Response of *Nicotiana alata* to Insertion of an Autoregulated Senescence-inhibition Gene

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ABSTRACT. *Nicotiana alata* Link and Otto (Jasmine tobacco) was transformed with an autoregulated senescence-inhibition gene construct P_{SAG12}-IPT encoding isopentenyl transferase via *Agrobacterium*-mediated transformation. Transformation was confirmed by polymerase chain reaction. Transgenic plants exhibited up to 2- to 4-fold fewer senesced leaves, 29% longer in situ flower life, 26% more shoot dry weight, and a 32% to 50% reduction in flowers per branch. Additionally, transgenics were 28% shorter and had up to 174% more branches, indicative of cytokinin overproduction and a lack of tight autoregulation of P_{SAG12}-IPT. Variation among independent transgenics suggests selection for enhanced P_{SAG12}-IPT is feasible. Our observations of increased branching and in situ flower longevity, as well as reduced plant height and flowers per branch provide new information on P_{SAG12}-IPT and its potential value for biological study and horticultural application.

Leaf senescence is a pressing problem for annual bedding plants like *Amaranthus* L., *Nicotiana* L., *Pelargonium* L. Her. Ex Ait., *Petunia* Juss., and *Zinnia* L. By middle to late summer, plant attractiveness is often compromised by mature senescing leaves, especially on stressed plants. Leaf senescence avoidance and chlorophyll retention is often associated with cytokinin level (Mok, 1994; Nooden, 1988a, 1988b; Van Staden et al., 1988). Recently, molecular methods have advanced our understanding of the role of cytokinins in plant development (Buchanan-Wollaston, 1997; Gan and Amasino, 1997; Klee, 1994; Weaver et al., 1997).

Lohman et al. (1994) identified senescence-associated genes (SAGs) from *Arabidopsis thaliana* (L.) Heynh., one of which has patterns of expression that are highly senescence specific. Messenger RNA from this SAG is detectable only during senescence. Its promoter (P_{SAG12}), was fused to a *Agrobacterium tumefaciens* gene encoding isopentenyl transferase (IPT). This construct is referred to as P_{SAG12}-IPT (Gan and Amasino, 1995). IPT catalyzes the first, rate-limiting step in cytokinin biosynthesis. This chimeric gene functions as an autoregulated senescence-inhibition system. P_{SAG12}-IPT transgenic *Nicotiana tabacum* L. were developmentally and morphologically normal, except for exhibiting delayed leaf senescence and increased seed yield and plant biomass (Gan and Amasino, 1995, 1996). Our study evaluated the potential use of P_{SAG12}-IPT to improve the ornamental qualities of *Nicotiana alata* (Jasmine tobacco).

Materials and Methods

Senescence inhibition system

Gan and Amasino (1995) describe the strategy used to create the autoregulatory senescence inhibition system.

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Tissue culture media

The media used in transformation and selection of transgenic plants included the following.

BASAL MEDIUM (B). MS medium (Murashige and Skoog, 1962) amended with 0.56 mM myo-inositol, 1.2 mM thiamine HCl, and 88 mM sucrose. Medium pH was adjusted to 5.7 before adding Difco Bacto agar at 7 g·L⁻¹ and autoclaving for 30 min at 120 °C and 104 kPa.

SHOOT PROLIFERATION MEDIUM (SP). B medium supplemented with 2.66 mM N⁶-benzyladenine (BA).

TISSUE SELECTION MEDIUM (TS). SP medium cooled to the touch after autoclaving, then supplemented with the antibiotics kanamycin and ticarcillin at 100 and 400 mg·L⁻¹, respectively.

SEED SELECTION MEDIUM (SS). Half-strength MS medium supplemented with 0.28 mM myo-inositol, 0.6 mM thiamine HCl, 14.7 mM sucrose, plus the antibiotics described above.

Transformation

Expanding *N. alata* leaves 3 to 4 cm long were excised from an 8-week-old male sterile double-flowered plant (Zainol et al, 1998) growing in the greenhouse, disinfested by stirring for 15 min in 5% Clorox (v/v, 0.26% NaOCl; Clorox Co., Oakland, Calif.) containing one drop Tween 20 (polyoxyethylenesorbitan) per 100 mL, and rinsed three times in sterile deionized water. Disinfested leaves were cut into 5 mm wide sections, turned on edge, and five sections were embedded halfway into ≈75 mL of SP medium per GA-7 container (Magenta Corp., Chicago, Ill.). Cultures were incubated in a growth chamber at 25 °C under a 16-h photoperiod provided by cool-white fluorescent lamps (30 μmol·m⁻²·s⁻¹) as measured with a photosynthesis system (LI-6400; LI-COR, Inc., Lincoln, Neb.) at shelf level. Explants were subcultured to fresh B medium 2 and 5 weeks after initial culture on SP medium. Eight weeks after initial culture, proliferating shoots were excised from callus and this callus without shoots (5 mm in diameter) was inoculated with *Agrobacterium tumefaciens* strain LBA4404 containing a binary vector carrying the P_{SAG12}-IPT gene construct (Horsch et al., 1985). Five shoots excised from the callus were rooted and grown to maturity for use as wild-type (WT) control plants. Inoculated calli were cocultivated with *A. tumefaciens* for 6 days on SP medium, then transferred to TS medium. Proliferating calli were subcultured to fresh TS medium after 4 weeks. After an additional 4 weeks of culture, independent green shoots were excised from the calli at their bases and the

Table 1. Primer designations and sequences.

Primer pair	Orientation ^a	Sequence (5' to 3')
SAG12	F	ACG GTT ATC ACT TCA GCC AAA T
IPT	F	GAC CTG CAT CTA ATT TTC G
IPT	R	CCT AAT ACA TTC CGA ACG G
nptII	F	GCT TGG GTG GAG AGG CTA TTC G
nptII	R	GCG ATA CCG TAA AGC ACG AGG

^aForward and reverse, respectively.

basal ends of the shoots were inserted 1 to 2 mm into TS medium without BA for rooting. After three weeks, eight rooted plants were transferred to a medium of 1 soil : 1 perlite : 1 sphagnum peat (by volume). At flowering, each of the eight putative transgenic plants and the five WT plants were crossed with *N. alata* 'Domino Crimson' (pollen donor). Seeds from these crosses were obtained from five putative transgenic plants (T1, T2, T3, T5, and T7) and the WT.

Selection for transgenics

Twenty-five milligrams of seed from WT, T1, T2, T3, T5, or T7 were disinfested by stirring for 12 min in a 30% Clorox (v/v, 1.58% NaOCl, Clorox Co.) solution containing one drop of Tween 20 per 100 mL then rinsing three times in sterile deionized water. Seeds were divided into three subsamples and each subsample was sown on the surface of ≈ 35 mL of SS medium in 100 $\times$ 20-mm petri dishes and the dish sealed with parafilm (American Natl. Can, Greenwich, Conn.). Cultures were maintained in a growth chamber in a completely random design under the conditions described previously. Green (kanamycin-resistant) and white (kanamycin-susceptible) seedlings were counted after 4 weeks in culture. Data were analyzed by chi-square goodness-of-fit tests.

Polymerase chain reaction (PCR) screen

Leaf tissue was harvested from *N. alata* and stored at -80°C for 7 d. DNA was extracted from 100 mg of young leaves of WT, T2, T3, T5, T7, and T2 $\times$ T5 using a plant miniprep kit (DNeasy; Qiagen, Mississauga, Ontario Canada). Tissue was ground with a 1.5-mL pellet pestle (Kontes Glass Co., Vineland, N.J.). No liquid nitrogen was used in extraction steps. Regions of P_{SAG12}-IPT that were amplified included a SAG12-IPT gene fragment using a SAG12 forward primer (22-mer) and an IPT reverse primer (19-mer), an IPT gene fragment using an IPT gene primer pair (both 19-mer), and an nptII gene fragment using an NPT II gene primer pair (22- and 21-mer) (Table 1). The PCR reaction was prepared containing a final concentration of 1 $\times$ PCR reaction buffer, 2.0 mM MgCl₂, 0.2 mM dNTP mix, 0.5 mM of each primer, 100 ng template DNA, 1.5 units of Taq DNA polymerase (Promega, Madison, Wisc.), and distilled water to a total volume of 30 mL. The amplification was carried out in a thermocycler (model 9700; Perkin Elmer Applied Biosystems, Inc., Foster City, Calif.) using 94 $^{\circ}\text{C}$ for 1 min, then 30 cycles of 94 $^{\circ}\text{C}$ for 30 s, 30 s at 54 $^{\circ}\text{C}$ for IPT or 56 $^{\circ}\text{C}$ for SAG12-IPT or 62 $^{\circ}\text{C}$ for nptII, 72 $^{\circ}\text{C}$ for 1 min (nptII and IPT) or 2 min (SAG12-IPT); and finally 72 $^{\circ}\text{C}$ for 10 min. Ten microliters from each PCR reaction was loaded on either a 1% (SAG 12 analysis) or 2% (IPT and nptII analyses) agarose gel and run at 120 V for 1.5 h. Gels were stained with ethidium bromide and the sizes of the amplified fragments were determined by comparison to a 1-kb DNA ladder (New England Biolabs, Beverly, Mass.).

Leaf senescence and flower longevity

TRIAL 1. Twenty green seedlings from the above study for each of WT, T1, T2, T3, T5, or T7 were planted on 30 Aug. 1995 in the

growing medium described above. These plants were acclimated and grown to flowering in a glasshouse at the University of Wisconsin, Madison (Ball, 1998). Plants were fertilized weekly with 200 mg-L⁻¹ of a 20N-8.8P-16.6K water soluble fertilizer (Peter's 20-20-20, Scott's-Sierra Hort. Products Co., Marysville, Ohio). Thirteen weeks after planting, fertilizer application ceased for 10 plants per accession and continued weekly for the remaining 10 plants per accession. These are henceforth called truncated and continuous fertilizer regimes, respectively. At 16 weeks of age (21 Dec. 1995), when flowering began, in situ flower longevity was determined. Flower longevity was measured as the number of days from flower opening to flower senescence (flower wilting) on the first two open flowers on each plant. At 23 weeks of age (7 Feb. 1996), senesced leaves (leaf 75% or more necrotic) were counted and the plants discarded.

TRIAL 2. Evaluation of leaf senescence was repeated in a polyhouse on a new population of plants for seeds sown 6 Feb. 1996. These plants were grown from the same seed lots as in Trial 1 and handled in the same manner. Physiological maturity of these plants (based on the date of first flower opening) occurred 5 weeks earlier than the plants in Trial 1 due to exposure to longer photoperiods, higher natural light levels, and higher temperatures in the greenhouse. Therefore, fertilizer application was stopped on one-half of the plants at 8 weeks of age, and leaf senescence data were collected at 18 weeks of age (5 July 1996).

TRIAL 3. Leaf senescence and in situ flower longevity were measured on a second population of plants grown in a polyhouse. Seeds were sown 13 Sept. 1996 and handled as described for Trial 1. Accession T3 was excluded due to lack of seeds. In situ flower longevity was determined starting at 11 weeks of age (26 Dec. 1996) on continuously fertilized plants. At 13 weeks of age, fertilizer application ceased on one-half of the plants. Senesced leaves were counted on 20 Mar. 1997 when the plants were 23 weeks old.

Leaf chlorophyll analysis

Discs were removed from each of the four oldest leaves on 23-week-old plants from Trial 3 using a 1 cm cork borer. Eight plants of WT, T1, T2, T5, or T7 were sampled from the continuous and truncated fertilizer regimes. Discs from each plant were placed in a test tube with 2 mL 96% ethanol in darkness at 4 $^{\circ}\text{C}$ for 48 h. Ethanol was decanted and saved. This process was repeated two more times to obtain a total of 6 mL of ethanol extract. Extracts were analyzed with a spectrophotometer (model DU-50; Beckman Instruments, Inc., Schaumburg, Ill.) at absorbencies of 665 and 649 nm, for chlorophyll *a* and chlorophyll *b*, respectively. Chlorophyll content was calculated using the following equations (Wintermans and DeMots 1965):

Table 2. Segregation in vitro for green (kanamycin resistant) and white (kanamycin susceptible) *Nicotiana alata* seedlings produced by crossing the parental genotype to *N. alata* 'Domino Crimson'.

Parental genotype ^a	Green (no.)	White (no.)	Expected ratio	χ^2	<i>P</i>
WT	0	201	0:1	0	1
T1	64	49	1:1	1.99	0.16
T2	132	121	1:1	0.48	0.49
T3	25	24	1:1	0.02	0.89
T5	29	27	1:1	0.07	0.79
T7	90	97	1:1	0.26	0.61

^aWT = wild type and T1, T2, T3, T5, and T7 = putative P_{SAG12}-IPT transgenic plants.

Fig. 1. PCR amplification products using primers that target SAG12-IPT, IPT and nptII regions of the transgene P_{SAG12} -IPT in *Nicotiana alata*. L = 1-kb ladder; T2, T3, T5, T7, and T2 x T5 = P_{SAG12} -IPT transgenics; WT = wild type; and C = a negative control with no DNA.

Chlorophyll a = $[13.7A_{665nm} - 5.76A_{649nm}] \times 6 \text{ mL ethanol} \times 4 \text{ cm}^2 \text{ leaf tissue}$.

Chlorophyll b = $[25.8A_{649nm} - 7.6A_{665nm}] \times 6 \text{ mL ethanol} \times 4 \text{ cm}^2 \text{ leaf tissue}$.

Total chlorophyll = chlorophyll a + chlorophyll b .

Growth analysis

Data were collected on plant height (growing medium surface to top of plant), number of branches, and number of flowers on 23-week-old plants under the truncated fertilizer regime from Trial 3. Shoot dry weight (DW) was recorded on shoots from 23-week-old continuously fertilized plants from Trial 3. Shoots were dried by forced air at 60 °C for 4 d before weighing.

Data analysis

Analysis of variance (ANOVA) procedures were used for analysis of quantitative data; the statistical computing package used was SAS Systems for Mixed Models (Littell et al., 1996). For analyses using data from a single trial only, the treatments were fertilizer and genotype; both of these factors were considered as fixed. For analyses using data from two trials (no analyses used data from all three trials), a factor for trial was added to the model. This factor was considered as random. This trial factor is viewed as a block; however, since blocks are not necessarily additive, interactions between trials and fixed factors were assessed for significance. Unless otherwise stated, $P = 0.05$ for all tests.

If the fertilizer by genotype interaction was significant, pairwise comparisons between all pairs of genotypes were performed for each fertilizer regime. If the interaction was not significant but the genotype main effect was, pairwise comparisons were conducted for the genotype means (averaged across the fertilizers). Pairwise comparisons, rather than a single comparison of WT versus the mean of the transgenics, were used since independent transgenics performed quite differently.

It was noted early, that use of original data resulted in heteroscedastic variances, in violation of usual ANOVA assumptions. A $\log_{10}$ transformation resulted in homogeneous variances. Thus, our analyses were conducted on the $\log_{10}$ scale; however, data in the tables and figures are presented as nontransformed data.

Results and Discussion

SELECTION FOR TRANSGENICS. Ratios for chi-square tests did not deviate significantly from expected 0:1 or 1:1 ratios (green : white seedlings) for the progeny of WT or the five putative transgenic genotypes, respectively, grown in vitro on selection medium (Table 2). Our predictions for segregation were based on results reported previously for P_{SAG12} -IPT acting as a monogenic dominant trait (Gan and Amasino, 1995).

PCR SCREEN. Successful amplification occurred using primers that target the SAG12-IPT region in genotypes T5, T7, and T2 x T5; the IPT region in T2, T5, T7, and T2 x T5; and the nptII region in T2, T3, T5, T7, and T2 x T5 (Fig. 1). No PCR-amplified products were detected for WT using any of the primer pairs, T3 using the IPT primer pair, or for T2 or T3 using the SAG12-IPT primer pair. Results for T5, T7, and T2 x T5 suggest integration of the full construct occurred, whereas results for T2 suggest it was missing the SAG12 promoter terminus. T3 appeared to be missing the SAG12 and IPT segments. All transgenics were nptII positive. Even though T1 was not confirmed to be transgenic by PCR and T2 and T3 were missing segments of the construct, T1, T2, T3, T5 and T7 will henceforth be referred to as transgenic.

LEAF SENESCENCE. In Trial 1, the transgenic plants showed little or no leaf senescence at 23 weeks of age, whereas the WT plants had four to eight senesced leaves at the base of the stem (Figs. 2 and 3). The number of senesced leaves from Trial 1 (Feb. 1996) was affected significantly by a fertilizer x genotype interaction (Table 3). WT and T3 had significantly more senesced leaves under the truncated fertilizer regime than plants that were continuously fertilized, whereas the response of T7 was reversed (Fig. 3A). Genotypes T1, T2, and T5 responded similarly to fertilizer regimes. Overall, the main effect of fertilizer was nonsignificant, whereas the main effect of genotype was significant (Table 3). WT plants had two to 14 times more senesced leaves than the transgenic plants (Fig. 3B). Significant variation existed among the transgenic genotypes for the number of senesced leaves, with mean values ranging from 0.4 to 2.6

Fig. 2. Basal leaf senescence on *Nicotiana alata* wild-type (left) and P_{SAG12} -IPT transgenic T7 (right) at 23 weeks of age from Trial 1 from the truncated fertilizer treatment.

Fig. 3. Senesced leaves on *Nicotiana alata* as influenced by (A) fertilizer and genotype, and (B) genotype in Trial 1. WT = wild type and T1, T2, T3, T5, and T7 = putative P_{SAG12} -IPT transgenic plants. In A error bars represent $\pm$ SE. Mean separation in B by pairwise *t* tests.

Table 3. Likelihood estimates for the number of senesced leaves on *Nicotiana alata* as influenced by fertilizer and genotype in Trial 1.

Source of variation ^z	Numerator df	Denominator df	F ^y
Fertilizer (F) ^x	1	101	0.08 ^{NS}
Genotype (G) ^w	5	101	22.95 ^{****}
F $\times$ G	5	101	4.34 ^{**}
Residual	101		0.05

^zEvaluation in February 1996 (Trial 1) at 23 weeks of age.

^yAnalysis on $\log_{10}$ transformed data.

^xContinuous or truncated.

^wWild type and P_{SAG12} -IPT transgenics T1, T2, T3, T5, and T7.

^{NS}, **, **** Nonsignificant or significant at $P \leq 0.01$ or 0.0001, respectively.

per plant. T3 had 2- to 6-fold more senesced leaves than the other transgenics. This finding is consistent with the PCR results for T3, which indicates the P_{SAG12} -IPT construct was not fully integrated into this genotype.

Leaf senescence in July 1996 (Trial 2) and March 1997 (Trial 3) was influenced significantly by fertilizer $\times$ genotype and trial $\times$ genotype interactions (Table 4), indicating genotypes responded differently to different fertilizer regimes and trials. The fertilizer $\times$ genotype interaction was due primarily to differences in magnitude of response to fertilizer regimes for WT and T7, compared to T1, T2, and T5 (Fig. 4A). Generally, there was a trend for plants under truncated fertilizer treatment to have more senesced leaves than those plants fertilized continuously. Transgenic plants given either fertilizer treatment had fewer senesced leaves than WT given continuous fertilizer. The main effects of fertilizer and genotype were significant (Table 4). Transgenic genotypes had from 2- to 5-

Table 4. Likelihood estimates for the number of senesced leaves on *Nicotiana alata* as influenced by fertilizer, genotype and trial in Trials 2 and 3.

Source of variation	Numerator df	Denominator df	F ^z
Fertilizer (F) ^y	1	159	8.07 ^{**}
Genotype (G) ^x	4	4.99	6.23 [*]
F $\times$ G	4	159	3.41 [*]

Covariance	Estimate
Trial (T) ^w	0 ^{NS}
T $\times$ F	0 ^{NS}
T $\times$ G	0.0099 ^{**}
T $\times$ F $\times$ G	0.0009 ^{NS}
Residual	0.0470

^zAnalysis on $\log_{10}$ transformed data.

^yContinuous or truncated.

^xWild type and putative P_{SAG12} -IPT transgenics T1, T2, T5, and T7.

^wEvaluation in July 1996 (Trial 2) and March 1997 (Trial 3) at 18 and 23 weeks of age, respectively.

^{NS}, **, ** Nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively.

Fig. 4. Senesced leaves on *Nicotiana alata* as influenced by (A) fertilizer $\times$ genotype, (B) genotype, and (C) fertilizer in Trials 2 and 3. WT = wild type and T1, T2, T5, and T7 = putative P_{SAG12} -IPT transgenic plants. In A error bars represent $\pm$ SE. Mean separation in B and C by pairwise *t* tests.

Table 5. Likelihood estimates for leaf chlorophyll content of *Nicotiana alata* leaves as influenced by fertilizer and genotype in Trial 3.

Source of variation	df	Chlorophyll ² (F values)			
		a	b	a : b	Total
Fertilizer (F) ^y	1	108.86****	103.66****	47.46****	111.98****
Genotype (G) ^x	4	34.10****	22.27****	35.84****	31.15****
G × F	4	2.44 ^{NS}	1.14 ^{NS}	10.76****	2.01 ^{NS}
Residual	63	3.98	1.03	0.02	8.66

²Evaluation at 23 weeks of age.

^yContinuous or truncated.

^xWild type and putative P_{SAG12}-IPT transgenics T1, T2, T5, and T7.

^{NS},**** Nonsignificant or significant at $P \leq 0.0001$, respectively.

Fig. 5. Leaf chlorophyll a : b ratio of *Nicotiana alata* as influenced by genotype × fertilizer in Trial 3. WT = wild type and T1, T2, T5, and T7 = putative P_{SAG12}-IPT transgenic plants. Error bars represent ± SE.

fold fewer senesced leaves than WT plants (Fig. 4B). Leaf senescence of T2, an apparent promoterless transgenic (Fig. 1) was similar to WT. Fertilizer truncation caused a 44.5% increase in senesced leaves over plants given continuous fertilizer (Fig. 4C).

Similar trends in number of senesced leaves were reported with the same chimeric gene in *N. tabacum* 'Wisconsin 38' (Gan and Amasino, 1995); with the IPT gene in *N. tabacum* 'Samsun' using the *Glycine max* L. heat shock promoter HS6871 (Smart et al., 1991); in *Solanum tuberosum* L. using the IPT gene and the native IPT promoter (Ooms et al., 1991); and *N. tabacum* 'Petit Havana' using a promoterless IPT gene inserted randomly into the genome (Hewelt et al., 1994).

Number of senesced leaves varied among genotypes of *N. alata* when trials were done at different times of the year (Table 4). Gan and Amasino (1997) suggested regulation of SAG expression is multifactorial. Thus, growing conditions could cause variation in gene expression. Expression of IPT in *G. max* under control of an auxin-inducible promoter resulted in differences in leaf senescence between plants grown in a growth chamber and in vitro (Li et al. 1992). Likewise, Scorza et al. (1994) reported response differences due to the *rolC* gene (gene product: cytokinin-b-glucosidase) in *N. tabacum* 'Wisconsin 38' grown under different photoperiods and irradiance. Thus, further study is warranted to characterize the effects of P_{SAG12}-IPT under different environmental conditions.

LEAF CHLOROPHYLL. The ratio of chlorophyll a to b was influenced significantly by a genotype × fertilizer interaction (Table 5). A higher chlorophyll a : b ratio was observed for WT,

T1, and T2 under continuous fertilizer application, whereas the ratio for T5 and T7 was similar under both regimes (Fig. 5). Genotype and fertilizer main effects influenced leaf chlorophyll a, b, a : b ratio, and total leaf chlorophyll (Table 5). Leaves of transgenic genotypes had higher chlorophyll a, b, a : b ratio, and total chlorophyll than WT (Fig. 6A–D). Significant variation was found among transgenic genotypes for chlorophyll measured. T7 had the greatest values followed by T5, T2, and T1, respectively.

Continuous fertilization produced leaf tissues with higher chlorophyll values than truncated treatments (Fig. 6E–H). The

Fig. 6. Content of leaf chlorophylls (A and E) a, (B and F) b, (C and G) ratio a : b, and (D and H) total of *Nicotiana alata* leaves as influenced by genotype and fertilizer. WT = wild type and T1, T2, T5, and T7 = putative P_{SAG12}-IPT transgenic plants. Cont. = continuous fertilizer and Trun. = Truncated fertilizer. Mean separation by pairwise *t* tests.

Table 6. Likelihood estimates for flower longevity in situ on *Nicotiana alata* as influenced by location/year and genotype in Trials 1 and 3.

Source of variation	Numerator df	Denominator df	F
Location/year (LY) ²	1	87	18.61****
Genotype (G) ^y	4	87	3.12*
LY × G	4	87	1.16 ^{NS}

Covariance	Estimate
Plant error ^x	9.75****
Residual	7.43

²December evaluation in a glasshouse (1995) and a polyhouse (1996).

^yWild type and P_{SAG12}-IPT transgenics T1, T2, T5, and T7.

^xError, used for F tests.

^{NS},**** Nonsignificant or significant at $P \leq 0.05$ or 0.0001, respectively.

Fig. 7. Flower longevity in situ of *Nicotiana alata* under continuous fertilization in Trials 1 and 3 as influenced by (A) genotype and (B) location/year. WT = wild type and T1, T2, T5, and T7 = putative P_{SAG12} -IPT transgenic plants. Mean separation by pairwise *t* tests.

higher chlorophyll content of transgenics compared to WT suggests the transgene is functioning to delay chlorophyll degradation whether or not the promoter is fully inserted. This information supports our leaf senescence results reported above.

FLOWER LONGEVITY. Comparison of flower longevity in situ of plants grown under continuous fertilizer in a glasshouse in December 1995 (Trial 1) and a polyhouse in December 1996 (Trial 3) resulted in significant main effects for genotype and location/year, and a nonsignificant interaction (Table 6). Flower longevity in situ varied between WT, and T1, T5, and T7, and among transgenics (Fig. 7A). WT and T2 flowers lasted ~18 d with longevity increasing 2 to 4 d for T1, T7, and T5, respectively. Flowers lasted 4 d longer in the glasshouse in December 1995 than in the polyhouse in December 1996 (Fig. 7B). Our results of increased in situ flower longevity provide new information on P_{SAG12} -IPT affecting floral organs.

GROWTH ANALYSIS. Transgenic plants appeared more compact and dense than WT plants (Fig. 8). Genotypes varied signifi-

Fig. 8. Phenotype of *Nicotiana alata* wild-type (left) and a P_{SAG12} -IPT transgenic plant T7 (right) at 23 weeks of age.

cantly for shoot dry weight (DW), plant height, number of branches, and flowers per branch, but the number of flowers per plant did not differ among the genotypes (Table 7). In addition, variation existed among WT and transgenics for shoot dry weight, plant height, and number of branches (Fig. 9A–C). Shoot DW ranged from 42 g for WT to 53.1 g for T7 (Fig. 9A). Increases in shoot DW were reported also with P_{SAG12} -IPT in *N. tabacum* (Gan and Amasino, 1995) and with the cytokinin biosynthesis gene *T-cyt* in *S. tuberosum* (Ooms et al., 1991). Plant height ranged from 46.8 cm for WT to 33.6 cm for T7 (Fig. 9B). Transgenic plants branched more (175.6 to 246.6 branches) than WT (89.6 branches) (Fig. 9C). Branching was more profuse in upper portions of transgenic plants than in WT plants (Fig. 10). Our results with P_{SAG12} -IPT in *N. alata* showed concurrent phenotypes of reduced plant height and increased branching. However, plant height and branching are developmental processes completed usually before the onset of tissue senescence. Our results thereby suggest P_{SAG12} -IPT in *N. alata* was not regulated tightly (i.e. expressed only during tissue senescence) but expressed sufficiently during plant development to cause a reduction of plant height and an increase in branching; responses typically associated with cytokinin overproduction (Klee, 1994).

Although the number of branches was greatest in transgenics, the number of flowers did not differ significantly from those observed on WT plants (Table 7 and Fig. 9D). More flowers existed per branch on WT plants (2.2), than on transgenic plants (1.1 to 1.5) (Fig. 9E). Despite these results, the increased number of branches observed on transgenic *N. alata* offers a potential for increasing numbers of flowers per plant. Transgenic *N. tabacum* expressing *rolC* produced more flowers per plant than wild type controls (Scorza et al., 1994). In our study, transgenics appeared to produce more flowers per plant than WT plants (Fig. 9D). Although means of wild type and transgenic plants were not significantly different, this effect might have been masked by the truncated fertilizer treatment, leading to inadequate nutrients, and less metabolites for allocation to flowering. Stress can promote young flower bud abortion and/or abscission while other organs are only slightly affected (Halevy, 1987). Therefore, further investigation of the influence of P_{SAG12} -IPT on flowering under normal fertilizer application is warranted.

Table 7. F values for shoot dry weight (DW), plant height, number of branches, number of flowers, and flowers/branch on *Nicotiana alata* as influenced by genotype and fertilizer regime in Trial 3.

Source of variation	df	F				
		Shoot DW ^z (g)	Plant ht ^y (cm)	Branches ^y (no.)	Flowers ^y (no.)	Flowers/branch ^y
Genotype ^x	4	5.41**	5.94**	6.70***	1.48 ^{NS}	4.61**
Residual	36 ^w , 31 ^v , 29 ^u	37.41	34.85	3729.67	4975.02	0.29

^{z,y}Data collected from plants grown under continuous or truncated fertilizer, respectively.

^xWild type and putative P_{SAG12}-IPT transgenic plants T1, T2, T5, and T7.

^{w,v,u}Error degrees of freedom for shoot DW; plant height; and number of branches, flowers, and flowers/branch, respectively.

^{NS,*,***}Nonsignificant or significant at $P \leq 0.01$ or 0.001, respectively.

Fig. 9. (A) Shoot dry weight (DW), (B) plant height, (C) number of branches, (D) number of flowers, and (E) number of flowers per branch on *Nicotiana alata* as influenced by genotype under a truncated fertilization regime in Trial 3. WT = wild type and T1, T2, T5, and T7 = putative P_{SAG12}-IPT transgenic plants. Mean separation by pairwise *t* tests.

PHENOTYPE EXPRESSION. Variation in phenotype existed among the five transgenic P_{SAG12}-IPT *N. alata* genotypes for all traits evaluated in this study, except for the number of flowers per plant. Similarly, phenotype of transgenic clones varied on promoterless IPT and *rolC* *N. tabacum* (Hewelt, 1994; Scorza et al., 1994), and T-cyt *S. tuberosum* (Ooms et al., 1991). The large amount of variation detected among the few transgenic genotypes evaluated in our study indicates potential for varying levels of P_{SAG12}-IPT expression.

In conclusion, our observations of increased branching, increased in situ flower longevity, reduced plant height, and fewer flowers per branch provide new information on the characteristics of P_{SAG12}-IPT transformants and their potential value for biological study and agricultural application. Suppression of leaf

senescence and increased in situ flower longevity through expression of P_{SAG12}-IPT in annual bedding plants and perennials would help maintain more aesthetically pleasing plants throughout the growing season. However, studies are needed to determine whether winter hardiness would be compromised in perennials by the transgene altering redistribution of nutrients during senescence and cold acclimation. Potentially, this chimeric gene could reduce leaf loss on indoor tropical foliage plants during acclimatization from high light crop production to low light interiorscapes as well. In addition, the shorter, more compact phenotype exhibited by the plants transformed with P_{SAG12}-IPT opens the possibility for its use in regulating plant growth.

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Fig. 10. Branching of *Nicotiana alata* wild-type (left) and a P_{SAG12}-IPT transgenic T7 (right) at 23 weeks of age.

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