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Research article

The constitutive expression of a two transgene construct enhances the abiotic stress tolerance of chrysanthemum

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ABSTRACT

Various abiotic stresses downgrade the quality and productivity of chrysanthemum. A construct carrying both *CcSOS1* (from *Chrysanthemum crassum*) and *CdICE1* (from *Chrysanthemum dichrum*) was constitutively expressed in the chrysanthemum variety 'Jinba'. The transgenic plants were superior to the wild type (WT) ones with respect to their sensitivity to low temperature, drought and salinity, as measured by visible damage and plant survival. Salinity stressed transgenic plants accumulated more proline, and their level of superoxide dismutase and peroxidase activity was higher than in WT plants. At the physiological level, they suffered less loss of viable leaf area, maintained a lower leaf electrolyte conductivity and retained more chlorophyll (a+b). The ratio between the K^+ and Na^+ content was higher in the root, stem and median leaves of salinity stressed transgenic plants than in those of WT plants.

1. Introduction

Abiotic stress caused by low temperature, drought or salinity represents a major constraint over crop growth and productivity (Sairam and Tyagi, 2004). Genetic variation for stress tolerance has been identified in some plant species, and a number of genes involved in such tolerances have been characterized. Among these genes is *ICE1,* which plays a role in determining low temperature tolerance in *Arabidopsis thaliana* (Chinnusamy et al., 2003; Liu et al., 2010); it encodes a MYC-like bHLH transcriptional activator and its over-expression enhances drought as well as low temperature tolerance (Chen et al., 2012). A second gene in this class is *SOS1,* which encodes a plasma membrane Na^+/H^+ antiporter involved in the long distance transport and exudation of Na^+ ions (Shi et al., 2000). The constitutive expression of *SOS1* increases the level of salinity tolerance in *A. thaliana* and tobacco (Feki et al., 2013; Yang et al., 2009; Yue et al., 2012).

Despite some successes in identifying specific genes underlying abiotic stress tolerance, it is well understood that the overall plant response to stress is governed by the action of a multiple gene network, and this has greatly compromised a single gene solution to achieving higher abiotic stress tolerance (RoyChoudhury et al., 2007). The introduction of gene constructs harboring two or even three transgenes has been achieved in some crop species, but these successes have to date involved biotic, rather than abiotic stress resistance/tolerance genes.

Chrysanthemum is a leading ornamental species, which is rather sensitive to low temperature, drought and salinity. The constitutive expression of either *CcSOS1* (from *Chrysanthemum crassum*) or *CdICE1* (from *Chrysanthemum dichrum*) both leads to a reduced plant sensitivity to salinity (Song et al., 2012), low temperature and drought (Chen et al., 2012). Here, the effect of constitutively expressing a *CcSOS1-CdICE1* construct in chrysanthemum is described.

2. Materials and methods

2.1. Plant material, transgene construct and transformation

The chrysanthemum cultivar 'Jinba' is maintained by the Chrysanthemum Germplasm Resource Preserving Centre, Nanjing Agricultural University, China. The two gene construct was built from the pre-existing single gene constructs *pCAMBIA1301-CcSOS1* (Song et al., 2012) and *pCAMBIA1301-CdICE1* (Chen et al., 2012). This was achieved by introducing a *Sal*I and a *Kpn*I restriction site into pMD19-GUS via a nested PCR based on the primer pairs F1/R1 and F2/R2 (sequences shown in Table 1). pMD19-GUS and *pCAMBIA1301-CdICE1* were then digested by *Nco*I and *Bst*EII respectively,

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Table 1
Sequences of PCR primers used.

Primer name	Primer sequence (5′-3′)
F1	GTCGACTAGATCTGAGGGTAAATTTCT
R1	CGGTACCTTTCGGTATAAAGACTTC
F2	CCCATGGTCGACTAGATCTGAGG
R2	GGGTCACCGGTACCTTTCGGTAT
HII-1	CGTCTGTCGAGAAGTTTC
HII-2	TACTTCTACACAGCCATC
SOS1-1	CATACCAAGTCTAGGCAGCATC
SOS1-2	GACTTTCACTTGCTATTTCTCCC
ICE1-1	CCAATAACCACGACCGCTAA
ICE1-2	CACAGTCCTCCCAAGTAA

and the resulting fragments ligated to one another to generate *pCAMBIA1301-CdICE1-GUS*. Both *pCAMBIA1301-CdICE1-GUS* and *pCAMBIA1301-CcSOS1* were subsequently digested by *Sal*I and *Kpn*I and the fragments ligated to one another to form *pCAMBIA1301-CdICE1-CcSOS1* (Fig. 1). To transform Chrysanthemum cv. 'Jinba', young leaves were cut into 0.5 cm diameter discs, which were then co-cultivated with *Agrobacterium tumefaciens* (strain EHA105) harboring the two gene construct, following Chen et al. (2012).

2.2. PCR and quantitative real time PCR (qRT-PCR) analysis

Genomic DNA of hygromycin resistant regenerated plants and non-transformed plants was extracted using the CTAB method (Porebski et al., 1997). The presence of the hygromycin resistance gene *hptII* was assayed by PCR using the primer pair HII-1/2 (Table 1). The reactions were subjected to an initial denaturation (94 °C/5 min), followed by 33 cycles of 94 °C/60s, 55 °C/45s, 72 °C/60s, and finally by an elongation step (72 °C/10min). RNA from the shoot apex of putative transgenic and wild type (WT) seedlings at 6–8 leaf stage was isolated using the RNAiso reagent (TaKaRa) and reverse transcribed to synthesize the first cDNA strand using M-MLV reverse transcriptase (TaKaRa). Each 20 μL qRT-PCR comprised 10 μL SYBR® Premix Ex Taq™ II (Takara), 0.4 μL of each primer (10 μM), 4.2 μL H_2O and 5 μL cDNA template. The temperature cycling regime comprised an initial denaturation (95 °C/2 min), followed by 40 cycles of 95 °C/10 s, 55 °C/15 s, 72 °C/20 s. A melting curve analysis was conducted following each assay to confirm the specificity of the amplicons. The primer pairs for the qRT-PCRs were *SOS1-1/2* and *ICE1-1/2* (Table 1). Relative transcript abundances were estimated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), and were normalized against the abundance of *CcSOS1* and *CdICE1* in WT plants.

2.3. Assessment of low temperature tolerance

Young leaves of transgenic and WT plants were exposed to low temperature stress following Chen et al. (2012) with minor modifications. Temperature gradient were −3 °C, −6 °C, −9 °C, −12 °C and −15 °C, each temperature gradient kept 1 h, followed by uniform cooling for 30 min using a Low-Temperature Cycler (Polyscience, Mode: 19610). The leaves were then left at 4 °C for 24 h, and immersed in distilled water for 15 h. The electrical conductivity (EC) of the water was then measured. A relative EC value (REC%) was derived from the ratio between the EC measured before and after boiling. The REC% values were fitted to the logistic function $K/(1+ae^{-bx})$, where x represented the freezing temperature and K the saturation capacity of cell damage rate calculated using Rcpsys (Gai, 2000). A semi-lethal temperature (LT_{50}) was calculated from the expression ln[(1/a)]/b. A whole plant low temperature assay was applied to both transgenic and WT plants at the 8-10 leaf stage. The plants were first acclimated at 4 °C for 12 h, then exposed either −6 °C or −9 °C for 1 h, returned to 4 °C for 4 h, and then allowed to recover for two weeks at 23 °C. A survival rate was calculated following Chen et al. (2012). Each assay comprised three replicates of four plants per line.

2.4. Drought tolerance assay

Both transgenic and WT plants at the 8-10 leaf stage were cultured in 20% aqueous PEG6000 for 36 h, after which the roots were rinsed and kept in water, The survival rate of the plants was calculated after one week's recovery following Hong et al. (2006).

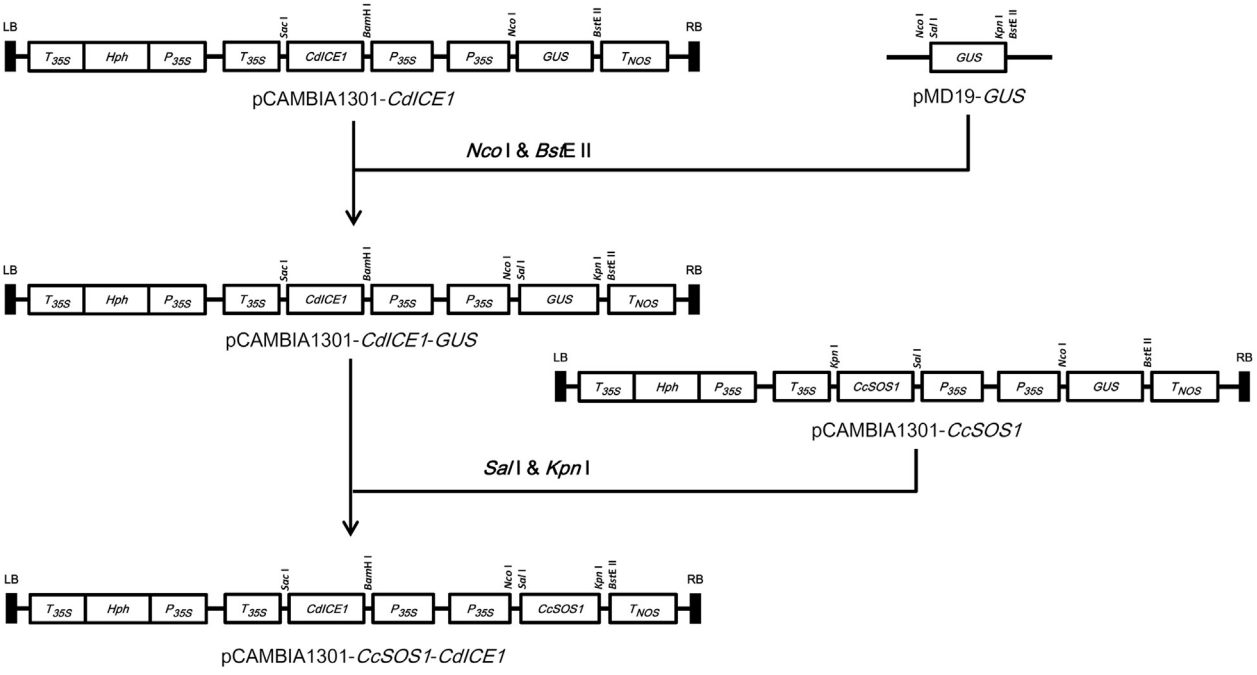

Fig. 1. Scheme for the construction of the expression vector carrying both *CcSOS1* and *CdICE1*.