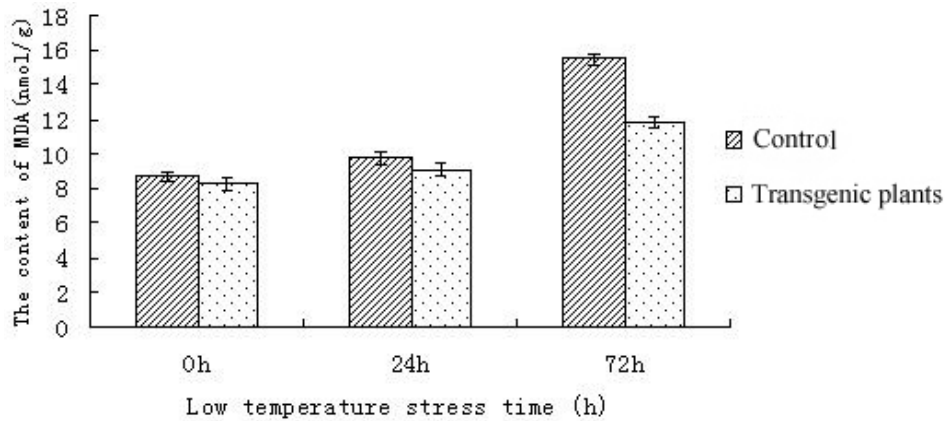


Figure 6-1. Malondialdehyde (MDA) content in transgenic ICE1 tomato plants under cold stress conditions.

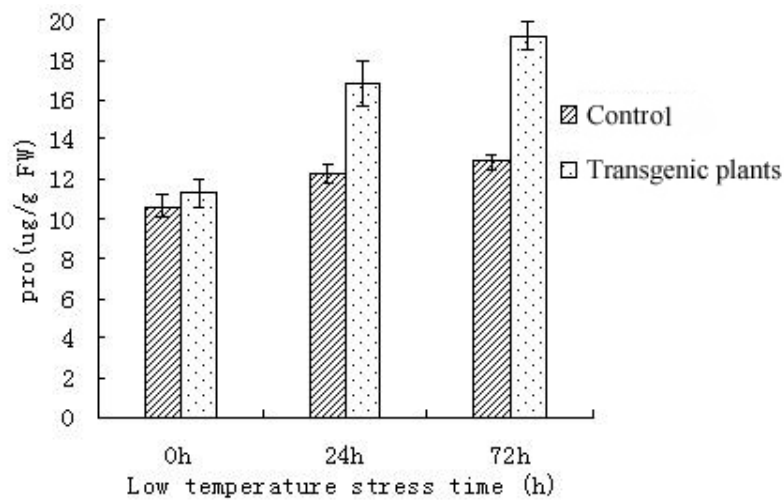


Figure 6-2. Proline (Pro) content in transgenic ICE1 tomato plants under cold stress conditions.

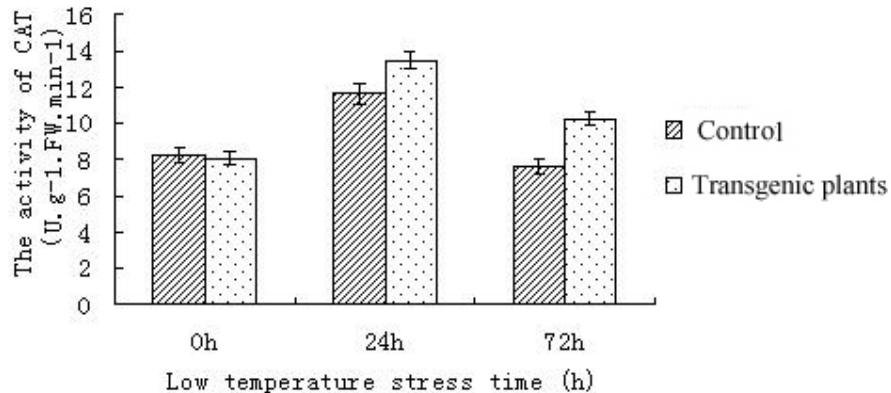


Figure 6-3. Effect of low temperature on catalase (CAT) activity in transgenic ICE1 tomato plants.

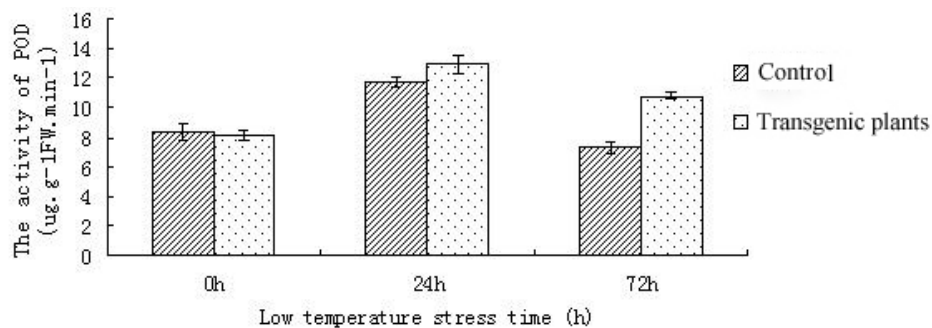


Figure 6-4. Effect of low temperature on peroxidase (POD) activity in transgenic ICE1 tomato plants.

## DISCUSSION

Chinnusamy et al. (2003) found that the constitutive expression of *ICE1* increases the transcriptional expression of *CBF3* under low temperatures and improves the ability of transgenic plants to resist the cold (Agarwal et al., 2006; Miura et al., 2007). Lee et al. (2005) found that overexpression of the *ICE1* gene does not affect normal growth of *A. thaliana* and significantly improves the frost resistance in transgenic *A. thaliana* seedlings. Many low-temperature response genes are controlled by *ICE1*, not only under low temperatures, but also at normal temperatures.

Ma (2007) genetically modified corn successfully using the expression vector pCambia3301. In the present research, *ICE1* was inserted into the binary vector successfully, using a two-step digestion with *Nco*I at 37°C and *Bst*EII at 60°C. During the selection process for transgenic calli, we found that Kan at 10 mg·L<sup>-1</sup> significantly inhibited the induction of the cotyledon's calli and the regeneration of adventitious buds. At higher Kan concentrations, the frequency of transgenic events decreased significantly, and growth vigor of the plant was diminished. When the Kan concentration was increased to 20 mg·L<sup>-1</sup>, the adventitious buds remained undifferentiated for untransformed calli. Therefore, Kan at 20 mg·L<sup>-1</sup> was chosen for transgenic callus selection.

In our experiments, transgenic *ICE1* tomato plants were subjected to low-temperature stress. The expression of the *ICE1* transgene resulted in reduced MDA content, increased

Pro content, and increased CAT and POD activities. The MDA level could be interpreted as a peroxidation indicator of membrane lipid, which may, to a certain extent, reflect the quantity of damaged cells. Pro is an important and effective osmotic adjuster in plant cells, and is shown to accumulate in biotically- and abiotically-stressed plants. CAT and POD are key enzymes that scavenge active oxygen radicals in plants, to reduce injury to the plant (Ma et al., 1998). We also found that MDA and Pro contents, and the CAT and POD activity levels, in the transgenic tomato were roughly the same as those in control plants before the cold stress. These data suggest that *ICE1* has no influence on the physiology and biochemistry of plants grown at a normal temperature. These results are consistent with those of Chinnusamy et al. (2003). With prolonged exposure to low-temperature, Pro content and CAT and POD activity levels in the transgenic *ICE1* plants were significantly higher than those in the control plants. This is probably due to the induction of the *ICE1* transgene overexpression by low temperatures for cold resistance. However, whether *ICE1* can effectively protect the tomato against low-temperature stresses and whether the low-temperature tolerance can be stably inherited remain to be investigated.

In conclusion, the present study shows that *ICE1* could effectively decrease cold-sensitivity in the tomato plant. This suggests that *ICE1*, as a promising transcriptional factor, could be targeted to improve cold-resistance in crops through genetic engineering. However, field trials are needed to determine the effectiveness of the transgenic *ICE1* plants for their cold resistance.

### Conflicts of interest

The authors declare no conflict of interest.

### ACKNOWLEDGMENTS

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