Marker-free transgenic cucumber expressing *Arabidopsis cbf1* gene confers chilling stress tolerance

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Abstract

Marker-free transgenic cucumber (*Cucumis sativus* L.) cv. Poinsett 76 SR plants were produced by *Agrobacterium* mediated transformation. A transformation efficiency of 1.62 was observed on using *Agrobacterium tumefaciens* strain LBA4404 harbouring *Arabidopsis cbf1* gene driven by the inducible promoter RD29A in a binary vector system pCAMBIA. Transgene integration and single copy insert in transgenic cucumber was confirmed by polymerase chain reaction (PCR) and Southern blot analysis in T_0 lines and also confirmed marker-free status in T_1 generation. Transgene expression was confirmed by reverse transcription (RT)-PCR in T_1 generation transgenic cucumber and advanced to T_2 generation. Upon exposure to chilling stress (4 °C), the T_2 generation transgenic plants survived up to 36 h; however, wild-type plants could not survive and gradually died. A significant decrease in membrane injury index (MII), increase in activities of antioxidant enzymes (SOD and CAT), free proline content and relative water content (RWC) in the leaves were observed in transgenic cucumber as compared to wild-type under chilling stress. Thus, the transgenic cucumber plants expressing *Arabidopsis cbf1* gene conferred protection against chilling stress.

Additional key words: Agrobacterium, c-repeat binding factor-1, *Cucumis sativus.*

Introduction

Abiotic stresses, such as chilling, drought and salinity are the major environmental factors that adversely affect plant growth and cause loss to productivity and yield quality (Djilianov *et al*. 2005). These abiotic stresses are interconnected and affect the plant water relations (Beck *et al*. 2007) leading to a series of morphological, physiological, biochemical and molecular changes (Baker *et al*. 1994). Low temperature causes dehydration of the cells and tissues, and crystallization of cellular water (Pearce 2001). Under low temperature, conductivity of membranes decreases, viscosity of water increases, and the closure of stomata is inhibited resulting in water stress (Sarad *et al*. 2004). It also retards metabolism, while stimulates formation of free radicals resulting in oxidative stress (Beck *et al*. 2007).

Cucumber is a very popular and widely cultivated, warm season, frost sensitive, annual vegetable crop. Thus, cultivation of cucumber in cold regions needs to improve its cold tolerance, *e.g*., by introducing stress tolerance gene(s). Development of reproducible tissue culture and transformation protocol in cucumber is the first step for genetic engineering (Vasudevan *et al*. 2007). Among the cucumber cultivars, Poinsett 76 SR was chosen for study because it is widely cultivated in the world.

C-repeat binding factor-1 (CBF1), a homolog of dehydration-responsive element (DRE)-binding proteins-1A (DREB1A), is a transcriptional activator that binds to the C repeat (CRT)/dehydration-responsive element (DRE), in the promoter region of cold responsive

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Abbreviations: BAP - 6-benzylaminopurine; CAT - catalase; CBF1 - c-repeat binding factor-1; CTAB - cetyltrimethylammonium bromide; IBA - indole-3-butyric acid; MII - membrane injury index; MS - Murashige and Skoog's medium; NBT - nitroblue tetrazolium chloride; *npt*II - neomycin phosphotransferase-II; RT-PCR - reverse transcription polymerase chain reaction; RWC - relative water content; SOD - superoxide dismutase.
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(COR) genes (Hsieh *et al*. 2002, Stockinger *et al*. 1997, Gilmour *et al*. 1998). Overexpression of *cbf1* gene in *Arabidopsis thaliana* and *Brassica napus* plants resulted in the induced expression of *COR* genes and increased freezing tolerance in the absence of cold acclimation (Jaglo-Ottosen *et al*. 1998), indicating the master role of the *cbf1* (Thomashow 2001).

The present study was designed to determine whether introgression of *Arabidopsis cbf1* by *Agrobacterium*-mediated transformation enhanced chilling stress tolerance in transgenic cucumber. Development of marker-free transgenics has become an important requisite in engineering the crops. Co-transformation enables integration of different transgenes at unlinked Mendelian loci (Afolabi *et al*. 2005), which shall segregate in progeny and hence generate marker-free plants. Our study led to development of marker-free transgenic cucumber through co-transformation method.

Materials and methods

Seeds of cucumber (*Cucumis sativus* L.) cv. Poinsett 76 SR (*US-Agriseeds*) were procured from *Seed Works India*, Hyderabad, India. The seeds were surface sterilized with 70 % ethanol and 0.1 % $HgCl_2$ followed by rinse with sterile double distilled water. Seeds were germinated on water agar medium. Shoot tips (3 - 4 mm) from 7-d-old *in vitro* grown seedlings were used as explants for co-transformation.

Agrobacterium tumefaciens strain *LBA4404* harbouring the plasmid pCAMBIA 0390 carrying the desired *cbf1* gene driven by inducible RD29A promoter and plasmid pCAMBIA 2300 carrying neomycin phosphotransferase (*nptII*) gene regulated by cauliflower mosaic virus (CaMV) promoter were used for co-transformation (Fig. 1*A*).

Shoot tip explants were pre-cultured on Murashige and Skoog (1962; MS) medium containing 0.1 mg dm^{-3} 6-benzylaminopurine (BAP) and 0.01 mg dm^{-3} indole-3-butyric acid (IBA) for 1 d under 16-h photoperiod, irradiance of 200 - 300 μmol m^{-2} s^{-1} and temperature of 25 ± 1 °C. *Agrobacterium* cultures harbouring *cbf*1 and *npt*II were grown separately in yeast extract + mannitol (YEM) medium containing 50 mg dm^{-3} kanamycin, 10 mg dm^{-3} rifampicin and 25 mg dm^{-3} streptomycin at 28 °C overnight. Bacterial cells were pelleted by centrifuging at 2 000 *g* for 10 min at room temperature and the pellet was suspended in liquid MS medium (MSS). The cultures were used to infect the pre-cultured explants for 15 min after diluting to the absorbance (A_{590}) 0.15 - 0.20. The infected explants were dried by a sterile blotting paper and cultured onto a freshly prepared pre-culture medium for 48-h in dark at 25 °C. Then, the explants were washed twice in liquid MSS containing 300 mg dm^{-3} cephotaxime, blot dried and cultured on a selection medium (the pre-culture medium supplemented with 150 mg dm^{-3} kanamycin and 300 mg dm^{-3} cephotaxime). The concentrations of kanamycin and cephotaxime were optimized in preliminary experiments (data not shown). Explants were sub-cultured to a fresh selection medium at week intervals. Elongated shoots were cultured on rooting medium (MS medium supplemented with 0.2 mg dm^{-3} IBA and 150 mg dm^{-3} kanamycin). The regenerated well rooted plantlets were hardened by transferring on a sterile mixture of vermiculite and peat moss in 1:1 ratio and placed in transgenic containment facility.

Genomic DNA was isolated from young leaf samples of transformed and wild type plants, using the cetyltrimethylammonium bromide (CTAB) method of Doyle and Doyle (1987). The transgene integration was confirmed by amplification using gene specific primers in PCR. About 100 ng of plant DNA from each sample was used for the 0.025 cm^3 PCR reaction mixture. PCR cycling was carried out to amplify the coding regions of transgenes using *cbf1* (forward: 5'-TTAATGGATCC AGACTGAGAACTCTAGTAACTACG-3′; and reverse: 5'-GTTTTGAGCTCAATTATTTTCCACTCGTTTCTA CAAC-3′) and, the *nptII* (forward: 5'-GAGGCTATT CGGCTATGACTG-3' and reverse: 5'-ATCCGGAGG GGCGATACCGGA-3′) gene-specific primers. PCR amplification was carried out using the cyclic parameters as: an initial denaturation at 94 ºC for 4 min, followed by 35 cycles of denaturation (94 °C for 1 min), annealing (55 ºC for 1 min) and primer extension at 72 °C, with a final extension step at 72 ºC for 5 min. The amplified PCR products were electrophoresed on 1 % agarose gel containing ethidium bromide and the bands were visualized using an *Alpha Imager EC*® (*Alpha Innotech*, San Leandro, USA).

Southern blot analysis was performed on PCR-positive plants. Genomic DNA (20 μg) was digested overnight with restriction enzyme (*Eco*RI) and the southern blots were prepared according to the procedure of Sambrook *et al*. (1989). The 830 bp amplicon of *cbf1*, labeled with [P^{32}]-dCTP using *Hexalab* ™ DNA labeling kit (*Fermentas*, Burlington, Canada) was used as the probe and hybridized to a *Hybond-N*$^+$ nylon membrane (0.45 μM; *Amersham*, Arlington Heights, USA). An autoradiograph was obtained by exposing the X-ray film to the membrane.

Total RNA of the transgenic plants was isolated using *RNeasy* plant mini kit (*Qiagen*, Hilden, Germany), and the reverse transcription (RT)-PCR was performed as follows: the reaction mixture [1× RT buffer (*Fermentas*), 10 mM dNTP mix, oligo dT primer and 1 μg RNA] was heated to 65 °C for 10 min, following which 10 U *MuLV*

RT was added and reverse transcription was performed at 37 °C for 60 min. The first strand cDNA was used for the PCR analysis using gene (*cbf1*)-specific primers to amplify the 830-bp fragment of the *cbf1* gene. The PCR conditions followed were the same as described above. The amplified PCR products were separated by electrophoresis on 1 % agarose gel containing ethidium bromide and the bands were visualized using *Alpha Imager EC*®.

Chi-square (χ^2) test was used for segregation analysis of T_1 generation transgenic lines.

Plants of the selected marker-free T_2 transgenic line and wild-type were incubated at 4 °C for different time intervals (0- to 36-h) and their potential to withstand chilling stress was assessed. Comparative analyses of some important physiological and biochemical parameters related to cold tolerance mechanisms were measured before and after exposure at 4 °C for 36 h.

Membrane injury index (MII) was recorded in terms of electrolyte leakage (EL) by recording electrical conductivity (*Cond 3151/SET*, *WTW*, Weilheim, Germany). Ten leaf discs of equal size were placed in 25 cm^3 distilled water and electrical conductivity (EC) was recorded after a 15 min of vacuum filtration (EC_1) and after autoclaved at 121°C for 30 min (EC_2). MII was calculated according to the following formula: MII [%] = $EC_1 / EC_2 \times 100$.

Relative water content (RWC) was measured as reported by Roberto *et al*. (2001) and calculated using formula: RWC [%] = (fresh mass - dry mass)/(water saturated mass - dry mass) × 100.

The enzyme extract was prepared by grinding 0.1 g leaf samples to a fine powder in liquid nitrogen and then with 100 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM EDTA. The extract was centrifuged at 14 000 *g* at 4 ºC for 10 min and the supernatant was used as the source of enzymes. Protein concentration was estimated according to the method of Bradford (1976).

SOD activity was estimated by UV-Vis spectrophotometer (*Labomed*, Culver City, USA) according to the method of Moaed *et al*. (2006). The 3 cm^3 reaction mixture contained 13 mM methionine, 25 mM NBT, 0.1 mM EDTA, 50 mM phosphate buffer, pH 7.1, 50 mM sodium carbonate and 0.1 cm^3 enzyme extract. Reaction was started by adding 2 μM riboflavin and placing the cuvettes under 30 W fluorescent lamps for 15 min. Reaction was stopped by switching off and putting the cuvettes under dark. The blue formazane produced by NBT photoreduction was measured at 560 nm. One SOD unit was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction. CAT activity was measured according to the method of Jaleel *et al*. (2007) with a slight modification. The 3 cm^3 assay mixture contained 0.1 M potassium phosphate buffer, pH 7.0, 15 mM H_2O_2 and 0.1 cm^3 of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm.

Free proline content in the leaf tissues was estimated by the method of Bates *et al*. (1973). The leaf tissues were extracted with 3 % sulphosalicylic acid and the filtered homogenate was reacted with glacial acetic acid and acid-ninhydrin reagent. The mixture was incubated in a boiling water bath for 30 min and snap cooled on ice to terminate the reaction. The formazon was extracted with toluene and the optical density was measured at 520 nm. The concentration of proline was estimated by referring to a standard curve.

Morphological data on plant growth habit, plant height, leaf type, fruit size, shape, mass, yield per plant, days to flowering, fruiting, first fruit set and first fruit harvesting were also recorded.

Data obtained from different sets of experiments were analyzed for significance ($P \leq 0.05$) using *t*-test.

Results and discussion

Development of reproducible regeneration protocol is a pre-requisite for successful genetic manipulation. There is no universally applicable method of culture, regeneration and transformation systems for all crop species. Tissues from different genotypes will differ in their response to culture. Choice of explant and seedling age plays an important role that makes difference between success and failure in inducing *in vitro* regeneration and transformation of any crop (Choi *et al*. 1994, Compton 2000, Ananthakrishnan *et al*. 2003). In the present study, 7-d-old *in vitro* grown cucumber seedlings were used as a donor of shoot tip explants for transformation experiment. Shoot tips are rich in meristematic tissue and have high regeneration potential. The use of shoot tip explants resulted in an efficient regeneration of the plantlets and was corroborated with earlier transformation experiments in cucumber cultivars (Chee 1990, Chee and Slighton 1991, Ganapathi and Perl-Treves 2000, Soniya and Das 2002). Recently, our laboratory has also generated an efficient *in vitro* regeneration protocol for long melon (Goyary *et al*. 2010) and successful transformation of summer squash with *cbf1* gene (Shah *et al*. 2008) using shoot tip explants. Pre-culture of explants is a critical step to achieve high frequency transformation. In the present study, a 1-d pre-culture in shoot regeneration medium (MS + 0.1 mg dm^{-3} BAP + 0.01 mg dm^{-3} IBA) was found suitable and produced higher number of transformed shoots as compared to different time intervals pre-cultured explants (data not shown). When the explants were co-cultivated without pre-culture, they were not able to survive the co-cultivation and eventually died. Similarly, more escapes were observed from the explants which are pre-cultured more than 1-d. *Agrobacterium* culture having absorbance

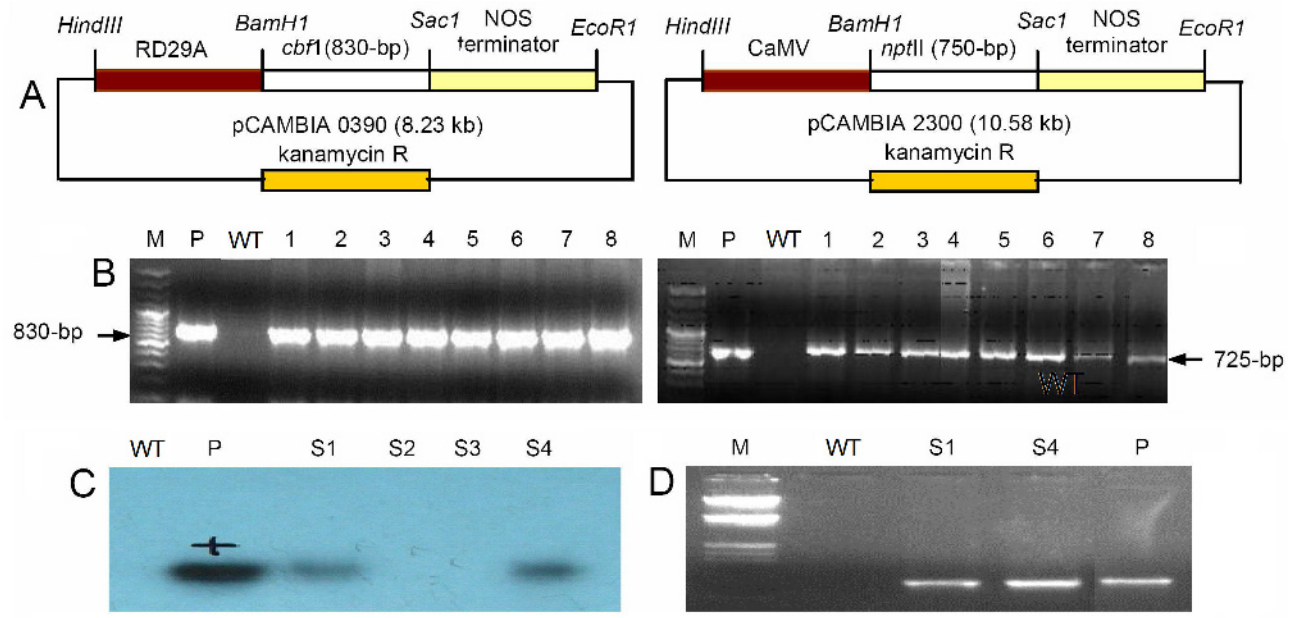

Fig. 1. *A* - Gene constructs of *cbf1* and *nptII* gene; *B* - PCR analysis from the DNA of leaves of T_1 generation transgenic plants, showing amplification of the *cbf1* gene (830 bp) and npt*II* gene (725 bp). *Lanes*: M - 100-bp ladder, P - plasmid DNA; WT - DNA sample from wild-type plant; 1 to 8 - DNA samples from transgenic plants; *C* - Southern blot analysis of genomic DNA from T_1 transgenic lines and wild-type plants. DNA was digested with *Eco*RI. *Lanes*: WT - DNA from wild-type plant; P - plasmid DNA; S1 to S4 - DNA samples from transgenic plants; *D* - Transcript expression analysis in cucumber transgenic plants (T_1 generation) by RT-PCR using the gene-specific primers. *Lanes*: M - 100-bp ladder; P - amplification from positive control; WT - amplification from wild-type; S1 and S4 - amplification from transgenic plants.

Table 1. Biochemical and physiological properties of marker-free cucumber transgenic (MT_2) and wild-type (WT) plants before and after exposure to cold stress (4 ºC for 36 h). Means ± SD of 5 independent experiments; * - statistically significant differences in cold-stressed transgenic lines relative to control plants ($P \leq 0.05$) as determined using the *t*-test.

	Control		Cold Stress	
	WT	MT_2	WT	MT_2
Electrolyte leakage [%]	7.00 ± 0.20	8.10 ± 0.25	18.00 ± 0.30	10.50 ± 0.24*
Relative water content [%]	85.00 ± 0.50	87.00 ± 0.60	65.00 ± 0.45	82.00 ± 0.63*
SOD activity [U mg^{-1}(protein)]	0.20 ± 0.01	0.22 ± 0.02	0.24 ± 0.01	0.42 ± 0.02*
CAT activity [µmol mg^{-1}(protein) min^{-1}]	0.27 ± 0.02	0.30 ± 0.02	0.38 ± 0.01	0.72 ± 0.03*
Proline content [µmol g^{-1}(f.m.)]	0.24 ± 0.02	0.28 ± 0.01	0.30 ± 0.03	0.49 ± 0.02*

of 0.2 at $\lambda_{590\ nm}$ was found optimal for co-cultivation of explants. This finding is corroborated with the previous results that A_{590} of 0.1 - 1.0 was essential for effective transformation in cucumber (Chee 1990, Chee and Slighton 1991, Soniya and Das 2002, Selvaraj 2002). A 1-d co-cultivation period was found suitable for effective transformation and more than 1-d led to bacterial over growth. The antibiotic kanamycin was used for selection of transformed explants in which the wild-type explants will show chlorosis and growth inhibition (Benveniste and Davies 1973, Brasileiro 1998). The minimal kanamycin doses to bleach the explants were carried out by supplementing different concentration in shoot regeneration medium and finally about 50 % bleaching was obtained by 100 mg dm^{-3} kanamycin. However, the effect of kanamycin was noticeable at 150 mg dm^{-3} in the medium as the explants started to bleach after 10 to 15 d and total loss of chlorophyll occurred after 30 d. There was an absolute arrest of shoot growth at this concentration. Hence, we supplemented 150 mg dm^{-3} kanamycin to MS medium for the selection of putative transformed explants in the subsequent experiments. Our result was justified by the previous studies where kanamycin, in the concentration of 50 - 150 mg dm^{-3}, applied for 4 - 6 weeks, was used as an efficient selection agent for cucumber transformation (Chee 1990, Nishibayashi *et al.* 1996, Ganapathi and Perl-Treves 2000). The bacterial overgrowth during the selection of transformed explants was controlled with 300 mg dm^{-3} cephotaxime fortified MS medium. After 12 weeks of

culture in the selection media, the non-bleached and green shoots of cucumber were elongated and were transferred to rooting media. A 37 % rooting rate was observed 30-d after transfer to rooting media. The regenerated plants with well developed roots were hardened in *Vermiculite* and peat moss in 1:1 ratio. The plants were then transferred to containment facility for further growth, fruiting, collection of seeds for advancement of generation. In this study, a complete *in vitro* regeneration with cucumber shoot tip explants were obtained in 40 d, however, 120 d was necessary in transformation experiment. This longer time period in transformation is due to the antibiotics present in the media used for the stringent selection of transformed explants.

Molecular analysis through PCR amplification confirmed the presence of *cbf1* and *nptII* gene in 36 plants (T_0 generation) with a transformation efficiency of 1.62 % (Fig. *1B*). Southern blot analysis was performed randomly on 8 PCR-positive (T_1-generation) plants to confirm the presence, integration and copy number of *cbf1* gene in transgenic plant. Out of 8 PCR-positive plants, only two transgenic plants displayed single southern positive band, which indicate a single copy insertion in the transformed plants (S_1 and S_4 lines). The genomic DNA from wild-type plant did not hybridize with probe (Fig. *1C*). Expression of the transgene (*cbf1*) was detected in both the two lines of transgenic plants under chilling stress condition (4 °C for 36 h), while the RNA from nontransgenic plants did not display any band in RT-PCR (Fig. *1D*). The expression of *cbf1* was observed under chilling stress because the promoter used in the construct (RD29A), is a stress inducible promoter.

Development of marker-free transgenics has become an important aspect in transgenic technology due to fear of horizontal gene flow of antibiotic resistance gene and also hindrances from further gene pyramiding in the transgenic crops. Recently it has been reported that multi-auto-transformation (MAT) vector system was used to excise the selectable marker gene from transgenic and produce marker-free *Petunia hybrida* plants (Khan *et al*. 2010). In the present study, co-transformation method was used which enables integration of different transgenes at unlinked Mendelian loci and segregate independently in progeny. One transgenic line (S_4 line) was tested for segregation analysis by applying χ^2- test in T_1 generation plants using PCR data. Of 36 plants analyzed, only 3 plants were *cbf1* positive, 8 plants were *cbf1* and *nptII* positive (both) and 17 plants were *nptII* positive. Out of 3 marker-free transgenic plants obtained after segregation analyses; one selected marker-free transgenic (MT_2) was studied for various biochemical and physiological parameters related to chilling stress tolerance in T_2 generation.

To elucidate the chilling stress tolerance, we exposed both transgenic and wild-type plants to 4 °C in a cold cabinet. The transgenic plants could survive up to 36-h; however, wild-type plants were not able to tolerate this chilling stress and gradually died. Thus, this finding showed a clear physical and phenotypical expression, indicating better chilling tolerance of transgenic plants than the wild-type plants. Besides, the physiological and biochemical changes connected with adaptation of the transgenic plants to harsh conditions were studied in marker-free transgenic plants in comparison to wild-type plants. The cell membrane is one of the first targets of many plant stresses (Roy *et al*. 2006). Chilling stress damages the structure of the cell membrane, making it leaky and resulting in the outflow of ions that are vital for proper cell functioning (Uemura *et al*. 1995, Jia *et al*. 2002). The integrity of intracellular organelles is also disrupted leading to the loss of compartmentalization, reduction and impairing of photosynthesis, protein assembly and general metabolic processes. In our study, the MII of transgenic and wild-type plants did not change at control (non-stress) condition. However, there was a significant increase of MII in wild-type plants after 36-h cold treatment. The transgenic plant showed an average of 40 % lower MII than the wild-type plants during chilling stress (Table 1). This lower MII in transgenic plants suggests that integration of *cbf1* in cucumber genome contributed to maintenance of membrane integrity at low temperature.

Relative water content (RWC) is a parameter to assess the drought tolerant capacity of plants. RWC of transgenic and wild-type plants were assayed before and after the chilling stress treatment. The RWC of transgenic and wild-type plants did not show any difference at control (non-stress) condition; however, transgenic plants showed significantly higher RWC (13 %) in chilling treated plants as compared to the wild-type (Table 1). This finding suggested the higher water holding capacity of the transgenic plants and thus making a better adaptive capacity in extreme environment.

The antioxidant enzymes SOD and CAT ensure the removal of the superoxide radical and the H_2O_2 produced during abiotic stresses (Scandalios 1993, Fadzilla *et al*. 1997, Baek *et al*. 2006). The SOD and CAT activity increased more in transgenic plants as compared to wild-type under chilling stress (Table 1), suggesting the effective detoxification reactive oxygen species in transgenic plants at low temperature stress. These findings also corroborated with the previous report that the up-regulation of antioxidant enzymes is a consequence of the overexpression of *cbf1* gene (Hsieh *et al*. 2002).

Many plants respond to abiotic stress by accumulating high concentration of compatible osmolytes, such as proline, mannitol, glycine-betaine (Hoekstra *et al*. 2001). It has been reported that the proline content is elevated in tomato transgenic plants overexpressing *cbf1* (Hsieh *et al*. 2002) and *Arabidopsis* transgenic plants overexpressing *cbf3* gene (Gilmour *et a*l. 2000). Recently, it has been reported that transformation with *P5CSF129A* gene,

which encodes Δ^1-pyrroline-5-carboxylate synthetase (P5CS) in *Eucalyptus saligna* induced proline content four times higher in transformed than in non-transformed plants (Dibax *et al*. 2010). In this study, the transgenic plants had significantly higher proline content (46.8 %; $P \leq 0.05$) under chilling stress as compared to wild-type plants; however, no change was observed in non-stress conditions (Table 1). Proline is a well known compatible osmolyte that protects membranes and proteins against the adverse effect of inorganic ions and temperature extremes by scavenging reactive oxygen species at high concentration. Proline also acts as a mediator of osmotic adjustment, stabilizer of subcellular structures, and also as a buffer of cellular redox potential in many plants. Our finding indicates that the transgenic cucumber expressing *cbf1* gene might have induced up-regulation of the genes involved in the pathway for proline synthesis in plants, corroborating with the previous report that the *P5CS* gene is up-regulated in transgenic *cbf1* tomato plants (Hsieh *et al*. 2002). Thus, elevated proline content might enhance the tolerance to drought, salinity and cold stress (Kavi *et al*. 1995).

In the present study, the biochemical and physiological parameters analyzed did not show any induction and/or change under non-stress conditions; however, the responses were observed under stress conditions, confirming that RD29A is a stress inducible promoter. The transgenic plants did not show any significant variations in morphological parameters like plant height, leaf type, fruit size, shape, mass, yield per plant, days to flowering, days to fruiting, days to first fruit set, *etc*., as compared to wild-type plants (data not shown). Thus, the results of the present study suggest the marker-free *cbf1* transgenic technology as an effective approach to impart chilling tolerance in cucumber.

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