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Development of a New Cold-Tolerant Maize (*Zea mays* L.) Germplasm Using the *ICE1* Gene from *Arabidopsis thaliana*

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ABSTRACT

To develop cold-tolerant maize germplasms and identify the activation of INDUCER OF CRT/DRE-BINDING FACTOR EXPRESSION (*ICE1*) expression in response to cold stress, RT-PCR was used to amplify the complete open reading frame sequence of the *ICE1* gene and construct the plant expression vector pCAMBIA3301-*ICE1*-Bar. Immature maize embryos and calli were transformed with the recombinant vector using *Agrobacterium tumefaciens*-mediated transformations. From the regenerated plantlets, three T1 lines were screened and identified by PCR. A Southern blot analysis showed that a single copy of the *ICE1* gene was integrated into the maize (*Zea mays* L.) genomes of the three T1 generations. Under low temperature-stress conditions (4°C), the relative conductivity levels decreased by 27.51%–31.44%, the proline concentrations increased by 12.50%–17.50%, the malondialdehyde concentrations decreased by 16.78%–18.37%, and the peroxidase activities increased by 19.60%–22.89% in the T1 lines compared with those of the control. A real-time quantitative PCR analysis showed that the *ICE1* gene was ectopically expressed in the roots, stems, and leaves of the T1 lines. *ICE1* positively regulates the expression of the *CBF* genes in response to cold stress. Thus, this study showed the successful transformation of maize with the *ICE1* gene, resulting in the generation of a new maize germplasm that had increased tolerance to cold stress.

KEYWORDS

Maize; *ICE1*; cold tolerance; inducer; maize; *Arabidopsis thaliana*

1 Introduction

Maize (*Zea mays* L.) is an important crop worldwide [1]. Frost in late spring severely impacts maize production in Northeastern China [2]. The germination of maize seeds has been examined using a series of enzymatic reactions to identify tolerance to this stress. The catalytic activities of enzymes are closely correlated with changes in temperature. Low temperatures during the sowing period delay emergence, with an average low temperature of 10°C, resulting in a reduction of the seed germination potential to 9.4% and the germination rate to 6.7%, compared with those of the control, thereby seriously affecting maize yield [3].

In 1998, Gilmour et al. [4] proposed that the presence of an inducer of the CRT/DRE-BINDING FACTOR (*CBF/DREB*) gene (INDUCER OF *CBF* EXPRESSION, *ICE*) plays an important role in the



regulation of *CBF/DREB1* gene expression. The *ICE1* gene encodes a bHLH transcription factor similar to the transcriptional activator MYC. At normal temperatures, *ICE1* exists in an inactive form that is unable to activate transcription. However, when plants suffer low-temperature stress, *ICE1* is activated and regulates *CBF/DREB1* expression by binding to a DRE/CRT element in the gene's promoter, initiating the *ICE1*–*CBF/DREB1*–*COR* cascade, and improving plant cold resistance. Post-transcriptional modifications of the *ICE1* protein, such as phosphorylation, dephosphorylation, ubiquitination, and a small ubiquitin-like modifier, are important for its activity. Low temperature activates MPK3/MPK6 activity, and activated MPK3/MPK6 inhibits the stability and transcriptional activity of the *ICE1* protein through the phosphorylation of its six conserved phosphorylation sites at low temperatures, thereby inhibiting the expression of the *CBF* gene and negatively regulating the tolerance of *Arabidopsis thaliana* to low-temperature stress [5]. The *ICE1* activity is negatively regulated by protease degradation through HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1 ligase of the ubiquitin E3 pathway [6,7]. The expression levels of a series of anti-stress-related genes may be regulated by the addition of an anti-stress-related transcription factor in plants that would be more effective than the insertion of a single anti-stress-related gene. In this study, the plant expression vector pCAMBIA3301-*ICE1* was constructed to include the *Bar* gene as the selective marker. Maize was transformed with this vector using *Agrobacterium tumefaciens*-mediated transformation. This work is important for the improvement of cold resistance in maize, and it lays a theoretical foundation for further studies of anti-stress mechanisms in maize.

2 Materials and Methods

2.1 Materials and Reagents

Immature embryos and embryogenic calli of the maize inbred line GSH9901 (a homozygous and stable inbred line covered by independent intellectual property rights bred by the research group), *Escherichia coli* (DH5 α), *Agrobacterium* (EHA105), the recombinant cloning vector plasmid pMD18T-*ICE1*, and the plant expression vector pCAMBIA3301 were provided by the Center for Plant Biotechnology of Jilin Agricultural University. PCR amplification reagents, DNA markers, and restriction enzymes were purchased from MBI. Other reagents were of domestic analytical grade.

2.2 Method

2.2.1 Construction of the Expression Vector pCAMBIA3301-*ICE1*

The complete open reading frame sequence of the *ICE1* gene (GenBank accession number: NM_113586.3) was cloned from wild-type *Arabidopsis*. Using seamless cloning technology, pMD18T-*ICE1* was used as the template to design the specific primers (P1S/P1AS) to amplify *ICE1* (Table 1). The 20- μ L PCR reaction comprised 10 μ L 2 \times Es Taq MasterMix, 1 μ L P1S, 1 μ L P1AS, 1 μ L *ICE1* plasmid, and 7 μ L dd water. The PCR conditions for *ICE1* amplification were as follows: 94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, followed by a final extension at 72°C for 10 min. The expression vector pCAMBIA3301 was double digested with *Nco*I and *Bst*EII and then incubated at 37°C for 3 h, followed by 65°C for 20 min. The PCR products and the enzyme digestion products were purified and recycled, respectively, and then combined using a seamless connection kit (CE Design Company) in accordance with the manufacturer's instructions. The reaction mixture comprised 50–200 ng vector fragment, 10–50 ng target fragment, 4 μ L 5 \times buffer, 2 μ L ligase, and dd water up to 20 μ L. The reaction was carried out at 37°C for 30 min, followed by 0°C for 5 min. The ligation products were transformed into *E. coli* DH5 α competent cells, and the positive clones were identified.

Table 1: Primer information

| Name | Sequence (5'-3') | Restriction site |
|---------|---|--|
| P1S | ACGGGGGACTCTTGACCATGGA GGTTCAGACTTTGGGGTA | Underlined is a homologous arm sequence with an <i>NcoI</i> restriction site and a pCAMBIA3301 sequence |
| P1AS | GGGGAAATTCGAGCTGGTCACCCT GCTTCAACAGATGCTTA | Underlined is a homologous arm sequence with a <i>BstEII</i> restriction site and a pCAMBIA3301 sequence |
| P2S | AGGTTTCAGACTTTGGGGTA | |
| P2AS | CTGCTTCAACAGATGCTTA | |
| P3BarS | TCAAATCTCGGTGACGGGC | |
| P3BarAS | ATGAGCCCAGAACGACGC | |
| P4S | TTAGTAGTAACCAACCACATC | |
| P4AS | GAGAACAAGAAGAAGAATC | |
| P5S | ATCTTGACTGAGCGTGGTTATTCC | |
| P5AS | GCTGGTCCTGGCTGTCTCC | |

2.2.2 *Agrobacterium*-Mediated Transformation of Maize

The recombinant plasmid was transformed into *Agrobacterium*, and the colonies containing the recombinant plasmid pCAMBIA3301-ICE1 were selected and inoculated into YEP liquid medium containing Kan following the method of Jiao et al. [8] with minor modifications. The vials containing the infection broth and calli were placed at 140 rpm on a shaker at 28°C during the infection process to allow greater contact between the infection fluid and calli, resulting in a greater infection rate.

2.2.3 Detection of Transgenic Plants

PCR Amplification

Genomic DNAs of the T1 lines were extracted using the CTAB method [9] and used as the templates for PCR detection of the *ICE1* and *Bar* genes. Transformed plants tested positive for the *ICE1* and *Bar* genes, whereas untransformed plants tested negative for these genes. The recombinant plasmid pCAMBIA3301-ICE1 was used as the positive control, and untransformed recipient maize plants were used as negative controls. The primers P2S, P2AS, P3BarS, and P3BarAS (Table 1) were designed on the basis of known sequences using Primer 5.0 software. The PCR reaction system and conditions for amplification of *ICE1* were as described earlier. PCR conditions for *Bar* gene amplification were as follows: 94°C for 5 min, 32 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 10 s.

Southern Blot Analysis of Transgenic Plants

DNA was extracted from the leaves of T₁ PCR-positive transgenic plants and digested with *Bam*HI. *ICE1* was used as the probe, and the biotin and DIG-labeled λ *Hind*III DNA (Beijing Bioco Laibo Technology Co., Ltd. (Beijing, China)) was used as the marker. Southern blotting was performed using a DIG DNA Labeling and Detection Kit I kit (Roche) in accordance with the manufacturer's instructions.

Quantitative Real-Time PCR Detection of Transgenic Lines

Transgenic plants determined to be positive for the transgene by Southern blotting were maintained at 4°C for 24 h. Total RNA was then extracted from the roots, stems, and leaves of these plants, reverse transcribed into cDNA, and diluted four-fold. Fluorescent quantitative real-time PCR (qRT-PCR) was carried out using the P4S, P4AS, P5S, and P5AS primers (Table 1). The maize β -*actin* gene (GenBank

accession number: NM_001252731.2) was used as a reference gene. The cDNAs of the cold-treated maize roots, stems, and leaves were analyzed using an Mx 3000 P FQ-PCR System (Jitai Biotech). A TaKaRa SYBR Premix Ex Taq™ kit was used in accordance with the manufacturer's instructions. Each 20- μ L PCR reaction comprised 10 μ L 2 \times SYBR Premix Ex Taq polymerase, 2 μ L primer P4S, 2 μ L primer P4AS, 2 μ L template, and dd water up to 20 μ L. Rapid two-step PCR conditions were used with an initial pre-denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 10 s for denaturation and 60°C for 30 s for the reaction. The relative expression of the target gene was analyzed using the $2^{-\Delta\Delta CT}$ method [10,11].

Determination of Physiological and Biochemical Indicators

The positive and untransformed plants were maintained at 4°C for 0, 24, or 72 h before changes in leaf relative conductivity, proline content, malondialdehyde (MDA) content, and peroxidase (POD) activity were measured. Each experiment was repeated three times to obtain an average. Relative conductivity was measured using the method of Petrov et al. [12]. The proline content was determined using the sulfosalicylic acid method, the MDA content by the thiobarbituric acid-spectrophotometric method, and POD activity using the guaiacol method [13].

Statistical Analysis

All results in this study were performed in more than three replicates. SPSS 19.0 software was used for statistical analysis of experimental measurement data. One way ANOVA were used to confirm the variability of results between treatments, respectively, $P < 0.05$ (*), $P < 0.01$ (**).

3 Results and Discussion

3.1 Construction and Identification of the pCAMBIA3301-ICE1 Plant Expression Vector

The plant expression vector pCAMBIA3301-ICE1 was digested with *Nco*I and *Bst*EII. The inducer sequence was amplified with its specific primer (Table 1). The products were separated by gel electrophoresis to identify the successful integration of the inducer sequence of the *ICE1* gene into the pCAMBIA3301 vector (Fig. 1).

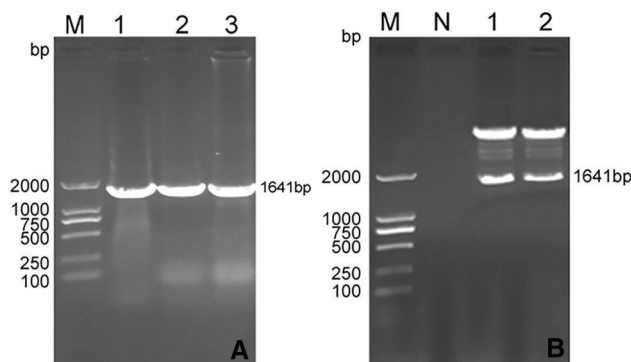


Figure 1: PCR and double digested verification recombinant plant expression vector. pCAMBIA3301-ICE1. (A): PCR to confirm the recombinant vector pCAMBIA3301-ICE1; M: DNA Marker DL 2000; 1–3: Recombinant plasmid; (B): double digested verification recombinant plant expression vector pCAMBIA3301-ICE1; M: DNA Marker DL 2000; N: water; 1–2: Recombinant plasmid

3.2 Production and Detection of Transgenic Plants on T₁ Generation

3.2.1 PCR Detection of T₁ Generation Plants

The plant expression vector pCAMBIA3301-ICE1 was transformed into the maize variety GSH9901, and three T₀-positive plants were detected by PCR. In total, 20 seeds of each of these T₀ plants were harvested and planted indoors to obtain T₁ plants. Genomic DNA was extracted from these plants and screened using PCR to determine the presence or absence of the *ICE1* gene (1,641 bp) and the herbicide-resistant *Bar* gene (552 bp). The recombinant plasmid pCAMBIA3301-ICE1 was used as the positive control, and untransformed recipient maize plants were used as negative controls. The results are shown in Fig. 2. Three transgenic positive maize plants GSH9901-1, -5, and -7 were successfully obtained.

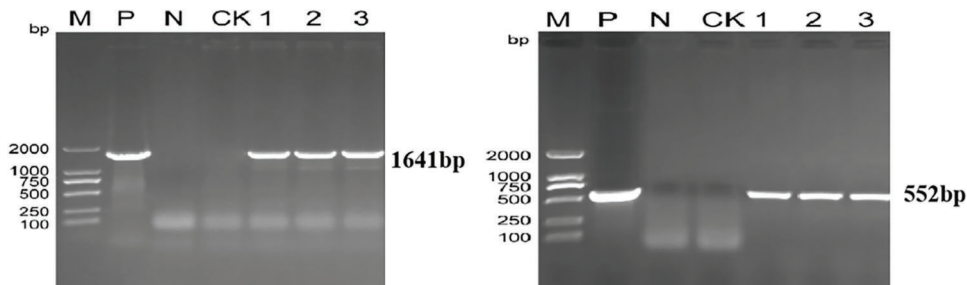


Figure 2: PCR detection of *ICE1* (A) and *Bar* (B) on T₁ generation plants. M: DNA Marker DL 2000; P: Positive control; N: water ; CK: Non-transformed plant genome; 1–3: T₁ transgenic plants

3.2.2 Southern Blot Analysis of T₁ Plants

Genomic DNAs were extracted from PCR positive plants and digested with *Bam*HI for 20 h before Southern blotting was performed using *ICE1* as the probe. As shown in Fig. 3, the untransformed plants did not show hybridization signals, whereas the three transgenic plants showed significant hybridization signals. This indicated that the *ICE1* gene was integrated into the maize genome as a single copy at different insertion sites.

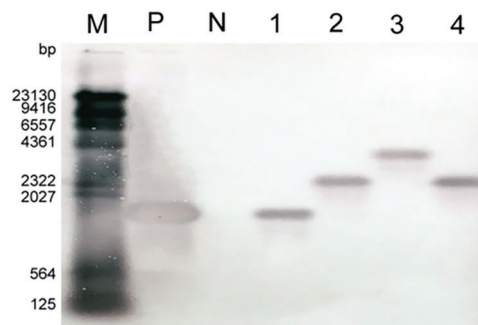


Figure 3: Southern blotting detection on T₁ generation of positive plants. M: λ *Hind*III DNA Marker; P: Positive control; N: Non-transformed plant genome; 1–3: T₁ transgenic plants

3.2.3 Detection of Physiological and Biochemical Indicators Related to Cold Tolerance in Transgenic Maize

First, the three-leaf stage overexpression (GSH9901-1, -5, and -7) and wild type (WT) plants were treated at 4°C for 0, 24, and 72 h (Fig. 4), and then, phenotypes were observed. After a 24-h cold treatment, less wilting and lodging were observed in the transformed than in the control plants.

The relative electrical conductivity, proline content, MDA content, and POD activity of the overexpressing transgenic and control plants were measured.

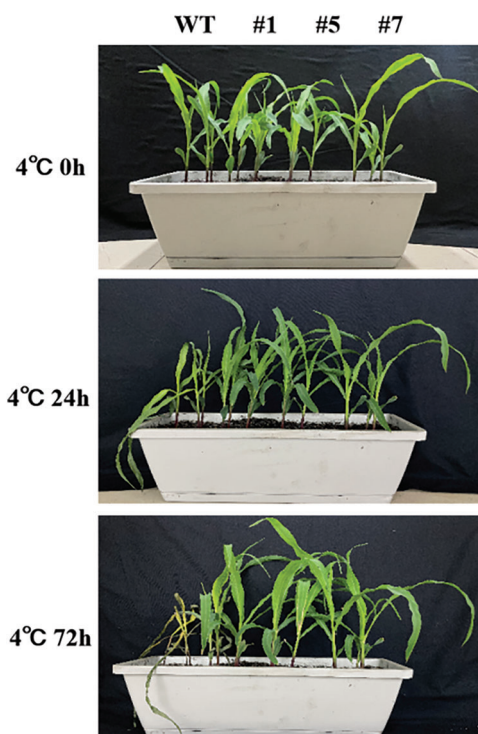


Figure 4: Phenotypic changes of maize under 4°C treatment

3.2.4 Determination of Relative Conductivity

Changes in the conductivity of plant tissue extravasation fluid reflect cell membrane damage and plant stress resistance. The greater the injury, the more likely the conductivity increases. As shown in Fig. 5, the relative electrical conductivities of *ICE1* transgenic plants at ambient temperatures did not differ significantly from the non-transformed plants. The relative electric conductivities of the leaves of transformed strains GSH9901-1, -5, and -7 increased by 21.04%, 35.59%, and 24.74%, respectively, after 24 h at 4°C and by 49.64%, 62.86%, and 48.16%, respectively, after 72 h at 4°C. The relative electrical conductivities of the WT leaves increased by 84.50% and 110.84% after 24 h and 72 h, respectively, at 4°C, indicating that the cell membranes of the transgenic lines were less damaged by the cold treatment.

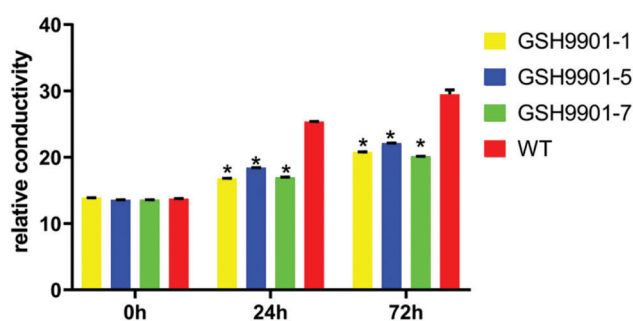


Figure 5: Relative conductivity changes on plants exposed to low temperature at different times (Duncan's method, $P < 0.05$)

3.2.5 Determination of Proline Concentrations

Proline is an important organic osmotic regulator in plant cells that accumulates under stress conditions, proline levels can, therefore, reflect the stress resistance of cells and the degree of cell damage. At room temperature, the proline concentrations of transformant leaves did not differ significantly from those of untransformed leaves (Fig. 6). After 24 h and 72 h at 4°C, the proline concentrations of the leaves of the transformed lines GSH9901-1 and -7 averaged $18.13 \mu\text{g}\cdot\text{g}^{-1}$ and $20.96 \mu\text{g}\cdot\text{g}^{-1}$, respectively, which were significantly higher than those of the untransformed WT line. The difference between $15.43 \mu\text{g}\cdot\text{g}^{-1}$ and $18.63 \mu\text{g}\cdot\text{g}^{-1}$ was significant at $P < 0.05$.

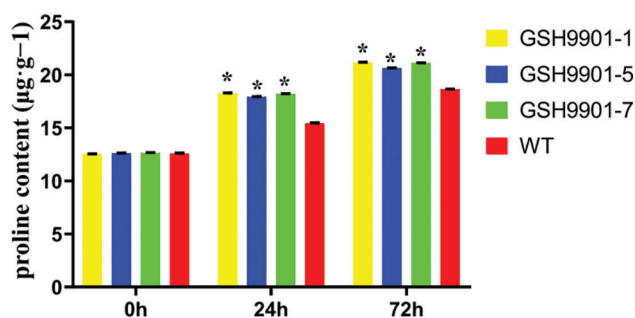


Figure 6: Proline concentrations ($\mu\text{g}\cdot\text{g}^{-1}$) of plants exposed to low temperature at different times (Duncan's method, $P < 0.05$)

3.2.6 Determination of Malondialdehyde Content

The MDA level reflects the degree of cell membrane peroxidation and a response to stress. After 24 h and 72 h at 4°C, the average MDA concentrations in the leaves of the transformed lines were $0.0500 \mu\text{mol}\cdot\text{g}^{-1}$ and $0.0513 \mu\text{mol}\cdot\text{g}^{-1}$ (Fig. 7), respectively, which were significantly lower than the $0.0598 \mu\text{mol}\cdot\text{g}^{-1}$ and $0.0628 \mu\text{mol}\cdot\text{g}^{-1}$, respectively, on the untransformed WT line. These differences were significant, but there were no significant differences at room temperature. Thus, the leaves of the transgenic lines exhibited reduced stress responses when compared with the leaves of the untransformed lines. The expression of *ICE1* in the leaves of the transgenic maize may, therefore, enhance membrane oxidization.

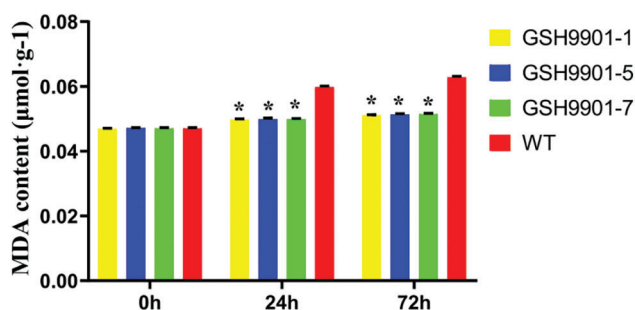


Figure 7: MDA concentration changes ($\mu\text{mol}\cdot\text{g}^{-1}$) on plants exposed to low temperature at different times (Duncan's method, $P < 0.05$)

3.2.7 Determination of Peroxidase Activity

In plants, POD is a key enzyme responsible for scavenging active oxygen free radicals. POD activity can, therefore, be used to measure the level of stress resistance in plants. At room temperature, there was no significant difference in the leaf POD activities of transformed and untransformed plants (Fig. 8).

After treatments at 4°C for 24 h and 72 h, the POD activity showed a trend of first increasing and then decreasing. The average POD activities of transformed plants increased by 19.60% and 22.89% at 24 h and 72 h, respectively, compared with the activities of untransformed plants, and the differences were significant. This indicates that the antioxidant capacity of the transgenic plants was greater than that of the non-transgenic plants.

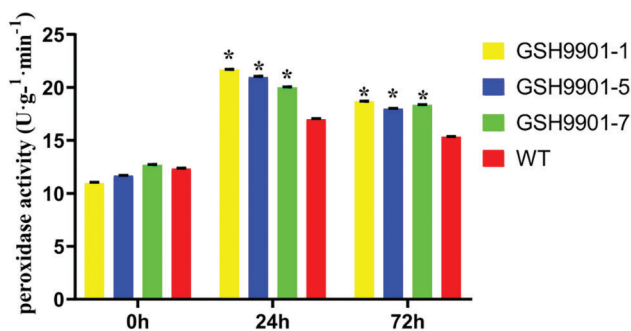


Figure 8: Peroxidase activity changes of plants exposed to low temperature at different times ($\text{U}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$) (Duncan's method, $P < 0.05$)

3.2.8 Detection of Transgenic Plants by qPCR

qRT-PCR with SYBR Green I was used to confirm that the Southern blot-positive transgenic plants contained the transgene. The relative expression levels of *ICE1* in the roots of transgenic maize plants GSHH9901-1, -5, and -7 were 5.19, 5.01, and 4.27, respectively, whereas the relative expression levels of *ICE1* in the stem tissue were 8.96, 9.09, and 7.62, respectively (Fig. 9). The relative expression levels of *ICE1* were the highest ($P < 0.05$) in the GSHH9901-1, -5, and -7 leaves, at 10.82, 10.42, and 11.26, respectively.

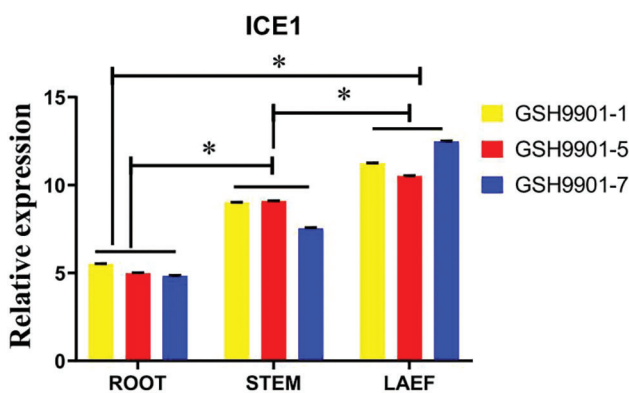


Figure 9: The relative expression of *ICE1* gene (Duncan's method, $P < 0.05$)

3.2.9 *ICE1* Positively Regulates *CBFs* Gene Expression under Cold Stress

To identify the relationships between *AtICE1* and the molecular components of the ICE–CBF pathway, we analyzed the *CBF* gene's expression pattern under cold conditions. Under normal conditions, the expression levels of the identified genes were relatively low in both WT and transgenic plants (GSH9901-1, -5, and -7). However, compared with those in the control group, all the family genes, such as *CBF1*, *CBF2*, and *CBF3*, were significantly ($P < 0.01$) up-regulated in the transgenic plants after 24 h of cold

treatment. After 72 h of cold treatment, the expression levels of these three genes decreased (Fig. 10). Thus, *ICE1* may positively regulate the expression of the *CBF* genes in response to cold stress and may, thereby, improve cold tolerance.

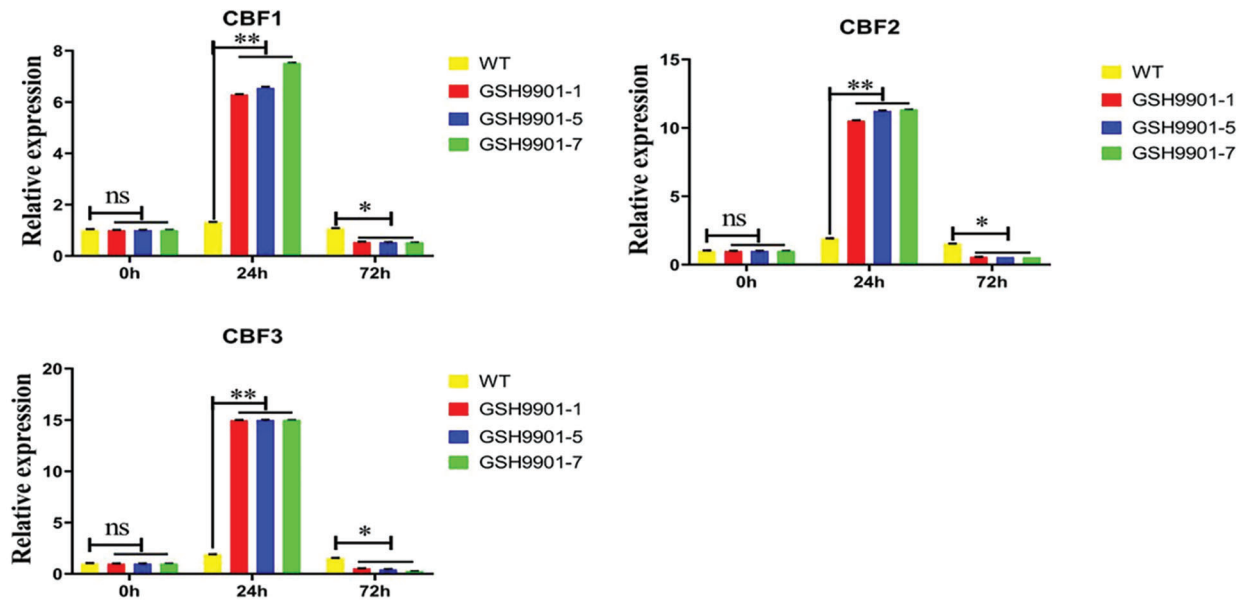


Figure 10: The effect of transgenic corn with *ICE1* on the transcription level of *CBFs* genes in the cold stress pathway (Duncan's method, * $P < 0.05$, ** $P < 0.01$)

4 Discussion

The generation of stress-resistant plants using genetic engineering generally involves the introduction of target genes, or the overexpression of existing genes of interest, in the plant species. The overexpression of *ICE1* in plants under normal growth conditions has a small effect on plant growth. Transgenic *ICE1*-expressing rice [14], tomato [15], *Arabidopsis* [16,17], tobacco [18], and lemon [7] plants show normal growth, with very few instances of mutation or dwarfing, indicating that the introduction of *ICE1* has fewer adverse effects on plant growth than the introduction of *CBF/DREB1*. When *CBF/DREB* expression increases in a plant, *COR* expression also increases, which regulates the expression of a series of genes and changes the physiological and biochemical indicators of the plant itself, resulting in the plant having a certain cold tolerance. Therefore, this study showed the successful transformation of maize with *AtICE1* resulting in the generation of a new maize germplasm that had increased tolerance to cold stress. However, Lu et al. [19] indicated that *ZmICE1* is the homolog of *Arabidopsis AtICE1/2* and plays an important role in the regulation of freezing stress responses. A comparative analysis indicated that the expression of the *ICE1* gene in *A. thaliana* changed more rapidly during the cold-response process. Therefore, compared with the transfer of the *ZmICE1* gene into maize, the transfer of the *AtICE1* gene into maize has a greater application value.

In this study, the pCAMBIA3301-*ICE1* plant expression vector was constructed using a rapid and seamless cloning technique and was transformed into maize using *Agrobacterium*-mediated transformation. Three T_1 -positive plants were obtained, with the Southern blotting indicating that the target gene was integrated into the maize genome. Under low-temperature stress conditions, the expression of *ICE1* decreased the relative conductivity of plant tissue extravasation fluid and the MDA concentrations, whereas the proline concentrations and POD activity increased. These results are

consistent with those of Zhou et al. [20] and Feng et al. [21]. Using qRT-PCR, the *ICE1* gene was determined to be expressed in the roots, stems, and leaves of transgenic maize plants under low-temperature conditions, indicating that low temperature induced *ICE1* expression and increased the cold tolerance of plants. At normal temperatures, the physiological indicators of transformed and untransformed plants were almost the same, indicating that *ICE1* was inactive under normal temperature conditions. This is consistent with the findings of Thomashow et al. [22]. Thus, further studies are required to confirm that *ICE1* transgenic maize plants can tolerate low-temperature stress and that the inserted *ICE1* is stable in the offspring.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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