April 2005

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Date of submission:

This manuscript contains three Tables and four Figures.

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 x hybrida* 'Marco Polo Odyssey' and *Dendranthema x 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^{o}C) after an initial exposure to a brief cold-induction period (4^{o}C for 72 hrs). 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^{o}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 vigor, 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 catalyzes 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 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 SAG_{12} 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 SAG_{12} 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 *SAG_{12}-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 deteriorate 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 glasshouse. In the study herein, we constructed an *ipt* fusion gene under the control of a cold-inducible promoter from the *cor15a* gene from *Arabidopsis thaliana*. 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 *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 *Eco*RI and *Sal*I and cloned into the cloning site of the *pBluescript*II *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 *Xho*I and *Sal*I were added to the ends of the *cor15a* primers 5'-GGCTCGAGAGATCTTGTCCGTTGAATTT-3' and 5'-GGTCGACGAGAGAGATCTTTA AGATGT-3'for PCR. The *cor15a* fragment was subcloned into the *pBluescript*II *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 *pBluescript*II *KS-cor15a-ipt-nos* and *pBin19* with *Kpn*I and *Sma*I 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^{o}C in the greenhouse in 3.8 l pots containing a peat-based substrate (Metro 510, Scotts Co., Marysville, OH). 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 were 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. Stem 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 50mg L^{-1} kanamycin and 25mg L^{-1} rifampicin. The suspension was incubated at 25^{o}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 re-suspended 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 3 d in the dark at 22-25^{o} C on plates with MS medium containing 2 mg L^{-1} of N^{6}-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 glasshouse 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'-GGCTCGAGAGATCTTGTCCGTTGAATTT-3' and reverse primer 5'-GGTCGACGAGAGAGATCTTTAAGATGT-3'; and for the 0.525 kb region of *ipt* gene (ii) forward primer 5'-GGTCCAACTTGCACAGGAAAG-3' and reverse primer 5'-GGCTTGCCTACTGGAAGCTTA-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 sec 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, Saint Louis, MO, USA). Finally, a 20µl 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 3h, 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-dioxigenin-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; 5mM of $MgCl_2$, 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-Omart 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. Austin, TX, USA) 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.) 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 10cm 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 re-suspended 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 and 663 nm according to the equation: 20.2 A_{645} + 8.02 A_{663} (Chory *et al.,* 1994).

Morphological analysis of transgenic plants

The effect of the transgene on growth and development of chrysanthemums 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^{o}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^{o}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 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 (cm^2) on 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, v/v/v, pH~2.5, -20°C). To each sample, 50 ρmol of each of 12 deuterium labelled standards ([$^2H_5$]Z, [$^2H_5$]ZR, [$^2H_5$]Z7G, [$^2H_5$]Z9G, [$^2H_5$]ZOG, [$^2H_5$]ZROG, [$^2H_6$]iP, [$^2H_6$]iPA, [$^2H_6$]iP7G, [$^2H_6$]iP9G, [$^2H_5$]DHZ, [$^2H_5$]DHZR; products of Apex Organics, Honiton, UK) were added. The extract was passed through 2 ml Si-C_{18} 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^{o}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^{n} equipped with an electrospray interface. Samples dissolved in 10% (v/v) acetonitrile (10 μl) were injected on an C_{18} 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 analyzed 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 3.3σ/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.

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 a 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^{o}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^{o}C treatment but not in the same lines grown at the 25^{o}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. A pre-treatment 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 *cor-15a-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 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 *p*mol/g 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 the transfer to the 25°C growth

permissive conditions for 3.5 d (averaging 16.8 *p*mol g^{-1} DW). But, 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 *p*mol 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-inductive conditions (averaging 1.5 *p*mol g^{-1} DW). The concentration of isopentenyladenine detected in non-induced wild type, non-induced *cor15a-ipt*, and cold induced wild type plants (4.1 *p*mol 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 *p*mol 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 *p*mol 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 *p*mol 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 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, 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$\pm$ 3.0 cm) and *cor15a-ipt* petunia plants (20.1$\pm$ 0.6 cm) were similar. Likewise, the average number of lateral shoots on the main stem (5.8$\pm$ 1.2 cm and 6.8$\pm$ 0.4 cm), and the average internode length on the main stem (1.7$\pm$ 0.3 cm and 1.5$\pm$ 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 to 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 to 2^{o}C over the temperature range of –4 to –8^{o}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 active in the shoots but not in the roots. 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, we carried out all morphological experiments under carefully controlled environmental conditions.

In our 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 to 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 *ipt* gene in petunia resulted in an immediate increase of zeatin and dihydrozeatin type cytokinins. We found 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). The increase in the concentration of isopentenyladenine, but not of its derivatives, indicates that cytokinin metabolism, including of 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 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 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 to non-induced leaves (Smigocki, 1991). The resulting transgenic plants were shorter, had 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 non-radioactive detection of ipt fragment in transgenic plants and Dr. Carol Auer (Plant Science Department, UCONN) for excellent scientific discussions concerning topic of this paper. This study was supported by a grant from Connecticut Innovation Inc.

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Figure legends.

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 *ipt* gene from putative-transgenic chrysanthemum lines. Lane 1: 1 kb molecular mark; 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-labeled 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 analyzed (lanes 1-3) but not from wild type petunia DNA (lane 4-5). Plasmid DNA was used as the positive control (lane 6).

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^{o}C), lane 2: wild type plant exposed to a cold-induction treatment (3.5 d at 4^{o}C), lane 3: transgenic plant (normal conditions, 25^{o}C), lane 4: transgenic plant exposed to a cold-induction treatment (3.5 d at 4^{o}C).

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^{o}C) or non-inducing (25^{o}C) temperatures prior to 18 d dark storage at 25^{o}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^{o}C) prior to dark storage did not (N).

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^{o}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, N^6–(Δ^2-isopenenyl)adenine; iPR, N^6–(Δ^2-isopentenyl)adenosine; iPRMP, N^6–(Δ^2-isopentenyl) 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} dry weight) 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
cor15a-ipt 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, isopentenyladenine, 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.

	Wild type		*cor15a-ipt*				
Temperature treatment before sampling [a]	*Duration of exposure (d)*						
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.

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 (3d at 4C) 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)
cor15a-ipt L9	21 (2.1)	10.1 (1.3)	4.8 (0.22)	0.32 (0.23)	0.72 (0.10)	5.29 (0.60)
cor15a-ipt 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)
cor15a-ipt L9	29 (3.7)	9.8 (1.7)	5.2 (0.55)	0.28 (0.31)	0.88 (0.12)	5.76 (0.79)
cor15a-ipt 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 x Cold (interaction)	NS	NS	NS	NS	NS	NS

[a] NS denotes non-significance, * denotes significant at P≤ 0.05, ** denotes significant at P≤ 0.01, *** denotes significant at P≤ 0.001.