



RESEARCH PAPER

Effects of *cor15a-IPT* gene expression on leaf senescence in transgenic *Petunia* × *hybrida* and *Dendranthema* × *grandiflorum*

Mariya Khodakovskaya^{1,2}, Yi Li¹, Jisheng Li¹, Radomíra Vaňková³, Jiří Malbeck³ and Richard McAvoy^{1,*}

¹ University of Connecticut, Plant Science Department, Agricultural Biotechnology Laboratories, 1390 Storrs Rd, U-4163, Storrs, CT 06269-4163, USA

² Institute Biology and Soil Sciences, Far-Eastern Branch, Russian Academy of Sciences, Vladivostok, Russian Federation

³ Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Received 13 August 2004; Accepted 20 December 2004

Abstract

To prevent leaf senescence of young transplants or excised shoots during storage under dark and cold conditions, the cytokinin biosynthetic gene isopentenyl transferase (*ipt*) was placed under the control of a cold-inducible promoter *cor15a* from *Arabidopsis thaliana* and introduced into *Petunia* × *hybrida* 'Marco Polo Odyssey' and *Dendranthema* × *grandiflorum* (chrysanthemum) 'Iridon'. Transgenic *cor15a-ipt* petunia and chrysanthemum plants and excised leaves remained green and healthy during prolonged dark storage (4 weeks at 25 °C) after an initial exposure to a brief cold-induction period (4 °C for 72 h). However, *cor15a-ipt* chrysanthemum plants and excised leaves that were not exposed to a cold-induction period, senesced under the same dark storage conditions. Regardless of cold-induction treatment, leaves and plants of non-transformed plants senesced under prolonged dark storage. Analysis of *ipt* expression indicated a marked increase in gene expression in intact transgenic plants as well as in isolated transgenic leaves exposed to a short cold-induction treatment prior to dark storage. These changes correlated with elevated concentrations of cytokinins in transgenic leaves after cold treatment. *Cor15a-ipt* transgenic plants showed a normal phenotype when grown at 25 °C.

Key words: Cold-inducible promoter, cytokinins, *ipt* expression, leaf senescence, ornamental plants.

Introduction

Cytokinins regulate a number of growth and developmental processes in plants, such as stimulating cell division, maintaining plant vigour, delaying plant senescence and post-harvest and low-temperature-induced leaf yellowing (Gan and Amasino, 1997). In practice, exogenous cytokinin applications are often not efficacious in commercial horticulture because they are expensive to use and are not readily assimilated (Hobbie *et al.*, 1994). Barry *et al.* (1985) generated cytokinin overproducing petunia plants using the *ipt* gene cloned from *Agrobacterium tumefaciens* under the control of the CaMV 35S promoter. The *ipt* gene encodes the enzyme isopentenyl transferase, which catalyses the rate-limiting step in cytokinin biosynthesis (Akiyoshi *et al.*, 1984). Excised leaves from cytokinin-overproducing *ipt*-transgenic plants displayed prolonged chlorophyll retention and delayed senescence (Li *et al.*, 1992). The delayed senescence observed in intact and excised leaves from *ipt*-transformed plants demonstrates the potential utility of the *ipt* gene in extending the storage life of ornamental plants, green vegetables, and fruits. Unfortunately, most *ipt*-transgenic plants exhibit morphological abnormalities, restricting the potential for use in commercial production. This occurs because overproduction of cytokinins during crop growth interferes with normal development (Gan and Amasino, 1997). For example, elevated cytokinins resulted in a reduction in stature, the release from apical dominance, changes in vascular development, and, in many cases, an inhibition of root growth (Klee *et al.*, 1987; Hobbie *et al.*, 1994). To achieve

* To whom correspondence should be addressed. Fax: +1 860 486 0534. E-mail: Richard.McAvoy@uconn.edu

better control of cytokinin overproduction, researchers fused the *ipt* gene to inducible promoters that were activated by stimuli such as heat (Medford *et al.*, 1989; Smigocki, 1991; Ainley *et al.*, 1993), wounding (Smigocki *et al.*, 1993), or light (Thomas *et al.*, 1995). However, in most cases, morphological changes in *ipt*-transgenic plants were observed even in the absence of an induction stimulus, suggesting leakage of the inducible system. Using the senescence-specific SAG₁₂ promoter to drive *ipt* expression in tobacco, Gan and Amasino (1995) demonstrated that a specific developmental response could be elicited through more precise control of *ipt* expression. The SAG₁₂ promoter activated *ipt* expression only at the onset of senescence, resulting in increased cytokinin concentrations in the senescing tissue and inhibition of the senescence process. Inhibition of leaf senescence by *ipt* expression led to the attenuation of the senescence-specific promoter, thus preventing cytokinin overproduction that would interfere with other aspects of development. In SAG₁₂-*ipt* tobacco, leaf senescence was effectively controlled without other developmental abnormalities (Gan and Amasino, 1997). Subsequently, this strategy was successfully used in rice (Fu *et al.*, 1998), cauliflower (Nguyen *et al.*, 1998), petunia (Clark *et al.*, 2004), and lettuce (McCabe *et al.*, 2001). For example, leaf senescence was retarded in mature 60-d-old lettuce plants that exhibited normal morphology with no significant differences in head diameter or fresh weight of leaves and roots (McCabe *et al.*, 2001).

In commercial horticulture, it is advantageous to be able to store whole live plants (seedlings) and excised shoots (cuttings) for extended periods of time without a loss of vitality. Plants and excised plant parts are typically stored under cool, dark conditions but the incidence of chilling injury and mortality increases with storage duration (Heins *et al.*, 1995). At warmer temperatures, leaves senesce and overall plant quality deteriorates rapidly in dark storage. Controlled expression of *ipt* during dark storage, but not during normal crop production, could potentially increase and extend the storage tolerance of commercial crops without adversely affecting subsequent production in the field or greenhouse. In this study, an *ipt* fusion gene under the control of a cold-inducible promoter from the *cor15a* gene from *Arabidopsis thaliana* was constructed. Petunia and chrysanthemum plants were used to test the effects of this unique construct on storage tolerance, chlorophyll stability, and growth and development of transgenic plants.

Materials and methods

Plasmid construction

Molecular cloning procedures were carried out as described by Sambrook *et al.* (1989). The *ipt* coding sequence used to construct the chimeric *cor15a/ipt* gene was derived from the *pUC19* vector containing the *ipt* under the control of the 35S promoter. A 1 kb fragment of *ipt-nos* was obtained by double digestion of *pUC19-35S-*

ipt-nos with *EcoRI* and *SalI* and cloned into the cloning site of the *pBluescriptII KS* vector. The 5' promoter and leader sequence from the *cor15a* gene (0.98 kb) was synthesized from genomic DNA of *Arabidopsis thaliana* by PCR reaction. The cloning sites for *XhoI* and *SalI* were added to the ends of the *cor15a* primers 5'-GGCTCGA-GAGATCTTGTCCGTTGAATTT-3' and 5'-GGTCGACGAGAG-AGATCTTTAAGATGT-3' for PCR. The *cor15a* fragment was subcloned into the *pBluescriptII KS-ipt-nos* vector by double digestion with *XhoI* and *SalI* to generate *pBluescriptII KS* with *ipt* under the control of the *cor15a* promoter. In the final step, the *cor15-ipt-nos* fragment was inserted into the multicloning site of the *pBin19* vector by digesting *pBluescriptII KS-cor15a-ipt-nos* and *pBin19* with *KpnI* and *SmaI* and then ligating the blunt ends. Thus, the binary *pBin19* vector containing *ipt* under the control of the *cor15a* promoter was generated. The binary plasmid was transformed into *Agrobacterium tumefaciens* strain LBA 4404 by electroporation. The identity and accuracy of the cloned *cor15a* promoter sequence was confirmed by DNA sequence analysis (WM Keck Biotechnology Laboratory, Yale University, New Haven, CT).

Transformation and regeneration of transgenic plants

Petunia cv. Marco Polo Odyssey and chrysanthemum cv. Iridon were grown at 25 °C in the greenhouse in 3.8 l pots containing a peat-based substrate (Metro 510, Scotts Co., Marysville, Ohio). Plants were fertilized weekly with 400 mg l⁻¹ N from a 20/4.3/16.6 N/P/K stock solution (Peter's 20-10-20, Scotts Co., Marysville OH). Plant shoots were cut at monthly intervals to induce new shoot growth. Leaf and stem tissue from young, newly developed shoots was used as explant tissue for plant transformation as follows.

Young, fully expanded petunia (cv. Marco Polo Odyssey) leaves were sterilized with 0.6% sodium hypochlorite (15–20 min) and then rinsed five times with sterile water. Stems from young, soft shoot tips of chrysanthemum plants (cv. Iridon) were washed for 60 s with 70% ethanol, rinsed three times with sterile water, and then sterilized in 0.3% sodium hypochlorite for 8 min before finally rinsing five times with sterile water. The bacterial suspension was cultured in LB medium supplemented with 50 mg l⁻¹ kanamycin and 25 mg l⁻¹ rifampicin. The suspension was incubated at 25 °C on a rotary shaker (220 rpm) until achieving an optical density of 0.4–0.7 (λ 600 nm). The suspension was then centrifuged and the pellet resuspended in a fresh liquid MS medium. Leaf explants of petunia or stem segments of chrysanthemum were soaked in the infection medium for 5 min, blotted dry and kept for 3 d in the dark at 22–25 °C on plates with MS medium containing 2 mg l⁻¹ of N⁶-benzyladenine (BA), 0.01 mg l⁻¹ of NAA for petunia explants or 0.225 mg l⁻¹ of BA, 2 mg l⁻¹ of IAA for chrysanthemum explants. After 2–3 d, explants were transferred to the respective selection media containing 50 mg l⁻¹ of kanamycin (for selection) and 200 mg l⁻¹ of timentin (to eliminate the *Agrobacterium*). Explants were transferred to fresh medium every 2–3 weeks, until shoots developed. Excised shoots were then transferred to phytohormone-free MS medium containing 50 mg l⁻¹ of kanamycin and 100 mg l⁻¹ of timentin until root induction was evident. Rooted explants were transferred to a peat-based medium (Metro 510, Scotts Co., Marysville, OH), and acclimated to the greenhouse environment.

Plant DNA extraction and polymerase chain reaction (PCR analysis)

Total DNA was isolated from leaf tissue using mini-prep kits (DNeasy Plant Mini Kit, Qiagen Inc., Valencia, CA, USA) and 250 ng of DNA was subjected to PCR reaction. The primers used to detect the *cor15a-ipt* locus were as follows: (i) for the full *cor15a* promoter (0.98 kb), forward primer 5'-GGCTCGAGAGATCTTGTCCGTT-GAATTT-3' and reverse primer 5'-GGTCGACGAGAGAGAT-CTTTAAGATGT-3'; and for the 0.525 kb region of the *ipt* gene (ii) forward primer 5'-GGTCCAACCTGCACAGGAAAG-3' and

reverse primer 5'-GGCTTGCTACTGGAAGCTTA-3'. PCR amplification was performed using a thermocycler (GeneAmp PCR System 2700, Applied Biosystems, Inc., Foster City, CA, USA). Cycling conditions for both genes were 3 min at 94 °C and then 30 cycles of 1 min at 94 °C, 1 min at 54 °C and 1 min 30 s at 72 °C, followed by extension at 72 °C for 5 min. The reactions included 200–250 ng of DNA template, 0.2 mM of dNTPs, 0.5 µM of each primer, *REDTaq* PCR buffer and 1.5 U of *REDTaq* DNA polymerase (Sigma, St Louis, MO, USA). Finally, a 20 µl aliquot of PCR product was observed under UV after electrophoresis on a 1% agarose gel with ethidium bromide. A 1 kb DNA molecular marker (Gibco BRL) was used as a reference to determine DNA fragment size.

Southern hybridization

Total genomic DNA was isolated from transgenic plants using DNeasy Plant Maxi Kits (Qiagen Inc., Valencia, CA, USA) in accordance with the recommended protocol. Total genomic DNA from putative transgenic and non-transformed control plants (10 mg samples) was digested at 37 °C overnight by double restriction with enzymes *Hind*III and *Eco*RI and *cor15a-ipt* fragment was released. Digested DNA from each line was separated through a 1% agarose gel prepared in TAE buffer, pH 8.5 (Sambrook *et al.*, 1989) and fragments were transferred from agarose gel to a nylon membrane (Amersham, Chalfont St Giles, UK) and cross-linked to the membrane under UV irradiation. The *ipt* gene probe (a 0.525 kb fragment of the *ipt* gene) was prepared with a PCR DIG Probe synthesis kit (Roche Molecular Biochemicals, Indianapolis, IN) in accordance with the recommended protocol. The DNA fixed on membranes was prehybridized using a prehybridization solution at 68 °C for 3 h, and then hybridized with the probe at 68 °C overnight, and finally triple-washed with the post-hybridization solution at 65 °C in a hybridization oven (HB-2D, Techne Ltd., Duxford-Cambridge, UK). Solutions for sample hybridization, and pre- and post-hybridization, and the buffers for the following steps were prepared as previously reported by Mercier (1998). Membranes were washed for 5 min in 50 ml of maleate buffer (0.1 M maleic acid, 3.0 M of NaCl, pH 8.0) at room temperature and then incubated for 1 h in 50 ml of blocking solution (maleate buffer plus 0.5% blocking reagent: Roche Molecular Biochemicals, Indianapolis, IN). Membranes were then incubated for 30 min in 20 ml of blocking solution with anti-digoxigenin-AP, Fab fragments (Roche Molecular Biochemicals, Indianapolis, IN) diluted to 1:10 000 and then washed four times for 10 min in 50 ml of the maleate buffer. As a final step, membranes were equilibrated for 5 min. in 50 ml of substrate buffer (100 mM of TRIS-HCl, 100 mM of NaCl, 5 mM of MgCl₂, pH 9.5) and then incubated at 37 °C for 10 min in 2 ml (sandwiched between two translucent plastic pages) of substrate buffer plus chemiluminescent substrate at a 1:100 dilution (CSPD, Roche Molecular Biochemicals, Indianapolis, IN). Membranes were exposed to autoradiographic film (Kodak X-Omat AR) for 4 h. X-ray films were developed with an automatic film processor.

Analysis of *ipt* expression in leaves of *petunia* and *chrysanthemum*

Using wild type and two *cor15a-ipt*-transgenic lines from both *petunia* and *chrysanthemum*, total RNA was isolated with TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) from samples that were frozen with liquid nitrogen and then ground in a mortar. For RT-PCR analysis, DNase treatment (DNA-free™, Ambion, Inc.) was used to eliminate DNA contamination from RNA samples, and then first-strand cDNA was synthesized from 1 µg of total RNA using First Strand Synthesis Kit RETROscript™ (Ambion Inc. Austin, TX, USA) following the manufacturer's recommended protocol. For PCR, 0.5 µl of RT-mix was used in a final volume of 25 µl. PCR reaction for the *ipt* gene fragment was carried out as described above. PCR reaction products along with

RT-mix and primers to 18S RNA were used as internal standards (QuantumRNA™ 18S Internal Standards, Ambion Inc.). PCR products (10 µl) were run on a 1% agarose gel.

Senescence of excised leaves and shoots

To determine transgenic plant tolerance to prolonged dark storage, excised leaves from *petunia* and *chrysanthemum* were surface-sterilized with 0.6% sodium hypochlorite for 60 s, rinsed five times and then placed on moist filter paper in a 10 cm Petri dish. Leaves were selected from individual transgenic lines and non-transformed wild-type plants. Each Petri dish contained two excised leaves from both an individual transgenic plant line and a non-transformed wild-type plant. For *petunia*, five transgenic lines were tested. For *chrysanthemum*, two transgenic lines were tested. Identical plates were assembled for each transgenic line of *petunia* and *chrysanthemum*. The plates were either exposed to (i) a cold induction period (3 d at 4 °C in the dark) followed by continuous dark storage at 25 °C, or (ii) continuous dark storage at 25 °C without a prior cold induction treatment. Plates in dark storage were checked daily over a 28 d period for evidence of leaf senescence. Chlorophyll concentration was assayed prior to the start of each experiment and after significant loss of chlorophyll was detected in the non-transformed wild-type tissue. Each treatment combination was replicated in triplicate and the experiment was repeated three times. In separate experiments, whole shoots were excised from both wild-type plants and individual transgenic lines of *petunia* and *chrysanthemum*. Shoots from individual plants were bundled in groups of five and wrapped in a moist paper towel. Bundles from each transgenic line and the wild type were enclosed in a plastic bag and subjected to the same treatments and experimental protocol as previously described.

Quantification of chlorophyll

Specific chlorophyll concentration was determined, as follows. Wild-type and transgenic leaves, from each treatment plate in the previously described study, were blotted dry and weighed, and 100 mg of tissue from each sample placed in a 1.5 ml microcentrifuge tubes. The samples were resuspended in 80% acetone, ground with a disposable pestle, and incubated in the dark for 30 min. Total chlorophyll (Chl µg ml⁻¹) was determined using absorbance at 645 nm and 663 nm according to the equation: 20.2 A₆₄₅ + 8.02 A₆₆₃ (Chory *et al.*, 1994).

Morphological analysis of transgenic plants

The effect of the transgene on growth and development of *chrysanthemum* was determined in growth chamber studies. Thirty shoots from each transgenic *cor15a-ipt* *chrysanthemum* plant lines 9 and 12, and from the wild-type cultivar 'Iridon' were excised and rooted in deep 606-cell packs (Kord Products, Bramalea, Ontario, Canada) containing a Metro 510 (Scotts Co., Marysville, Ohio) peat-lite medium. After shoots were well rooted (3 weeks), the rooted cuttings were transferred to the growth chamber (EGC model S10, EGC, Chargrin Falls, OH) at 25/20 °C day/night (16 h at 300 µmol m⁻² s⁻¹). Plants were allowed to acclimate to the growth chamber conditions for 2 weeks and then 10 plants from each line were exposed to a cold induction period (3 d at 4°C) and then returned to the growth chamber while 10 plants from each line remained in the growth chamber without exposure to a cold induction period. In the growth chamber, plants were watered as needed and fertilized once per week with N at 5.3 mmol (75 mg l⁻¹) from a 20/4.3/16.6 N/P/K stock solution (Peter's 20-10-20, Scotts Co., Marysville OH). After 6 weeks in the growth chamber five plants from each treatment were harvested and the following data recorded: shoot fresh weight (g), number of lateral shoots, lateral shoot length (cm), number of secondary shoots on each lateral, leaf area (cm²) on the uppermost lateral shoot, number of nodes on the uppermost lateral shoot, and

total number of lateral shoots on the main stem. These parameters were used to calculate the average internode length and the average area per leaf on the uppermost lateral shoot. These data were used to determine difference in vegetative growth habit between transgenic and wild-type plants with or without exposure to a cold-induction period. In a separate study, the remaining 10 rooted chrysanthemum cuttings from each line were exposed to short-day conditions to induce flowering and the number of flower buds on each plant were recorded at anthesis.

Plants were arranged in a randomized complete block design with 10 replicated blocks. Statistical effects were determined using a 2-way analysis of variance with genetic line and cold-treatment as the main effects.

Cytokinin analysis

Cytokinins were extracted and purified according to the method of Dobrev and Kaminek (2002). Freeze-dried samples were homogenized with mortar and pestle in liquid nitrogen and extracted overnight with 10 ml methanol/water/formic acid (15/4/1, by vol., pH ~2.5, -20 °C). To each sample, 50 pmol of each of 12 deuterium labelled standards ($[^2\text{H}_5]\text{Z}$, $[^2\text{H}_5]\text{ZR}$, $[^2\text{H}_5]\text{Z7G}$, $[^2\text{H}_5]\text{Z9G}$, $[^2\text{H}_5]\text{ZOG}$, $[^2\text{H}_5]\text{ZROG}$, $[^2\text{H}_6]\text{iP}$, $[^2\text{H}_6]\text{iPA}$, $[^2\text{H}_6]\text{iP7G}$, $[^2\text{H}_6]\text{iP9G}$, $[^2\text{H}_5]\text{DHZ}$, $[^2\text{H}_5]\text{DHZR}$; products of Apex Organics, Honiton, UK) were added. The extract was passed through 2 ml Si-C₁₈ columns (SepPak Plus, Waters, USA) to remove interfering lipophilic substances. After organic solvent evaporation *in vacuo*, the aqueous residue was applied to an Oasis MCX mixed mode (cation exchange and reverse-phase) column (150 mg, Waters, USA). Adsorbed cytokinins were eluted stepwise with 5 ml of 0.35 M ammonium in water (cytokinin nucleotides) and 0.35 M ammonium in 60% methanol (v/v) (cytokinin bases, ribosides, and glucosides). The eluted fractions were evaporated *in vacuo*. Nucleotide samples were dephosphorylated with acid phosphatase (0.6 U per sample) for 1 h at 37 °C. LC-MS analysis was performed using a Rheos 2000 HPLC gradient pump (Flux Instruments, Basel, Switzerland) and HIS PAL autosampler (CTC Analytics, Zwingen, Switzerland) coupled to an Ion Trap Mass Spectrometer Finnigan MAT LCQ-MS[®] equipped with an electrospray interface. Samples dissolved in 10% (v/v) acetonitrile (10 µl) were injected on a C₁₈ column (Aqua 125A, 2 mm/250 mm/5 µm) and eluted with a linear gradient of B from 10% to 50% in 26 min (mobile phase: water (A), acetonitrile (B), and 0.001% (v/v) acetic acid in water (C) at a flow rate 0.2 ml min⁻¹). Under these chromatographic conditions all analysed cytokinins were separated. Detection and quantification were carried out using a Finnigan LCQ operated in the positive ion, full-scan MS/MS mode using a multilevel calibration graph with deuterated cytokinins as internal standards. The levels of 19 different cytokinin derivatives were measured. The detection limit was calculated for each compound as 3.3 σ/S , where σ is the standard deviation of the response and S the slope of the calibration curve. For each treatment, samples were collected from each of three independent plants and each sample was injected at least twice.

Results

Gene construction and plant transformation

Transformation of petunia and chrysanthemum with the *cor15a* gene promoter-*ipt* gene (*cor15a-ipt*) construct resulted in more than 30 kanamycin-resistant putative transformants for each species. PCR and Southern hybridization analysis confirmed recombinant DNA integration into the genome of individual putative-transgenic petunia

and chrysanthemum lines (Fig. 1). PCR amplification of both plasmid DNA and the genomic DNA from chrysanthemum lines produced the expected 0.98 kb fragment of the *cor15a* promoter (Fig. 1A) and the 0.52 kb fragment of the *ipt* gene (Fig. 1B). No amplification of DNA was detected in non-transgenic plants. Southern blot analysis of petunia genomic DNA revealed the integration of the *ipt* gene into the genome of several primary transformants, while no signal was detected in control plants (Fig. 1C). Transgenic plants of petunia were also confirmed by PCR reaction, and PCR positive lines of petunia and chrysanthemum were used for all subsequent experiments.

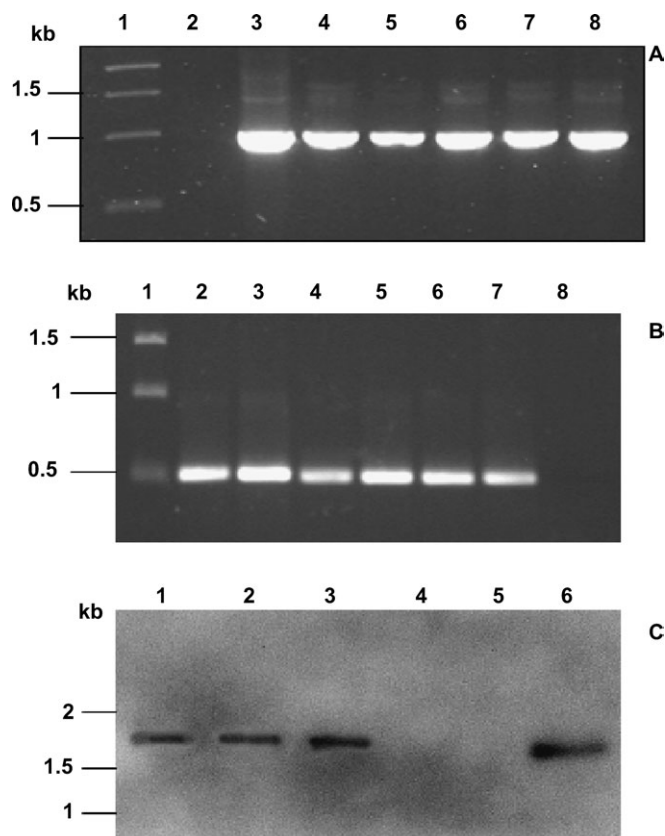


Fig. 1. (A, B, C) PCR analysis of DNA from putative transgenic chrysanthemum lines and Southern blot analysis of genomic DNA isolated from petunia plants. (A) PCR analysis showing the presence of the expected 0.98 kb fragment of the *cor15a* promoter from putative-transgenic chrysanthemum lines. Lane 1: 1 kb molecular marker; lane 2: negative control (untransformed chrysanthemum plant); lane 3: positive control (plasmid *cor15a-ipt-nos*); lanes 4–8: putative-transgenic chrysanthemum plants. (B) PCR analysis showing the presence of the expected 0.52 kb fragment of the *ipt* gene from putative-transgenic chrysanthemum lines. Lane 1: 1 kb molecular marker; lane 2: positive control (plasmid *cor15a-ipt-nos*); lanes 3–7: transgenic chrysanthemum plants; lane 8: negative control (untransformed chrysanthemum plant). (C) Southern blot analysis of genomic DNA from *cor15a-ipt* petunia lines. A DIG-labelled 0.52 kb fragment of the *ipt* gene from the *cor15a-ipt-nos* plasmid DNA was used as a probe. An *ipt* fragment was detected from each of the transgenic lines analysed (lanes 1–3) but not from wild-type petunia DNA (lanes 4–5). Plasmid DNA was used as the positive control (lane 6).

Molecular analysis of transgenic plants expressing *cor15a-ipt*

Reverse transcription-PCR (RT-PCR) analysis was used to confirm *ipt* expression in transgenic lines in response to cold-induction signal. Total RNAs were extracted from the leaves of wild-type and selected transgenic lines of chrysanthemum (lines 9 and 12) and petunia (lines 7 and 9) that were grown under normal conditions or first exposed to a 3 d cold-induction (4 °C) treatment. RT-PCR analysis showed that the 0.52 kb *ipt* DNA fragment was amplified in both *cor15a-ipt* chrysanthemum (line 9) and *cor15a-ipt* petunia (line 7) exposed to the 4 °C treatment, but not in the same lines grown at the 25 °C and not exposed to the cold-induction treatment (Fig. 2). Similar results were obtained with line 9 of petunia and 12 line of chrysanthemum (data not shown). Wild-type plants showed no evidence of *ipt* gene expression regardless of temperature treatment. These data demonstrate that *ipt* expression in *cor15a-ipt* plants could be up-regulated with a cold-induction signal, but remained suppressed at normal growing temperatures.

Leaf senescence

The leaf senescence response of chrysanthemum and petunia under long-term dark storage conditions differed markedly between *cor15a-ipt* and wild-type plants (Fig. 3). Overall, leaves from cold-induced *cor15a-ipt* plants remained green and healthy in prolonged dark storage while leaves from non-induced *cor15a-ipt* plants and from wild-type plants, regardless of cold-induction treatments, did not. Similar results were observed with excised leaves of both chrysanthemum and petunia, and excised shoots and whole intact plants of chrysanthemum. For example, excised leaves of both wild-type chrysanthemum and wild-type petunia showed a dramatic loss of chlorophyll and advanced tissue senescence after 28 d in continuous darkness at 25 °C.

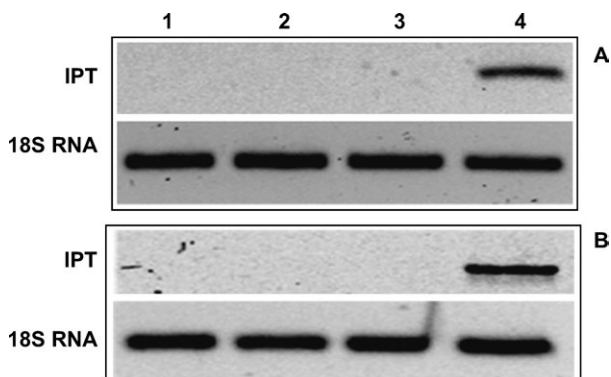


Fig. 2. RT-PCR analysis of *ipt* expression in *cor15a-ipt* and wild-type chrysanthemum (A) and petunia (B) plants exposed to normal growing temperatures or a cold-induction treatment. Lane 1: wild-type plant (normal conditions, 25 °C), lane 2: wild-type plant exposed to a cold-induction treatment (3.5 d at 4 °C), lane 3: transgenic plant (normal conditions, 25 °C), lane 4: transgenic plant exposed to a cold-induction treatment (3.5 d at 4 °C).

A pretreatment of cold-induction temperatures had little effect on the course of tissue senescence under these conditions. However, when excised leaves of *cor15a-ipt* chrysanthemum and *cor15a-ipt* petunia were exposed to a cold-induction treatment (4 °C for 3 d) and then stored in the dark for 28 d, the tissue showed little or no visible symptoms of chlorophyll loss or tissue senescence. Leaves of *cor15a-ipt* chrysanthemum and *cor15a-ipt* petunia that were not exposed to a cold-induction treatment prior to dark storage developed symptoms of chlorophyll loss or tissue senescence that approached those observed in wild-type leaves. With both excised shoots of *cor15a-ipt* chrysanthemum and whole intact *cor15a-ipt* chrysanthemum plants, a cold-induction treatment was required to produce the delayed onset of leaf senescence response under prolonged dark storage conditions.

Quantitative analysis revealed that leaf chlorophyll concentrations in *cor15a-ipt* petunia lines and wild-type plants were similar under normal greenhouse growing conditions and showed a similar decline under prolonged dark storage conditions (Fig. 4). However, when plants were first exposed to a cold-induction treatment, the chlorophyll concentration in the *cor15a-ipt* lines remained at the level of normal grown plants even when exposed to prolonged dark storage. Cold induction had no beneficial effect on chlorophyll stability in the wild-type plants and chlorophyll concentrations showed a precipitous decline in response to dark storage. Experiments with excised leaves of wild-type and *cor15a-ipt* chrysanthemum produced a similar response (data not shown).

Changes in endogenous concentrations of cytokinins

Analysis of endogenous cytokinins, in freeze-dried petunia shoot tips from *cor15a-ipt* plants, revealed a dramatic increase after a cold-induction treatment compared to concentrations from wild-type plants (Table 1). Expression of the *cor15a-ipt* gene in petunia especially affected zeatin and dihydrozeatin type cytokinins. In *cor15a-ipt* plants, exposure to a cold-induction period (3.5 d at 4 °C) resulted in the increase of the physiologically active cytokinin *trans*-zeatin and its riboside (>4-fold and >18-fold, respectively), as well as of the storage cytokinins zeatin nucleotide (>10-fold increase), zeatin *O*-glucoside (5-fold increase) and the cytokinin deactivation products zeatin 7-glucoside (>10-fold increase) and zeatin-9-glucoside (>7-fold increase). The dihydrozeatin type cytokinins followed the same trend, but the increase was less dramatic. From the isopentenyladenine type cytokinins only the level of the active base (isopentenyladenine) was slightly increased during *ipt* expression at 4 °C. The concentration of isopentenyladenosine was considerably elevated under growth permissive conditions (25 °C) in both wild-type and transformed plants.

In *cor15a-ipt* chrysanthemum plants, cold-induced *ipt* gene expression produced marked increases in both the

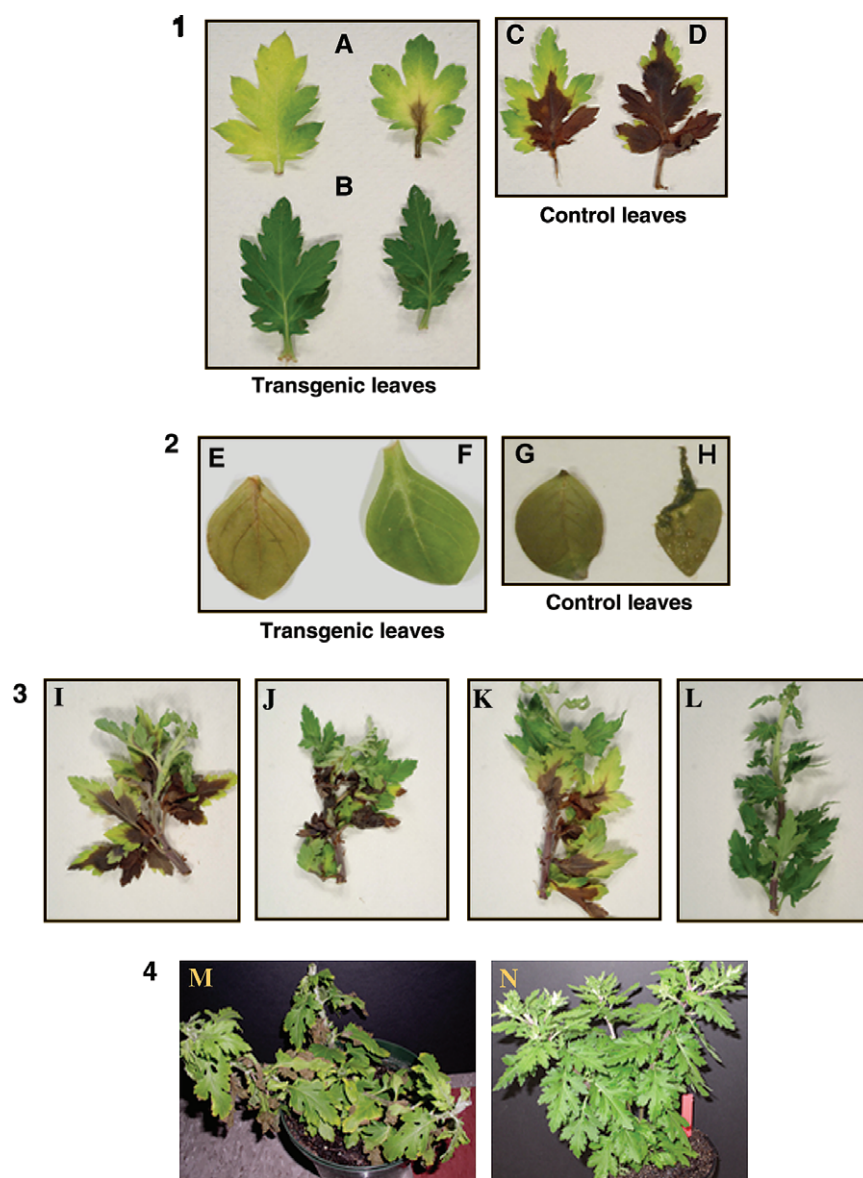


Fig. 3. Senescence in excised leaves, stems, and whole plants of wild-type and *cor15a-ipt* plants. Row 1: excised *cor15a-ipt* and wild-type chrysanthemum leaves were either exposed to cold-inducing or non-inducing temperatures prior to dark storage. Leaves from *cor15a-ipt* plants that did not receive a cold-induction treatment prior to storage senesced (A) while leaves that received a cold-induction treatment prior to storage remained green (B). Wild-type chrysanthemum leaves senesced in dark storage whether first exposed to cold-temperatures (C) or not (D). Row 2: similarly, *cor15a-ipt* petunia leaves that did not receive a cold-induction treatment prior to storage senesced (E) while leaves that received a cold-induction treatment prior to storage remained green (F). Wild-type petunia leaves senesced in dark storage whether first exposed to cold temperatures (H) or not (G). Row 3: excised chrysanthemum shoots from wild-type and *cor15a-ipt* chrysanthemum were either exposed to cold (3 d at 4 °C) or non-inducing (25 °C) temperatures prior to 18 d dark storage at 25 °C. Shoots from wild-type plants senesced when stored in the dark regardless of cold induction treatment (I, no cold induction; J, cold-induced). Shoots from *cor15a-ipt* plants stored without cold-induction also senesced in dark storage (K), but shoots stored after exposure to cold-induction temperatures remained healthy (L). Row 4: intact *cor15a-ipt* chrysanthemum plants after 24 d of incubation in dark conditions. Transgenic plants that were not exposed to cold prior to dark storage senesced (M), but plants that were exposed to a cold-induction period (3.5 d at 4 °C) prior to dark storage did not (N).

storage cytokinin pool ($P \leq 0.05$) and the pool of physiologically active cytokinins ($P \leq 0.05$), but the total cytokinin pool (active, deactivated, and storage forms combined) was not substantially altered (Table 2). In more detail, *trans*-zeatin concentrations were similar in wild-type chrysanthemum, under both non-inducing (25 °C) and cold-inducing (4 °C) temperatures, and in non-induced

cor15a-ipt plants (averaging 8.2 pmol g⁻¹ DW). However, concentrations increased ($P \leq 0.05$) in *cor15a-ipt* plants exposed to a prolonged period (14 d) at 4 °C and those exposed to a short cold induction period (3.5 d) followed by transfer to the 25 °C growth-permissive conditions for 3.5 d (averaging 16.8 pmol g⁻¹ DW). However, 10.5 d after transfer to 25 °C the concentration of *trans*-zeatin in

cold-induced *cor15a-ipt* plants decreased to non-induced concentrations. In *cor15a-ipt* plants exposed to 4 °C for either 7 d or for 3.5 d followed by 3.5 d at 25 °C, the concentration of *trans*-zeatin riboside (averaging 6.6 pmol g⁻¹ DW) was measurably higher ($P \leq 0.05$) than in non-induced *cor15a-ipt* plants and wild-type plants in both inductive and non-

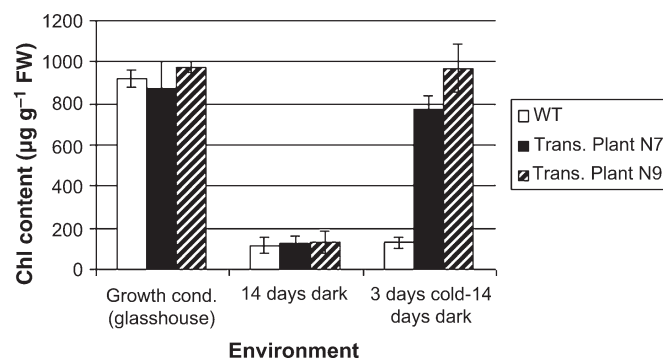


Fig. 4. Chlorophyll concentrations in leaves of wild-type (control) and *cor15a-ipt* petunia plants (from lines 7 and 9) under growth conditions (in the greenhouse), or following 14 d dark storage without prior cold-induction, or exposed to a cold-induction treatment (3 d at 4 °C) prior to 14 d dark storage. Each value represents the mean of seven observations. Error bars represent the standard error of means.

Table 1. Cytokinin concentrations in wild-type and *cor15a-ipt* transgenic petunia plants exposed to cold-induction or non-inducing conditions

Values represent the mean of samples from three different plants from the same transgenic line. Z, *trans*-zeatin; ZR, *trans*-zeatin 9-riboside; ZRMP, *trans*-zeatin 9-riboside-5'-monophosphate; ZOG, *trans*-zeatin O-glucoside; Z7G, *trans*-zeatin 7-glucoside; Z9G, *trans*-zeatin 9-glucoside; DHZ, dihydrozeatin; DHZR, dihydrozeatin 9-riboside; DHZROG, dihydrozeatin 9-riboside O-glucoside; iP, N⁶-(Δ²-isopenenyl)adenine; iPR, N⁶-(Δ²-isopenenyl)adenosine; iPRMP, N⁶-(Δ²-isopenenyl) 9-riboside-5'-monophosphate. All plants were rooted and then grown for 2 weeks at 25 °C prior to the start of temperature treatments.

Duration of cold-induction period before sampling (d)	Concentrations (pmol g ⁻¹ DW) of various cytokinin species												
	Z	ZR	ZRMP	Z7G	Z9G	ZOG	DHZ	DHZR	DHZROG	iP	iPR	iPRMP	Active cytokinins
Wild-type petunia													
0	9.4	<0.1	4.5	5.8	0.1	3.6	0.4	0.4	1.2	3.1	15.9	11.0	29.2
3.5	6.1	0.4	6.6	8.8	<0.1	3.4	0.4	0.6	2.9	3.5	5.3	15.6	16.2
<i>cor15a-ipt</i> petunia													
0	5.7	0.7	5.8	5.1	0.1	2.8	0.7	1.4	1.4	4.2	12.0	11.7	24.2
3.5	24.4	13.1	61.2	52.1	2.0	14.1	5.1	0.9	10.8	4.9	4.6	9.8	53.0

Table 2. Concentrations of physiologically active cytokinins (*trans*-zeatin, isopenenyladenine, dihydrozeatin, and the corresponding ribosides), O-glucosides (of *trans*-zeatin, *trans*-zeatin riboside, and dihydrozeatin riboside) and total cytokinins in leaves of wild-type and *cor15a-ipt* chrysanthemum plants exposed to different inductive and non-inductive temperature conditions prior to sampling

Temperature treatment before sampling ^a	Wild type		<i>cor15a-ipt</i>				
	Duration of exposure (d)		0	3.5	14	3.5	3.5
Duration of cold-induction (4 °C) period (d)	0	3.5	0	3.5	14	3.5	3.5
Days at 25 °C after cold-induction	0	0	0	0	0	3.5	10.5
Cytokinin pool	Cytokinin concentration (pmol g ⁻¹ DW)						
Total active species	22.5	16.6	29.7	12.4	27.2	40.4	25.1
O-glucosides	86.8	96.3	102	123.5	129.9	77.1	81.6
Total cytokinins	137.2	127.5	164.6	154.4	181.0	145.2	151.0

^a Plants not subjected to a cold treatment were raised at 25 °C and sampled at the beginning of the study period.

inductive conditions (averaging 1.5 pmol g⁻¹ DW). The concentration of isopenenyladenine detected in non-induced wild-type, non-induced *cor15a-ipt*, and cold-induced wild-type plants (4.1 pmol g⁻¹ DW), was higher ($P \leq 0.05$) than the concentration found in *cor15a-ipt* plants exposed to cold for between 3.5 d and 14 d, or in plants exposed to cold for 3.5 d and then returned to 25 °C for 3.5 d (2.7 pmol g⁻¹ DW). By contrast, the concentration of the corresponding riboside (iP7R) significantly increased ($P \leq 0.05$) in *cor15a-ipt* plants induced in cold for 3.5 d and then returned to growth conditions for either 3.5 d or 10.5 d (13.1 pmol g⁻¹ DW) compared to the average concentration found in cold-induced wild-type plants, non-induced wild-type plants and non-induced *cor15a-ipt* plants (7.1 pmol g⁻¹ DW). The concentration of iP7R was dramatically lower ($P \leq 0.01$) in *cor15a-ipt* plants after 3.5 d of cold-induction than when similar plants were transferred to growth conditions for 3.5 d or 10.5 d. The concentration of dihydrozeatin was low in all plants held at 4 °C, but the concentration of dihydrozeatin riboside increased with *ipt* expression.

Plant morphology

The overall growth habit of *cor15a-ipt* plants under growth chamber conditions (25 °C) was not substantially different

Table 3. Growth characteristics of wild-type and *cor15a-ipt* transgenic *chrysanthemums*

Plants were grown in the vegetative state in the growth chamber under 25/20 °C 16/8 h day/night temperature conditions. Half the plants received a cold-induction treatment (3 d at 4 °C) and 1 week 25 °C dark storage prior to the growth study and the remaining plants were not exposed to cold induction temperatures.

Genetic line	Shoot fresh weight (g ±SE)	Average lateral shoot length (cm ±SE)	Average laterals per plant (No. ±SE)	Secondary branches per lateral shoot (No. ±SE)	Average internode length on top lateral (cm ±SE)	Average area per leaf on top lateral (cm ² ±SE)
Cold-induction (3 d at 4 °C) prior to growing in the growth chamber						
Wild type	28 (1.4)	11.5 (1.1)	5.4 (0.27)	0.60 (0.27)	0.94 (0.18)	5.64 (0.83)
<i>cor15a-ipt</i> L9	21 (2.1)	10.1 (1.3)	4.8 (0.22)	0.32 (0.23)	0.72 (0.10)	5.29 (0.60)
<i>cor15a-ipt</i> L12	27 (3.4)	14.6 (1.0)	4.8 (0.55)	0.76 (0.35)	1.08 (0.20)	5.68 (0.37)
No cold-induction prior to growing in the growth chamber						
Wild type	44 (4.5)	12.5 (0.8)	6.8 (1.29)	1.09 (0.49)	1.06 (0.16)	7.39 (0.52)
<i>cor15a-ipt</i> L9	29 (3.7)	9.8 (1.7)	5.2 (0.55)	0.28 (0.31)	0.88 (0.12)	5.76 (0.79)
<i>cor15a-ipt</i> L12	35 (4)	16 (0.2)	5 (0)	0.64 (0.3)	1.02 (0.07)	7.81 (0.71)
Source of variation	Statistical effects ^a					
Genetic line	**	***	NS	NS	NS	NS
Cold treatment	***	NS	NS	NS	NS	**
Genetic×cold (interaction)	NS	NS	NS	NS	NS	NS

^a NS denotes non-significance, * denotes significant at $P \leq 0.05$, ** denotes significant at $P \leq 0.01$, *** denotes significant at $P \leq 0.001$.

from the wild-type *chrysanthemum* line (Table 3). In addition, the overall growth response of both *cor15a-ipt* lines and wild-type plants that were first exposed to a cold-induction treatment remained similar, indicating that the increase in *ipt* expression in cold-induced plants did not have a long-lasting effect on subsequent plant growth. Of the growth parameters observed, only shoot fresh weight and average lateral shoot length were affected by genotype. Shoot fresh weight for *cor15a-ipt* line 12 was similar to the wild type while shoot fresh weight for *cor15a-ipt* line 9 was lower. However, average lateral shoot length for *cor15a-ipt* line 12 was greater than either the wild type or *cor15-ipt* line 9. Shoot fresh weight and average leaf size (on the uppermost lateral branch) were both affected by cold-induction treatment, but both the *cor15a-ipt* lines and the wild-type plants responded in the same way to this treatment. Most significantly there was no interactive effect of genotype and environmental treatment on any of the growth responses observed, indicating that any increase in cytokinin that resulted from a cold-induction period did not persist during plant development at normal greenhouse temperatures. The average number of lateral branches on each plant, the number of secondary branches on each lateral shoot, and average internode length on the top lateral branch were all unaffected by genotype or temperature treatment.

No differences were observed between non-induced wild type and *cor15a-ipt* *petunia* lines grown in the 25 °C growth chamber. For example, the average length of the main stem of non-induced wild-type (21 ± 3.0 cm) and *cor15a-ipt* *petunia* plants (20.1 ± 0.6 cm) were similar. Likewise, the average number of lateral shoots on the main stem (5.8 ± 1.2) and (6.8 ± 0.4), and the average internode length on the main stem (1.7 ± 0.3 cm and 1.5 ± 0.1 cm)

were also similar for wild-type and *cor15a-ipt* *petunia* plants, respectively. Even when exposed to an initial cold treatment, growth response was similar for the wild-type and the *cor15a-ipt* transgenic *petunia* lines for four of the six parameters measured (length of the main stem, number of leaves on the main shoot, leaf area on the main shoot, and number of lateral branches on the main shoot). Compared with the wild type, shorter internodes were observed on two of the three transgenic lines tested and one transgenic line displayed a leaf area increase on the first lateral shoot. None of these anatomical features were consistent with the type of changes associated with constitutive *ipt* gene expression.

Discussion

In the horticulture trade, billions of plugs (e.g. whole plants used for transplant) and vegetative cuttings are produced annually for sale to commercial growers. Stockpiling plugs and cuttings for later use is advantageous because it increases productivity. However, long-term storage can only be successful if the majority of the plants survive and remain vigorous. Such storage requires cold temperatures to minimize respiration and the rapid deterioration of chlorophyll that results when plants are exposed to low light and warm temperatures (Heins *et al.*, 1995).

The control of *ipt* expression, and consequently delayed leaf senescence, under specific stress conditions using the cold-inducible promoter from the *cor15a* gene from *Arabidopsis thaliana*, is demonstrated here. This promoter was selected so that gene expression would occur only after the plants were exposed to a brief but specific environmental stress. The *cor15a* gene is a member of the COR

(cold-regulated) gene family. *Cor15a* encodes a 15 kDa polypeptide that is targeted to the chloroplasts. Upon transit into the organelle, the *cor15a* peptide is processed to a 9.4 kDa polypeptide designated as *cor15am*. The constitutive expression of *cor15a* in non-acclimated transgenic *Arabidopsis* plants increases the freezing tolerance of both chloroplasts frozen *in situ* and isolated leaf protoplasts frozen *in vitro* by 1–2 °C over the temperature range of –4 to –8 °C (Thomashow, 1999). Baker *et al.* (1994) showed that the *cor15a* promoter is inactive, or very weakly active, in most of the tissues and plant organs maintained under temperatures associated with active growth and, that in response to low temperature, it becomes highly activate in the shoots but not in the roots (Baker *et al.*, 1994). Root expression of *ipt* is a concern with asexually propagated species because of the potential for cytokinins to impede root development. Analysis of the *cis*-elements within the *cor15a* promoter indicated that the 5' region between nucleotides –305 and +78 imparted ABA- and drought-regulated gene expression in addition to cold-regulated expression (Baker *et al.*, 1994). Therefore, to avoid undesirable stress, which could also affect *ipt* gene expression, all morphological experiments were carried out under carefully controlled environmental conditions.

In these experiments, RT-PCR analysis confirmed that *ipt* expression was a result of cold-activation and no transcript was detected in either wild-type plants or transgenic plants that were not exposed to cold temperature conditions. These results are in accordance with the data reported by Hajela *et al.* (1990), who detected *cor* transcripts (regulated by the *cor15a* promoter) 1–4 h after *Arabidopsis* plants were exposed to cold temperatures. The amount of transcripts continued to increase for about 12 h and then remained elevated as long as the plants remained in the cold (up to 14 d in their study). However, when the plants were returned to normal growth temperatures, transcripts decreased rapidly and returned to concentrations found in the non-transgenic plants after 8 h.

These experiments showed that expression of the *ipt* gene in petunia resulted in an immediate increase of zeatin and dihydrozeatin type cytokinins. A 5.3-fold increase of physiologically active forms (*trans*-zeatin, dihydrozeatin and their ribosides), a 3.6-fold increase of storage forms (*O*-glucosides of *trans*-zeatin and dihydrozeatin and *trans*-zeatin nucleotide) and a 5.4-fold increase of deactivation forms (N-glucosides of *trans*-zeatin and dihydrozeatin) were found. The increase in the concentration of isopentenyladenine, but not of its derivatives, indicates that cytokinin metabolism, including the hydroxylation of the isoprenoid side chain, is very fast in this species. High concentrations of isopentenyladenosine under growth permissive conditions is in accordance with the results of Auer *et al.* (1999), who found an increase in isopentenyladenine/isopentenyladenosine in *Petunia hybrida* explants during shoot induction and especially in the shoot developmental

phase. When considering the relatively moderate increase of endogenous cytokinins that followed cold-induced *ipt* expression, it is also necessary to consider that the increase in cytokinin biosynthesis probably stimulated additional cytokinin oxidase/dehydrogenase activity, in a way similar to what was detected in petunia after application of BA (Auer *et al.*, 1999).

In chrysanthemum plants, *ipt* expression led to the accumulation of storage cytokinins (*O*-glucosides), and only after prolonged cold induction (more than 7 d), an increase in active cytokinins occurred. Short induction (3.5 d) followed by the plant transfer to growth permissive conditions (25 °C) resulted in the marked increase of all physiologically active cytokinins, accompanied by the decrease of cytokinin *O*-glucosides. This difference between petunia and chrysanthemum plants may be due to specific differences in the adaptation to cold temperatures. In chrysanthemum the concentration of active cytokinins seems to be tightly regulated in response to the temperature. A decrease in concentration of active cytokinin species during prolonged incubation at 25 °C would be expected if the storage temperature led to a decrease in transcript in conjunction with continued cytokinin turnover (affected by cytokinin oxidase/dehydrogenase).

In this study, an overall increase in cytokinin concentrations in cold-induced *cor15a-ipt* petunia and chrysanthemum plants did not reach the level reported after *ipt* overexpression in other systems. For example when *ipt* was placed under the control of a *Drosophila* heat-inducible promoter (*hsp70*) and introduced in *Nicotiana plumbaginifolia*, the resulting increase in cytokinin concentration ranged from 140- to 200-fold compared with non-induced leaves (Smigocki, 1991). The resulting transgenic plants were shorter, had an underdeveloped root system, and reduced leaf width. Another transgenic tobacco containing the maize *hsp70-ipt* gene exhibited an after-heat-treatment increase in zeatin and zeatin riboside concentrations of 52- and 23-fold, respectively (Medford *et al.*, 1989). In these studies, a consistent, low level of expression was observed even under non-inducing conditions and plant phenotype was dramatically affected, especially at the higher cytokinin concentrations (Medford *et al.*, 1989). When a more tightly regulated soybean heat-inducible promoter was used to regulate *ipt* expression, *ipt* transcription was not detected in plants exposed to normal temperatures and plants did not display the phenotypic characteristics associated with constant *ipt* expression. After heat shock, zeatin riboside concentration increased only 5-fold and the plants developed with shorted internodes, crinkled and down-folded leaves and enlarged stems. Transgenic plants also displayed delayed leaf senescence and flower bud development (Ainley *et al.*, 1993). Still others have reported that a sharp, transient increase in cytokinins was sufficient to promote plant cell division (Redig *et al.*, 1996; Dobrev *et al.*, 2002), and even a temporary increase in cytokinin triggered

changes in organ initiation and differentiation (Kaminek *et al.*, 1997).

Regardless of the magnitude of changes in cytokinin concentrations observed in *cor15a-ipt* petunia and chrysanthemum lines, the cold-induced plants in this study displayed a dramatic increase in chlorophyll retention and a dramatic delay in senescence under warm, dark storage conditions. For example, following exposure to a 72 h activation period at 4 °C, leaves from *cor15a-ipt* petunia and chrysanthemum remained healthy and green even after 3 weeks of dark incubation at 25 °C. Leaves of non-transformed plants senesced under the same storage regime. Similar responses were observed for shoot tip cuttings and whole plants. In addition, actively growing *cor15a-ipt* lines exhibited growth and development characteristics that were similar to the wild-type petunia and chrysanthemum phenotypes. A normal phenotype was observed even when *cor15a-ipt* lines were initially exposed to cold activation temperatures before growing in the 25 °C environment. Thus, up-regulation of *cor15a-ipt* in response to cold-induction appeared to be sufficient to alter leaf senescence properties of petunia and chrysanthemum but, under light and temperature conditions associated with active growth, the presence of the *cor15a-ipt* gene did not elicit the type of undesirable phenomic responses associated with constitutive *ipt* expression.

Acknowledgements

The authors thank Dr Richard Mercier (Plant Science Department, UCONN) for help in the non-radioactive detection of the *ipt* fragment in transgenic plants and Dr Carol Auer (Plant Science Department, UCONN) for excellent scientific discussions concerning the topic of this paper. This study was supported by a grant from Connecticut Innovation Inc.

References

- Ainley WM, McNeil KJ, Hill JW, Lingle WL, Simpson RB, Brenner ML, Nagao RT, Key JL. 1993. Regulatable endogenous production of cytokinins up to toxic levels in transgenic plants and plant tissues. *Plant Molecular Biology* **22**, 13–23.
- Akiyoshi DE, Klee H, Amasino RM, Nester EW, Gordon MP. 1984. T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proceedings of the National Academy of Sciences, USA* **81**, 5994–5998.
- Auer CA, Motyka V, Brezinova A, Kaminek M. 1999. Endogenous cytokinin accumulation and cytokinin oxidase activity during shoot organogenesis of *Petunia hybrida*. *Physiologia Plantarum* **105**, 141–147.
- Baker SS, Wilhem KS, Thomashow MF. 1994. The 5'-region of *Arabidopsis thaliana* *cor15a* has *cis*-acting elements that confer cold-, drought-, and ABA-regulated gene expression. *Plant Molecular Biology* **24**, 701–713.
- Barry GF, Rogers SG, Hein M, Niedermeyer J, Hoffman N. 1985. Identification of cytokinin genes and transfer into plants. *Current Topics of Plant Biochemistry and Physiology* **4**, 101–109.
- Chory J, Reinecke D, Sim S, Washburn T, Brenner M. 1994. A role for cytokinins in de-etiolation in *Arabidopsis*. *Plant Physiology* **104**, 339–347.
- Clark DG, Dervinis C, Barret JE, Klee H, Jones M. 2004. Drought-induced leaf senescence and horticultural performance of transgenic P-SAG12-IPT petunias. *Journal of the American Society for Horticultural Science* **129**, 93–99.
- Dobrev PI, Kaminek M. 2002. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. *Journal of Chromatography* **950**, 21–29.
- Dobrev P, Motyka V, Gaudinová A, Malbeck J, Trávníčková A, Kaminek M, Vankova R. 2002. Transient accumulation of *cis*- and *trans*-zeatin type cytokinins and its relation to cytokinin oxidase activity during cell cycle of synchronized tobacco BY-2 cells. *Plant Physiology and Biochemistry* **40**, 333–337.
- Fu Y, Ding Y, Liu X, Sun C, Cao S, Wang D, He S, Wang X, Li L, Tian W. 1998. Rice transformation with a senescence-inhibition chimeric gene. *Chinese Science Bulletin* **43**, 1810–1815.
- Gan S, Amasino RM. 1995. Inhibition of leaf senescence by autoregulated production of cytokinin. *Science* **270**, 1986–1988.
- Gan S, Amasino RM. 1997. Making sense of senescence: Molecular genetic regulation and manipulation of leaf senescence. *Plant Physiology* **113**, 313–319.
- Heins RD, Kaczperski MP, Wallace Jr TF, Lange NE, Carlson WH, Flore JA. 1995. Low-temperature storage of bedding plant plugs. *Acta Horticulturae* **396**, 285–296.
- Hajela RH, Horvath DP, Gilmour SJ, Thomashow MF. 1990. Molecular cloning and expression of *cor* (cold-regulated) genes in *Arabidopsis thaliana*. *Plant Physiology* **93**, 1246–1252.
- Hobbie L, Timpte C, Estelle M. 1994. Molecular genetics of auxin and cytokinin. *Plant Molecular Biology* **26**, 1499–1591.
- Kaminek M, Motyka V, Vankova R. 1997. Regulation of cytokinin content in plant cells. *Physiologia Plantarum* **101**, 689–700.
- Klee H, Horsch R, Rogers S. 1987. *Agrobacterium*-mediated plant transformation and its further applications to plant biology. *Annual Review of Plant Physiology* **38**, 467–486.
- Li Y, Hagen G, Guilfoyle TJ. 1992. Altered morphology in transgenic tobacco plants that overproduce cytokinins in specific tissues and organs. *Developmental Biology* **153**, 386–395.
- McCabe MS, Garratt LC, Schepers F, Jordi WLRM, Stoopen GM, Davelaar E, van Rhijn JHA, Power JB, Davey MR. 2001. Effects of P_{sag12}-*ipt* gene expression on development and senescence in transgenic lettuce. *Plant Physiology* **127**, 505–516.
- Medford JI, Horgan R, El-Sawi X, Klee HJ. 1989. Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. *The Plant Cell* **1**, 403–413.
- Mercier RW. 1998. Apoplastic invertase: a dissection of its role in photosynthate translocation and partitioning. PhD thesis, University of Connecticut, Storrs, CT, USA.
- Nguyen KHT, Kane EJ, Dix PJ. 1998. Hormonal regulation of senescence in cauliflower (*Brassica oleracea* var. *Botrytisa*). In: *Plant biotechnology and in vitro biology in the 21st century*. Proceedings of the IX International Congress on Plant Tissue Culture. Dordrecht, The Netherlands: Kluwer Academic Publishers, abstract no. 96.
- Redig P, Shaul O, Inzé D, Van Montagu M, Van Onckelen H. 1996. Levels of endogenous cytokinins, indole-3-acetic acid and abscisic acid during the cell cycle of synchronized tobacco BY-2 cells. *FEBS Letters* **391**, 175–180.
- Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.

- Smigocki AC.** 1991. Cytokinin content and tissue distribution in plants transformed by a reconstructed isopentenyl transferase gene. *Plant Molecular Biology* **16**, 105–115.
- Smigocki AC, Neal Jr JW, McCanna I, Douglass L.** 1993. Cytokinin-mediated insect resistance in *Nicotiana* plants transformed with the *ipt* gene. *Plant Molecular Biology* **23**, 325–335.
- Thomashow MF.** 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**, 571–599.
- Thomas JC, Smigocki AC, Bohnert HJ.** 1995. Light-induced expression of IPT from *Agrobacterium tumefaciens* results in cytokinin accumulation and somatic stress symptoms in transgenic tobacco. *Plant Molecular Biology* **27**, 225–235.