Table 3. Final substrate pH and electrical conductivity determined on extracted solutions on samples taken from Experiment One and Two. Samples were taken using a saturated media extract method. In Experiment One, New Guinea impatiens' 'Anaea' were grown with 3.3, 6.6, or 9.9 g of 13N-5.7P-10.8K Nutricote incorporated per 1.5-liter pot. In Experiment Two, New Guinea impatiens 'Anaea' were grown with 6.6 or 13.2 g of 13N-5.7P-10.8K Nutricote top-dressed at transplanting (AT) or 1 week (+1WAT) or 2 weeks (+2WAT) after transplanting. All values are means of five replications.

Fertilizer	Time of application	pH	Electrical conductivity (dS/m)
	Experiment One		
3.3		7.18	0.76
6.6		7.07	0.96
9.9		6.63	1.62
Fertilizer		NS	0.0001
Linear		NS	0.0001
Quadratic		NS	NS
	Experiment Two		
6.6	AT	6.86	0.40
6.6	+1WAT	7.00	0.62
6.6	+2WAT	7.34	0.54
13.2	AT	6.27	1.45
13.2	+1WAT	6.08	1.22
13.2	+2WAT	5.67	1.27
Fertilizer		NS	0.0001
Time		NS	NS
Linear		NS	NS
Fertilizer* Time		NS	NS

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PREVENTION OF LEAF SENESCENCE IN PETUNIA VIA GENETIC TRANSFORMATION WITH SAG-*IPT*

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Abstract. **Leaf senescence leads to a decline in the aesthetic value of ornamental plants, and limits their salability. Both exogenous and endogenous cytokinins have been shown to delay leaf senescence in plants. Using a promoter from a senescence-associated gene (SAG 12) fused to the isopentenyl transferase (*IPT*) gene, which catalyzes the rate-limiting step of cytokinin production, we were able to produce transgenic petunia plants with delayed leaf senescence. Due to temporal and spatial regulation of SAG 12-*IPT*, transformed plants showed only slight adverse side effects in terms of their horticultural performance due to the presence of the transgene.**

Introduction

Senescence is the final developmental stage in the life of a leaf. It is characterized by the degradation of proteins, nucleic acids and other macromolecules and a mobilization of the metabolites to growing cells (Buchanan-Wollaston, 1997; Guarente et al., 1998). Studies with *Arabidopsis* have shown that senescence processes are highly regulated and are dependent upon *de novo* synthesis of many senescence-associated genes (SAGs). These SAGs encode the enzymes responsible for the metabolism and mobilization of the senescing leaf cell's contents (Buchanan-Wollaston, 1997; Hensel et al., 1993; Weaver et al., 1998). Although leaf senescence can be triggered by many different environmental stim-

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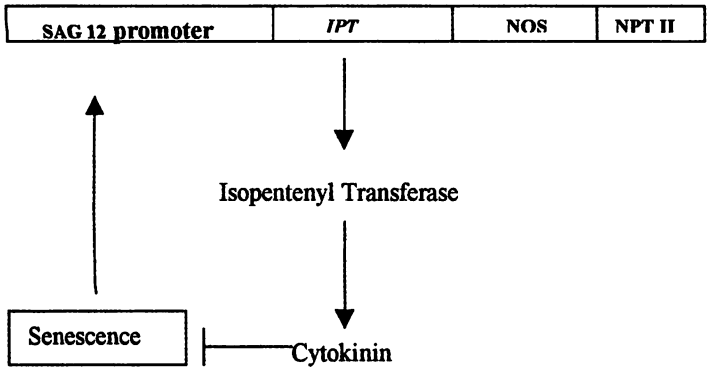

Figure 1. SAG 12-IPT construct. At the onset of senescence, the SAG 12 promoter drives expression if IPT, which results in increased cytokinin production that inhibits senescence (Gan and Amasino, 1995).

uli, such as leaf age, drought stress, nutrient limitations, and hormone concentrations, the SAGs do not respond to these cues in the same manner. Different SAGs can be regulated at different times in response to the various senescence-inducing events (Buchanan-Wollaston, 1997; Park et al., 1998; Weaver et al., 1998). Of all the SAGs, SAG 12 is thought to have the best specificity to natural senescence. It shows no detectable expression in young leaves and does not accumulated to an appreciable level until the leaves become about 20% senescent (Weaver et al., 1998).

The characteristic "yellowing" or chlorosis of the senescent leaves of an ornamental plant can often decrease its aesthetic appearance and diminish its economic value. For this reason, researchers have focused on ways to stop or delay the onset of leaf senescence. One way that this has been accomplished has been through the use of cytokinins. Cytokinins are an important class of plant hormones that influence almost all aspects of plant growth and development. They have been shown to stop and, in some cases, reverse leaf senescence processes (Gan and Amasino, 1996; Kaminek et al., 1997; Kende and Zeevaart, 1997). Research has shown a reverse correlation between cytokinin levels and senescence, which can be dramatically illustrated by the formation of green islands in areas of infection by pathogens that produce cytokinins, such as powdery mildew (*Erysiphe graminis* D.C.) (Gan and Amasino, 1996). The limitations to the use of cytokinins to delay leaf senescence become a question of delivery of the hormone to the site where it is needed when it is needed. Exogenously applied cytokinins may not enter the cells and can be quickly conjugated or metabolized into non-active forms (Gan and Amasino, 1996; Kaminek et al., 1997). Scientists have recently turned to metabolic engineering in order to circumvent the problems associated with exogenous applications.

Isopentenyl transferase (*IPT*) is encoded on the Ti (tumor-inducing) plasmid of *Agrobacterium tumefaciens* (E.F. Sm. & Town.) Conn. It catalyses the transfer of an isopentenyl group from Δ^2-isopentenyl pyrophosphate to adenosine-5'-phosphate (AMP) to form isopentenyladenine ribotide, which is then quickly converted to different cytokinins. This is thought to be the rate limiting step in the cytokinin biosynthetic pathway since expression of this one gene can cause an overproduction of cytokinins (Gan and Amasino 1995; Gan and Amasino, 1996). Attempts have been made to use this gene to increase cytokinin production and stop senescence. The results of these studies were often complicated. Studies that used constitutive promoters (e.g. CaMv-35S) produced abnormally growing plants and studies that used inducible promoters (e.g. heat shock promoters, Cu^{2+}-inducible promoter) could not rule out the possibility that the inducing treatment (e.g. high temperature) was causing the phenotypic effects (Buchanan-Wollaston, 1997; Gan and Amasino, 1995; McKenzie et al., 1998).

In an attempt to overcome the problems of past transgenic work, Gan and Amasino (1995) developed a construct that used the highly specific SAG 12 promoter to drive the expression of *IPT*. The SAG 12 promoter gave three important features: *IPT* was spatially regulated, temporally regulated, and quantitatively regulated (Gan and Amasino 1995; Gan and Amasino, 1996). In other words, cytokinin production was limited to senescing leaves, and when hormone levels rose high enough to inhibit senescence, the gene was turned off (Fig. 1). The SAG 12-*IPT* construct was used to transform tobacco (*Nicotiana Tabacum* L. cv. Wisconsin). Transformed plants displayed a wild type growth and development except that leaf senescence was inhibited and there was a significant increase in the flower number and seed yield (Gan and Amasino, 1995).

In order to demonstrate the benefits of this biotechnology and the use of genetic engineering to the floriculture industry, we transformed petunia ('V26') with the SAG 12-*IPT* construct. Transformed plants were evaluated for a delayed senescence phenotype and other horticultural performance characteristics such as numbers of flowers produced and adventitious root formation.

Materials and Methods

Inbreed 'V26' petunias were transformed with the SAG 12-*IPT* construct (Fig. 1) using the protocol of Jorgensen et al. (1996). Transformants were selected on the basis of the ability to grow on kanamycin and the resulting plants were deemed T_0. T_0 plants were grown in Fafard 2 mix (Fafard Co., Apopka, Fla.) in the greenhouse at a day/night regime of 22°C/16°C in 5.2-liter pots, and fertilized with 300 ppm Peters 15N-0.22P-0.12K (Scotts Inc., Marysville, Ohio) weekly. T_0 plants were self-pollinated to produce the T_1 generation. T_1 and 'V26' control seeds were germinated in Farad 2 under intermittent mist with a day/night temperature of 22°C/16°C. After 2 weeks, the seedlings were taken out of mist and placed in a greenhouse and given weekly fertilization with 300 ppm 15N-0.22P-0.12K (Peters) for 1 month. Seedlings were transplanted to 1.5-liter pots in Fafard 2 and weekly fertigation was continued until flowering. Of the 16 separate T_0 transformation events that were recovered, two T_1 families (I98-3 and I98-14) were selected based on a demonstrated phenotype of delayed leaf senescence. These plants were used in subsequent studies to determine horticultural performance.

T_1 plants were confirmed as positive (containing the SAG 12-*IPT* construct) or negative through the use of polymerase chain reaction (PCR). Genomic DNA was extracted from plants using the method of Doyle and Doyle (1990). One hundred nanograms of DNA was used for a PCR reaction using primers for the NPT II gene (kanamycin resistance) that is linked to the SAG 12-*IPT* construct (Fig. 1). PCR was performed using a kit from Life Technologies Inc. (Gaithersburg, Md.) under the recommended conditions. Reactions were electrophoresed on a 1.2% agarose gel and the presence (positive) or absence (negative) of the 750bp NPT II band was noted.

To determine the effect of the SAG 12-*IPT* gene on adventitious root formation, vegetative cuttings were taken from 6-week-old positive and 'V26' control plants just prior to flowering. Cuttings were then randomly stuck in 6-packs (60ml/cell) containing perlite: Fafard 2 (2:1, by volume) and then placed under intermittent mist of 5 sec/20 min. After 7 days, the mist was reset to 5-sec/30 min. Seven days later (14 days total), the cuttings were rated on a 0 to 5 scale for amount of roots. The rates were designated as follows: 0-no roots, 1-less than 3 roots, 2- between 4 and 10 roots, 3- between 11 and 20 roots, 4 - between 21 to 30 roots, and 5- greater than 30 roots.

For leaf senescence experiments, T_1 plants and 'V26' wild type plants were arranged in a randomized complete block in the greenhouse and grown with 22°C/16°C day/night temperatures until flowering. At a marketable stage, the plants were allowed to wilt for 24 hours. After wilting, the plants were fertigated again with 300 ppm 15N-0.22P-0.12K and allowed to recover. Five days later, a leaf-yellowing rate was taken on a 0 to 5 scale. The ratings were assigned as follows: 0–no visible sign of chlorosis, 1–slight lightening but no yellowing of the lower leaves, 2–visible chlorosis on at least 2 leaves, 3–visible chlorosis on 3 to 4 leaves, 4–visible chlorosis on five to 6 leaves and 5–visible chlorosis on more than 7 leaves. Fourteen days after recovery, the plants were allowed to wilt again and the same rating scheme was applied. The total number of open flowers and total number of branches were also recorded after the second wilt at the same time the leaf rating was taken.

Results and Discussion

In order to delay senescence via the SAG 12-*IPT* construct, 16 independent transformed lines of 'V26' petunias were generated. Of these 16 lines, two were selected based on a delayed leaf senescence phenotype. These two lines (designated I98-3 and I98-14) were self-pollinated and the resulting T_1 generation was used to study the horticultural performance of the SAG 12- *IPT* plants.

The ability to vegetatively propagate ornamental plants is of key importance to their commercial success. Previous studies with transgenic plants have shown that over-expression of cytokinins can lead to a reduction of root initiation and growth (Gan and Amasino, 1996; Mckenzie et al., 1998). Weaver et al. (1998) has shown that detached leaves of *Arabidopsis* plants slightly accumulated SAG 12 mRNA after 2 days in the light. To determine if the SAG 12-*IPT* construct would effect rooting, vegetative cuttings of wild type and transgenic SAG 12-*IPT* were rooted as described. The 'V26' control cuttings had more roots on average than did the SAG 12-*IPT* cuttings (Table 1). The injury incurred by taking vegetative cuttings likely induced premature senescence and in turn may have induced the SAG 12-*IPT* construct. The resultant cytokinin production may have slightly suppressed root initiation, but the levels may not have been high enough to completely inhibit the process.

Drought stress is known to induce leaf senescence in plants (Weaver et al., 1998). Floriculture crops have been known to become drought stressed during all aspects of production, retail, and in the care of the consumer. For these reasons, we decided to determine if drought stress could be used as an inducer of leaf senescence. Wild type and transgenic SAG 12-*IPT* plants were allowed to wilt and were observed for the onset of leaf chlorosis and senescence. After a 14-day recover period, the plants were wilted again and observed for leaf chlorosis and senescence. In transformed plants, visible leaf chlorosis was greatly reduced in the plants containing the SAG 12-*IPT* gene, as compared to the 'V26' (Table 1) in both wilts. This is consistent with the results obtained from Gan and Amasino (1995) with transgenic tobacco, which showed a significant delay in the onset of leaf senescence, via transformation with SAG 12-*IPT*.

Table 1. Effects of SAG 12-*IPT* on the horticultural performance characteristics of 'V26' petunias.

Cultivar	Root rating[1]	Leaf chlorosis rating[2]	Branch number[3]	Flower number[3]
'V26'	3.72 ± 0.09	1.86 ± 0.17	8.85 ± 0.36	10.38 ± 0.76
I98-3	2.5 ± 0.13	0.05 ± 0.05	13.7 ± 0.84	18.1 ± 1.84
I98-14	3.33 ± 0.14	0.13 ± 0.09	9.83 ± 0.95	12.0 ± 1.13

[1]Vegetative cuttings of 6-week-old 'V26' wild type and SAG 12-*IPT* were placed under intermittent mist for 14 days. Ratings were given as described in materials and methods. Higher ratings indicate better rooting. Values are means ± standard errors.

[2]Wild type 'V26' and SAG 12-*IPT* plants were wilted for 24 hours to induce senescence. The plants were then allowed to recover for 14 days, after which they were allowed to wilt a second time and re-hydrated. Leaf chlorosis rating was taken 5 days later. Higher ratings indicate more leaf chlorosis. Values are means ± standard errors.

[3]Branch numbers and flower number were counted on the date the leaf chlorosis ratings were taken. Values are means ± standard errors.

Studies with other transgenic plants which constitutively expressed cytokinins have shown that plants had a decrease in apical dominance which resulted in increased growth of the lateral branches (Gan and Amasino, 1996). Increased branching is of importance to the horticultural performance of ornamental plants since increased branching leads to a better habit and more flowers. In an effort to determine if SAG 12-*IPT* had an effect on apical dominance petunia, branches were counted on the day the second leaf rating was taken. The line I98-3 did have an increased number of branches but I98-14 did not when compared to the 'V26' controls (Table 1). Gan and Amasino (1995) did not report any differences in the branching pattern of their SAG 12-*IPT* tobacco. The increased number of branches in I98-3 observed in this study may be due to tissue culture effects, or this line may be 'leaky' in expressing SAG 12-*IPT*, i.e., SAG 12-*IPT* in I98-3 may show a basal level of expression under non-induced conditions, and slight amount of cytokinins would accumulate.

The amount of flowers that an ornamental plant produces has an effect on its economic value. Gan and Amasino (1995) showed that tobacco transformed with the SAG 12–*IPT* displayed an increased number of flowers. In order to determine if the SAG 12-*IPT* petunias produced a modified number of flowers as compared to wild type 'V26' plants, the number of open flowers were noted on the day the second leaf rating was taken. The SAG 12-*IPT* plants had more open flowers than wild type plants (Table 1), which is consistent with the results obtained in SAG 12-*IPT* tobacco (Gan and Amasino, 1996).

Conclusion

Leaf senescence has a negative impact on the salability of ornamental crops. Recently Gan and Amasino (1995) transformed tobacco (*Nicotiana tabacum* cv. Wisconsin) with the SAG 12-*IPT* construct, which significantly delayed the onset of leaf senescence and increased the number of flowers with no

deleterious effects reported on the plants. To demonstrate the benefits of this biotechnology and the use of genetic engineering to the floriculture industry, we transformed 'V26' petunia with the SAG 12-*IPT* construct. Transformed plants showed a significant delay in senescence and an increased number of flowers as compared to wild type plants. One line, I98-3, showed a decrease in apical dominance. The only deleterious effect observed was a slight decrease in rooting of vegetative cuttings. We feel that the potential benefits of this genetic construct to petunia, and perhaps the floriculture industry may be significant. However, further work must be conducted to see if other important horticultural performance and reproductive characteristics are altered by the presence of SAG 12-*IPT*.

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EXTENDING THE NATURAL FLOWERING CYCLE OF *SPATHIPHYLLUM* THROUGH SELECTION

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Abstract. **The natural flowering season for most cultivars of mature *Spathiphyllum* (Schott) is Feb. through June. In an attempt to increase natural *Spathiphyllum* flower production through breeding, a population of hybrids was created and grown, and flowers harvested weekly over a period of one year. The seedlings with the highest total flower counts were then intercrossed and a second hybrid population was grown and screened. Plants with the most flowers were again selected while all others were discarded. Then the original parent plants plus the hybrids selected for high flower counts from the two succeeding generations were asexually propagated by division into single shoots. Once established, these plants (three commercial cultivars and five CFREC-A hybrids) were tested for flower production for 1 year in a replicated experiment. The results indicate that flower production in *spathiphyllum* can be increased through selection.**

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Introduction

Spathiphyllum is the top selling ornamental tropical foliage plant because, it addition to beautiful foliage, mature plants produce attractive white flowers that last several weeks. The presence of flowers greatly enhances the salability of *Spathiphyllum.* The natural flowering period for *Spathiphyllum is* generally Jan. through June. Natural flower production begins to decline over the summer and is at its lowest level during the fall months.

The ability to induce *Spathiphyllum* to flower by applying foliar sprays of gibberellic acid (GA3) has made it possible to have a continual supply of blooming plants for sale (Henny, 1981). GA3-treatment is reliable and allows growers to program their crops throughout the year. Treated plants typically flower within 9-12 weeks after treatment, depending upon time of year or cultivar.

However, there are some side effects from treating plants with GA3 to induce flowering. Some flowers will have distorted spathes and/or spadices. Foliage of treated plants may have an altered appearance since new leaves produced after GA3-treatment are narrower than original leaves. In addition plants that have flowered heavily following GA3-treatment will be stunted in later growth compared to untreated plants. The extent of these adverse effects varies among cultivars (Henny and Fooshee, 1985). It also should be noted that chemicals and their proper application can be expensive, and their use may become more restricted as environmental regulations increase. Therefore, other means need to be developed to insure a continuous supply of flowering plants.