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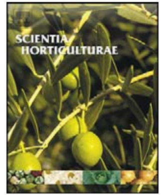

# Cucumber (*Cucumis sativus* L.) over-expressing cold-induced transcriptome regulator *ICE1* exhibits changed morphological characters and enhances chilling tolerance

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ABSTRACT

Cold is an environmental factor that limits the growing season of cucumber and adversely affects fruit quality and productivity. *ICE1* is a positive regulator of *CBF3* and has a critical role in cold stress (Chinnusamy et al., 2003). To test the function and potential use of cold-induced transcriptome regulator *ICE1* (inducer of *CBF* expression) in improving the chill tolerance of cucumbers, the *Arabidopsis ICE1* gene driven by *super* promoter was introduced into cucumbers by Agrobacterium-mediated transformation. Transgenic plants were obtained, and were confirmed by PCR and southern blot, then the expression of *ICE1* was tested by RT-PCR. Transgenic plants exhibited dwarf phenotypes, and shorter internodes, with higher nodes in the first female flowers and a lower female flower rate of every node than wild type plants. Under cold stress, the chilling index of four transgenic lines (IF3, IF6, IF7, and IF10) was decreased significantly, indicating their improved chilling tolerance. Over-expression of *ICE1* in cucumbers could induce the expression of cold-responsive genes, further activating the accumulation of soluble sugars and free proline, and inhibiting malondialdehyde (MDA) accumulation. This leads to electrolyte leakage and regulation of osmotic potential to osmotic stress under cold stress.

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## 1. Introduction

The C-repeat binding factors (*CBF*) are among the stress related transcription factors that have a critical role in the regulation of low temperature stress response in *Arabidopsis* and other plant species (Thomashow, 2001). *ICE1* is a positive regulator of *CBF3* and has a critical role in cold stress. *ICE1* encodes an MYC-like *bHLH* transcriptional activator and binds specifically to the *MYC* recognition sequences in the *CBF3* promoter (Chinnusamy et al., 2003). The *CBF* transcriptional activators, named as *CBF1*, *CBF2*, and *CBF3* (*DREB1b*, *DREB1c*, and *DREB1a*), bind to *CRT*/*DRE* elements found in the regulatory regions of many cold inducible genes, and induce their transcription activation, which governs the plant responses to low temperature (Stockinger et al., 1997; Liu et al., 1999; Medina et al., 1999). Cold-responsive genes encode a diverse array of proteins such as the enzymes involved in respiration and the metabolism of carbohydrates, lipid, antioxidants, antifreeze proteins and similar substances (Guy, 1990; Thomashow, 1999).

Cucumbers belong to the Cucubitaceae family, and are widely cultivated in many areas of the world (Esquinas-Alcazar and Gulick, 1983; Lawrence, 1990). Cucumbers are a typical chill-sensitive vegetable crop. Their tissues, fruits and even whole plants suffer physiological obstacles chilling stress were making, especially when they are cultivated in greenhouses during the winter (Cabrera and Saltveit, 1990). Recent research on enhancing the chill resistance of cucumbers suggests that one of the most effective approaches is varietal breeding (Singla-Pareek et al., 2007) and the acquisition and introduction of germplasm with better chilling resistance. It may be necessary to create germplasm that includes improved chilling other plants or animals (Yin et al., 2004).

Many studies have indicated that cold-responsive genes are critical in plants that are chill tolerant (Gong et al., 2002; Hsieh et al., 2002). Some cold-responsive genes have been introduced into crops to induce or improve the responses to biotic and abiotic stimulus (Kasuga et al., 2004; Głodek et al., 2008). Over-expression of *ICE1* in wild type *Arabidopsis* enhances the expression of *CBF* under low temperature and improves the freezing tolerance of transgenic plants. Whether *ICE1* over-expression can improve the chilling tolerance of cucumber is unknown, so in this study, *ICE1*

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driven by *super* promoter was introduced to cucumbers, and the cold tolerance of the resulting transgenic plants was evaluated.

## 2. Materials and methods

### 2.1. Materials and cucumber transformation

*Cucumis sativus* L. cv. S516 (from Dr. H.Z. Ren, China Agricultural University) was multiplied by artificial self-pollination for use in this experiment. pBIB-super promoter-*ICE1*, from Z.Z. Gong Lab, College of Biological Science of China Agricultural University, plasmid was integrated into *Agrobacterium rhizogenes* EHA3101. Sterilized, cucumber seeds were sown on MS medium containing 30 g/l sucrose and 2.5 g/l gelrite (Sigma, American). One day after the seeds germinated, cotyledons were separated and cut into a 1 cm × 1 cm squares, and used as explants. The explants were dipped in the Agrobacteria-diluted suspension with MS liquid medium for 15 min. These explants were placed onto shoot induction medium containing 2 mg/l BA and 1 mg/l ABA, then cultured for 3 d at 25 °C under dark conditions. The materials were transferred to germinate mediate medium containing 2 mg/l BA, 1 mg/l ABA, 25 mg/l kanamycin and 100 mg/l claforan, then cultured for 14 d at 25 °C, 100 $\mu$M m$^{-2}$ s$^{-1}$. The regeneration shoots were placed in kanamycin selection medium containing 2 mg/l BA, 1 mg/l ABA, 100 mg/l kanamycin, and 100 mg/l claforan, for 14 d. The shoots with kanamycin resistance were cultured in MS medium with 100 mg/l kanamycin and 50 mg/l claforan, for development into whole plants.

### 2.2. DNA isolation, PCR and southern blot analysis

Total genomic DNA was isolated from cucumber leaves according to Saghai-Maroof et al. (1984). PCR was performed using primer pairs specific for *ICE1* gene, with forward primer 5′-TGCTCGGTCACTTCTTGC-3′; and reverse primer 5′-TGGTAGCGAG-CAACAGAC-3′. The PCR program was as following: 94 °C, 5 min; 30 cycle, 94 °C, 30 s; 60 °C, 30 s; 72 °C, 60 s; 72 °C, 10 min. Genomic DNA was digested with XbaI and KpnI, and DNA probes were purified from PCR-amplified fragment of *ICE1* gene. Southern blot was carried out according to the DIG mark and blot detection reagent box.

### 2.3. Isolation of RNA and RT-PCR

The leaf samples were collected under 6 °C treatment for 24 h. The RNA was isolated from cucumber leaves using trizol box (Invitrogen, American). The superScript$^{TM}$ III reverse transcriptase (Invitrogen, American) was used in the first-strand cDNA synthesis. The cDNA was used in the second-strand synthesis, PCR program was as follows: 94 °C, 2 min; 30 cycles, 94 °C, 30 s; 60 °C, 90 s; 72 °C, 90 s; 72 °C, 10 min.

### 2.4. Investigation of transgenetic cucumber in the greenhouse

The $T_1$ generation of transgenic cucumbers was sown in soil and cultured in a greenhouse. The greenhouse is special for transgenic plants. 3 repeats were set, 1 line per repeat, 15 plants per lines. Starting with the 5th weeks after the seeds were sown, plant height, leaf number, the node of the first female flower, female flower number were observed and recorded every week for 3 times.

### 2.5. Assessment of chilling tolerance

The $T_1$ generation of the transgenic and control plants was used for cold assessment. Plants of 6 transgenic $T_1$ generation transgenic lines were grown in the greenhouse to the 3-leaf stage. These plants were kept in the growth chamber in the light at 28 °C for 12 h, and in the dark at 18 °C for 12 h. Then these transgenic plants were exposed to cold stress at 5($\pm$0.5) °C for 4 d, with 100 $\mu$M m$^{-2}$ s$^{-1}$ light for 12 h every day. After this chilling stress, statistics of chilling tolerance were gathered and indexed. Cucumber leaves were taken, then frozen in liquid nitrogen to store for the detection of electrolyte leakage rate, MDA content, activity of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). The contents of compatible solutes and free proline in plants were recorded.

Chilling tolerance was indexed following Semeniuk et al. (1986): after chilling stress cucumber material was rated based on visible symptoms: 0, no injury; 1, trace; 2, slight; 3, moderate; 4, severe; 5, extensive. Electrolyte leakage rate was measured according to Murray et al. (1989); soluble sugar was determined based on Yem and Willis (1954); proline was measured according to Troll and Lindsley (1955); and MDA measurements were based on Heath and Parker (1968). POD and CAT activities were determined according to the methods of Cakmak et al. (1993), by monitoring the rates of $H_2O_2$ decomposition and guaiacol oxidation, respectively. The measurement of activity of SOD was determined by the nitroblue tetrazolium (NBT) method of Dhindsa (1980).

### 2.6. Statistic analysis

The date was analysed by SPSS 11.5 for Windows. And least significantly difference (LSD) procedure was used to determine which means are significantly different from which others ($p \leq 0.05$).

## 3. Results

### 3.1. Confirmation of integration of Super-ICE1 in transgenic cucumber

The four transgenic lines (IF3, IF6, IF7, and IF10) were detected by polymerase chain reaction (PCR) and southern blot, and the results showed that *Super-ICE1* was integrated into the cucumber genome. In order to detect the expression of *Super-ICE1* in transgenic plants under normal conditions, RT-PCR was used. The expression was detected in all four transgenic lines (Fig. 1).

Transgenic cucumbers exhibited dwarf phenotypes, shorter internodes, and higher nodes of first female flowers and a lower female flower rate for every node than control plants: plant height, leaf number and length of the biggest leaf were measured 7 weeks after sowing the seeds, and the internode length was the mean of three measurements. The $T_1$ generation transgenic lines were shorter (Fig. 2; Table 1). The internode length of the transgenic lines was shorter than those of wild type plants. The biggest leaf of the wild type plants was bigger than those of the transgenic lines. However, there was no significant difference between leaf numbers.

The expression of *ICE1* affected both the morphology of the transgenic cucumbers and their development. The first female flower node of the transgenic plants was significantly higher than those of the wild types, which lead to later flowering for the transgenic plants. The female flower rate of per node of transgenic plants was lower than wild types, which decreased production.

### 3.2. Over-expression of ICE1 gene enhanced the cold resistance of transgenic plants

Under cold stress, four transgenic lines (IF3, IF6, IF7, and IF10) showed lighter injuries (Fig. 3), and their chilling injury indices significantly lower than wild type, and were decreased by14%, 33%, 20%, 16% respectively (Table 2). Wild type plants exhibited severe injury in their cotyledons; the first and second true leaves, and

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