



***Agrobacterium*-mediated transformation of tomato with the *ICE1* transcription factor gene**

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ABSTRACT. *ICE1* genes play a very important role in plants in cold conditions. To improve the cold resistance of tomato, the *ICE1* gene of *Arabidopsis thaliana* was used to construct the plant expression vector p3301-*ICE1*, and was overexpressed in tomato through *Agrobacterium*-mediated transformation. Five strains of resistant plants were obtained. PCR and half-quantitative results showed that the *ICE1* gene was transferred to tomato; three strains tested positive. After low-temperature stress treatment, praline content and peroxide and catalase

activities in the transgenic tomato plants were higher compared with non-transgenic controls, while malondialdehyde content was clearly lower.

Key words: Cold transcription factor; *ICE1* gene; Tomato; Plant expression vector

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is a highly cold-sensitive crop. Its growth is hindered when the temperature drops below 10°C, gradually decreases at 8°C, and completely stops when the temperature reaches 5°C. Because of low temperatures, tomato plants often show a reduced growth period, growth retardation, dwarfism, and yield decreases (Lin et al., 2000). In recent years, plant freezing tolerance has been much studied, and researchers have found that cold-induced genes play an important role in plant resistance to low temperature and cold adaptation processes (Thomashow, 1999). The transcription factor *CBF* (CRT/DRE-binding factor) combines with a low-temperature-induced gene promoter in the CRT/DRE control element and regulates gene expression over a range of low temperatures to improve plant cold resistance (Stockinger et al., 1997; Liu et al., 1998). *CBF* has been widely used in genetic engineering to improve crop resistance (Hsieh et al., 2002; Savitch et al., 2005) since it regulates the expression of multiple genes with functions related to drought, high-salt and low-temperature tolerance. Li et al. (2010) found that the *CBF1* gene was expressed in cucumber plants under low-temperature and salt stress, but not under drought stress or in response to ABA.

ICE1 (inducer of *CBF* expression 1) induces the expression of *CBF*, which regulates cold-regulated (*COR*) gene expression upstream of the transcription factor. *ICE1* encodes a bHLH transcription factor similar to the transcription activation gene *MYC*, inactive at normal temperature, which specifically binds to *CBF3* promoter sequences at low temperature and induces the expression of *CBF3*, which then binds to the downstream gene promoters of DRE sequences, inducing a series of downstream *COR* genes and other genes that play a role in plant cold adaptation. Thus, it improves the cold resistance of transgenic plants, without causing growth abnormalities (Chinnusamy et al., 2003). *ICE1* gene transformation has been shown to improve cold resistance compared with non-transgenic plants in *Arabidopsis* (Chinnusamy et al., 2003), citrus (Huang, 2005), *Populus suaveolens* Fisch (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). Therefore, in this experiment we used the cold resistance gene *ICE1* to construct a plant expression vector, which we transformed into the tomato cultivar “rhubarb” by *Agrobacterium*-mediated transformation. Our results not only have important significance for improving the cold resistance of tomato but also lay the foundation for further study of *ICE1* transcription factors in cold-resistance mechanisms of tomato.

MATERIAL AND METHODS

This study was conducted at the Northeast Agricultural University and Cold Region Vegetables Biology Key Laboratory of Heilongjiang Provincial Education Department from 2007 to 2009.

Tomato materials

The tomato variety used for experiments was the cultivar “rhubarb”, which was provided by the Cold Region Vegetables Biological Key Laboratory of the College of Horticulture in Northeast Agricultural University. Seeds were sown in the laboratory’s tissue culture incubator to establish a tomato genetic transformation system.

Carriers and reagents

The recombinant plasmid pMD18-ICE1 was a gift from Associate Professor Zheng Yinying of the College of Life Science at Shihezi University. The *Agrobacterium* EHA105 strains and plant expression vector pCAMBIA3301 (referred to as p3301) were provided by Associate Professor Wang Yong at Northeast Agricultural University. Restriction endonucleases (*Nco*I and *Bst*EII), T4 DNA ligase, kanamycin (Kan), and carboxybenzyl penicillin (Carb) were purchased from TaKaRa Co. (Dalian, China). Standard DNA molecular weight markers Trans2k and Trans15k were purchased from Wholly-Gold Biotechnology Co. (Beijing, China). *Escherichia coli* DH5 α was maintained in our laboratory.

Culture medium

The preliminary medium was MS + 2.0 mg/L 6-BA + 0.2 mg/L IAA. The co-culture medium was MS + 2.0 mg/L 6-BA + 0.2 mg/L IAA. The selective medium was MS + 2.0 mg/L 6-BA + 0.2 mg/L IAA + 20 mg/L Kan + 300 mg/L Carb. The rooting medium was 1/2 MS + 0.5 mg/L IAA + 10 mg/L Kan + 300 mg/L Carb.

Primer design and polymerase chain reaction (PCR) system

According to the *Arabidopsis thaliana* *ICE1* gene mRNA sequence (GenBank accession No. AY195621) reported by Chinnusamy et al. (2003), we designed a pair of PCR amplification primers, namely upstream primer P1: 5'-CCATGGGTCTTGACGGAAACAAT-3' and downstream primer P2: 5'-GGTAACCTCAGATCATACCAGCATACCCTG-3'. The upstream and downstream primers included *Nco*I and *Bst*EII restriction enzyme sites at their 5'-ends, based on the plasmid pMD18-ICE1 as a template. PCR conditions were as follows: 94°C preliminary denaturation for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR mixture included 1 μ L template, 2.5 μ L 10X Taq buffer, 2.5 μ L 2.5 mM dNTPs, 1 μ L forward and reverse primers, 0.1 μ L Taq DNA polymerase, and water to make up the volume to 25 μ L. After agarose gel electrophoresis, PCR products were recovered using the DNA Gel Extraction kit from the Harbin Marine Biotechnology Co. (Harbin, China).

Construction of the expression vector p3301-ICE1

For *Nco*I and *Bst*EII double digestion of *ICE1* and p3301, 1 μ L *Nco*I was added to each 50- μ L reaction mixture and incubated at 37°C for 4 h. After gel extraction and purification, 1 μ L *Bst*EII was added and the reaction mixture was incubated at 60°C for 4 h. After electrophoresis and recovery, the double-digested plant expression vector p3301 and gene

fragment were ligated together by T4 DNA ligase according to manufacturer instructions. The reaction mixture included 6 μL *ICE1*, 2 μL p3301 plasmid, 1 μL 10X T4 DNA connection buffer, 1 μL T4 DNA ligase, and ddH₂O to make the volume up to 10 μL ; this was incubated at 16°C overnight. Competent *E. coli* DH5 α cells were transformed by the freeze-thaw method, and positive recombinants expressing the plasmid were screened and sequenced.

***Agrobacterium* transformation**

The positive recombinant cells were used to transform the plasmid into *Agrobacterium* EHA105 using liquid nitrogen by the freeze-thaw method. The *Agrobacterium* was grown on YEB agar culture medium containing 50 mg/L Kan for 48 h. The plasmid was extracted after picking single white colonies and resuspending the bacteria in medium. The plasmid was used as the template with the specific primers P1 and P2 for PCR amplification, and the PCR product was purified by 1% agarose gel electrophoresis. After colony propagation, the transformed *Agrobacterium tumefaciens* EHA105 was kept at -80°C for future use.

Establishment of the tomato genetic transformation system

Under sterile conditions, tomato cotyledons were cut into 0.5 x 0.5-cm sections and placed on pre-culture medium. The tomato cotyledons were preconditioned for 2 days, and then infected with *A. tumefaciens* EHA105 containing the recombinant plasmid p3301-ICE1 that was diluted 1:100 (v/v) to an OD₆₀₀ of 0.4 to 0.6; the infection time was 8-10 min, and excess bacterial solution was blotted. The infected explants were placed on co-culture medium for 36 h, and then transferred to selective co-culture medium, which was changed every 14 days. When the cotyledon callus differentiated into small buds, the buds were excised and transferred to rooting medium to continue resistance screening. Ultimately, resistant seedlings were acquired.

Genetically modified tomato detection

Genomic DNA from the genetically modified tomatoes was extracted and used as a template for PCR amplification to screen and identify positive plants. Cultivated rhubarb tomatoes that were not transformed with the *ICE1* gene were used as a negative control (because RNA was extracted from the transgenic plants for RT-PCR, and no housekeeping genes could be used as a positive control). Using the Trizol method, total RNA was extracted from PCR-positive plants kept in the dark condition at 4°C of low temperature treatment for 12 h in the incubator and RT-PCR was performed. To assess cold resistance, the transformed and control plants were kept at 4°C for low-temperature treatment for 0, 24, or 72 h, and then physiological indicators related to cold resistance were measured in the leaves, including the malondialdehyde (MDA) and proline (Pro) content, and peroxidase (POD) and catalase (CAT) activities. This experiment was repeated three times, and the average values were used for data analysis. The ninhydrin colorimetric method was used to measure Pro content (Zou, 1998), the MDA content was measured by the thiobarbituric method (Zhang, 1989), POD activity was measured using the guaiacol method (Cakmak and Marschner, 1992), and CAT activity was measured using the hydrogen peroxide decomposition volume method (Zhao et al., 1998).

Data analysis

The Microsoft Excel software was used for statistical analysis.

RESULTS

Effect of *Agrobacterium* infection concentration on tomato genetic transformation

Table 1 shows that when the bacterial suspension concentration was $OD_{600} = 0.2$, adventitious bud differentiation rate was low, only 1.7%, and there was no *Agrobacterium* growth in the bacteriostatic medium. With increased concentration of *Agrobacterium*, adventitious bud differentiation rate significantly increased; at $OD_{600} = 0.4$, tomato adventitious bud differentiation rate was the highest, about 5.0%. Also, *Agrobacterium* growth was moderate and explants less contaminated. At $OD_{600} = 0.5$, *Agrobacterium* growth was rapid, with severe medium contamination, tomato explant browning and eventual death due to bacterial toxicity and lowered conversion rate. Therefore an *Agrobacterium* concentration of $OD_{600} = 0.4$ was deemed appropriate for the working bacterial suspension concentration.

Table 1. Influence of *Agrobacterium* concentration on tomato genetic transformation.

Bacteria liquid concentration (OD_{600})	Explant. (No. piece)	Adventitious bud (No. piece)	Adventitious bud differentiation rate (%)
0	60	0	0 ^{ab}
0.2	60	1	1.7 ± 0.04 ^c
0.3	60	2	3.3 ± 0.16 ^{bb}
0.4	60	3	5.0 ± 0.11 ^{ba}
0.5	60	1	1.7 ± 0.14 ^c
0.6	60	0	0 ^{ab}

Capital letters indicate that significant difference exists at the level of $\alpha = 0.01$.

Influence of *Agrobacterium* infection time on tomato genetic transformation

Table 2 shows that in explants with an *Agrobacterium* infection time of 10 min, adventitious bud differentiation rate was significantly higher than in other treatments, and other infection times did not show a significant difference in adventitious bud differentiation rate. In explants with an *Agrobacterium* infection time of 5 min, co-cultivation did not show any *Agrobacterium* growth, and there was no transformation. With an infection time of 10 min, tomato adventitious bud differentiation rate was 1.7%, when co-cultured with *Agrobacterium*, but there was less contamination. With infection of more than 20 min, co-culture medium was overgrown with *Agrobacterium*, there was contamination pollution, and explant browning was difficult to control, resulting in a drop in transformation rate. Therefore, 10 min was the appropriate infection time.

Influence of kanamycin on adventitious bud differentiation in cotyledons

Table 3 shows that tomato cotyledon explants were more sensitive to Kan, where 10 mg/L Kan already had a significant influence on cotyledon callus induction and adventitious bud differentiation, where callus induction frequency and adventitious bud differentiation rate significantly decreased, with growth potential also weakened. At Kan concentration up

to 20 mg/L, there were no cotyledon explants with adventitious bud differentiation. At Kan concentration greater than 20 mg/L, only part of the cotyledon and explants at the incision place was slightly swollen, and after 10 days, browning death gradually occurred. Therefore, 20 mg/L was the best Kan screening concentration for transgenic tomato cotyledons.

Table 2. Influence of *Agrobacterium* infection time on tomato genetic transformation.

Infection time (min)	Explant (No. piece)	Adventitious bud (No. piece)	Adventitious bud differentiation rate (%)
0	60	0	0 ^{bb}
5	60	0	0 ^{bb}
10	60	1	1.7 ± 0.07 ^{aa}
20	60	0	0 ^{bb}
30	60	0	0 ^{bb}

Capital letters indicate that significant difference exists at the level of $\alpha = 0.01$.

Table 3. Influence of kanamycin (Kan) on tomato cotyledon adventitious bud differentiation.

Kan (mg/L)	Explant (No. piece)	Callus (No. piece)	Callus induction rate (%)	Adventitious bud (No. piece)	Adventitious bud differentiation rate (%)
0	60	60	100 ± 0 ^{aa}	53	88.3 ± 0.87 ^{aa}
10	60	40	66.7 ± 0.96 ^{bb}	20	33.3 ± 0.31 ^{bb}
20	60	14	23.3 ± 1.37 ^{cc}	0	0 ^{cc}
30	60	0	0 ^{dd}	0	0 ^{cc}
40	60	0	0 ^{dd}	0	0 ^{cc}

Capital letters indicate that significant difference exists at the level of $\alpha = 0.01$.

Influence of different concentrations of Carb bacteriostatic effect and tomato cotyledon adventitious bud differentiation

Table 4 shows that bacteriostatic effect increased with Carb concentration between 100 and 400 mg/L, along with a rise in callus induction. However, adventitious bud differentiation rate in explants was very sensitive to Carb, when Carb concentration was greater than 400 mg/L, adventitious bud differentiation rate significantly decreased but was still higher than with other processing. During the basic experiment period, *Agrobacterium* growth was effectively hindered, and adventitious bud differentiation rate was the highest, about 5%. Thus, bacteriostatic agent Carb could effectively limit *Agrobacterium* growth and have a minimal impact on explant adventitious bud differentiation, and 300 mg/L Carb would be the best inhibitory concentration for transgenic tomato cotyledons.

Table 4. Screening for the effective concentration of the antibiotic carboxybenzyl penicillin (Carb) to inhibit *Agrobacterium* growth.

Carb (mg/L)	Explant (No. piece)	Pollution rate (%)	Callus (No. piece)	Callus induction rate (%)	Adventitious bud (No. piece)	Adventitious bud differentiation rate (%)
0	60	100	0	0 ^{ff}	0	0 ^{cc}
100	60	60	24	40 ± 1.27 ^{ee}	0	0 ^{cc}
200	60	28	43	71.6 ± 0.79 ^{dd}	1	1.7 ± 0.03 ^{bb}
300	60	8	55	91.7 ± 0.77 ^{aa}	3	5.0 ± 0.14 ^{aa}
400	60	0	54	90 ± 1.08 ^{bb}	0	0 ^{cc}
500	60	0	52	86.6 ± 0.60 ^{cc}	0	0 ^{cc}

Capital letters indicate that significant difference exists at the level of $\alpha = 0.01$.

PCR amplification of the target gene *ICE1*

Using the plasmid pMD18-*ICE1* as a template and the P1 and P2 primers, the cDNA coding region of the *ICE1* gene was obtained by PCR. The amplified band was 1500 bp, which was consistent with the expected results (Figure 1).

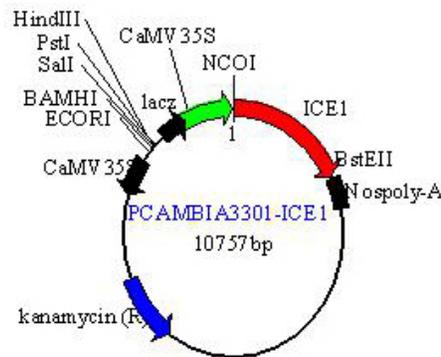


Figure 1. Expression vector pCAMBIA3301-*ICE1*. p3301-*ICE1* (approximately 11 kb) contains an approximately 1500-bp fragment of the *ICE1* gene of *Arabidopsis* under the CaMV35S promoter. The selectable marker gene is the NPTII gene driven by the *Nos* promoter. For genetic transformation of tomato, the plasmid was transformed into *Agrobacterium* EHA105 through the freeze-thaw method.

p3301-*ICE1* vector construction

PCR and *NcoI*/*BstEII* double digestion were used to identify the p3301-*ICE1* recombinant plasmid, and we obtained specific bands at 1500 bp on the gel (Figure 2, lanes 1 and 2). *BglII* (located in the *ICE1* gene at the internal position of 169 bp) restriction endonuclease digestion was used to confirm the identity of the PCR product. We obtained a fragment of nearly 169 bp as expected (Figure 2, lane 3), indicating that construction of the expression vector p3301-*ICE1* containing the *ICE1* gene fragment was successful.

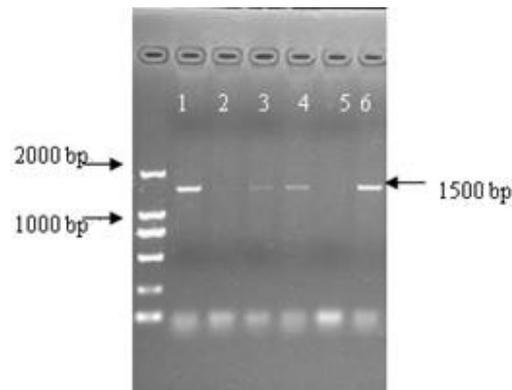


Figure 2. PCR detection of transgenic tomato. Lanes 1-5: PCR amplification of transgenic tomato genomic DNA; lane 6: positive control. The Kan-resistant tomato plants and positive controls both showed amplification of the expected band; the size of the *ICE1* primer amplification product was 1500 bp.

Transfer of the plant expression vector p3301-ICE1 into *Agrobacterium* EHA105

With the primers P1 and P2, PCR was performed on the recombinant plant expression vector p3301-ICE1, which had been transformed into *Agrobacterium*. A 1500-bp fragment was amplified (Figure 3), which indicated that the recombinant plasmid had been successfully transferred to *Agrobacterium* EHA105.

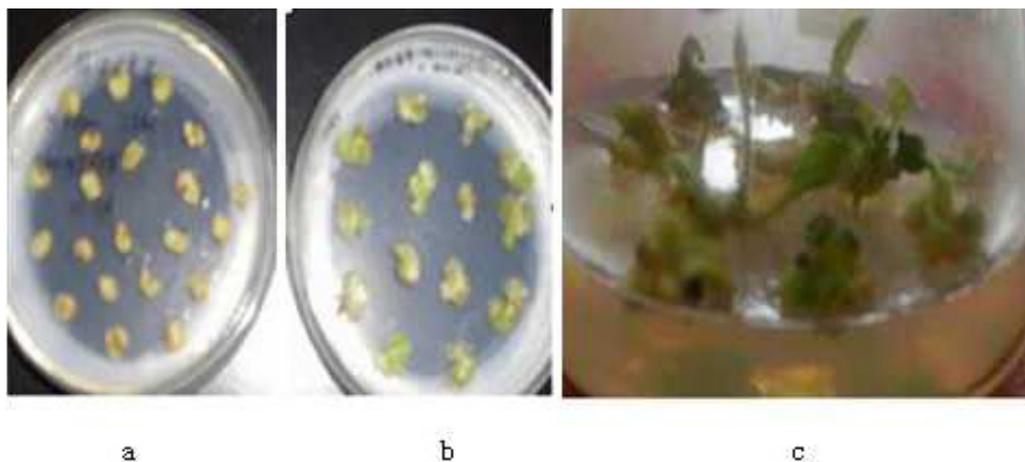


Figure 3. Tomato regeneration resistant buds of Kan screening medium. (a) Control, (b) resistant callus, (c) resistant buds. Three hundred explants were infected and 80 Kan-resistant calli were obtained, including 20 differentiation-resistant buds with a differentiation rate of 6.7%. PCR detection was conducted on five resistant plants, of which three were positive (a positive rate of 60%). The false-positive results are presumably because of an insufficient selective concentration of Kan.

Transgenic tomato plant generation

Transformed tomato cotyledons were placed on the selection medium, and after about 14 days, callus began to appear (Figure 3a). At about 42 days, the callus tissue began to differentiate into cluster buds (Figure 3b). When the callus buds reached about 2 cm (Figure 3c), they were excised and placed on rooting medium; after approximately 10 days, the tomato plants began to take root (Figure 3c). Ultimately, five tomato plants grew normally, which suggested that the *ICE1* gene had been transferred to the tomato.

Molecular detection of transgenic tomato plants

We used CTAB to extract the tomato genomic DNA (Figure 4, lane 1), which was amplified by PCR with the *ICE1* primers P1 and P2. Three Kan-resistant tomato plants and the positive control amplified the expected band; the size of the amplification product using the *ICE1* primers was 1500 bp (Figure 4, lane 2). These three PCR-positive plants were given a low-temperature treatment at 4°C for 12 h, and then RT-PCR detection was performed. All three plants produced an amplification product of 1500 bp (Figure 4, lane 3).

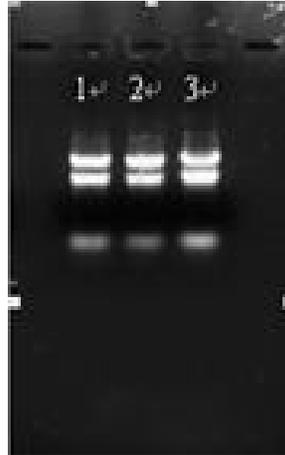


Figure 4. Electrophoresis of tomato genome. Lanes 1-3: GM *ICE1* genomic DNA (genomic DNA of transgenic *ICE1* tomato plants).

Physiological indices related to cold resistance of transgenic tomato plants

The MDA content in plants showed an increasing trend with low-temperature stress. Early in the low-temperature treatment (0 and 24 h), MDA content in transgenic and control plants changed only slightly. However, after 72 h of low-temperature stress, MDA content in the genetically modified plants was significantly lower than in control plants (Figure 5, lane 1).

Pro content in transgenic and control plants increased with low-temperature stress. Compared with control plants, Pro content in the transgenic plants increased with prolonged stress time (Figure 5, lane 2).

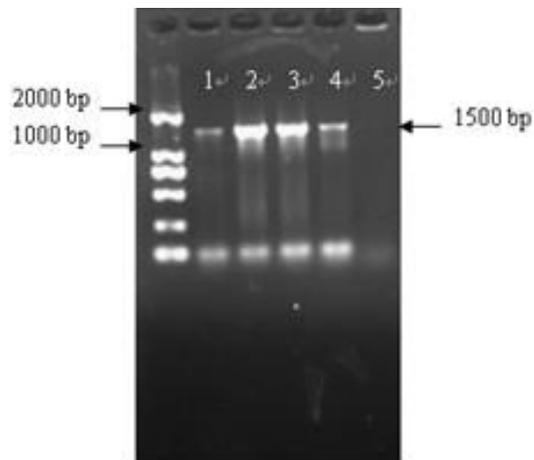


Figure 5. RT-PCR detection of transgenic tomato plants. Lanes 1-3: GM tomato; lane 4: positive control; lane 5: negative control. These three strains of PCR-positive plants we kept at a low temperature of 4°C for 12 h before RT-PCR; consequently, 1500-bp bands were amplified. These preliminary results showed that the *ICE1* gene was transferred to tomato.

CAT and POD activities showed a consistent trend, i.e., CAT and POD activities in the transgenic and control plants showed an initial increase followed by a decrease with continued low-temperature stress. After low-temperature stress, compared with control plants, CAT and POD activities in the transgenic plants increased significantly, and with the extension of stress time, differences in CAT and POD activities increased (Figure 5, lanes 3 and 4).

DISCUSSION

Chinnusamy et al. (2003) found that constitutive expression of the *ICE1* gene increased the transcription of *CBF3* at low temperature and improved the ability of transgenic plants to resist cold (Agarwal et al., 2006, Miura et al., 2007). Lee et al. (2005) found that overexpression of the *ICE1* gene did not affect the normal growth of *A. thaliana*, but significantly improved the frost resistance of transgenic seedlings. Many low-temperature response genes are not only controlled by *ICE1* at low temperature, but are also influenced by it at normal temperatures. The pCAMBIA3301 expression vector has been successfully used to genetically modify corn plants. In this study, we also tried to insert the *ICE1* gene into the binary vector pCAMBIA3301, but the two enzymes we used were *NcoI* and *BstEII*. *NcoI*'s optimum temperature is 37°C, while *BstEII*'s is 60°C, so we had to perform enzyme digestion step-by-step. Through trial and error, we found that enzyme digestion with *BstEII* first and then *NcoI* gave the best results, since no non-specific banding was observed. Thus, we eventually obtained the *ICE1* gene expression vector. Testing of the callus screening process showed that tomato cotyledons were sensitive to Kan. A concentration of 10 mg/L had a significant influence on cotyledon callus induction and adventitious bud regeneration; callus induction frequency and adventitious bud differentiation frequency decreased significantly, and growth vigor was also weakened. When the Kan concentration was increased to 20 mg/L in the medium, the cotyledon adventitious buds were still undifferentiated. Therefore, a Kan concentration of 20 mg/L was appropriate for the adventitious bud differentiation stage.

In this study, low-temperature stress treatments of the GM *ICE1* tomato plants showed that *ICE1* gene transformation reduced MDA content and increased proline content and CAT and POD activities. MDA content can be used as an indicator of the degree of membrane lipid peroxidation, which to a certain extent reflects the degree of cell damage. Proline is an important and effective organic osmotic adjustment substance in plant cells, and almost all types of stress lead to the accumulation of proline in plant cells. CAT and POD are key enzymes in eliminating reactive oxygen radicals in plants, and they function by scavenging reactive oxygen to reduce active oxygen damage to the plant (Ma et al., 1998). In this experiment, we found that MDA and proline contents and CAT and POD activities in the transgenic tomato were roughly the same as in control plants at the start of the stress treatment (0 h), indicating that *ICE1* was inactive at normal temperatures and had no influence on the physiology and metabolism of the plants, which was consistent with the study by Chinnusamy et al. (2003). As the duration of the low-temperature stress increased, proline content and CAT and POD activities in the transgenic plants significantly increased compared with the control plants; this was probably caused by overexpression of the exogenous *ICE1* gene induced by low temperature, thereby increasing the plant's cold resistance. However, these are only preliminary test results; whether the *ICE1* gene can truly withstand the test of low-temperature stress and whether this low-temperature tolerance is stably inherited requires further study.

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