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Over-Expression of *ICE1* Gene in Transgenic Rice Improves Cold Tolerance

XIANG Dian-jun[1,3], HU Xiang-yang[2], ZHANG Yu[1], YIN Kui-de[1]

([1]*School of Life Science and Biotechnology, Heilongjiang August First Land Reclamation University, Daqing 163319, China;* [2]*Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China;* [3]*Heilongjiang Agricultural Economy Professional College, Mudanjiang 157032, China*)

Abstract: *ICE1*, an *Arabidopsis thaliana* transcription factor gene, was cloned by RT-PCR and successfully transformed into rice variety Kenjiandao 10 by the *Agrobacterium*-mediated transformation method. PCR amplification and Southern blot analysis indicated that *ICE1* had been integrated into rice genome. Compared with the non-transgenic plants, the transgenic plants exhibited high resistance to hygromycin B and were consistent with the Mendelian inheritance of a single copy of the transgenic *ICE1*. Under the low temperature stress, the transgenic plants showed the lower mortality rate and the increased proline content. These results suggest that the *Arabidopsis ICE1* is functional in rice and the over-expression of *ICE1* improves the tolerance to cold stress in rice.

Key words: *Arabidopsis thaliana* transcription factor gene; rice; genetic transformation; cold tolerance; proline content

As one of the staple food resources for human being, rice occupies an important position in national economy in some countries of the East and Southeast Asia. However, the damages of cold, chilling and freezing are big problems in cold regions of these countries, which block rice seed germination, seriously affect rice production and grain quality, and hinder the introduction of superior varieties with long growth duration. Breeding cold tolerant rice variety is an effective and economical approach to overcome these problems. Besides conventional breeding approach, genetic engineering offers another effective way to improve the cold tolerance of rice. It is possible to enhance the cold tolerance by over-expression of extraneous cold-resistance genes in rice.

The activation of transcriptional activator gene *ICE* (inducer of *CBF* expression) in *Arabidopsis thaliana* under low temperature stimulates the *CBFs/DREBs* expression. Subsequently, the activated *CBFs/DREBs* binds to the CRT/DRE *cis* element (CCGAC) in promoter regions, together interacts with other proteins (RNA polymerase and so on), and finally induces the expression of downstream cold-responsive genes (*COR*) as well as the other cold acclimation genes [1]. This process changes the contents of soluble sugar and proline, and finally enhances the plant tolerance to cold stress [2]. Chinnusamy et al [3] isolated the *ICE1* gene from *A. thaliana*. The constitutive expression of *ICE1* improved the cold tolerance in *A. thaliana* [3-5]. Dubouzet et al [6] cloned the homologous genes of *Arabidopsis CBFs/DREBs* in rice designated as *OsDREB1A*, *OsDREB1B*, *OsDREB1C*, *OsDREB1D* and *OsDREB2A*. Chen et al [7] cloned *OsDREBL* in rice, and Tian et al [8] cloned three transcriptional factors (*OsDREB1-1*, *OsDREB4-1* and *OsDREB4-2*) in rice. Most of the cloned genes could bind to the CRT/DRE *cis* element in *COR* promoter regions. Jin et al [9-10] successfully transformed an *Arabidopsis CBF1* into rice and reported that the over-expression of *CBF1* enhanced the cold tolerance in rice. Till now, most of rice *COR* genes have been isolated and identified [11], but the homologous genes of *Arabidopsis ICE1* have not been reported in rice. Moreover, the transcriptional level of *CBF* genes can't be regulated by themselves under cold stress, which provides a potential molecular biological basis for improving rice cold tolerance by transforming *Arabidopsis ICE1*. In this study, we successfully transferred the *Arabidopsis thaliana* transcription factor gene *ICE1* into a rice variety Kenjiandao 10 by the *Agrobacterium*-mediated

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Corresponding author: YIN Kui-de (yinkuide@sohu.com)
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transformation method, and studied the effects of *ICE1* transformation on the cold tolerance of rice variety Kenjiandao 10 under low temperature.

MATERIALS AND METHODS

Plant materials

Test materials were *Arabidopsis thaliana* L. (Columbia ecotype) and a japonica rice variety Kenjiandao 10 (*Oryza sativa* L.). They were provided by the Heilongjiang August First Land Reclamation University, China. Seeds of Kenjiandao 10 were used to prepare the mature seed-derived embryogenic calli.

Bacterial strain and plasmid

The *Agrobacterium* strain was LBA4404, and the *Escherichia coli* strain was DH5α. The intermediate vectors were pRT104, pBluescript and pCAMBIA1300. The plant expression vector named pCAMBIA1300-35S-*ICE1*-polyA (Fig. 1) was constructed, which contained the *ICE1* cDNA fragment and the selective marker gene *HPT* that conferres the resistance to hygromycin under the control of the CaMV35S promoter. The plant expression vector could be digested by *Bam*H I and *Sal* I to get an approximate 2200 bp fragment, and digested by *EcoR* I to get an approximate 1800 bp fragment.

Enzymes and reagents

Trizol and D2000 marker were from TIANGEN Company; the perfect DNA 100 bp marker was from Ambiogen; the reverse transcriptional enzyme reagent kit was from Sigma; LA *Taq* DNA polymerase, restriction enzyme, T_4 DNA ligation enzyme, 1 kb DNA Ladder were from TaKaRa. According to the *ICE1* sequence from GenBank, we designed a pair of primers: $ICE1_f$ (5′-CgaattcGATGGGTCTTGACGGA A-3′, with *Eco*R I digestion site) and $ICE1_r$ (5′-Gctcta gaTCATACCAGCATACCCT-3′, with *Xba* I digestion site). The acetosyringone and hygromycin B were from the Beijing Dingguo Company; DIG DNA Labeling and Detection Kit, the hybridization solution and the nylon membrane for Southern blot were Roche products.

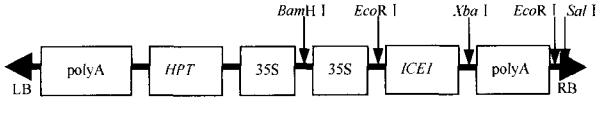

Fig. 1. Schematic diagram of the plant expression vector pCAMBIA1300-35S-*ICE1*-polyA.

ICE1, Inducer of *CBF* expression gene; 35S, CaMV35S promoter; *HPT*, Hygromycin phosphotransferase gene; LB, Left border; RB, Right border.

Culture medium

MS medium was used as the basic medium in rice tissue culture and genetic transformation. The callus induction medium: MS medium+2 mg/L 2, 4-D +0.5 mg/L KT+0.5 mg/L NAA; the regeneration medium: MS medium+2 mg/L 6-BA; the rooting medium: 1/2 MS+0.5 mg/L NAA.

Gene cloning and plant expression vector construction

A. thaliana seedlings (10 days old) were treated at 0°C for 12 h before extracting the total RNA according to the kit instructions. RT-PCR was performed with the reverse transcriptase (M-MLV) using $Olig(dT)_{18}$ as a primer, and with the LA *Taq* DNA polymerase using $ICE1_f$ and $ICE1_r$ as primers.

The PCR amplification was performed as follows: pre-denatured at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s and extension at 72°C for 1 min. After checked by enzyme digestion and sequence analysis, the PCR product of *ICE1* was digested with *Eco*R I and *Xba* I, then inserted between CaMV35S and polyA in the pRT104 vector, and guaranteed the correct reading frame. After digested with *Pst* I, the CaMV35S-*ICE1*-polyA fragment was inserted into the pBluscript plasmid; then digested with *Bam*H I and *Sal* I, the CaMV35S-*ICE1*-polyA fragment was inserted into the pCAMBIA1300 vector [12]. The fusion constructs were transformed into the competent cells of DH5α, and the positive clones were screened. The final constructs were transferred into LBA4404 for rice callus transformation.

Agrobacterium-mediated callus transformation

The rice calli induced from the Kenjiandao 10 mature embryos were transformed by the *Agrobacterium*-mediated method reported by Hiei et al [13]. After the second selection, the hygromycin-resistant calli were transferred to the regeneration medium and cultured

under the photoperiod of 15 h illumination. The regenerated plants about 2 cm height were transferred to the rooting medium. When the plants reached 10 cm height, the tube lids were pulled. After two days culture, the plants were transferred to the soil in pots. The transgenic rice plants were tested to screen the positive ones.

Detection of transgenic rice plants

The total genomic DNA was extracted from 2 g rice leaves and tested by the PCR amplification [14]. We randomly selected the positive plants from PCR detection, and extracted the total genomic DNA for Southern blot. The DNA was digested with *Eco*R I, subjected to electrophoresis, transferred on the membrane, and fixed. Meanwhile, the wild type rice DNA and the expression vector (there was no *Eco*R I enzyme restriction site between *ICE1* and polyA, Fig. 1) were used as the negative and positive controls, respectively. The probe of *ICE1* gene was synthesized and detected according to the DIG DNA Labeling and Detection Kit instructions. The membrane with the fixed single strand DNA was immersed in pre- hybridization buffer. After prehybridization, the membrane was transferred to hybridization solution containing the labeled probe.

The seeds of T_1 generation and non-transgenic plants were surface sterilized, and inoculated on MS medium with 100 mg/L hygromycin. The segregations of T_1 transgenic generations on hygromycin resistance were calculated according to the formula: $\chi^2=(|A-3a|-2)^2/3(A+a)$ (A is the number of hygromycin resistant seeds, a is the number of hygromycin sensitive seeds, df=1, α=0.05, $\chi^2_{(0.05,\ 1)}$=3.84). And then the hygromycin resistant seeds were sown in the soil. When the seedlings were about 10 cm height, they were subjected to cold treatment in a refrigerator at 4°C for 3 days and then back to the constant environments of 23°C. The performances of the plants were observed, and the withering rate after the low-temperature treatment was calculated.

The proline content in rice leaves of T_1 generation and non-transgenic plants at the seedling age of 13 days (before cold treatment) and 16 days (after cold treatment) were determined by the method according to Gao et al [15]. Four replicated measurements were performed for each sample.

RESULTS

PCR product of *ICE1*

The electrophoresis showed that the PCR product of *ICE1* was about 1500 bp (Fig. 2), consistent with the length of *ICE1* (1485 bp) announced in GenBank. The PCR product was further confirmed by enzyme digestion and sequence analysis.

Construction and identification of plant expression vector pCMBIA1300-35S-*ICE1*-polyA

The above cloned *ICE1* gene was used to construct the vectors. The fragment of 35S-*ICE1*-polyA (about 2200 bp) was inserted into the pCMBIA1300 binary vector and transferred into DH5α. The positive clones named pCMBIA1300-35S-*ICE1*-polyA were screened by colony PCR (Fig. 3). After digested with *Bam*H I and *Sal* I, a fragment of about 2200 bp (Fig. 4) was cut from the expression vector, proving that the *ICE1* gene had already been successfully inserted into the plant expression vector pCMBIA1300.

PCR detection of transgenic rice plants

A total of 43 individual transgenic rice plants in the study were checked by PCR. The *ICE1* gene about 1500 bp was presented in 12 individual transgenic plants (Fig. 5), but was absent in the negative CK and the other individual plants, indicating that the *ICE1*

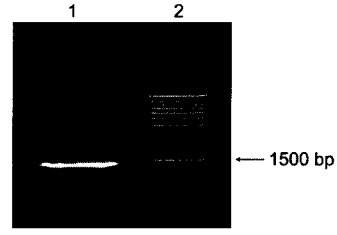

Fig. 2. PCR product of *ICE1*.
Lane 1, PCR product; Lane 2, 1 kb DNA Ladder.

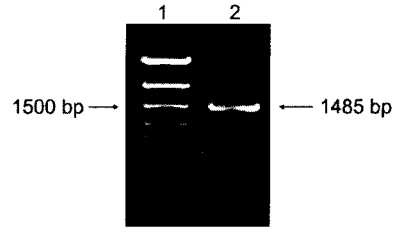

Fig. 3. Colony PCR detection of the plant expression vector.
Lane 1, Perfect DNA 100 bp Marker; Lane 2, Colony PCR product.

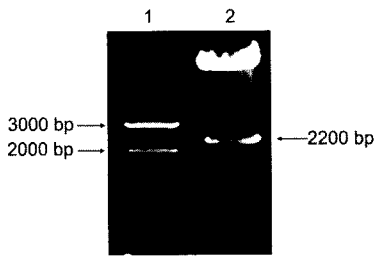

Fig. 4. Enzyme digestion detection of plant expression vector.
pCMBIA1300-35S-*ICE1*-polyA were digested with *Bam*H I and *Sal* I. Lane 1, Perfect DNA 100 bp Marker; Lane 2, Double enzyme digestion product.

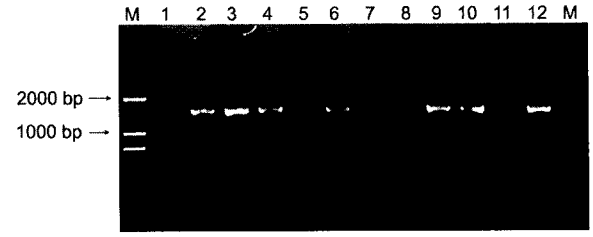

Fig. 5. PCR detection of some transgenic plants.
M, D2000 Marker; 3, Plasmid positive CK; 7, Non-transgenic plants, negative CK. The rest lanes are transgenic plants: 1, T_0-43; 2, T_0-39; 4, T_0-14; 5, T_0-37; 6, T_0-24; 8, T_0-6; 9, T_0-1; 10, T_0-19; 11, T_0-38; 12, T_0-11.

gene was integrated into rice genome with the transformation rate of 28%.

Southern blot analysis of transgenic rice plants

The Southern blot analysis of some transgenic rice plants showed that the band around 1800 bp was presented in the positive plants, no bands could be detected from the negative CK, and two bands were presented in the transgenic rice line T_0-24 (Fig. 6). These results further confirmed that the *ICE1* gene was integrated into the rice genomic DNA with 1–2 copies.

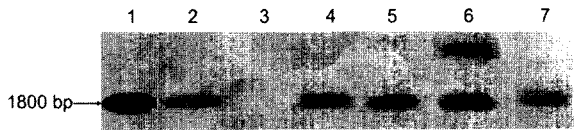

Fig. 6. Southern blot analysis of some transgenic plants.
Rice genomic DNA was digested with *Eco*R I, and hybridized with the probe of *ICE1* gene. Lane 1, Positive CK; Lane 3, Non-transgenic rice plant, negative CK; Lanes 2, 4 to 7, Transgenic plants (T_0-39, T_0-14, T_0-1, T_0-24 and T_0-11).

Segregation in T_1 transgenic generation of rice for hygromycin resistance

Fig. 7 shows the germination of untransformed control seeds were suppressed on the medium with 100 μg/mL hygromycin, whereas some seeds of T_0 generation exhibited high resistance to hygromycin and germinated well. The χ^2 test (Table 1) indicated that *ICE1* was inherited in T_1 generation. The segregation ratios for hygromycin resistance were 3:1 for most of T_0 transgenic lines, which suggested that the *ICE1* gene was integrated into the rice genome with single copy. One transgenic rice line (T_0-24) did not show the segregation ratio of 3:1, indicating that the *ICE1* gene was integrated into the rice genome in multi-copy or multi-site.

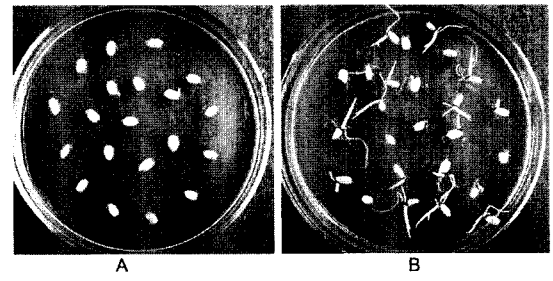

Fig. 7. Detection of hygromycin resistance in transgenic rice seeds (T_1 generation).
A, Non-transgenic rice seeds; B, Transgenic rice seeds.

Cold tolerance in T_1 transgenic generation of rice

The cold treatment showed that the mortality rate was approximately 80% for non-transgenic plants, while about 10% for transgenic rice lines T_0-11-1 and T_0-39-1, and about 50% for the other three transgenic lines. Compared with the untransformed control plants, the transgenic plants showed stronger cold tolerance.

Table 1. Segregations of T_1 transgenic generation for hygromycin resistance.

Plant line	No. of T_0 seeds for detection	No. of hygromycin resistant seeds	No. of hygromycin sensitive seeds	χ^2 (3:1)	Fitness
T_0-39	61	44	17	0.14	H_0
T_0-24	68	41	27	7.08	H_A
T_0- 4	43	30	13	1.74	H_0
T_0-1	71	50	21	0.59	H_0
T_0-11	48	34	14	0.25	H_0
Wild type (CK)	69	0	69		

As shown in Fig. 8, the transgenic lines T_0-11-1 and T_0-39-1 presented weaker wilting, withering and lodging under low temperature. These results suggest that the over-expression of *ICE1* gene improved the cold tolerance of transgenic Kenjiandao 10 to a certain level.

Proline content in T_1 transgenic generation of rice

Proline is a stronger hydrophilic amino acid. Higher proline content can help cells maintain water and biological macromolecular structure, and enhance the plant environmental stress tolerance. After the low-temperature treatment, the proline content in transgenic rice plants had a higher increasing amplitude compared with non-transgenic ones. As shown in Fig. 9, the proline contents in the transgenic lines T_0-11-1 and T_0-39-1 were 1.1 times and 0.8 times as those in non-transgenic ones before the low-temperature treatment, respectively, and were 2.16 times and 2.23 times as those in non-transgenic ones, respectively after the low-temperature treatment. This suggests that the over-expression of the *ICE1* gene in rice promoted the proline synthesis.

DISCUSSION

The cold tolerance of plants, a complex property controlled by the multiple factors, is mainly genetically determined and simultaneously affected by environment. There are many functional genes (e.g. *COR*) and regulatory genes (e.g. transcriptional factors) regulating the plant response to cold stress, however, their expression levels are very low under normal conditions, therefore, the gene expression regulation system plays a vital role in cold tolerance. Compared with the transformation of single functional gene, the transformation of transcription activator is a more effective way to improve the cold tolerance in plants.

Gilmour et al [4] obtained transgenic *A. thaliana* with the over-expression of *CBF3/DREB1A*, and observed that the over-expression of *CBF3* regulated the expression of downstream target genes and increased the accumulations of proline and sugar. Therefore, they speculated that the *CBF3/DREB1A* gene regulated not only *COR* genes, but possibly other stress tolerance genes conferring cold tolerance improvement in plants.

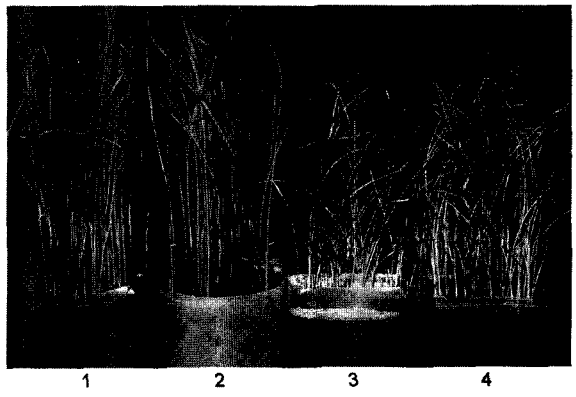

Fig. 8. Detection of cold tolerance in transgenic rice plants (T_1 generation).

1, Transgenic T_1 generation rice plants, serial number T_0-11-1; 2, Transgenic T_1 generation rice plants, serial number T_0-39-1; 3 and 4, Non-transgenic plants.

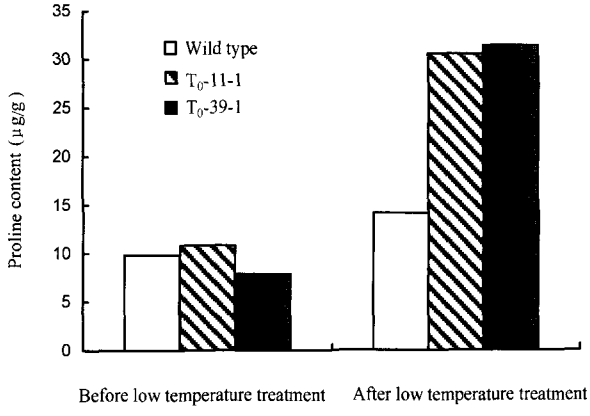

Fig. 9. Proline content in transgenic rice plants (T_0-11-1 and T_0-39-1) and wild type before and after low-temperature treatment.

Chinnusamy et al [5, 16-17] isolated the *ICE1* gene from *A. thaliana* and reported that the *ICE1* gene regulated *COR* through *CBF3*. Jing et al [9-10] got the transgenic rice plants containing *Arabidopisis CBF1* and noted that the over-expression of *CBF1* improved the cold tolerance and increased the proline content in rice plants. In this study, we also found that the over-expression of *ICE1* enhanced the cold tolerance and the proline content in rice. It is postulated that the *ICE1* gene activated rice *OsDREBs*, the transcriptional factors homologous to *CBFs/DREBs* in *Arabidopsis*, then the activated *OsDREBs* regulated *COR* and other correlative genes in rice. Certainly, more experiments should be carried out to investigate the expression patterns of *OsDREB* and *COR* genes in the transgenic rice under cold stress.

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