Improving low-temperature tolerance in sugarcane by expressing the *ipt* gene under a cold inducible promoter

N.G. BELINTANI[1], J.T.S. GUERZONI[1], R.M.P. MOREIRA[2] and L.G.E. VIEIRA[1]*

Plant Biotechnology Laboratory, IAPAR, Londrina, 86001-970 PR, Brazil[1]
State University of Londrina, General Biology Department, Londrina, 86051-990 PR, Brazil[2]

Abstract

Sugarcane is cultivated in tropical and subtropical regions where cold stress is not very common, but lower yields and reduced industrial quality of the plants are observed when it occurs. In our efforts to enhance cold tolerance in sugarcane, the gene encoding the enzyme isopentenyltransferase (*ipt*) under control of the cold inducible gene promoter AtCOR15a was transferred *via* biolistic transformation into sugarcane (*Saccharum* spp.) cv. RB855536. Semi-quantitative RT-PCR using *GAPDH* encoding glyceraldehyde-3-phosphate dehydrogenase as the normalizer gene showed the increased expression of the *ipt* gene under cold stress. The detached leaves of genetically modified plants subjected to low temperatures showed visible reduction of leaf senescence in comparison to non-transgenic control plants. Induced overexpression of *ipt* gene also enhanced cold tolerance of non-acclimated whole plants. After being subjected to freezing temperature, leaf total chlorophyll contents of transgenic plants were up to 31 % higher than in wild type plants. Also, lower malondialdehyde content and electrolyte leakage indicated less damage induced by cold in transgenic plants. Thus, the expression of *ipt* driven by the stress inducible *COR15a* promoter did not affect plant growth while providing a greater tolerance to cold stress.

Additional key words: cold stress, *COR15a* promoter, cytokinins, *Saccharum officinarum*, senescence.

Introduction

Sugarcanes (*Saccharum* spp. - hybrids of *S. officinarum* and *S. spontaneum*) are tropical grasses widely grown in both hemispheres in over 120 countries worldwide. Sugarcane is considered a cold-sensitive plant (Tai and Lentini 1998) and occupies the tropical and subtropical areas where frost is not a usual phenomenon. However, when it occurs there are crop failures accompanied by reduced capacity for sucrose accumulation in the stalks (D'Hont *et al.* 2008). Cold damage of mature plants can kill re-growth of young plants and ratoons. The optimum temperature for sugarcane growth is about 35 ºC, and temperatures below 20 ºC significantly limit growth and yield (Moore 1987).

Low temperature stress, including temperatures above (chilling) and below 0 ºC (freezing), causes severe losses in most crops (Xin and Browse 2000). The accumulation of ice in the intercellular spaces can potentially result in the physical disruption of cells and tissues. The injury also involves effects on membrane structure and function caused by dehydration (Uemura *et al.* 1995). Low temperatures alter phytohormone contents, prolong cell cycle and trigger cold acclimation signaling pathways. However, it is still unclear how the cross-talk in these signaling pathways proceeds (Xia *et al.* 2009). The cytokinins (CKs) have a profound effect on the senescence of plant organs (Mikkelsen *et al.* 2004) and participate in the modulation of metabolism and morphogenesis in response to environmental stimuli (Sakakibara 2006).

Most of the research done to determine the role that CKs play in plant growth and development analyzed the effects of applied cytokinin, but it is not always clear that the effects of exogenous hormones are indicative of the actual physiological role of the endogenous hormone (Kieber 2002). To avoid these problems, endogenous

contents of CKs have been increased by creating transgenic plants with increased activity of isopentenyl transferase encoded by the *ipt* gene (Akiyoshi *et al.* 1984). Cytokinin overproduction in these plants provided lower leaf abscission, maintenance of photosynthetically active leaves (Květoň 2006), delay in flowering (Gan and Amasino 1995, McCabe *et al.* 2001), and increased tolerance to biotic and abiotic stresses (Rivero *et al.* 2007, Swartzberg *et al.* 2008, Pasquali *et al.* 2009).

As most of the transgenic plants with constitutively expressed *ipt* exhibit some delay in leaf senescence, as well as a variety of morphological and developmental abnormalities (Wang *et al.* 1997), different promoters have been used to manipulate the expression of *ipt*, including heat, wound and light-inducible promoters, and tissue or development specific promoters. Using the senescence-specific *SAG12* promoter (Gan and Amasino 1995), delayed yellowing was reported in lettuce and broccoli both before and after harvest (Chen *et al.* 2001, McCabe *et al.* 2001). Leaf senescence has also been successfully delayed when the *ipt* gene was under copper-inducible (McKenzie *et al.* 1998), organ-specific (Pasquali *et al.* 2009) or stress inducible (Rivero *et al.* 2007) promoters. Remarkably, the suppression of leaf senescence due to the production of cytokinins contributed to the efficient scavenging of reactive oxygen species and, as consequence, protected the photosynthetic

apparatus during drought stress, leading to increased tolerance and suppression of cell death program in senescent cells (Rivero *et al.* 2007). Studies with transgenic petunia and chrysanthemum containing the cold inducible promoter of the *Arabidopsis thaliana* gene *COR15a*, that encodes a 15-kD cryoprotective polypeptide targeted to the chloroplasts (Thomashow 1994), fused to *ipt* gene (AtCOR15a:*ipt*) resulted in reduction of senescence of these plants after a period of cold acclimation (Khodakovskaya *et al.* 2005).

Many of the *Arabidopsis* cold-responsive or *COR* genes are regulated by the C repeat/dehydration-responsive element (CRT/DRE). It is a *cis*-acting element containing the CCGAC motif, which activates transcription in response to both low temperature and water deficit, including the cold-inducible expression of the gene *COR15*a (Cheong *et al.* 2002).

Besides the reduced genetic variability for cold tolerance in the *Saccharum* complex, conventional breeding would require at least a decade just to introgress alleles that confer tolerance to cold (Ming *et al.* 2006). In this context, this work aimed to transform sugarcane with the *ipt* gene, which encodes the enzyme isopentenyl transferase from *A. tumefaciens*, under control of the *A. thaliana* cold inducible gene promoter COR15a and evaluate the response of transgenic plants to low temperatures.

Materials and methods

Tissue culture and plant transformation: Leaf segments of 6- to 8-month-old plants of *Saccharum officinarum* L. cv. RB855536 were surface sterilized in 70 % (v/v) ethanol for 1 min and in 2.5 % (v/v) sodium hypochlorite for 20 min. Transverse segments 2 - 3 mm wide were excised from the apical meristem and cultured on Murashige and Skoog (1962; MS) medium supplemented with 20 g dm^{-3} sucrose, 3 mg dm^{-3} 2,4-dichlorophenoxy-acetic acid (2,4-D) and 8 g dm^{-3} *Bacto* agar (MSC3 medium). The explants were kept in the dark for 4 weeks and then the selected embryogenic calli were cultured on Petri dishes with the same basal medium containing 1 mg dm^{-3} 2,4-D (MSC1 medium) for 4 d prior to bombardment.

The expression cassette AtCOR15a:*ipt*:nos, with the *ipt* gene of *A. tumefaciens* under control of a cold inducible promoter AtCOR15a and the *nos* terminator, was removed from pBIN19 vector (kindly provided by Dr. Richard McAvoy, University of Connecticut, Storrs, USA). A *Xho*I/*Sma*I fragment was cloned into the *pBluescript II KS*(+/-) by sticky ends to obtain the pIBI34 vector. This vector and the plasmid pEmuKN, which contains the selectable marker gene *nptII* fused to the recombinant promoter Emu (Last *et al.* 1991), were used at equimolar amount for co-bombardment.

The transformation was performed essentially as

described by Molinari *et al.* (2007). Following bombardment, calli were transferred to induction medium MSC1 at 25 °C for 3 d in the dark. Calli were then transferred to selection medium MSC1 containing 50 mg cm^{-3} geneticin under 16-h photoperiod at 30 μmol m^{-2} s^{-1} of PAR and subcultured every two weeks. After this selection process, calli with about 5 - 10 mm diameter were transferred to regeneration medium (MSC3 without 2,4-D and geneticin) under the same conditions described above until plant regeneration. The transformants and non-transformed plants were micropropagated according to Lee (1987) and then transferred to pots in greenhouse for hardening.

Polymerase chain reaction: Genomic DNA was prepared from young sugarcane leaves using the procedure developed by Doyle and Doyle (1987). The *ipt*-specific primers F: 5'-ACGACGACCGCGATAGCT CT-3' and R: 5'-AAGCGGCTGCGTTAACTTGG-3' and *nptII*-specific primers F: 5'-GAGGCTATTCGGCTA TGACTGG-3' and R: 5'-ATCGGGAGCGGCGTAACC GTA-3' were used to amplify 643 bp and 650 bp fragments, respectively. The PCR reactions were carried out in a total volume of 0.02 cm^3 comprising 50 ng genomic DNA, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.0 mM MgCl$_2$, 0.1 mM of each dNTP, 1 U *Taq* DNA

polymerase and 10 mM of each primer. The amplifications were performed with an initial cycle of 3 min at 94 ºC, followed by 35 cycles of 1 min at 94 ºC, 45 s at 58 ºC, 1.5 min at 72 ºC, and a final extension cycle of 5 min at 72 ºC.

Cold tolerance experiments: Three transgenic events (M1, E3 and J5) were chosen to determine leaf senescence after an incubation period at different temperatures. Four excised leaf segments (5 cm-long) were surface sterilized and then placed on moist filter paper in Petri dish. The plates were exposed to different treatments for a 7-d period: *1*) continuous dark at 27 ºC without a prior cold induction treatment; *2*) a cold period of 2 d at 4 ºC in the dark followed by continuous dark at 27 ºC; *3*) one day at 4 ºC, followed by 6 h at 0 ºC, and then 27 ºC, always in the dark. Leaf chlorophyll content, assayed prior to the start and at the end of the experiment, were obtained in triplicate.

Plants of four transformation events (M1, F1, I2 and E3) and a non-transformed control (wild type, WT) were used to access cold tolerance in a growth chamber in a 16 h-photoperiod and irradiance of 220 µmol m^{-2} s^{-1}. The plants were multiplied *via* organogenesis *in vitro* and acclimatized in greenhouse. Four plants (replicates) of each event were transplanted in 3 dm^3 pots and the experiment was carried out when the plants were four months old. First, leaves were collected under conditions of greenhouse (28 ºC) 24 h prior transferring the plants to an antechamber at 20 ºC. After 6 h in this condition, the plants were placed in the growth chamber with the initial temperature set at 15 ºC. Sampling of leaf tissue for transcriptional expression analysis of *ipt* gene and physiological analysis (leaf chlorophyll, MDA and ion leakage) were performed at the following points: *1*) day 0 - plants in greenhouse, 24 h before transferred to the growth chamber, 2) post-freezing - 8 h after reaching 0 ºC, and *3*) post-stress - plants in greenhouse (28 ºC) 24 h after the cold stress.

Measurement of chlorophyll and MDA contents and electrolyte leakage: Chlorophyll (Chl) content was determined in 80 % acetone extract spectrophoto-metricaly (*UV-1650 PC, Shimadzu*, Kyoto, Japan) at 645 and 663 nm according to Lichtenthaler (1987). For determination of malondialdehyde (MDA) content according to Heath and Packer (1968), 100 mg of leaf tissue were homogenized with 3 cm^3 of 80:20 (v/v) ethanol:water, followed by centrifugation at 3 000 *g* for 10 min. Absorbances were read at 532 and 600 nm.

The degree of membrane integrity was assessed by

the leakage of electrolytes from the leaves of plants according to Rizhsky *et al.* (2002). Five leaf discs for every treatment group were immersed in 5 cm^{-3} of *MiliQ* water for 3 h. The results are expressed as a percentage of total ion content after disrupting the cell membranes by incubation in a bathing solution at 98 ºC for 25 min. The amount of electrolyte leakage was measured conductometrically (*Micronal* model *B331*, SP, Brazil).

Semi-quantitative RT-PCR: Total RNAs were extracted from leaves using *Trizol®* reagent (*Invitrogen*, Carlsbad, CA, USA). RNA quality was verified on 1 % agarose gel stained with ethidium bromide (0.5 mg cm^{-3}) in 1× TAE buffer. RNA content was quantified on a *NanoDrop 1000* spectrophotometer (*Thermo Scientific*, Wilmington, DE, USA). RNA (5 µg) was treated with DNAse and cDNAs were synthesized using the kit *ThermoScript*^{TM} in RT-PCR system (*Invitrogen*) according to the manufacturer's protocol. The *ipt* primers described previously and *GAPDH* primers F: 5'-TCTTGCTGACTGCTTATGGA-3' and R: 5'-TGTGAGGTCAACCACTGAGACAT-3' were used for the analysis of the transgene expression. To accomplish the amplification of the *ipt* and the normalizer glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) genes in exponential phase, previous analysis of amplification products in different conditions and numbers of PCR cycles were tested. The cycling conditions for *ipt* gene were: initial denaturation at 94 ºC for 1 min, followed by 35 cycles of 1 min at 94 ºC, 1 min at 56 ºC, 1.5 min at 72 ºC and final extension at 72 °C for 5 min. For the normalizer gene, the cycling conditions were 94 ºC for 5 min followed by 25 cycles of 94 ºC for 1 min, 59 ºC for 1 min and final extension at 72 °C for 5 min. The DNA was subjected to electrophoresis on agarose gel and stained with ethidium bromide. The intensity band signals were analyzed with *Kodak Digital Science 1D* (*KDS v. 2.0.3*) with the image background subtracted. The amplifications of each treatment were performed in triplicate.

Data analysis: The senescence assay and the experiment with plants in growth chamber were arranged in a completely randomized block design, in split-plot scheme over time where the main plots were represented by control and transgenic plants and sub-plots by temperature conditions. Each of the four replications for chlorophyll, conductivity and MDA analysis was constituted by a pool of leaves from four transgenic and non-transformed control plants. Three technical replicates were performed. Differences among treatments were analyzed by one-way *ANOVA* using Tukey's multiple range test at *P* < 0.05.

Results

Approximately 3500 explants were grown in induction medium and produced enough embryogenic calli to

bombard 150 plates. After subcultivation in selective medium, 70 putative transgenic plants were regenerated

and the plantlets subsequently hardened in greenhouse (Fig. 1). The sugarcane transformation protocol here resulted in 70 regenerated plantlets. Only in 32 from them amplified *nptII* fragment of 650 bp was confirmed, in 17 *ipt* amplification fragment of 643 bp, and in 15 both *ipt* and *nptII* fragments, from which plantlets were chosen for cold tolerance experiments. No apparent morphological differences were observed in transgenic plants when compared to non-transformed plants four months after the acclimatization.
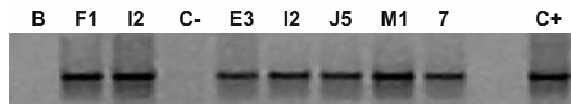

Fig. 1. PCR analysis of selected transformation events using *ipt* gene specific primers showing the presence of the expected 643 bp fragment. *Lane B: blank; lanes* F1, I2, E3, J5, M1: DNA from transgenic plants selected for cold stress experiments, C-: non-transformed control plant; 7: transgenic plant; C+: positive control (DNA of control plant + pIBI34 plasmid).

Table 1. Chlorophyll content [mg g^{-1}(f.m.)] in sugarcane leaf segments incubated in the dark for 7 d. M1, E3, J5 - transgenic plants, WT - non-transformed control. Means ± SE, $n = 4$. Lowercase letters refer to the comparison of WT and transgenic plants; capital letters refer to the comparison of different temperature treatments. The same letters represent no significant differences between means at $P < 0.05$ determined by Tukey's test.

	Day 0	27 °C - day 7	4 °C - day 7	0 °C - day 7
M1	5.7 ± 0.08 [aA]	1.9 ± 0.06 [aC]	2.2 ± 0.07 [aC]	5.7 ± 0.08 [aB]
E3	5.5 ± 0.12 [abA]	1.6 ± 0.05 [aC]	1.7 ± 0.04 [bC]	3.6 ± 0.09 [aB]
J5	5.4 ± 0.12 [abA]	0.9 ± 0.03 [bC]	2.4 ± 0.04 [aB]	2.3 ± 0.05 [bB]
WT	3.1 ± 0.13 [bA]	0.6 ± 0.06 [cD]	1.1 ± 0.06 [cC]	2.1 ± 0.09 [bB]

Total chlorophyll content was determined in detached leaves incubated in dark for 7 d at different temperatures. There were no significant differences in Chl content of detached leaves between transgenic plants and wild type (WT) plant at the beginning of the experiment (day 0) (Table 1). When incubated in the dark for 7 d at 27 °C, the leaf segments of the transgenic plants had higher chlorophyll content than WT. At this condition, the plant J5 had the lowest Chl amount among the three plants tested, but still higher than WT (Table 1). The exposure of the leaf segments to 4 °C for 2 d before placing in the dark at 27 °C for the remaining 5 d of the treatment caused a higher ($P < 0.05$) decline in the total Chl in the control in comparison with the transgenic leaves. The same could be verified when the leaf segments were incubated in the dark at 0 °C for 6 h after 1 d acclimation period at 4 °C (Table 1).

The whole transgenic plants showed higher Chl content in comparison to WT plants before being subjected to low temperature stress (day 0, Table 2). A large increase of leaf chlorophyll content in all *ipt* transformants was observed when plants were subjected to low temperature stress (post freeze treatment). Significant differences between the different transgenic plants and WT were maintained also 24 h after the end of the stress, even though the Chl content declined (Table 2).

There were no differences in MDA content in

Table 2. Chlorophyll content [mg g^{-1}(f.m.)], MDA content [nmol g^{-1}(f.m.)] and electrolyte leakage [%] in sugarcane leaves under different temperatures. M1, F1, I2, E3 - transgenic plants, WT - non-transformed control. Means ± SE, $n = 4$. Lowercase letters refer to the comparison of control and transgenic plants; capital letters refer to the comparison of different temperatures. The same letters represent no significant differences between means at $P < 0.05$ determined by Tukey's test.

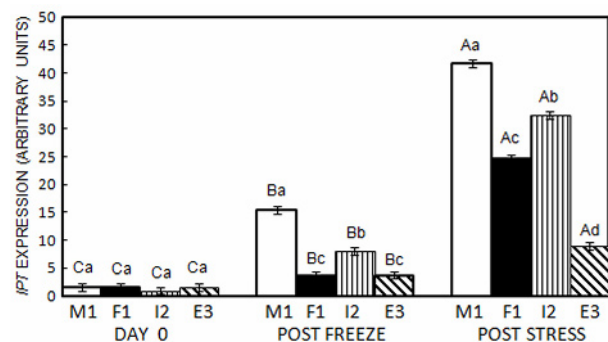|		Day 0	Post freezing	Post stress
Chl	M1	2.3 ± 0.04 [bcB]	8.2 ± 0.52 [aA]	2.9 ± 0.06 [abA]
	F1	3.6 ± 0.04 [aB]	6.1 ± 0.47 [aA]	3.6 ± 0.31 [abA]
	I2	2.8 ± 0.03 [abC]	7.3 ± 0.41 [aA]	4.7 ± 0.15 [aB]
	E3	2.3 ± 0.08 [bcC]	8.3 ± 0.31 [aA]	4.1 ± 0.47 [aB]
	WT	1.1 ± 0.01 [cB]	1.9 ± 0.03 [bB]	1.2 ± 0.25 [bB]
MDA	M1	13.42 ± 0.70 [aB]	35.97 ± 3.65 [bcA]	37.93 ± 1.04 [bA]
	F1	13.03 ± 2.26 [aB]	38.19 ± 2.00 [bcA]	36.62 ± 1.22 [bA]
	I2	11.73 ± 0.52 [aC]	30.76 ± 4.00 [cA]	23.33 ± 3.00 [cB]
	E3	12.51 ± 1.82 [aC]	41,97 ± 2.26 [bA]	32.58 ± 2.26 [bB]
	WT	14.47 ± 2.09 [aC]	74.29 ± 1.56 [aA]	46.92 ± 0.78 [aB]
Leakage	M1	2.69 ± 0.30 [aA]	19.75 ± 0.99 [bA]	8.00 ± 0.28 [aA]
	F1	4.36 ± 0.80 [aC]	16.49 ± 1.75 [bB]	2.87 ± 0.27 [aC]
	I2	2.31 ± 0.19 [aB]	22.18 ± 1.41 [bA]	3.46 ± 0.14 [aB]
	E3	2.29 ± 1.34 [aB]	12.40 ± 0.60 [bAB]	5.08 ± 0.06 [aB]
	WT	2.28 ± 0.75 [aC]	40.56 ± 0.13 [aB]	4.41 ± 0.29 [aC]

Fig. 2. RT-PCR analysis of *ipt* expression in sugarcane transgenic plants normalized with *GAPDH*. The lowest ratio of expression of the *ipt* gene (*ipt/GAPDH* of event I2 on day 0) was arbitrarily defined as 1 for comparison. Values are presented by means ± SE, $n = 4$. Lowercase letters refer to the comparison of control and transgenic plants; capital letters refer to the comparison of different temperatures. The same letters represent no significant differences between means at $P < 0.05$ determined by Tukey's test.

transgenic and WT plants before stress. However, the WT plants showed significantly higher MDA content (lipid peroxidation) when compared to the transformed plants during cold stress. There was a slight decrease in the MDA content in the transgenic plants 24 h after the cold stress, whereas it remained increased in WT plants (Table 2).

After the low temperature treatment, the plants expressing the *ipt* gene exhibited less electrolyte leakage compared to WT plants, demonstrating that the four transgenic lines were more tolerant than the control plants. No significant difference was found between the transgenic lines after the stress, with all plants exhibiting similar low levels of leakage (Table 2).

Discussion

Exposure of sugarcane plants to chilling temperatures for three weeks results in reduced number, length and mass of stalks and sugar production, and extended cold stress can kill young and developing shoots during re-growth (Tai and Lentini 1998). Increment in cold temperature tolerance of even a few degrees would be of considerable benefit to sugarcane crop exposed to low temperatures during the winter period. As progress in sugarcane breeding programs to develop cultivars with improved low temperature tolerance has been slow due to the limitation of naturally occurring genetic variability in cold-hardy traits, the use of genetic engineering as part of a combined strategy might improve cold tolerance.

An interaction between cytokinin signaling and cold response has been observed in *Arabidopsis* (Lee *et al.* 2005). The role of CKs in low temperature tolerance can be studied by using transgenic plants with enhanced endogenous cytokinin production due to the insertion of the *ipt* gene from *Agrobacterium tumefaciens* T-DNA. However, plants expressing the *ipt* gene constitutively exhibit some delay in leaf senescence, as well as a variety of morphological and developmental abnormalities (Gan and Amasino 1995, Wang *et al.* 1997). Also, constitutive expression of traits that are normally induced in response to environmental stress will sometimes lead to costs in the absence of that stress (Su and Wu 2004). Therefore, it is desirable to use stress-regulated promoters since responses to low temperatures are not constitutive but triggered by specific signals. Petunia and chrysanthemum transformed with the *ipt* gene under control of the cold inducible promoter AtCOR15a showed positive regulation of the isopentenyltransferase gene after a short period of cold stress. Excised leaves from plants with elevated CK content remained green and healthy during prolonged dark storage after cold treatment (Khodakovskaya *et al.* 2005).

In this work, we assessed the ability of the *ipt* gene driven by the AtCOR15a promoter to increase cold tolerance in *Saccharum officinarum*, a species adapted to

The expression of *ipt* gene in the sugarcane plants during the experiment in growth chamber was measured by semi-quantitative RT-PCR analyzing the band intensity of both *ipt* and *GAPDH* genes (Fig. 2). Low expression was detected in all transgenic plants before the beginning of the low temperature stress. After being subjected to freezing temperature, the plant M1 showed the highest expression, with about 9-fold increase in comparison to the plants grown at 28 ºC in the greenhouse. One day after the stress, all transgenic plants showed increased transcriptional activity of the *ipt* gene, the plant M1 still presenting the highest *ipt* mRNA content in leaves, with approximately 25-fold increase compared to day 0.

warm regions. For this purpose, we obtained 70 putative transgenic plants that resulted in 32 positive plantlets confirmed *via* PCR. In a microprojectile-transformation study with sugarcane, Bower *et al.* (1996) were able to obtain 67 - 79 % of co-transformed plants using the Emu promoter and the geneticin resistance gene *aphA*. In this work, 47 % (15 out of 32) of the regenerated plants selected on geneticin were co-transformed. It is important to mention that all the plants obtained in this work showed no morphological abnormalities under normal growth temperatures, similarly to previous report with plants containing the AtCOR15a:*ipt*:nos transformation cassette (Khodakovskaya *et al.* 2005).

Chlorophyll content, lipid peroxidation and ion leakage, well established markers of senescence and abiotic stresses (Lim *et al.* 2007), were used to evaluate the effect of the enhanced transcription of the *ipt* gene in sugarcane leaves under low temperatures. In the senescence assay with detached leaf segments, there was a small but significant increase in chlorophyll contents in both transgenic and WT plants at 27 °C, a temperature that supposedly should not induce the *COR15a* promoter. One possible reason for this is the presence of CRT/DRE sequences in the *AtCOR15a* gene promoter region (Baker *et al.* 1994). Cold-responsive (*COR*) genes are controlled by transcription factors, including the CBF/DREB family that bind the CRT/DRE motif of *COR* genes, that appear to be ubiquitously present in plants regardless of freezing tolerance capacity (Benedict *et al.* 2006, Agarwal and Jha 2010). These transcription factors are involved in cross-regulation of genes responding to different stresses, with the CBF/DREB family among the early responsive genes after injury (Cheong *et al.* 2002). As the leaf segments have been excised for plating, the CRT/DRE sequences present in the *AtCOR15a* gene promoter could have caused a basal induction of the *COR15a* promoter in the transgenic sugarcane plants by dehydration and/or wounding even at higher temperatures. The higher chlorophyll content in detached leaf segments of

transgenic plants in comparison with WT when the plants were exposed to low temperature treatments (4 and 0 °C) can be explained by the well-established *COR15a* promoter induction in response to cold. The higher activity of this promoter would increase the *ipt* gene transcription and, consequently, reduce the senescence by increasing CK content in the transformed plants.

In the growth chamber assay, the level of *ipt* transcripts measured by semi-quantitative RT-PCR increased when plants were subjected to the cold stress, reaching even higher values 24 h after stress, indicating the maintenance of transcriptional activity of the promoter *COR15a* for a long period in the transgenic plants. No hybridization signal was detected with *ipt* gene radioactive probe in Northern blotting (data not shown). It suggests that transgene expression activated by promoter *COR15a* at low temperatures cannot be considered as being drastically up-regulated in the transgenic plants. However, the increased transcriptional expression observed in the transgenic plants, when compared to the expression detected in plants grown at normal temperature, was enough to increase the cold tolerance of the sugarcane plants.

Electrical conductivity, as an indirect measure of cell membrane damage, and production of MDA from the peroxidation of polyunsaturated fatty acids mediated by reactive oxygen species (ROS) are commonly used as a stress indicator in plant membranes under cold stress (Zhang *et al.* 2010). In this work, cold stress-induced lipid peroxidation and electrolyte leakage were significantly higher in WT plants when compared with transformed plants in both freezing and post-stress treatments, indicating greater protection to cell membrane in the leaves of *ipt* transformed plants. As overproduction of CKs seems to affect directly the antioxidant enzymatic defense (Synková *et al.* 2006), the capacity of the

transgenic plants to better cope with cold stress could be attributed to the high activity of antioxidant enzymes.

The higher expression of *ipt* transcripts after return of transgenic plants to non-stressful temperatures did not correspond to the changes observed in chlorophyll, MDA and ion leakage. Such result can be explained by post-transcriptional regulatory mechanisms, such as: *1)* the negative regulation of isopentenyltranferase activity effected by binding proteins, which are involved in negative feedback mechanisms and act in CK signaling pathways regulation (To and Kieber 2007), *2)* CK degradation or metabolic inactivation (Sakakibara 2006), and *3)* differences in biochemical properties of CK receptors and in ligand preference (Werner and Schmulling 2009). Analysis of CK content and identification of the most active forms during cold stress in transgenic plants are under way to help understanding the uncoupling of *ipt* gene expression and the senescence indicator in the post stress conditions.

In summary, the cold induced expression of the isopentenyltransferase gene under the *AtCOR15a* promoter was an effective strategy to increase the tolerance of sugarcane plants to low temperatures, as demonstrated by the altered senescence of leaf segments *in vitro*. Also, transgenic plants exposed to a short period of cold temperature conditions presented increase in chlorophyll retention and lower damage in cell membrane. Finally, due to the fact that up-regulation of the *ipt* gene in sugarcane plants by the cold-inducible *AtCOR15a* promoter did not cause unwanted phenotypic changes usually associated with constitutive *ipt* expression, this approach could represent a promising strategy in avoiding severe productivity losses and freeze-deterioration observed in higher latitudes sugarcane growing regions.

References

Agarwal, P.K., Jha, B.: Transcription factors in plants and ABA dependent and independent abiotic stress signaling. - Biol. Plant. **54**: 201-212, 2010.

Akiyoshi, D., Klee, H., Amasino, R., Nester, E., Gordon, M.: T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. - Proc. nat. Acad. Sci. USA. **81**: 5994-5998, 1984.

Baker, S.S., Wilhelm, K.S., Thomashow, M.F.: The 5'-region of *Arabidopsis thaliana cor15a* has *cis*-acting elements that confer cold, drought and ABA-regulated gene expression. - Plant mol. Biol. **24**: 701-713, 1994.

Benedict, C., Skinner, J.S, Meng, R., Chang, Y., Bhalerao, R., Huner, N.P.A., Finn, C.E., Chen, T.H.H., Hurry, V.: The CBF1-dependent low temperature signaling pathway, regulon and increase in freeze tolerance are conserved in *Populus* spp. - Plant Cell Environ. **29**: 1259-1272, 2006.

Bower, R., Elliott, A.R., Potier, B.A.M., Birch, R.G.: High-efficiency, microprojectile-mediated cotransformation of sugarcane, using visible or selectable markers. - Mol. Breed. **2**: 239-249, 1996.

Chen, L.F.O., Hwang, J.Y., Charng, Y.Y., Sun, C.W., Yang, S.F.: Transformation of broccoli (*Brassica oleracea* var. *italica*) with isopentenyltransferase gene via *Agrobacterium tumefaciens* for post-harvest retardation. - Mol. Breed. **7**: 243-257, 2001.

Cheong, Y.H., Chang, H.-S., Gupta, R., Wang, X., Zhu, T., Luan, S.: Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in *Arabidopsis*. - Plant Physiol. **129**: 661-677, 2002.

D'Hont, A., Souza, G.M., Menossi, M., Vincentz, M., Van-Slyuis, M.-A., Glaszmann, J.C., Ulian, E.: Sugarcane: a major source of sweetness, alcohol and bioenergy. - In: Moore, P.H., Ming, R. (ed.): Genomics of Tropical Crop Plants. Pp. 483-513. Springer, New York 2008.

Doyle, J.J., Doyle, J.L.: Isolation of plant DNA from fresh

tissue. - Focus **12**: 13-15, 1987.

Gan, S., Amasino, M.: Inhibition of leaf senescence by autoregulated production of cytokinin. - Science **270**: 1986-1988, 1995.

Heath, R.L., Packer, L.: Photoperoxidation in isolated chloroplast I. Kinetics and stoichiometry of fatty acid peroxidation. - Arch. Biochem. Biophys. **125**: 189-198, 1968.

Khodakovskaya, M., Li, Y., Vankova, R., Malbeck, J., Mcavoy, R.: Effects of *cor15a-ipt* gene expression on leaf senescence in transgenic *Petunia × hybrida* and *Dendranthema × grandiflorum*. - J. exp. Bot. **56**: 1165-1175, 2005.

Kieber, J.J.: Cytokinins. - In: Somerville, C., Meyerowitz, E. (ed.): The *Arabidopsis* Book. Pp. 2-25. American Society of Plant Biologists, Rockville 2002.

Květoň, J.: Extent of *ipt* gene expression and resulting amount of cytokinins affect activities of carboxylation enzymes in transgenic plants. - Biol. Plant. **50**: 21-30, 2006.

Last, D.I., Brettell, R.I.S., Chamberlain, D.A., Chaudhury, A.M., Larkin, P.J., Marsh, E.L., Peacock, W.J., Dennis, E.S.: *pEmu*: an improved promoter for gene expression in cereal cells. - Theor. appl. Genet. **82**: 582-588, 1991.

Lee, B., Henderson, D.A., Zhua, J.-K.: The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1. - Plant Cell **17**: 3155–3175, 2005.

Lee, T.S.G.: Micropropagation of sugarcane (*Saccharum* spp.). - Plant Cell Tissue Organ Cult. **10**: 47-55, 1987.

Lichtenthaler, H.K.: Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. - Meth. Enzymol. **148**: 350-382, 1987.

Lim, P.O.K., Kim, H.J., Nam, H.G.: Leaf senescence. - Annu. Rev. Plant Biol. **58**: 115-136, 2007.

McCabe, M.S., Garrat, L.C., Schepers, F., Jordi, W.J.R., Stoopen, G.M., Davelaar, E., Van Rhijn, H.A., Power, J.B., Davey, M.R.: Effects of P_{SAG12}-*ipt* gene expression on development and senescence in transgenic lettuce. - Plant Physiol. **127**: 505-516, 2001.

McKenzie, M.J., Mett, V., Reynolds, P.H.S., Jameson, P.: Controlled cytokinin production in transgenic tobacco using a copper-inducible promoter. - Plant Physiol. **116**: 969-977, 1998.

Mikkelsen, M.D., Naur, P., Halkier, B.A.: *Arabidopsis* mutants in the C-S lyase of glucosinolate biosynthesis establish a critical role for indole-3-acetaldoxime in auxin homeostasis. - Plant J. **37**: 770-777, 2004.

Ming, R., Moore, P.H., Wu, K.-K., D'Hont, A., Glaszmann, J., Thew, T.L.: Sugarcane improvement trough breeding and biotechnology. - Plant Breed. Rev. **27**: 15-118, 2006.

Molinari, H.B., Marur, C.J., Daros, E., Campos, M.K.F., Carvalho, J.F.R.P., Bespalhok Filho, J.C., Pereira, L.F.P., Vieira, L.G.E.: Evaluation of the stress-inducible production of proline in transgenic sugarcane (*Saccharum* spp.): osmotic adjustment, chlorophyll fluorescence and oxidative stress. - Physiol. Plant. **130**: 218-229, 2007.

Moore, P.H.: Breeding for stress resistance. - In: Heinz, D.J. (ed.): Sugarcane Improvement through Breeding. Pp. 503-542. Elsevier, Amsterdam 1987.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-479, 1962.

Pasquali, G., Orbović, V., Grosser, J.W.: Transgenic grapefruit plants expressing the $P_{APETALA3}$-IPT_{gp} gene exhibit altered expression of PR genes. - Plant Cell Tissue Organ Cult. **97**: 215-223, 2009.

Rivero, R.M., Kojima, M., Gepstein, A., Sakakibara, A., Mitler, R., Gepstein, S., Blumwald, E.: Delayed leaf senescence induces extreme drought tolerance in a flowering plant. - Proc. natl. Acad. Sci. USA **104**: 19631-19636, 2007.

Rizhsky, L., Hallak-Herr, E., Van Breusegem, F., Rachmilevitch, S., Barr, J.E., Rodermel, S., Inze, D., Mittler, R.: Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase. - Plant J. **32**: 329-342, 2002.

Sakakibara, H.: Cytokinins: activity, biosynthesis, and translocation. - Annu. Rev. Plant Biol. **57**: 431-449, 2006.

Su, J., Wu, R.: Stress-inducible synthesis of proline in transgenic rice confers faster growth under stress conditions than that with constitutive synthesis. - Plant Sci. **166**: 941-948, 2004.

Swartzberg, D., Kirshner, B., Rav-David, D., Elad, Y., Granot, D.: *Botrytis cinerea* induces senescence and is inhibited by autoregulated expression of the *ipt* gene. - Eur. J. Plant Pathol. **120**: 289-297, 2008.

Synková, H., Semorádová, Š., Schnablová, R., Witters, E., Hušák, M., Valcke, R.: Cytokinin-induced activity of antioxidant enzymes in transgenic *Pssu-ipt* tobacco during plant ontogeny. - Biol. Plant. **50**: 31-41, 2006.

Tai, P.Y.P., Lentini, R.S.: Freeze damage of Florida sugarcane. - In: Anderson, D.L. (ed.): Sugarcane Handbook. Florida Cooperative Extension, Gainesville 1998.

Thomashow, M.F.: *Arabidopsis thaliana* as a model for studying mechanisms of plant cold tolerance. - In: Meyerowitz, E., Somerville, C. (ed.): *Arabidopsis*. Pp. 807-834. Cold Spring Harbor Laboratory Press, Cold Spring Harbor - New York 1994.

To, J.P.C., Kieber, J.J.: Cytokinin signaling: two-components and more. - Trends Plant Sci. **13**: 85-92, 2007.

Uemura, M., Joseph, R.A., Steponkus, P.L.: Cold acclimation of *Arabidopsis thaliana* (Effect on plasma membrane lipid composition and freeze-induced lesions). - Plant Physiol. **109**: 15-30, 1995.

Wang, J., Letham, D.S., Cornish, E., Wei, K., Hocart, C.H., Michael, M., Stevenson, K.R.: Studies of cytokinin action and metabolism using tobacco plants expressing either the *ipt* or *gus* gene controlled by a chalcone synthase promoter: II *ipt* and *gus* gene expression, cytokinin levels and metabolism. - Aust. Plant Physiol. **24**: 73-683. 1997.

Werner, T., Schmulling, T.: Cytokinin action in plant development. - Curr. Opin. Plant Biol. **12**: 527-538, 2009.

Xia, J., Zhao, H., Liu, W., Li, L., He, Y.: Role of cytokinin and salicylic acid in plant growth at low temperatures. - Plant Growth Regul. **57**: 211-221, 2009.

Xin, Z., Browse, J.: Cold comfort farm: the acclimation of plants to freezing temperatures. - Plant Cell Environ. **23**: 893-902, 2000.

Zhang, X.-Y., Liang, C., Wang, G.-P., Luo, Y., Wang, W.: The protection of wheat plasma membrane under cold stress by glycine betaine overproduction. - Biol. Plant. **54**: 83-88, 2010.