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ORIGINAL PAPER

Enhancement of flowering and branching phenotype in chrysanthemum by expression of *ipt* under the control of a 0.821 kb fragment of the *LEACO1* gene promoter

Mariya Khodakovskaya · Radomira Vaňková · Jiři Malbeck · Aizhen Li · Yi Li · Richard McAvoy

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Abstract The cytokinin biosynthesis gene, isopentenyl transferase (*ipt*), under the control of an 821 bp fragment of the *LEACO1* gene promoter (from *Lycopersicon esculentum*) was introduced into *Dendranthema* × *grandiflorium* '*Iridon*' (chrysanthemum). LEACO1$_{0.821kb}$-ipt transgenic lines grown in the vegetative state, exhibited a range of phenotypic changes including increased branching and reduced internode lengths. LEACO1$_{0.821kb}$-ipt transgenic lines grown in the generative state, exhibited increased flower bud count that ranged from 3.8- to 6.7-times the number produced by wild-type plants. Dramatic increases in flower number were associated with a delay of flower bud development and a decrease in flower bud diameter. RT-PCR analysis indicated differences in *ipt* gene expression between individual transgenic lines that exhibited a range of phenotypes. Within an individual transgenic line, RT-PCR analysis revealed changes in *ipt* gene expression at different stages of generative shoot development. Expression of *ipt* in transgenic lines correlated well with high concentrations of the sum total to bioactive cytokinins plus the glucosides and phosphate derivatives of these species, under both vegetative and generative growth conditions. In general, transgenic lines accumulated higher concentrations of both storage-form cytokinins (*O*-glucosides) and deactivated-form cytokinins (*N*-glucosides) in generative shoots of than in vegetative shoots. Based on the range of phenotypes observed in various transgenic chrysanthemum lines, we conclude that the *LEACO1*$_{0.821kb}$-*ipt* gene appears to have great potential for use in ornamental crop improvement.

Keywords *ipt* Gene · Cytokinins · Transgenic chrysanthemum · *LEACO1*-promoter · Branching · Number of flower buds

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M. Khodakovskaya (✉)
Department of Applied Science,
University of Arkansas at Little Rock,
Little Rock, AR 72204, USA
e-mail: mvkhodakovsk@ualr.edu; m_khod@yahoo.com

A. Li · Y. Li · R. McAvoy
Department of Plant Science, University of Connecticut,
Storrs, CT 06269, USA

M. Khodakovskaya
Institute of Biology and Soil Science, Far-Eastern Branch,
Russian Academy of Sciences, Vladivostok, Russian Federation

R. Vaňková · J. Malbeck
Institute of Experimental Botany, v.v.i.,
Academy of Sciences of the Czech Republic,
Prague, Czech Republic

Introduction

It is well known that growth and development of plants can be greatly affected by inducing variations in hormone concentrations. In ornamental horticulture, exogenously applied growth regulating chemicals are routinely used to enhance the aesthetic appearance of plants by increasing branching and/or reducing stem elongation. Although similar growth effects can be achieved through traditional breeding methods, molecular genetic modification (GM) can produce this phenotype by manipulating specific plant hormone levels. Thus, GM represents a tool that holds great potential for improving ornamental crops (Chandler 2003).

The GM approach to hormone manipulations offers several distinct advantages over classical exogenous-treatment techniques. First, since the hormones are produced endogenously, limitations associated with uptake and subsequent transport, are overcome. Second, by using appropriate transcriptional promoters both the timing and tissue specificity of hormone perturbation can be more precisely controlled (Hiatt 1993). One strategy for increasing endogenous cytokinin levels is based on the integration of the *ipt* gene in the plant genome. The *ipt* gene encodes the enzyme isopentenyltransferase that catalyzes the rate-limiting step in cytokinin biosynthesis (Akiyoshi et al. 1984). Non-targeted cytokinin overproduction associated with transgenic *ipt* plants results in a variety of undesirable morphological abnormalities (Ainley et al. 1993; Klee et al. 1987). In order to more precisely control cytokinin expression levels researchers fused the *ipt* gene to a variety of promoters including those inducible by heat (Ainley et al. 1993; Medford et al. 1989; Smigocki 1991) wounding (Smigocki et al. 1993), light (Thomas et al. 1995), cold (Khodakovskaya et al. 2006a), and induction of ethylene synthesis (Khodakovskaya et al. 2006b). Gan and Amasino (1995) provided an elegant example of targeted gene expression when they used a senescence-specific promoter to drive *ipt* gene expression. Here the onset of leaf senescence triggered an increase in cytokinin in the senescing organ. The upsurge in cytokinin retarded senescence that subsequently attenuated *ipt* gene expression in the senescence-retarded organs. However, even in this example plant morphological development was affected as evidenced by differences in flower production between P_{sag12}-ipt transgenic and WT tobacco (*Nicotiana tabacum*). In a more comprehensive investigation it was reported that targeted cytokinin manipulation in P_{sag12}-ipt tobacco produced both direct and indirect affects on plant metabolism that subsequently affected morphological development (Jordi et al. 2000). Here, they report that the bioactive cytokinins zeatin and zeatin-riboside increased more in senescing leaves (15×) than young leaves (2×) of P_{sag12}-ipt tobacco compared to the WT and that the only observed phenotypic difference was a 5–10% reduction in height of the transgenic lines. Flower number and lateral branching were not affected. In contrast, extensive phenotypic differences were reported when P_{sag12}-*ipt* was expressed in ornamental tobacco *Nicotiana alata* (Schroeder et al. 2001). Here, transgenic plants displayed increases in flower longevity, shoot dry weight and branching but reductions in flower number per shoot and plant height. The authors suggested P_{sag12}-*ipt* has potential value for commercial horticultural applications. However, the widely different phenotypic response of *N. tabacum* and *N. alata* to P_{sag12}-*ipt* underscores the importance to testing chimeric gene constructs in more than one species and in selecting the promoter that most effectively produces the desired phenotype in a target plant species especially when commercial applications are being considered.

In this study we use the *ipt* gene under the control of an 821 bp fragment of the *LEACO1* gene promoter. 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 ethylene biosynthesis pathway (Yang and Hoffman 1984). The *LEACO1* promoter is active in response to aging, wounding, ethylene, pathogen infection and treatment with methyljasmonate and α-amino butyric acid (Blume and Grierson 1997) and contains a number of elements associated with stress-inducible genes (the TCA motif) (Goldsbrough et al. 1993), and ethylene-response gene promoters (Itzhaki et al. 1994; Montgomery et al. 1993). In addition, Khodakovskaya et al. (2006b) demonstrated that plants transformed with $LEACO1_{0.821kb}$-*GUS* showed increased expression in the presence of auxin and decreased expression when auxin was inhibited.

Previously we transformed *N. tabacum* with the $LEACO1_{0.821kb}$-*ipt* fusion gene and characterized several interesting phenotypic changes in transgenic tobacco lines including increases in flower bud number or lateral branching (Khodakovskaya et al. 2006b). Interestingly, transgenic lines that exhibited increased flower bud counts showed normal branching (relative to the wild type) and lines with increased branching did not exhibit increased bud counts. Cytokinin concentrations were not reported.

As previous studies have demonstrated, constitutive up-regulation of plant hormones produces too many adverse side effects to be of use in commercial crop improvement. However, hormonal expression patterns tied to specific physiological events have the potential to overcome specific crop production problem such as leaf senescence (Schroeder et al. 2001). With $LEACO1_{0.821kb}$-*GUS* expression patterns indicated that this promoter responded to specific endogenous hormonal signals (Khodakovskaya et al. 2006b) and the phenotypic modifications observed in $LEACO1_{0.821kb}$-ipt tobacco could be beneficial in economically important crops. For instance, in chrysanthemum plants typically are pinched manually or treated with plant growth regulators to increase branching and flower number, and to produce the desirable compact mound-shaped form. If $LEACO1_{0.821kb}$-*ipt* chrysanthemeum would express phenotypic characteristics similar to those previously reported in tobacco, then this construct could be used to reduce the costs of labor and materials in production. However, the phenotypic response of different species (chrysanthemum vs. tobacco) to a gene construct based on a hormone responsive promoter can not be assumed and therefore must be tested.

The objective of this study was to test whether the phenotypic responses observed in *LEACO1$_{0.821kb}$-ipt* tobacco could be replicated in the commercially important species chrysanthemum (*Dendranthema* × *grandiflorun*).

We report that many LEACO1$_{0.821kb}$-ipt chrysanthemum lines exhibited dramatic increases in cytokinin concentration along with increases in the number of flower buds and/or lateral branches relative to the wild-type cultivars. However, these plants failed to develop the detrimental side effects normally associated with cytokinin over-expression. We concluded that the *LEACO1$_{0.821kb}$-ipt* fusion gene appears to be a useful tool for crop development where increased branching and flower bud number are considered desirable traits.

Materials and methods

Binary vector construction, transformation and production of transgenic chrysanthemum plants

The *LEACO1$_{0.821kb}$-ipt* construct that we have previously cloned (Khodakovskaya et al. 2006b), was used in this study. The binary plasmid (*pBin19-LEACO1$_{0.821kb}$-ipt*) was transformed into *Agrobacterium tumefaciens* strain *LBA 4404* by electroporation. The young, soft stems of chrysanthemum plants (cv. *Iridon*) were washed briefly (30 s) with 70% ethanol, rinsed three-times with sterile water, and then sterilized in 5% Clorox for 8 min before finally rinsing five-times with sterile water. The bacterial suspension was cultured in LB medium supplemented with 50 mg/l kanamycin and 25 mg/l rifampicin. The suspension was grown for 24 h at 25°C on a rotary shaker (220 rpm) until achieving an optical density of 0.4–0.7 (λ 600 nm) than centrifuged and diluted at a 3:1 ratio using fresh MS medium (Murashige and Skoog 1962). Stem segments of chrysanthemum were soaked in the infection MS medium for at least 5 min, blotted dry and kept 3 days in the dark at 22–25°C on plates with MS medium containing 0.225 mg/l of 6-BA, 2 mg/l of IAA for chrysanthemum explants. After 2–3 days explants were transferred to the respective selection media containing 50 mg/l of kanamycin (for selection) and 200 mg/l 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 of kanamycin and 100 mg/l timentin until root induction was evident. Rooted explants were transferred to a peat-based medium, Metro 510 (Scotts Co., Marysville, OH, USA), and then gradually acclimated to the greenhouse.

Plant DNA extraction and polymerase chain reaction (PCR analysis)

Total DNA was isolated from leaf tissue of greenhouse plants by DNeasy Plant Mini Kit Qiagen (Qiagen Inc., Valencia, CA, USA) and 250 ng of DNA was subjected to PCR reaction. The primers used to detect the *LEACO1-ipt* locus were (1) forward primer 5′-GTGAACCTCTCATG TATTCC-3′ and reverse primer 5′-TCTTAATTTCTTGG TAAAGTGTTT-3′, specific for 0.821 kb part of *LEACO1* promoter; (2) forward primer 5′-GGTCCAACTTGCAC AGGAAAG-3′ and reverse primer 5′-TAACAAACAACA TGGCATATC-3′, specific for the 0.525 kb region of *ipt* gene (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 54°C and 1 min 30 s at 72°C; and extension at 72°C for 5 min. The reactions involved 200 ng of DNA template, 0.2 mM of dNTPs, 20 mM of each primer, *REDTaq* PCR Buffer and 1.5 U of *REDTaq* DNA polymerase (Sigma, Saint Louis, MI, USA) in a final reaction volume of 20 µl. PCR products were observed under UV after electrophoresis on a 1% agarose gels with ethidium.

Analysis of ipt expression in leaves

Total RNA was isolated from chrysanthemum samples by grinding previously frozen tissue in a mortar with TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). For RT-PCR analysis, potential DNA contamination was removed from RNA samples by DNase treatment (DNA-free^{TM}, Ambion, Inc.), and then first-strand cDNA was synthesized from 1 µg of total RNA using First Strand Synthesis Kit RETROscript^{TM} (Ambion Inc. Austin, TX, USA) following the manufacturer's instruction. For PCR, 0.5 µL of RT-mix was used in a final volume of 25 µl. PCR reaction for fragment of the *ipt* gene was carried out as described above. As internal standards the products of PCR reaction with RT-mix and primers to 18S RNA were used (QuantumRNA^{TM} 18S Internal Standards from Ambion Inc.). PCR products (10 µl) were run on a 1% agarose gel.

Plant growth conditions and morphological analysis of transgenic phenotype

The effect of the transgene on growth and development of chrysanthemum plants was determined in a series of glasshouse and growth chamber studies. Shoots from the transgenic LEACO1$_{0.821kb}$-ipt chrysanthemum lines N1, N5 and N14 and the wild-type cultivar 'Iridon' and were rooted in deep 606-cell packs (Kord Products, Brampton, Ontario,

Canada) containing the peat-lite medium Metro 510 (Scotts Co., Marysville, Ohio, USA). Rooted cuttings were transplanted to 10-cm pots and transferred to the growth chamber (EGC model S10, EGC, Chagrin Falls, OH, USA) at 25°C day (16 h at 300 μmol/m^2 per s) and 20°C night. Plants were allowed to acclimate to the growth chamber conditions for 2-weeks and then exposed to either vegetative growth conditions or generative growth conditions. Chrysanthemum is a qualitative flowering plant, with distinct vegetative and generative growth stages that are controlled by photoperiod. Vegetative growth conditions consisted of 25°C day/20°C night temperatures and a 16-h photoperiod. Generative growth conditions consisted of 25°C day/20°C night temperatures and a 10-h photoperiod. Plants under both vegetative and generative growth conditions received a total of 10 h of photosynthetically active radiation at 300 μmol/m^2 per s during each 24-h cycle. For the vegetative treatment however, photosynthetic radiation was delivered in one 9-h block followed by four 15-min events that were distributed intermittently so that the longest continuous dark period was 8-h (note: night-interruption with a brief light period is a common commercial practice used to control flowering response in photoperiodic species). Plants were watered as needed and fertilized once per week with N at 5.3 mmol (75 mg/l) from 20 N-4.3P-16.6 K (Peter's 20-10-20, Scotts Co., Marysville OH, USA).

Using multiple growth chambers, plants were arranged in a randomized complete block array with each of the transgenic lines and the wild type represented in each replicated block. Each block was replicated six times under either vegetative or generative conditions. The experiment was repeated. In a separate chamber, ten plants from each transgenic line and the wild type were allowed to progress to flower senescence. These plants were used to determine flower longevity. After 72 days in the growth chamber vegetative plants were harvested and the following data recorded: total shoot fresh weight (g), number of lateral shoots on the main stem, length of each lateral shoot (cm), number of leaves on each lateral shoot, number of secondary shoots on each lateral, and the length of each secondary shoot. Generative plants were harvested 63 days after the beginning of short-day conditions and the number of flower buds on each lateral branch, the number of nodes on each lateral producing a generative bud, the date of initial anthesis, flower bud diameter at initial anthesis, and the date of initial flower senescence were recorded in addition to the aforementioned data. Flower longevity was determined by recording the date of anthesis and the date of senescence. In a follow up study, twelve vegetative tip cutting were harvested from selected transgenic LEAC-$O1_{-0.821kb}$-ipt mum lines and the wild-type cultivar 'Iridon' and rooted in Fafard 3B medium. Once all genetic lines were rooted, cuttings were transferred to 1.6-L pots in Fafard 3B. After a short acclimation period, plants were sorted for size and plants of equal size were placed into each of eight-replicated blocks. Each block included one plant from each genotypic line and the wild type. Plants were allowed 21-days to acclimate and establish roots in the new container and then blackout cloth was pulled daily from 6 pm to 8 a.m. to create short-day conditions and induce flower formation. Plants were fertilized at weekly intervals with 500 mg/l N from a 20 N-10 P_2O_5-20 K_2O formulation.

As individual plants reached first bloom (defined as the time when the first petal lifted off the floral disk) bud diameter and date of bloom were recorded. After all plants reached first bloom, plants were harvested and the following data collected: number of lateral branches, length of each lateral branch, number of nodes on each lateral branch, and the number of flower buds on each lateral branch. These data were used to calculate the number of days to bloom, flower bud diameter at first bloom, average shoot weight, total number of lateral branches per plant, total number of flower buds per plant, average lateral branch length, average internode length per lateral, and average number of buds per lateral branch. Overall flower diameter was recorded when blooms were fully opened. Phenotypic characteristics between genotypic lines were compared using single-factor ANOVA procedures.

Senescence of excised leaves and quantification of chlorophyll

To determine senescence characteristics of transgenic chrysanthemum lines, excised leaves from transgenic lines N1, N5 and N14 and the WT plant were surface sterilized with 0.6% sodium hypochlorite for 60 s, rinsed five times with sterile water and then placed on moist filter paper in a 10 cm Petri dish. Each Petri dish contained four excised leaves, one from each transgenic line and the non-transformed wild type. The plates were placed in continuous dark at 25°C and checked daily over a 28-day period for evidence of leaf senescence. Chlorophyll concentration was assayed prior to the start of dark conditions 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.

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 tube. 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) 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).

Cytokinin analysis

Cytokinins were extracted and purified according to the method of Dobrev and Kaminek (2002). Freeze-dried samples were homogenized with mortar and pestle in liquid nitrogen and extracted overnight with 10 ml methanol/water/formic acid (15/4/1, by vol., pH ~2.5, −20°C). To each sample, 50 pmol of each of 12 deuterium labelled standards ([2H5]Z, [2H5]ZR, [2H5]Z7G, [2H5]Z9G, [2H5]ZOG, [2H5]ZROG, [2H6]iP, [2H6]iPR, [2H6]iP7G, [2H6]iP9G, [2H5]DHZ, [2H5]DHZR; products of Apex Organics, Honiton, UK) were added. The extract was passed through 2 ml Si-C18 columns (SepPak Plus, Waters, USA) to remove interfering lipophilic substances. After organic solvent evaporation in vacuo, the aqueous residue was applied to an Oasis MCX mixed mode (cation exchange and reverse-phase) column (150 mg, Waters, USA). Adsorbed cytokinins were eluted stepwise with 5 ml of 0.35 M ammonium in water (cytokinin nucleotides) and 0.35 M ammonium in 60% methanol (v/v) (cytokinin bases, ribosides, and glucosides). The eluted fractions were evaporated in vacuo. Nucleotide samples were dephosphorylated with alkaline phosphatase (0.6 U per sample) for 1 h at 37°C. LC-MS analysis was performed using a Rheos 2000 HPLC gradient pump (Flux Instruments, Basel, Switzerland) and HIS PAL autosampler (CTC Analytics, Zwingen, Switzerland) coupled to an Ion Trap Mass Spectrometer Finnigan MAT LCQ-MSn equipped with an electrospray interface. Samples dissolved in 10% (v/v) acetonitrile (10 µl) were injected on a C18 column (Aqua 125A, 2 mm/250 mm/5 µm) and eluted with a linear gradient of B from 10 to 50% in 26 min (mobile phase: water (A), acetonitrile (B), and 0.001% (v/v) acetic acid in water (C) at a flow rate 0.2 ml/min. 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. A total of 27 different cytokinin derivatives were measured. The detection limit was calculated for each compound as 3.3 σ/S, where σ is the standard deviation of the response and S the slope of the calibration curve. For each treatment, samples were collected from each of three independent plants and each sample was injected at least twice.

Results

Establishment of transgenic plants of *Dendranthema* × *grandiflorium* harboring the *LEACO1*$_{0.821kb}$-*ipt* fragment

Transformation of chrysanthemum with the 0.821 kb *LEACO1* gene promoter-*ipt* gene (*LEACO1*$_{0.821kb}$-*ipt*) construct resulted in 52 kanamycin-resistant putative transformants. PCR analysis confirmed recombinant DNA integration into the genome of 26 individual putative-transgenic chrysanthemum lines (Supplemental Fig. S1). PCR amplification of both plasmid DNA and the genomic DNA from chrysanthemum lines produced the expected 0.821 kb fragment of the *LEACO1* promoter (Supplemental Fig. S1A) and the 0.52 kb fragment of the *ipt* gene (Supplemental Fig. S1B). No amplification of DNA was detected in non-transgenic plants. Southern blot analysis of chrysanthemum genomic DNA revealed the integration of the *ipt* gene into the genome of all primary transformants, while no signal was detected in wild-type control plants (data not shown). Selected PCR positive chrysanthemum lines were used for molecular and cytokinin analysis and for phenotype characterization. Copies of individual transgenic lines were generated by asexual propagation (e.g. rooting of vegetative shoot-tip cuttings).

Morphological and molecular analysis of transgenic chrysanthemum plants expressing the *LEACO1*$_{0.821k}$-*ipt* fragment

Branching and flowering characteristics in transgenic LEACO1$_{0.821kb}$-ipt chrysanthemum plants differed markedly from the wild type. Overall transgenic lines displayed a range of phenotypic characteristics (Fig. 1; Table 1). Some lines showed dramatically increased branching and flower bud counts while others exhibited a more moderate increase in branching and flower bud count. Additionally several PCR positive transgenic lines (such as line N3) displayed a phenotype similar to the wild type.

Reverse transcriptional-PCR (RT-PCR) analysis was used to assess the level of *ipt* expression in transgenic lines (Fig. 1b) that represented the range of phenotypes observed (Fig. 1a). Total RNAs were extracted from leaves of wild-type and transgenic lines N1, N3, N5, N14 at the same growth stage. All plants were grown under the same conditions. RT-PCR analysis showed that in general increased branching and flower bud counts corresponded with the level of *ipt* expression. For instance, both transgenic line N3 and the wild type showed no evidence of *ipt* expression and both groups of plants displayed similar branching and flowering characteristics. In contrast, transgenic lines N1 and N14 exhibited high *ipt* expression levels and increases in lateral branching and flower bud counts. By comparison, transgenic line N5 exhibited a lower *ipt* expression level than transgenic lines N1 and N14, and a more moderate branching and flowering phenotype.

Under generative growth conditions in the greenhouse shoot morphology and flowering response of WT plants differed greatly from the transgenic lines N1, N5, and N14 (Table 1; Fig. 1c). In addition, differences were also

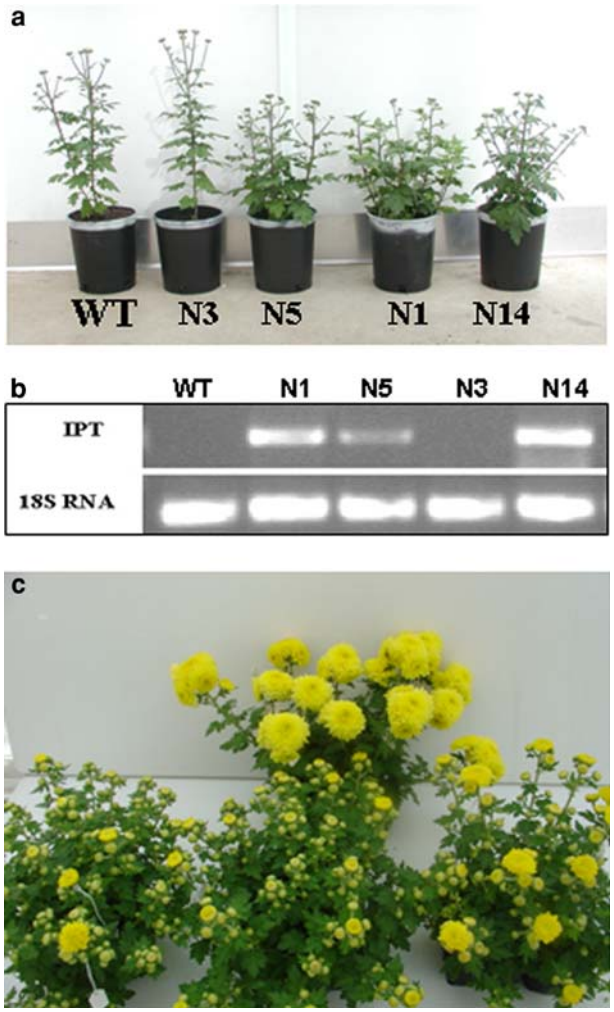

Fig. 1 Wild-type and LEACO1$_{0.821kb}$-ipt transgenic plant lines in the generative stage of development. **a** Branching of wild type and LEACO1$_{0.821kb}$-ipt transgenic lines N1, N3, N5, and N14 at mature bud stage. **b** Expression of the *ipt* gene (RT-PCR) of transgenic and wild-type plants. *Lanes 1* Wild type, *lines 2–4* transgenic lines N1, N5, N3 and N14 expressing the *ipt* gene under control of a 0.821 kb of fragment of the *LEACO1* promoter. **c** Generative phenotype of wild-type (back) and selected transgenic chrysanthemum lines at the open bud stage show a range of flowering and branching phenotypes when grown under glasshouse conditions

observed between transgenic lines. Specifically, wild-type plants produced greater shoot fresh weights than transgenic plants ($P \leq 0.01$) but transgenic lines produced more lateral branches ($P \leq 0.001$). For example wild-type plants averaged 2.9 laterals per plant but the LEACO1$_{0.821kb}$-ipt lines N1, N5 and N14 averaged 5.5, 4.1 and 5.8 lateral branches per plant, respectively. In addition, these same transgenic lines produced shorter lateral shoots ($P \leq 0.001$), shorter internodes ($P \leq 0.001$), and dramatically higher total bud counts ($P \leq 0.001$) compared to wild-type plants (Table 1). The WT averaged 23.1 buds per plant but lines N1, N5 and N15 averaged 154.6, 88 and 148.5 buds per plant, respectively. The number of nodes

Table 1 Flower response in wild-type chrysanthemum (cv. Iridon) and transgenic lines containing the *LEACO1$_{0.821kb}$-ipt* construction

Construct	Line	Shoot fresh weight (g)	Lateral shoots per plant (#)	Total buds per plant (#)	Average lateral length (cm)	Average nodes per lateral (#)	Average internode length per lateral (mm)	Length of apical lateral (cm)	Number of nodes on apical lateral (#)	Internode length on apical lateral (mm)	Number of buds per apical lateral (#)
cv. Iridon	WT	87.0 ± 4.9*	2.9 ± 0.1	23.1 ± 1.5	23.6 ± 0.3	17.9 ± 0.5	13.3 ± 0.3	23.2 ± 0.5	18.5 ± 0.5	12.6 ± 0.3	9.6 ± 0.9
LEACO1$_{0.821}$-IPT	N1	71.8 ± 4.2	5.5 ± 0.3	154.6 ± 7.7	18.6 ± 0.9	19.5 ± 0.4	9.5 ± 0.3	20.7 ± 0.6	20.4 ± 0.5	10.2 ± 0.2	46.0 ± 2.0
LEACO1$_{0.821}$-IPT	N5	67.0 ± 2.6	4.1 ± 0.4	88.0 ± 5.3	20.5 ± 0.9	18.1 ± 0.4	11.3 ± 0.3	22.7 ± 0.5	19.3 ± 0.5	11.8 ± 0.3	32.9 ± 2.0
LEACO1$_{0.821}$-IPT	N14	62.6 ± 5.3	5.8 ± 0.2	148.5 ± 9.4	18.8 ± 0.9	17.8 ± 0.3	10.5 ± 0.4	19.6 ± 0.5	18.9 ± 0.5	10.4 ± 0.2	43.3 ± 4.1
		Statistical effects									
		$P \leq 0.01$	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.05$	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.05$	$P \leq 0.001$	$P \leq 0.001$

* ±SE, Standard error

per lateral was similar for the WT and transgenic lines N5 and N14 but higher in transgenic line N1 ($P \leq 0.05$). The growth and flowering response observed in the apical lateral branch of the WT and transgenic lines N1, N5 and N14 was similar to that of the entire shoot. Note: the apical-most lateral branch receives the most light and tends to be the most dominant on the plant. Here the WT lateral was longer than N1 and N14 but similar to N5. The number of nodes of the WT lateral was similar to N5 and N14 but less than N1. The WT had longer internodes than any of the transgenic lines and transgenic line N5 had longer internodes than both N1 and N14. The most dramatic difference was observed in the number of flower buds on the apical lateral branch. While the WT averaged only 9.6 buds per lateral, N5 average 32.9 and both N1 and N14 produced more than either the WT or N5 lines at 46 and 43.3, respectively.

From plants grown under generative conditions in the growth chamber, increased bud counts on transgenic plants were associated with reduced bud size (Fig. 2a) and delayed bud development (Fig. 2b). Transgenic lines that produced dramatically higher bud counts than the wild type had a longer interval from flower initiation to anthesis compare to the wild type however, flower longevity for transgenic lines was similar to the wild type (Fig. 2b). Buds of transgenic lines were otherwise normal.

Leaf senescence in LEACO1$_{0.821kb}$-ipt transgenic chrysanthemum

We found that senescence of detached chrysanthemum leaves differed markedly between LEACO1$_{0.821kb}$-ipt and wild-type plants when plated on wet filter paper and held in the dark at 25°C (Fig. 2c). Quantitative analysis revealed that leaf chlorophyll concentration in transgenic lines and wild-type plants were similar under normal greenhouse condition (e.g. time 0 in Fig. 2c). After 14 days of dark storage the chlorophyll concentration declined by 90% in wild-type leaves, while detached leaves of lines N1, N5, and N14 remained green and appeared healthy. After 20 days of dark storage chlorophyll concentration declined to 67% of the initial level in line N1 but only to 30% and 44% of the initial level in lines N5 and N14, respectively.

Changes in endogenous cytokinin concentrations in transgenic chrysanthemum lines

Analysis of endogenous cytokinins in freeze-dried chrysanthemum shoot tips revealed differences in bioactive cytokinins, storage-form cytokinins (*O*-glucosides), deactivated-form cytokinins (*N*-glucosides), active cytokinin phosphates, and total *cis*-Zeatin derivatives between the LEACO1$_{0.821kb}$-ipt and WT lines tested (Table 2). On

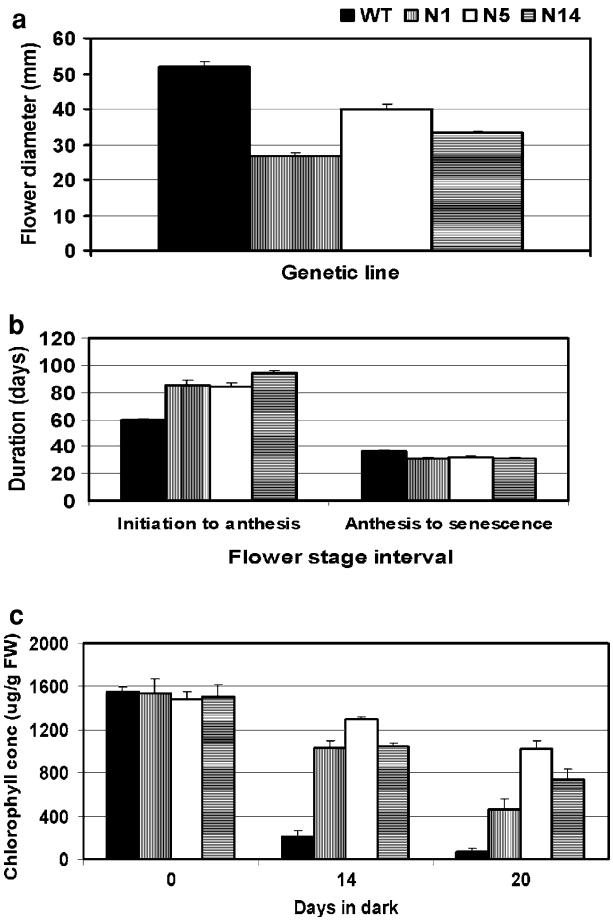

Fig. 2 Physiological traits associated with wild-type (WT) and LEACO1$_{0821kb}$-ipt transgenic chrysanthemum lines. **a** Diameter of fully open blooms of WT and LEACO1$_{0.821kb}$-ipt lines (N1, N5, and N14). **b** Time intervals for bud development stages for WT and LEACO1$_{0.821kb}$-ipt transgenic lines (N1, N5, and N14). Bud development stages include; bud initiation to anthesis and anthesis to senescence. **c** Chlorophyll concentrations in leaves of WT and LEACO1$_{0.821kb}$- ipt chrysanthemum lines (N1, N5 and N14) during dark incubation. Chlorophyll concentrations were measured in leaves from glasshouse grown plants and then again after 14 and 20 days of dark storage. Each value represents the mean of ten observations. *Error bars* represent standard error of the mean

average, these cytokinin pools were higher in LEACO1$_{0.821kb}$-ipt lines than the WT. Differences in these same cytokinin pools, with the exception of the bioactive forms, were observed between plants in the vegetative stage of development and those in the generative growth stage (Table 2). Here, cytokinin concentrations were higher in gerenative plants than in vegetative plants.

Under generative conditions bioactive cytokinins, *N*-glucosides, and total active cytokinins (including the associated glucosides and phosphates) were higher in N5 than the other lines ($P \leq 0.05$). Under vegetative conditions the active cytokinin phosphate pool was higher in N5 and the total active cytokinin pool (including associated

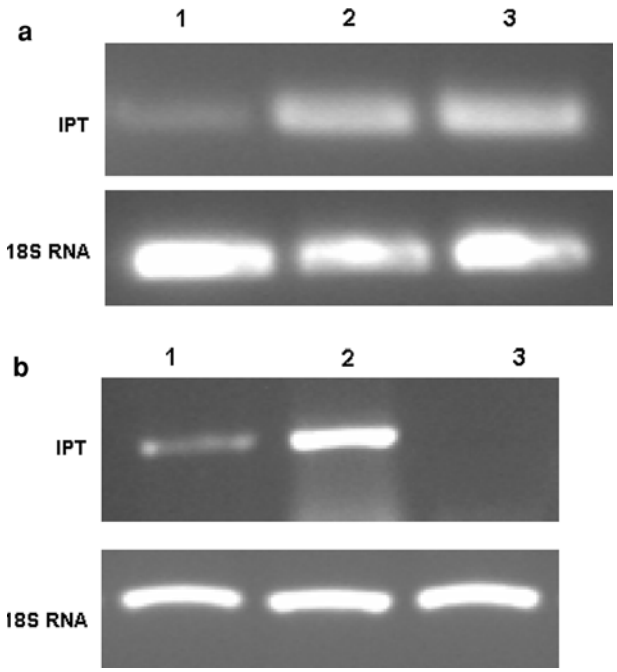

Fig. 3 Expression of *ipt* in LEACO1$_{0.821kb}$-ipt chrysanthemum is dependent on grown stage (vegetative or generative) (**a**) and the presence of auxin (**b**). **a***ipt* expression in LEACO1$_{0.821kb}$-ipt plant (line N5) in vegetative stage (*lane 1*), in young flower bud (5 mm) stage (*lane 2*), or in mature flower bud (12 mm) stage (*lane 3*). **b** RT-PCR analysis of *ipt* gene expression in LEACO1$_{0.821kb}$-ipt plants with the apical shoot intact (*lane 1*), or the apical shoot excised and 0.01% IAA applied to the cut apex (*lane 2*), or the apical shoot excised and no auxin applied (*lane 3*)

glucosides and phosphates) were higher in N5 than in WT but not in N1 and N14.

In general, cytokinin sample-to-sample variance was high for all plant material tested and thus large differences were required to statistically distinguish between individual genetic lines. However by pooling the data for the N1, N5 and N14 transgenic lines, and comparing it to the cytokinin profiles observed from the WT samples, clear trends emerge. Here bioactive cytokinins (87.7 vs. 31.4 pmol/g dry tissue), deactivated cytokinins (149.7 vs. 29.9 pmol/g dry tissue), and total bioactive cytokinins plus glucosides and phosphates (300.6 vs. 124.8 pmol/g dry tissue) were all higher ($P \leq 0.05$) in transgenic shoots than WT shoots (respectively) when grown under generative conditions. Under these same conditions only the active cytokinin phosphate pool was lower ($P \leq 0.05$) in transgenic shoots than WT shoots (15 vs. 32.2 pmol/g dry tissue, respectively). For plants in the vegetative growth stage, bioactive cytokinins (79 vs. 33.1 pmol/g dry tissue), storage-form cytokinin *O*-glucosides (31.3 vs. 10.7 pmol/g dry tissue), deactivated *N*-glucoside cytokinins (23.6 vs. 5.9 pmol/g dry tissue), and total bioactive cytokinins plus the associated glucosides and phosphates (142 vs. 55.6 pmol/g dry tissue) were higher ($P \leq 0.05$) in transgenic shoots than WT shoots, respectively.

Changes in ipt expression in generative LEACO1$_{0.821kb}$-ipt transgenic tissues

RT-PCR analysis was conducted to further characterize changes in *ipt* expression in generative LEACO1$_{0.821kb}$-ipt transgenic tissue (Fig. 3a). Here *ipt* expression was monitored in vegetative shoots as well as in young developing flower buds (5 mm) and more mature buds (12 mm in diameter) of the transgenic line N5. Expression of the *ipt* gene showed a marked increase in flower bud tissue compared to vegetative shoot tissue. RT-PCR analysis showed *ipt* gene expression in LEACO1$_{0.821kb}$-ipt plants with the apical shoot intact and even higher expression in shoots with the apical bud excised and 0.01% IAA applied to the excised tip. No *ipt* expression was detected when the apical bud was excised and no exogenous auxin applied (Fig. 3b).

Discussion

LEACO1$_{0.821kb}$-ipt chrysanthemum plants displayed a range of potentially useful traits including increased lateral branching, reduced internode lengths, increased bud counts and delayed leaf senescence. Such morphological characteristics dramatically affect aesthetic appearance and plant form is very important in the ornamental industry. In agronomic species such traits bear a direct relationship to yield potential and are of economic interest. The phenotypic variation observed in LEACO1$_{0.821kb}$-ipt plants suggests that variations in the time and/or level of cytokinin biosynthesis affected shoot morphology in such a way as to alter plant form. For example, high *ipt* expression levels during the early stages of flower bud induction were detected in transgenic lines N1 and N14 which displayed the most highly branched phenotype (Fig. 1a, b). Further, cytokinin concentration was highest during the later stages of bud development in line N5 which displayed only a modest increase in branching during the early stages of shoot development but a high flower bud number and large final flower bud diameters (Fig. 2a).

The desired balance between vegetative and generative development depends upon the intended commercial use of the crop and this balance can be manipulated with exogenous cytokinin. For example, in *Hatiora gaetneri* flower bud number more than doubled in response to a cytokinin spray (Boyle 1995; Boyle et al. 1988). In glasshouse grown *Glycine max*, exogenous cytokinin stimulated increased seed production (Nagel et al. 2001). In *Pisum sativum*, release of lateral buds from apical dominance, as well as

Table 2 Cytokinin concentrations in wild-type and transgenic LEACO1$_{0.821kb}$-ipt chrysanthemum plants (lines, 1, 5, 14)

Genetic line	Cytokinin pool					
	Bioactive cytokinins [Concentration (pmol/g of freeze dry tissue) ±SE]	Storage forms: active cytokinin *O*-glucosides [Concentration (pmol/g of freeze dry tissue) ±SE]	Deactivated forms: active cytokinin *N*-glucosides [Concentration (pmol/g of freeze dry tissue) ±SE]	Active cytokinin phosphates [Concentration (pmol/g of freeze dry tissue) ±SE]	Total *cis*-Z derivatives (active, storage, deactivated and phosphates) [Concentration (pmol/g of freeze dry tissue) ±SE]	Total bioactive cytokinins, their glucosides and phosphates [Concentration (pmol/g of freeze dry tissue) ±SE]
Vegetative stage of development						
WT	33.1 ± 4.0	10.7 ± 3.1	5.9 ± 2.0	5.9 ± 0.3	860 ± 99	55.6 ± 7.0
N1	86.0 ± 23.1	30.7 ± 10.3	18.6 ± 1.7	4.8 ± 3.8	682 ± 48	140 ± 25.3
N5	106.8 ± 29.9	42.9 ± 21.8	33.3 ± 19.9	17.8 ± 6.3	581 ± 146	200.8 ± 65.2
N14	44.1 ± 13.8	20.4 ± 6.5	19.2 ± 5.2	1.4 ± 1.5	626 ± 19	85.1 ± 24.9
Generative stage of development						
WT	31.4 ± 13.3	31.2 ± 2.6	29.9 ± 7.8	32.3 ± 4.4	1244 ± 156	124.8 ± 15.7
N1	46.5 ± 4.2	26.2 ± 7.1	68.8 ± 21.2	6.9 ± 5.6	562 ± 115	147.8 ± 23.8
N5	138.5 ± 16.4	68.7 ± 22.9	304.9 ± 102.5	28.7 ± 1.4	1264 ± 62	540.9 ± 114
N14	78.1 ± 20.9	49.9 ± 13.8	75.8 ± 21.1	9.4 ± 8.3	931 ± 154	213.2 ± 59.4
Statistical summary: ns = nonsignificant, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$						
Genetic line	****	*	***	***	***	****
Growth stage	ns	*	***	***	***	***
Interaction	ns ($P = 0.07$)	ns	**	*	**	**

Cytokinin pool:

Bioactive cytokinin species: Z, Z9R, DHZ, Z9R, DHZ9R, iP, iP9R

Storage cytokinin species: ZOG, DHZOG, Z9ROG, DHZ9ROG

Deactivated cytokinin species: Z7G, DHZ7G-1, DHZ7G-2, Z9G, DHZ9G, iP7G, iP9G

Cytokinin phosphates species: ZRP, iPRP, DHZRP

Total *cis*-Z derivatives: c-Z, c-Z9R, c-ZOG, c-Z9ROG, c-Z7G, c-Z9G, c-ZRP

increased IAA synthesis and export, were induced by cytokinin (Li and Bangerth 2003). The efficacy of exogenous applications is limited however, because flowers and leaves do not readily absorb cytokinins and topically applied cytokinins are relatively immobile in the plant (Hobbie et al. 1994). Our data demonstrate that by integrating the *ipt* gene into the plant genome, endogenous levels of cytokinins can be modulated to induce similar growth responses.

Here, we demonstrate a method to dramatically increase both branching and flower bud number in chrysanthemum by regulating *ipt* gene expression with an 821 bp fragment of the ACC oxidase gene promoter from tomato (*LEACO1*). Since ACC oxidase catalyzes the last step of ethylene biosynthesis, this promoter is activated under conditions where ethylene biosynthesis is stimulated (Blume and Grierson 1997).

In transgenic tobacco both endogenous and exogenous auxin stimulated $LEACO1_{0.821kb}$-promoter activity (Khodakovskaya et al. 2006b). In $LEACO1_{0.821kb}$-ipt chrysanthemum plants, we found that *ipt* gene expression was inhibited by the removal of the endogenous auxin source (the shoot apex) and *ipt* gene expression was restored when exogenous IAA was applied to the excised shoot tip (Fig. 3b). The 821 bp fragment of the LEACO1 promoter that we used to drive *ipt* expression contains multiple copies of the auxin responsive element (AuxRE) required TGTCTC-sequence [or the inverse GAGACA-sequence, or the TGTCTt sequence with one substitution in position six (substituted bases in lower case)] found in genes regulated by auxin response factors (Ulmasov et al. 1995). This fragment also contains the G-box motif (CtCGTG G-Box motif with one substitution in the two position) and multiple copies of the ACGT G-box core element (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).

Elevated concentrations of both auxin and cytokinin have been associated with generative development in species such as *Dendrobium* (Campos and Kerbauy 2004) but similar observations have not been reported for chrysanthemum. Here, we report that total active cytokinins including phosphates and *O*-glucosides, increased from 55.6 pmol/g dry tissue in vegetative shoots to 124.8 pmol/g dry tissue in generative shoots from the wild-type chrysanthemum, and from 142 pmol/g dry tissue in vegetative shoots to 300.6 pmol/g dry tissue in generative shoots from the transgenic chrysanthemum lines.

Delayed senescence was observed in excised transgenic chrysanthemum leaves but not in wild-type leaves (Fig. 2c). Blume and Grierson (1997) reported *LEACO1*-promoted *GUS* expression increased in plants exposed to ethylene, and that increased GUS expression was observed even when shorter fragments of the *LEACO1* gene promoter (396 or 1825 bp) were used. The 1825 bp fragment contained a number of short motifs involved in regulating expression of many genes that respond during senescence (Goldsbrough et al. 1993) or upon exposure to ethylene (Itzhaki et al. 1994; Montgomery et al. 1993). Delayed senescence is one of the earliest reported plant responses to cytokinin. This trait appears to have great potential for horticultural application and has been observed in many *ipt* transgenic species when various promoters were used (Chang et al. 2003; Gan and Amasino 1995, 1997; Khodakovskaya et al. 2005; McCabe et al. 2001; Schroeder et al. 2001).

Since a variety of hormonal signals are always present in plants, it is not surprising that a range of phenotypes was observed in $LEACO1_{0.821kb}$-ipt transgenic chrysanthemum lines grown under both generative and vegetative conditions. A comparison of the large increase in flower bud number observed in transgenic chrysanthemum lines with the 50% reduction reported in P_{sag12}-*iptN. alata* (Schroeder et al. 2001) illustrates how expression of the same gene under the control of different promoters can affect plant development differently. This also reinforces the importance of selecting a promoter most suited for each individual application.

Although cytokinin concentrations varied between $LEACO1_{0.821}$-ipt chrysanthemum lines (Table 2), overall concentrations of bioactive and deactivated cytokinin forms were higher in transgenic lines than in the wild type. Khodakovskaya et al. (2006a) reported a similar response in chrysanthemum expressing *ipt* under the control of a cold-inducible promoter. Furthermore, RT-PCR analysis of $LEACO1_{0.821}$-ipt plants demonstrated that both branching and flower response correlated with *ipt* expression level. However, in transgenic lines increases in flower bud counts were accompanied by delayed bud development and a decrease in bud size. The inverse correlation between flower (or fruit) number and flower (or fruit) size is a well-recognized phenomenon in horticulture that results when an increased number of carbohydrate-sinks compete for limited photosynthetic resources. For example, a common practice in commercial chrysanthemum culture involves the manual removal of lateral flower buds to increase the size of the remaining apical bud. A similar phenomenon was reported when P_{sag12}-*ipt* was expressed in *N. tabacum* (Jordi et al. 2000).

Under generative conditions, increased cytokinin content in transgenic chrysanthemum lines could result from elevated auxin content in the developing floral meristem, or other endogenous factors capable of stimulating *ipt* activity. Increased expression of several *AtIPT* genes (*AtIPT1*,

4, *6* and *8*) during various stages of flower development (Miyawaki et al. 2004), as well as elevated cytokinin concentration in the wild-type chrysanthemum under generative conditions suggest that chysanthemum *IPTs* might contribute to the observed effect on the cytokinin pool. In *Arabidopsis*, tobacco and *Sinapis alba* elevated cytokinin content in phloem and apical meristem was reported after flower induction by a single long day (Corbesier et al. 2003; Dewitte et al. 1999; Jacqmard et al. 2002; Lejeune et al. 1994). Nevertheless, changes in flowering response in transgenic chrysanthemum indicate considerable impact from the *LEACO1*$_{0.821kb}$*-ipt* transcript.

The effects of *ipt* expression (driven by the *LEACO1*$_{0.821kb}$ promoter) on chrysanthemum morphology and endogenous cytokinin content illustrates the complexity of hormone interactions in plants but also suggests some of the potential uses for this specific construct in commercial plant development especially when increased lateral branching and flower bud counts are desirable.

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