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

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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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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.

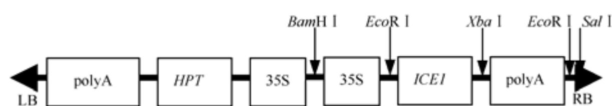

Fig. 1. Schematic diagram of the plant expression vector pCAMBIA1300-35S-*ICE1*-polyA.

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 0ºC 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 94ºC for 3 min, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 56ºC for 45 s and extension at 72ºC 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

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