June 2006

Expression of *ipt* Gene Controlled by an Ethylene and Auxin Responsive Fragment of the LEACO1 Promoter Increases Flower Number in Transgenic *Nicotiana tabacum*

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Abstract Cytokinins play important roles in regulating plant growth and development. A new genetic construct for regulating cytokinin content in plant cells was cloned and tested. The gene coding for isopentenyl transferase (*ipt*) was placed under the control of a 0.821 kb fragment of the ACC oxidase gene promoter from *Lycopersicon esculentum* (LEACO1) and introduced into *Nicotiana tabacum* (*cv. Havana*). Some $LEACO1_{0.821kb}$-*ipt* transgenic plant lines displayed normal shoot morphology but with a dramatic increase in the number of flower buds compared to non-transgenic plants. Other transgenic lines produced excessive lateral branch development but no change in flower bud number. Isolated leaves of transgenic tobacco plants showed a significantly

prolonged retention of chlorophyll under dark incubation (25^{o} C for 20 days). Leaves of non-transformed plants senesced gradually under the same conditions. Experiments with LEACO1$_{0.821kb}$-*gus* transgenic tobacco plants suggested auxin and ethylene involvement in induction of LEACO1$_{0.821kb}$ promoter activity. Multiple copies of nucleotide base sequences associated with either ethylene or auxin response elements were identified in the LEACO1$_{0.821kb}$ promoter fragment. The LEACO1$_{0.821kb}$-*ipt* fusion gene appears to have potential utility for improving certain ornamental and agricultural crop species by increasing flower bud initiation and altering branching habit.

Keywords transgenic *Nicotiana tabacum*, fragment of LEACO1 promoter, *ipt* expression, leaf senescence, number of flower buds.

Introduction

Cytokinins play a role in many growth and developmental processes in plants, such as apical dominance, shoot cell proliferation, cell differentiation, flowering, fruit set and ripening, leaf senescence and seed germination (Mok and Mok 1994; Swarup et al. 2002). The effects of cytokinins on plants can be exploited for agricultural and horticultural purposes through either exogenous application of cytokinin or endogenous manipulation of cytokinin metabolism. In *Hatiora gaetneri* for example, flower bud number more than doubled in response to a spray application of synthetic cytokinins (Boyle 1988; Boyle 1995). The efficacy of exogenous spray applications is limited however, because flowers and leaves do not readily absorb cytokinins and movement of cytokinins within the plant is limited (Hobbie et al. 1994). Alternatively, by integrating the *ipt* gene into the plant

genome endogenous levels of cytokinins can be modified. The *ipt* gene encodes the enzyme isopentenyl transferase, which catalyzes the rate-limiting step in cytokinin biosynthesis (Akiyoshi et al. 1984). A number of promoters, including those inducible by heat (Medford et al. 1989; Smigocki 1991; Ainley et al. 1993), wounding (Smigocki et al. 1993), or light (Thomas et al. 1995), have been used to drive IPT gene expression. Unfortunately, most of the resulting *ipt* transgenic plants exhibited morphological abnormalities since overproduction of cytokinins interferes with so many developmental processes (Gan and Amasino 1997). Transgenic plants that overproduce cytokinins show reduced stature, release of apical dominance, changes in vascular development, and in some cases, inhibited root growth (Klee et al. 1987; Ainley et al. 1993). In one study, Li et al. (1992) fused the *ipt* gene to the auxin-inducible SAUR promoter. This promoter is primarily active in elongating tissue and SAUR-*ipt* plants expressed elevated levels of cytokinins in these tissues. SAUR-*ipt* plants displayed reduced stature, increased axillary bud development, reduced root initiation and growth, and exhibited complex and variable changes in senescence. Recently, Khodakovskaya et al. (2005) used the cold inducible promoter cor15a to drive expression of the *ipt* gene in petunia and chrysanthemum, and demonstrated that IPT expression could be regulated in response to a short (3-d) cold treatment. In that study, IPT expression did not affect plant morphology under normal growth temperatures but did produce increased cytokinin concentrations and delayed leaf senescence in cold induced shoots.

The *LEACO1* gene encodes the ACC oxidase enzyme in *Lycopersicon esculentum.* ACC oxidase catalyzes the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, the last step in the biosynthesis of this plant hormone (Yang and Hoffman

1984). ACC is synthesized from S-adenosylmethionine (SAM) by the enzyme ACC synthase. Blume and Grierson (1997) reported that the ACC oxidase promoter from tomato (LEACO1) is active in response to aging, wounding, ethylene, pathogen infection and treatment with methyljasmonate and α-amino butyric acid. The *LEACO1* gene is inducible in a number of organs at various stages of the plant life cycle. In addition, the ACC oxidase transcripts are reported to be spatially regulated throughout flower development (Barry et al. 1996), and LEACO1 promoter activity detected in leaves and flowers and can be induced by external factors that stimulate ethylene pathway activity such as wounding, exogenous ethylene or pathogen infection (Blume and Grierson 1997).

Blume and Grierson (1997) found that fragments of the LEACO1 promoter (396 bp and 1825 bp) were sufficient to drive strong GUS expression during both leaf and flower senescence, but a 124 bp fragment was less effective. Based on these responses, they concluded that the essential cis-acting elements are located in the region between –396 and –124 upstream of the transcription start site. However, the 1825 bp sequence contained a repetitive element found in the tomato genome that resembles the long terminal repeats (LTRs) of copia-like retrotransposons (Blume et al. 1997) while the 396 bp sequence did not include this element. Further, sequences with high homology (70-75%) to the 5' flanking sequences of the ACC oxidase genes PHACO1, PHACO3 and PHACO4 (Tang et al. 1993) are located within 500 bp of the transcription start site. Also, at least two different stress-responsive short motifs were present in the 1825 bp fragment but not in the 396 bp fragment. For instance, Blume and Grierson (1997) report the 10 bp TCA motif (5'-TCATCTTCTT-3') occurs seven times (allowing two substitutions) in the LEACO1 promoter between nucleotides –667 and –1447, and an 8 bp element (5'-

AA/TTTCAAA-3') is present in three copies between nucleotides –473 and –1662. The *TCA* motif is present in the 5' upstream region of over 30 stress- and pathogen-inducible genes (Goldsbrough et al. 1993) while the 8 bp element is reportedly necessary for ethylene-response in the carnation GST1 (Itzhaki et al. 1994) and the tomato E4 (Montgomery et al. 1993) gene promoters.

The LEACO1 promoter fragment used in our study, contained only a portion of the LTR-like element (Blume et al. 1997) but two copies of the TCA motif and one of the 8 bp 5'-AA/TTTCAAA-3'ethylene responsive element. In addition, the promoter fragment used in our study contained multiple copies of the auxin responsive element (AuxRE) required TGTCTC- (or inverse GAGACA-) sequence found in genes regulated by auxin response factors (Ulmasov et al. 1995). TGTCTC/GAGACA AuxREs are found in many auxin response genes (Guilfoyle et al. 1998). Composite AuxREs may contain both the TGTCTC element and a coupling element such as a G-box motif (Guilfoyle et al. 1998; Hong et al. 1995). G-box motifs are regulatory elements found in many genes including auxin responsive genes such as GmAux28 (Hong et al. 1995). A common feature of genes in the auxin/IAA family, are regions containing multiple putative AuxREs (Remington et al. 2004). These putative AuxREs contain motifs with at least five out of six nucleotides matching the consensus TGTCTC sequence in forward or reverse orientation (Ulmasov et al. 1997; Ulmasov et al. 1999a; Ulmasov et al. 1999b; Remington et al. 2004). Ulmasov et al. (1997, 1999b) reported that the nucleotides TGTC (positions +1 to +4) of the TGTCTC element are essential for binding auxin response factors, while substitutions at +5 are tolerated, and the importance of position +6 is variable. In the LEACO1 gene promoter fragment used in our study, we identified single

copies of the TGTCTC, and inverse GAGACA, sequences in reverse orientation as well as three copies of the sequence TGTCTC motif with one substitution in position six (TGTCTt) in forward orientation (substituted bases in lower case). In addition, we identified two copies of the CACGTG G-Box motif with one substitution (CtCGTG) and three copies of the ACGT G-box core element.

Here, we selected an 821 bp fragment of the LEACO1 gene promoter to drive both IPT and GUS gene expression. Our objectives were to (1) test relative changes in $LEACO1_{0.821kb}$ promoter activity in response to various auxin and ethylene promoting and inhibiting agents on $LEACO1_{0.821kb}$–*gus* plants, and (2) characterize the *in situ* phenotypic response of selected $LEACO1_{0.821kb}$–*ipt* transgenic tobacco lines in the absence of an exogenous hormonal agent.

Materials and methods

Binary vector constructions

Using standard molecular cloning procedures (Sambrook et al. 1989) and the primers (forward) 5'-GGTTGAGTTGTTTCCCTCTG-3' and (reverse) 5'-GGTAAAGTGTTTTCCTAAGTG-3', an 821 bp fragment from the promoter region plus a 97 bp of 5' UTR leader sequence for a total fragment length of 918 bp of the untranscribed sequence of the *LEACO1* gene was synthesized by PCR reaction. Figure 1 schematically depicts $LEACO1_{0.821kb}$ -*ipt*-nos vector (Fig.1 A) and 0.821 kb fragment of LEACO1 promoter (Fig.1 B). This fragment of the LEACO1 promoter contains part of the LTR element (between nucleotides -821 to –590) and includes two 10 bp TCA motifs

(starting from -739 and –667 upstream) and an 8 bp ethylene responsive element (starting from -473). In reverse orientation, single copies of the consensus AuxRE TGTCTC- and the inverse GAGACA-sequences are located at –776 and –592, respectively. In forward orientation, the TGTCTC element (with one substitution in position six, TGTCTt) appears at –187, –666, and –688. The G-box motif CACGTG (with one substitution CtCGTG) appears at –552 and –656. G-box core ACGT elements were found at –483, -703 and –726. The $LEACO1_{0.821kb}$ promoter fragment was cloned into the *Hin*dIII-*Sal*I sites of the pBin19 binary vector, replacing the CaMV promoter fragment from the 35S-*ipt*-nos construct in this plasmid (initial binary vector pBin19 containing *ipt* gene under control of 35S promoter was provided by Dr. Li, University of Connecticut). The DNA sequence of the $LEACO1_{0.821kb}$ promoter fragment in the established plasmid was confirmed by DNA sequence analysis (W.M. Keck Biotechnology Laboratory, Yale University, New Haven, CT). The binary plasmid was transferred into *Agrobacterium tumefaciens* strain LBA 4404 by electroporation.

To construct a gene containing *gus* under the control of the $LEACO1_{0.821kb}$ fragment, the pBin19- $LEACO1_{0.821kb}$-*ipt* vector was cut by *Sal*I and *SacI* releasing the *ipt* gene. Similarly, the *gus* gene was released from the pUC19-*gus*-nos construct by digestion with *Sal*I and *Sac*I. $LEACO1_{0.821kb}$ -nos and *gus* fragments were ligated together. The plasmid containing the whole $LEACO1_{0.821kb}$-*gus*-nos fragment in pBin19 binary vector was transferred into *Agrobacterium tumefaciens* strain LBA 4404 by electroporation.

Plant transformation and T_1/T_2 plant production

Tobacco plants were transformed using the *Agrobacterium*-mediated transformation method (An et al. 1988). Briefly, young tobacco leaves were surface-sterilized, cut into discs and co-cocultivated with *Agrobacterium tumefaciens* LBA 4404 bearing the LEACO1-*ipt* construct or LEACO1-*gus* construct. Following co-cultivation, the explants were transferred to the MS medium (Murashige and Skoog 1962) supplemented with 0.1 mg/l α-naphthaleneacetic acid and 1 mg/l 6-benzylaminopurine. Kanamycin at 300 mg/l was used for selection and timentin at 400 mg/l was used to suppress *Agrobacterium*. Explants were transferred to fresh medium at 2-3 week intervals until shoots began to emerge from the transgenic calli. Excised shoots were then transferred to MS medium containing 100 mg/l of kanamycin until roots developed. Finally, rooted plantlets were transferred to pots containing Metro 510 (Scotts, Co., Marysville, OH, USA) and acclimated to the growth environment at 25^{o}C. Individual, transgenic lines were then analyzed by PCR for foreign genes integration. Seeds (T_1) were germinated on MS medium supplemented 100mg/l kanamycin to determine the segregation patterns of the transgene. Progeny obtained from T_1 plants were analyzed and homozygous lines (T_2) were used in future experiments.

Plant DNA extraction and polymerase chain reaction (PCR) analysis

Total DNA was isolated from leaf tissue of greenhouse grown plants (T_0 and T_1 generations) using DNeasy Plant Mini Kits (Qiagen Inc., Valencia, CA, USA) and 250 ng of DNA was subjected to PCR reaction. The primers used to detect the recombinant DNA were (i) forward primer 5'-GGTTGAGTTGTTTCCCTCTG-3' and reverse primer 5'-

GGTAAAGTGTTTTCCTAAGTG-3', specific for the 0.918 kb fragment (nucleotides –1-918) of LEACO1 promoter; (ii) forward primer 5'-GGTCCAACTTGCACAGGAAAG-3' and reverse primer 5'-GGCTTGCCTACTGGAAGCTTA-3', specific for the 0.525 kb region of the *ipt* gene (full size - 0.745 kb); 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, 30 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min 30 sec at 72°C, and extension at 72°C for 5 min. The reactions involved 200 ng of DNA template, 0.2 mM of dNTPs, 0.5 μM of each primer, REDTaq PCR Buffer and 1unit of REDTaq DNA polymerase (Sigma, Saint Louis, MO, USA). Finally, a 10μl 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, Carlsbad, CA, USA) was used as a reference to determine DNA fragment size. Only PCR positive plants transgrnic plants were used for further tests.

Southern hybridization

Genomic DNA was isolated from transgenic plants using a DNeasy Plant Maxi Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. A 10 μg sample of total genomic DNA from putative transgenic and non-transformed control plants was digested overnight by restriction with *Hind*III at 37° C. Digested DNA of each line was separated through a 1% agarose gel prepared in 1X TAE (Sambrook et al. 1989) and fragments were transferred from the agarose gel to a nylon membrane (Amersham, Chalfont St Giles, UK) and cross-linked to the membrane by UV treatment. The *ipt* gene probe (0.525 kb fragment of the 0.745 kb *ipt* gene) was prepared with a PCR DIG Probe

Synthesis Kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. DNA fixed on membranes was pre-hybridized with a pre-hybridizing solution at 68^{o}C for 3 hours, hybridized with probe at 68^{o}C overnight and washed by post-hybridization solution 4 times for 15 min. at 65^{0} C in a hybridization oven. Solutions used for sample hybridization, pre- and post-hybridization, and buffers for the ensuing steps were prepared as previously reported by Mercier (1998). Membranes were washed for 5 min. in 50 ml of maleate buffer (0.1 M of maleic acid and 3.0 M of NaCl) at room temperature and then incubated for 1 hour in 50 ml of blocking solution [maleate buffer plus 0.5% blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA). Next, membranes were incubated for 30 min. in 20 ml of blocking solution plus anti-dioxigenin-AP, Fab fragments (Roche Molecular Biochemicals, Indianapolis, IN, USA) diluted to 1:10,000 and then washed 4 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 $MgCl_2$) and then incubated at 37^{o}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, USA). Membranes were exposed to autoradiographic film (Kodak X-Omart AR) for 4 hours. X-ray films were developed with an automatic film processor.

Application of chemicals and histochemical analysis of GUS activity

Individual transgenic LEACO1$_{0.821kb}$-*gus* tobacco seedlings (lines N3 and N5 of T_1 generation), that showed positive β–glucuronidase activity, were treated with either IAA, the ethylene synthesis inhibitor aminooxyacetic acid (AOA), the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA), the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), or the ethylene generating compound ethephon. AOA, ACC, IAA, TIBA were from Sigma (Sigma-Aldrich, Inc., St.Louis, MO, USA). Ethephon was from Rhone-Poulenc Ag Company (Monterey, CA, USA). In the initial experiment, 100mg/l IAA in lanolin was applied to the apex of an excised shoot, 2.5 g/l TIBA was applied in a lanolin ring directly below the intact apex, and 0.5 mM AOA was brushed on the stem surface below the apex. In a second experiment, ACC (10^{-5} M), ethephon (500 mg/l) and AOA were applied as sprays but TIBA was applied as previously described. ACC was applied to seedlings that had the shoot apex removed, and the other compounds were applied to plants with the apex intact. All chemicals were reapplied every two days, and after six days the seedlings were harvested and histochemical analysis was conducted. In both experiments, untreated seedlings with the apex intact or with the apex removed were used as control treatments. For histochemical assays of β–glucuronidase activity, the stems of young LEACO1$_{0.821kb}$-*gus* tobacco seedlings were sectioned by hand. Stem sections were vacuum infiltrated with a staining solution containing 1mM X-GLUC (5-bromo-4-chloro-3-indolyl–β-D-glucuronic acid) and incubated at 37^0 C for 24 h. After staining, samples were rinsed with water and fixed in 70% ethanol. Each treatment was sampled in triplicate. Results of GUS-expression were documented using digital photography (Olympus 3X optical zoom and Olympus light microscope (SZH10) with 0.7X magnification).

Analysis of IPT expression in leaves of tobacco

Total RNA was isolated from tobacco plants by flash freezing tissue in liquid nitrogen and then grinding in a mortar with TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). For Reverse Transcription-PCR (RT-PCR) analysis, possible DNA contamination was removed from RNA samples using a DNase treatment (DNA-free™, Ambion Inc., Austin, TX). The first-strand cDNA was then synthesized from 1 μg of total RNA using a RETROscript™ First Strand Synthesis Kit (Ambion Inc., Austin, TX) following the manufacturer's instruction. For PCR, 0.5 μL of RT-mix was used in a final volume of 25 μL. The PCR reaction for the *ipt* gene fragment was conducted as previously described. 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.

Plant growth conditions and morphological analysis of transgenic plants

The effect of the transgene on growth and development and number of flower buds on two selected transgenic lines was compared to the wild-type tobacco line. The transgenic lines were selected to represent the overall phenotypic range, from plants with normal vegetative growth but enhanced flower development to plants with unusual vegetative development. The study was conducted as follows. Shoots from each transgenic LEACO1-*ipt* tobacco plant lines N2 and N4 of T_1, T_2 generation and from the wild-type

cultivar '*Havana*' were rooted in deep 606-cell packs (Kord Products, Bramalea, Ontario, Canada) containing a Metro 510 (Scotts Co., Marysville, OH, USA) peat-lite medium. After six weeks, plants from each line were harvested and the following data recorded: plant height, number of nodes, leaf area per plant, number of flower buds on main stem, number of lateral branches. These parameters were used to calculate the average internode length and the average area per leaf on the upper-most lateral shoot. These data were used to determine difference in growth habit between transgenic and wild-type plants. Plants were arranged in a randomized complete block design with 10 replicated blocks. Statistical effects were determined using a one-way analysis of variance to compare the responses of genetic lines.

The effects of ethylene on the IPT gene expression in two transgenic tobacco plant lines (N2 and N4) was investigated by spraying plants in the greenhouse with ethephon (500 mg/l) and then sampling tissue after 1.5 days. RNA was isolated from the tissue samples, and RT-PCR analysis was conducted as previously described.

Quantification of Chlorophyll

Specific chlorophyll concentration was calculated as follows. Leaf tissue from each sample was blotted dry, weighed, and placed in a 1.5-mL Eppendorf tube. 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 according to the equation: 20.2 A_{645} + 8.02 A_{663} (Chory et al. 1994). Average of chlorophyll content was

determined from 10 independent samples representing 10 leaves from each transgenic line and the wild type.

Results

Establishment and molecular analysis of transgenic plants of *Nicotiana tabacum* harboring the LEACO1$_{0.821kb}$ -*ipt* and LEACO1$_{0.821kb}$ -*gus* fragments

The *ipt* gene positioned under the transcriptional control of a 0.821 kb fragment of LEACO1 promoter was introduced to wild-type tobacco plants by *Agrobacterium* transformation. More than 30 kanamycin-resistant, putative transformants were regenerated, transferred to pots and successfully acclimated to glasshouse conditions.

PCR analysis confirmed the integration of the foreign gene into the genome of the putative transgenic tobacco plants (Fig.2) and plants of generations T_1 and T_2 (data not shown). PCR amplifications produced the expected 0.918 kb fragment (the LEACO1$_{0.821kb}$ promoter plus the 5' UTR leader sequence) (Fig.2A) and a 0.52 kb fragment of the *ipt* gene (Fig. 2B) from both the putative transgenic lines and the plasmid DNA used as a positive control. DNA was not detected in non-transgenic wild type plants. Southern blot analysis (Fig. 2C) confirmed integration of the chimeric LEACO1$_{0.821kb}$-*ipt* gene into the genomic DNA isolated from transgenic tobacco lines (T_1 generation) representing the range of phenotypes observed (lines N1, N2, N4 and N8).

Ten kanamycin-resistant LEACO1$_{0.821kb}$-*gus* plants were also confirmed positive for the presence of the LEACO1$_{0.821kb}$ promoter by PCR and stained positive in the GUS histochemical assay.

GUS expression under the control of the LEACO1$_{0.821kb}$ promoter.

LEACO1$_{0.821kb}$-*gus* tobacco seedlings (lines N3 and N5) were used to assess the effects of IAA and ethylene pathway activity on LEACO1$_{0.821kb}$-controlled gene regulation. Both lines produced similar responses. Under glasshouse conditions, GUS expression was observed in leaves and stems of young LEACO1$_{0.821kb}$-*gus* tobacco seedlings (Fig. 3A). GUS expression was not observed in roots of plants from the same line (Fig.3A). Strongest GUS expression is found in the apical and lateral meristems in vegetative shoots and surrounding the developing flower bud and in lateral meristems in generative shoots (Fig.3A: I and II).

To assess the involvement of auxin and ethylene in inducing the LEACO1$_{0.821kb}$ promoter, we applied IAA, AOA, ACC, or ethephon to plants with the apical buds either intact or removed (Fig. 3 B, C, D). Intact apical buds produce endogenous IAA but shoots with excised apical buds do not. With apical buds intact, plants showed increased GUS gene expression while shoots with the apical bud removed showed reduced GUS expression (Fig. 3). Elevated GUS expression was observed in shoots with the apical bud removed when exogenously IAA was applied in a lanolin paste to the cut shoot apex. However, GUS expression remained suppressed in the presence of IAA (applied to the cut shoot apex) when the auxin transport inhibitor TIBA was applied directly below the

IAA lanolin paste. In shoots with intact apical buds, normal high GUS expression was inhibited by TIBA and by the ethylene biosynthesis inhibitor AOA. These results suggest involvement of both auxin and ethylene in stimulating LEACO1$_{0.821kb}$ promoter activity.

In an ensuing experiment, young LEACO1$_{0.821kb}$-*gus* seedlings (line N3 and line N5) were treated with a spray of either AOA, ethephon, or the ethylene precursor ACC, or lanolin containing TIBA (Fig. 3. C, D). In plants with intact apical buds, normal high GUS expression was inhibited by both TIBA and AOA. But intact shoots exposed to ethephon continued to show high GUS expression. Seedlings with apical buds removed showed a lower level of GUS gene expression compare with plants with intact apical buds but GUS expression in plants with excised apical buds was stimulated by the ethylene precursor ACC.

Transgenic plants expressing *ipt* under control of the LEACO1$_{0.821kb}$ -promoter exhibit distinctive phenotypic characteristics

T_1 generation LEACO1$_{0.821kb}$-*ipt* tobacco lines displayed a range of growth habits from plants that appeared similar to the wild type in overall stature and branching habit but produced a greater number of flower buds (lines N2 and N8) to plant lines that expressed shoot characteristics typical of constitutive cytokinin expression (lines N1 and N4) (Table 1 and Fig. 4). Southern hybridization analysis of genomic DNA digested with *Hin*dIII confirmed the integration of the *ipt* gene into the genome of the T_1 transgenic lines, while no signal was detected in the wild type plants (Fig. 2C). The increase in flower bud initiation associated with LEACO1$_{0.821kb}$-*ipt* lines N2 and N8 was found to be stable in

successive seed generations. For example, the trend toward increased flower bud numbers in transgenic line N2 also was observed in the T_2 seed generation (in homozygous line). In a separate experiment, the average number of flowers per plant in the wild type was 44±3.1. In comparison, T_2 generation transgenic tobacco plants averaged 84.5±6.4 flowers per plant for line N2 and only 34.3±5.5 flowers per plant for line N4.

RT-PCR analysis with total RNAs extracted from the leaves of wild type and transgenic lines N2 and N4 growing under normal glasshouse conditions, and the same lines treated with the ethylene-releasing compound ethephon, showed differential levels of expression (Fig. 5). The 0.52 fragment of the *ipt* cDNA derived from mRNA was amplified in both transgenic lines under normal greenhouse conditions but the level of expression was much higher in the line (N4) that showed the more extreme cytokinin phenotype. Gene expression was not detected in wild type plants or in transgenic lines sprayed with ethephon (500 mg/L). These data demonstrate that the range of phenotypes observed in the various LEACO1$_{0.821kb}$ -*ipt* tobacco lines corresponded with the level of IPT expression and that IPT expression could be suppressed by exogenous application of ethephon.

LEACO1$_{0.821kb}$ -*ipt* transgenic tobacco lines exhibit delayed leaf senescence.

Leaf senescence was dramatically delayed in LEACO1$_{0.821kb}$-*ipt* transgenic plants compared to wild type tobacco plants (Fig. 6). Excised leaves from wild type tobacco and transgenic lines N2 and N4 were stored under dark conditions (25° C) for up to 40

days. Chlorophyll content in wild type leaves declined to less than 5% of the initial concentration after the 10^{th} day of dark storage and disappeared completely by the 15^{th} day. Leaves of transgenic lines showed tolerance to dark storage and maintained approximately 50% of the initial chlorophyll concentrations after 20 days in the dark. Leaves from LEACO1$_{0.821kb}$-*ipt* line N4 retained approximately 25% of the initial chlorophyll concentrations for up to 40 days in the dark. Resistance to leaf senescence appeared to be positively correlated with differences in IPT gene expression.

Discussion

Plant growth, development and metabolic processes such as ethylene biosynthesis are regulated by complex interactions of antagonistic and coordinately regulating hormones including auxin, cytokinin and ethylene (Swarup et al. 2002; Kim et al. 2001). Auxin stimulates ethylene production by inducing the synthesis of ACC (Yang and Hoffman 1984). Auxin induced up-regulation of ACC synthase in *Arabidopsis*, tomato, and lupin (Abel et al. 1995; Abel and Theologis 1996; and Beckman et al. 2000). In mungbean, auxin enhanced both ACC synthase and ACC oxidase expression (Yu et al. 1998). Elevated ACC oxidase levels are associated with plant structures containing rapidly dividing cells such as the shoot apical meristem (Liu et al. 1997). Using the LEACO1$_{0.821kb}$-*gus* gene to assess LEACO1$_{0.821kb}$-regulated gene activity, we found that removal of the endogenous auxin source (the shoot apical meristem) inhibited GUS expression. However, applying exogenous IAA to the excised shoot tip restored GUS expression. In shoots with the apical meristem intact, the auxin transport inhibitor TIBA suppressed GUS expression. Shoots with the apical bud removed continued to show GUS

expression when exposed to the ethylene precursor ACC but GUS expression was suppressed in shoots with the apical bud intact when exposed to the ethylene pathway inhibitor AOA.

These data suggest that the $LEACO1_{0.821kb}$ promoter is induced by ethylene pathway activity, and also demonstrate a likely role for endogenous auxin in triggering $LEACO1_{0.821kb}$ promoter activity. Blume and Grierson (1997) reported an increase in LEACO1 promoted GUS expression in plants exposed to ethylene gas. In their study, GUS gene expression was similar when either a 396 bp or an 1825 bp fragment of the LEACO1 gene promoter was used. While the 1825 bp fragment contained a number of short motifs that have been reported to be important for regulating expression of many genes involved in stress response (Goldsbrough et al. 1993) during senescence or for ethylene response (Itzhaki et al. 1994; Montgomery et al. 1993) the 396 bp fragment promoter sequence did not include any of these short motifs. In our study, the 821 bp fragment of the LEACO1 promoter sequence that we used to drive IPT (and GUS) expression included two copies of the TCA motif (Goldsbrough et al. 1993) and one copy of the 8 bp ethylene responsive element (Itzhaki et al. 1994; Montgomery et al. 1993), plus multiple copies of the TGTCTC sequence that is essential for AuxREs and the G-box motif associated with some auxin-responsive genes (Menkens et al. 1995; Guilfoyle et al. 1998). Thus, it is also possible that the $LEACO1_{0.821kb}$ promoter fragment used in this study is activated by auxin directly via auxin responsive factors that are able to bind with AuxREs. $LEACO1_{0.821kb}$-*ipt* transgenic plants displayed a range of phenotypic characteristics. Some lines produced normal shoot morphology but with an increased number of flower buds compared to wild type plants. These lines showed a low level of

IPT expression (RT-PCR) in the vegetative plant under glasshouse conditions. However, other lines showed an extreme phenotype characterized by excessive lateral branch development and fewer floral buds. The phenotypic appearance of these lines appeared to be typical of plants that over-express cytokinin, and IPT gene expression as determined by RT-PCR analysis, was higher in lines that exhibited this phenotype. In addition, transgenic plants that overproduce cytokinins typically display resistance to leaf senescence under stressful conditions (Li et al. 1992; Gan and Amasino 1997; McCabe et al. 2001; Schroeder et al. 2001; Clark et al. 2004). We found that detached leaves from LEACO1$_{0.821kb}$-*ipt* plants displayed chlorophyll retention characteristics that correlated with the range of IPT expression observed in the transgenic lines. Schroeder et al. (2001) noted that the delayed leaf senescence trait enhances the aesthetic value of ornamental plants. This would be especially true when fresh, excised plant parts are of economic important such as cut foliage. The increase in flower bud initiation that we observed in lines N2 and N8 suggests that IPT expression increased in these lines as the growth phase switched from vegetative to generative. Under generative growth conditions, a direct increase in ethylene biosynthetic pathway activity or a change in endogenous IAA concentration could trigger an increase in ethylene pathway activity, resulting in a subsequent increase in expression of the LEACO1$_{0.821kb}$-*ipt* gene. In contrast to the increased flower bud counts observed in lines N2 and N8, lines N1 and N4 showed a dramatic increase in lateral branch number but a flower bud number similar to the wild type. This response may reflect a difference in the timing and strength of *ipt* up-regulation between the two groups of transgenic plants. Schroeder et al. (2001) observed increased branching in *Nicotiana alata* expressing *ipt* under the control of P$_{sag12}$ but no

increase in flower bud number compared to wild type plants. They noted that IPT was not tightly regulated in this construct and the increased expression during the early stages of shoot development produced the increased branch effect. Indeed, Boyle et al. (1988) reported that topical treatments with benzyladenine resulted in increased branching when applied to plants (*Phipsalidopsis gaertneri*) in the vegetative growth stage but increased flower bud number when applied in the generative stage. The phenotypic variations that we observed in this study suggest that increases in endogenous cytokinin concentrations peaked at different stages of shoot development in transgenic lines that exhibited excessive branching (N1 and N4) compared to those that exhibited normal shoot morphology but increased flower bud numbers (N2 and N8). Differences in the timing and strength of IPT expression could explain the variations in shoot development and flower initiation that we observed. From RT-PCR analysis we know that the level of mRNA transcripts were higher in lines that exhibited the excessive branching phenotype than in lines that exhibited normal shoot morphology and increase flower bud number (Fig. 5).

Our observation with LEACO1$_{0.821kb}$-*gus* and LEACO1$_{0.821kb}$-*ipt* in response to the ethylene generating compound ethephon raises an interesting questions about the interplay of various plant hormones in regulating LEACO1$_{0.821kb}$ activity. In LEACO1$_{0.821kb}$-*gus* with intact apical buds, GUS expression remained high when plants were exposed to ethephon. While this observation did not suggest an increase in GUS expression (GUS expression was normally high in LEACO1$_{0.821kb}$-*gus* with intact apical buds, Fig. 3), ethephon certainly did not reduce GUS expression. However, when LEACO1$_{0.821kb}$-*ipt* plants were exposed to ethephon, expression of the IPT gene was

suppressed (based on RT-PCR analysis, Fig. 5). One would expect ethephon to stimulate ethylene pathway activity in general, and thus increase expression in genes under the control of the $LEACO1_{0.821kb}$ promoter. However, Liu (1997) reported that ethephon did not affect ACC oxidase mRNA levels in sunflower hypocotyls, while in mung bean hypocotyls Kim et al. (2001) observed increased ACC oxidase transcription levels and decrease ACC synthase transcription levels in response to ethylene gas.

Simple negative feedback or autoinhibition of ethylene could explain the response observed in our study. Autoinhibition of endogenous ethylene production has been described in banana fruit tissue (Vendrell and McGlasson 1971), fruits of sycomore fig (Zeroni and Galil 1976), citrus peel discs (Riov and Yang 1982), and avocado (Zauberman and Fuchs 1973). Autoinhibition of ethylene synthesis results from the reduction in ACC availability (Riov and Yang 1982). In the case of $LEACO1_{0.821kb}$-*ipt* plants, perhaps initial increases in cytokinin concentrations upon an ethephon treatment served to stimulate ethylene biosynthesis, but additional ethylene generation could have produced a negative feedback on ethylene biosynthesis and a subsequent down regulation of the $LEACO1_{0.821kb}$-*ipt* gene. However, if autoinhibition of endogenous ethylene production were responsible for down regulating $LEACO1_{0.821kb}$ promoter activity, one might expect that GUS expression would decrease in the presence of ethephon as well.

The fact that 24 hours after ethephon treatment, plants carrying the $LEACO1_{0.821kb}$-*ipt* showed a marked decrease in mRNA from IPT gene expression may be explained with another more likely mechanism: autoinhibition of the $LEACO1_{0.821kb}$-*ipt* gene by increased cytokinin concentrations. Although cytokinin can induce ethylene biosynthesis in plants (Vogel et al. 1998a; Vogel et al. 1998b), it has been shown that

cytokinin, an antisenescence hormone, often produces physiological effects opposite of ethylene, a senescence hormone. It is therefore possible that expression of the LEACO1$_{0.821kb}$-*ipt* gene, which leads to an increase in cytokinin concentrations in plants, may result in an inhibition of LEACO1$_{0.821kb}$ gene promoter activity, that is similar to the expression of the SAG12-*ipt* (Gan and Amasino 1995). Kim et al. (2001) found reduced ACC oxidase activity and a progressive reduction in ethylene production in mung bean hypocotyl tissue exposed to increasing concentrations of BA, and Coenen et al. (2003) reported cytokinin inhibited auxin-induced ethylene synthesis in tomato hypocotyl segments. However, it should be noted that in intact tomato seedlings, cytokinin stimulated ethylene synthesis (Coenen and Lomax 1998), and in mungbean hypocotyls exposed to the synthetic cytokinin BA, a synergistic increase in ethylene was observed in the presence of IAA (Lau et al. 1997; Yoshii and Imaseki 1982).

Exposing LEACO1$_{0.821kb}$-*ipt* plants to ethephon during shoot development and observing phenotype would be an approach that one might consider to elucidate how ethephon affects LEACO1$_{0.821kb}$-*ipt* gene expression. However, this approach would be unlikely to yield useful results since we know from commercial use (Gent and McAvoy 2000) that exposure to ethephon produces a phenocopy of the cytokinin over-expression phenotype. Therefore, if plants show reduced internode lengths, increased lateral branching, or variations in flower bud development, it would be impossible to discern whether the morphological traits resulted solely from ethephon exposure or from increased cytokinin expression, or some combination of both.

Although elucidating the complex cross talk of endogenous hormones involved in plant development was not the objective of our study, our observation on plant

development in response to $LEACO1_{0.821kb}$ regulated IPT expression does provide another example of the complexity of hormone interactions. Our data also suggests some of the potential uses for this specific construct in commercial plant development. For example, increases in the number of flower buds and lateral branches, on plants that otherwise display a normal phenotype, can be useful in a number of commercial crop plants from asexually propagated ornamental species to sexually propagated agronomic species. With asexually propagated crop species, unlike in sexually propagated species, trait stability in the primary transformants is important while stability in the seed progeny is not.

Acknowledgements

The authors thank Dr. Richard Mercier (Plant Science Department, UCONN) for help in non-radioactive detection of *ipt* fragment in transgenic plants. Authors acknowledge the Russian Science Support Foundation for support provided Dr. Khodakovskaya in the conduct of this study. Funding for this study was provided by CII (Connecticut Innovation Inc.), the Storrs Agricultural Experiment Station, and the University of Connecticut Research Foundation.

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Table 1. Growth characteristics of wild type plants and selected LEACO1$_{0.821kb}$ -*ipt* tobacco lines under greenhouse conditions. T_1 generation LEACO1$_{0.821kb}$ -*ipt* tobacco lines were selected that represented the range of observed phenotypes.

	Growth characteristic*						
Genetic line	Plant height (cm)	Average number of nodes (no#)	Average internode length (cm)	Average leaf area per plant (cm2)	Average area per leaf (cm2)	Number of flower buds on main stem	Average number of lateral branches
Wild type	86.8 ± 6.0a	18.2 ± 1.9a	4.8 ± 0.5a	2357 ± 488a	130 ± 16.8a	46.2 ± 5.6b	0b
LEACO1$_{0.821kb}$ -*ipt* N2	84 ± 2.6a	24 ± 3.2a	3.7 ± 0.5b	3070 ± 383a	132 ± 20.2a	133 ± 16.3a	0b
LEACO1$_{0.821kb}$ -*ipt* N4	73.8 ± 6.5a	23.4 ± 3.8a	3.2 ± 0.1b	2298 ± 172a	98 ± 8.8a	27.6 ± 5.9b	10.4 ± 0.6a

* Means in columns followed by same letters are not significantly different according to Tukey's multiple comparison test with a family error rate of 0.05.

Figures

Figure 1. Structures of the LEACO1$_{0.821kb}$ -*ipt*-nos construct (A) and 0.821 kb fragment of the LEACO1 promoter (B).

A: Scheme showing the structure of the LEACO1$_{0.821kb}$ -*ipt*-nos construct. From left to right: RB - right border of pBin19; P-nos nopaline synthase promoter; NPTII - neomycin phosphotransferase (nptII) gene from Tn5; T -nos - nopaline synthase terminator; *LEACO1*$_{0.821kb}$ fragment of *LEACO1* promoter from tomato; U- 0.097kb 5' UTR sequence; IPT - *ipt* gene from *Agrobacterium tumefaciens* ; LB - left border of pBin19 vector.

B: Scheme showing location of stress-responsive short motifs, TGTCTC /GAGACA-elements and G-box motifs in 0.821 kb fragment of LEACO1 promoter from *Lycopersicon esculentum*. The LEACO1 promoter fragment contained part of the LTR element (between nucleotides -821 to –590 upstream from the coding start site), two 10 bp TCA motifs (↓) and an 8 bp ethylene responsive element (∎). Single copies of the consensus AuxRE TGTCTC- and the inverse GAGACA-sequences are located at –776 and –592, respectively (⋮). In forward orientation the TGTCTC element (with one substitution TGTCTt) appears at –187, –666, and –688 (✦). The G-box motif CACGTG (with one substitution CtCGTG) appears at –552 and –656 (↕). G-box core ACGT elements were found at –483, -703 and –726(⋮).

Figure 2. PCR analysis of putative transgenic LEACO1$_{0.821kb}$-*ipt* tobacco plants and Southern blot analysis of genomic DNA isolated from tobacco plants. As expected,

transgenic plants carried the 0.918 kb fragment of the LEACO1$_{0.821kb}$ promoter plus the 0.097kb UTR leader sequence (A) and also showed a 0.52 kb fragment of the *ipt* gene (B). Lane 1: 1 kb DNA ladder; Lane 2: Positive control (plasmid LEACO1$_{0.821kb}$ -*ipt*-nos); Lane 3: Negative control (wild type tobacco); Lane 4-8: Putative transgenic tobacco lines. Southern blot analysis (C) of genomic DNA isolated from the wild type tobacco and LEACO1$_{0.821kb}$-*ipt* transgenic tobacco lines (T_1 generation) representing the range of phenotypes observed. The DIG-labeled 0.52 kb fragment of the *ipt* gene from LEACO1$_{0.821kb}$-*ipt*-nos plasmid DNA was used as a probe. Two copies of the *ipt* gene were detected in some lines (such as N1 and N4, in lanes 2 and 3 respectively) while a single copy of the gene was detected in other lines (lines N2 and N8 in lanes 4 and 5 respectively). Non-transformed wild type *Nicotiana tabacum* DNA (Lane 1) served as a negative control and the *ipt* fragment was not detected. Plasmid DNA was used as a positive control (Lane 6).

Figure 3. Tobacco seedlings transformed with the LEACO1$_{0.821kb}$ -*gus* reporter gene. The blue color indicates increased expression of the LEACO1$_{0.821kb}$ -*gus* gene and reduced blue color indicates a reduction in gene expression. A: GUS expression in young leaf, stem and root of a LEACO1$_{0.821kb}$ -*gus* transgenic seedlings (left). GUS expression viewed in longitudinally sectioned shoot tips from LEACO1$_{0.821kb}$ -*gus* transgenic plants (right). Note that the strongest GUS expression (indicated by arrows) is found in the apical and lateral meristems in vegetative shoots (I) and surrounding the developing flower bud and in lateral meristems in generative shoots (II); B, C & D: GUS-response of transgenic LEACO1$_{0.821kb}$ -*gus* plants following exposure to IAA, ethephon, the IAA

transport inhibitor TIBA, the biosynthetic precursor of ethylene ACC, or the ethylene biosynthesis inhibitor AOA in plants N5 and N3 with the shoot apex either intact (I) or removed (II). In the initial experiment, (B) IAA and TIBA applied in a lanolin paste, but AOA was applied in solution using a paintbrush to spread a thin film over the stem surface. IAA was applied to the shoot apex and TIBA was applied as a ring on the stem just below the shoot apex. In the following experiment (C and D), ACC, AOA, and ethephon were applied as sprays but TIBA was again applied in a lanolin paste.

Figure 4. $LEACO1_{0.821kb}$ -*ipt* tobacco lines displayed morphological traits that ranged from relatively normal to a form associated with excessive cytokinin expression. $LEACO1_{0.821kb}$ -*ipt* plants from lines N2 and N8, displayed a relatively normal shoot morphology but with an increased number of flower buds (A) relative to the wild-type line (B). But, $LEACO1_{0.821kb}$ -ipt plants such as lines N1 and N4, displayed short internodes, increased lateral branching and poor root development (D) compared to the wild type (C).

Figure 5. Ethephon suppressed IPT expression in $LEACO1_{0.821kb}$ -*ipt* tobacco lines. RT-PCR analysis was used to detect changes in IPT expression in transgenic lines after treatment with the ethylene-generating compound ethephon. Lane 1: $LEACO1_{0.821kb}$ -*ipt* (line N2) without ethephon treatment; Lane 2: $LEACO1_{0.821kb}$ -*ipt* (line N2) 24h after ethephon treatment; Lane 3: $LEACO1_{0.821kb}$ -*ipt* (line N4) without ethephon treatment; Lane 4: $LEACO1_{0.821kb}$ -*ipt* (line N4) 24h after ethephon treatment; Lane 5: wild type

tobacco without ethephon treatment; Lane 6: wild type tobacco 24h after ethephon treatment.

Figure 6. Excised leaves of LEACO1$_{0.821kb}$ -*ipt* tobacco lines showed delayed senescence under prolonged dark conditions. Leaves from wild type tobacco (*cv. Havana*) and LEACO1$_{0.821kb}$ -*ipt* transgenic lines were detached, plated onto wet filter paper, and stored in the dark at 25^{o}C. The chlorophyll content (μg/g FW) was measured immediately after leaves were detached and then periodically over a 40-day period. Chlorophyll in wild type leaves declined rapidly during the first 10-day period, but in leaves from LEACO1$_{0.821kb}$ -*ipt* plants, 50% of the initial chlorophyll concentration remained after 20 days in the dark. Leaves from the LEACO1$_{0.821kb}$ -*ipt* line that showed the highest IPT expression levels (N4) retained 30% of initial chlorophyll levels for up to 40 days. Means at each sample date labeled with the same letters are not significantly different according to Tukey's multiple comparison test with a family error rate of 0.05.