Inhibition of Leaf Senescence by Autoregulated Production of Cytokinin

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Controlling expression of *IPT*, a gene encoding isopentenyl transferase (the enzyme that catalyzes the rate-limiting step in cytokinin biosynthesis), with a senescence-specific promoter results in the suppression of leaf senescence. Transgenic tobacco plants expressing this chimeric gene do not exhibit the developmental abnormalities usually associated with *IPT* expression because the system is autoregulatory. Because sufficient cytokinin is produced to retard senescence, the activity of the senescence-specific promoter is attenuated. Senescence-retarded leaves exhibit a prolonged, photosynthetically active life-span. This result demonstrates that endogenously produced cytokinin can regulate senescence and provides a system to specifically manipulate the senescence program.

Leaf senescence is a type of programmed cell death that constitutes the final phase of leaf development (*1*). During senescence leaf cells undergo coordinated changes in cell structure, metabolism (*1*, *2*), and gene expression (*3*), resulting in a sharp decline in photosynthetic capacity (*2*). The cytokinin class of plant hormones may play a role in controlling leaf senescence because a decline in leaf cytokinin levels occurs in senescing leaves and external application of cytokinin often delays senescence (*4*–*6*). Transgenic plants that express *IPT* exhibit cytokinin overproduction and some delay of leaf senescence (*7*–*10*). However, a variety of developmental and morphological alterations are associated with these plants, which complicate the interpretation of the role of cytokinins in leaf senescence. We now report the development of a senescence-inhibition system in which cytokinin production is (i) specifically targeted to senescing leaves and (ii) is negatively autoregulated to prevent hormone overproduction. Transgenic plants containing this system display efficient retardation of leaf senescence without other developmental abnormalities.

The strategy used to create this autoregulatory senescence-inhibition system is shown in Fig. 1. We have identified senescence-associated genes (SAGs) from *Arabidopsis thaliana* (*3*) and found that the expression of one of these genes, *SAG12*, is highly senescence specific (*11*). The *SAG12* promoter (P_{SAG12}) was joined to the coding region of *IPT* to form the chimeric gene P_{SAG12}-*IPT* (*12*). At the onset of senescence this promoter should activate *IPT* expression and increase the cytokinin content to a level that prevents the leaf from senescing. The prevention of senescence would in turn attenuate expression from the promoter to prevent overproduction of cytokinin.

P_{SAG12}-*IPT* was introduced into leaf cells of tobacco (*Nicotiana tabacum* cv. Wisconsin 38) by *Agrobacterium*-mediated transformation (*13*), and eight independent transgenic plants were obtained. All of these transgenic plants were identical to wild-type plants in growth and development except for a significant delay of leaf senescence. In the next generation resulting from self-pollination, all of the plants containing P_{SAG12}-*IPT* showed the delayed leaf senescence phenotype regardless of the transgene copy number. A comparison of the development of one of these transgenic lines with wild type is presented in Fig. 2. Seedlings of transgenic and wild-type plants had equally developed shoot and root systems (Fig. 2A), and both lines produced flower buds after forming an identical number of nodes and reaching the same height (Fig. 2B). In both lines, the first flowers reached anthesis at the same time, and by 12 weeks both transgenic and wild-type plants had produced a similar number of flowers (Fig. 2C). There were no significant differences in overall plant height and leaf number on the main stems (Table 1). However, there was a prominent difference in the progression of leaf senescence in transgenic plants compared to wild-type plants. As the wild-type plants aged, leaf senescence progressed sequentially from the bottom to the top leaves. During this time, there was no visible sign of senescence in any leaves of P_{SAG12}-*IPT* plants of identical age and development (Fig. 2, A to C). At the time that the uppermost leaves in wild-type plants had undergone senescence and flowering had terminated, the oldest leaves of age-matched P_{SAG12}-*IPT* plants were still green, and these plants continued to produce flowers (Fig. 2D). The P_{SAG12}-*IPT* plant produced more than 300 flowers compared to ~180 flowers in wild type (Table 1). Senescence in young, healthy leaves that were detached from plants was also evaluated. Leaves from P_{SAG12}-*IPT* plants showed no signs of senescence for more than 40 days after detachment, whereas leaves of wild-type plants started senescing 10 days after detachment (Fig. 2E).

The normal development of P_{SAG12}-*IPT* plants indicated that the autoregulatory system operated only in senescing leaves without modifying the development of other parts of the plant. This was specifically examined in reciprocal grafts of P_{SAG12}-*IPT* plants and wild-type plants (*14*). In either graft orientation, senescence progressed normally in leaves of wild-type parts of the chimeric plants but not in leaves containing P_{SAG12}-*IPT* (Fig. 2F), demonstrating that the cytokinin produced in P_{SAG12}-*IPT* leaves was not translocated in amounts suf-

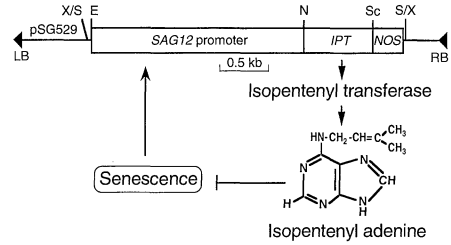

Fig. 1. Rationale of the autoregulatory senescence-inhibition system. The senescence-specific *SAG12* promoter (GenBank accession number U37336) was fused to *IPT* (*12*). At the onset of senescence P_{SAG12} directs the expression of *IPT*, which results in an increased level of a cytokinin (isopentenyl adenine) that in turn inhibits senescence. The inhibition of senescence attenuates expression from P_{SAG12} to prevent cytokinin overproduction. LB, RB, left and right T-DNA border, respectively. Restriction sites: E, Eco RV; N, Nco I; Sc, Sac I; and S/X or X/S, ligated Spe I and Xba I sites.

Table 1. Comparison of P_{SAG12}-*IPT* transgenic and wild-type tobacco plants. Biomass is the dry weight of the plant above the soil excluding seeds (*17*). Plant height was measured from the soil surface to the top of the highest floral stalk. Data presented are means and standard deviations for the 8 wild-type and 13 transgenic plants.

Trait	Wild-type plants	P_{SAG12}-*IPT* plants
Flowers produced*	178.3 ± 28.1	327.5 ± 46.3
Seed yield (g/plant)	20.44 ± 4.18	31.15 ± 4.10
Biomass (g/plant)	107.51 ± 14.41	150.79 ± 20.15
Plant height (cm)	176.3 ± 14.3	180.2 ± 7.9
Leaf number on main stem	33.3 ± 0.5	33.5 ± 1.4

*At the time of termination of the experiment, P_{SAG12}-*IPT* plants were still producing flowers.

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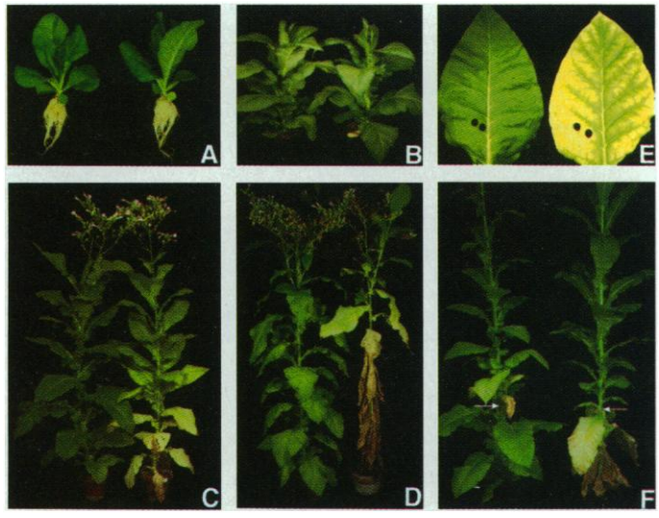

Fig. 2. (A to D) Phenotype of P_{SAG12}-*IPT* transgenic and wild-type tobacco plants. In each panel, the transgenic plant is on the left and the wild type on the right. Plants are shown at various stages of development, which occur approximately 3.5 weeks (**A**), 6 weeks (**B**), 12 weeks (**C**), and 20 weeks (**D**) after transplanting seedlings into soil (*18*). (**E**) Retarded senescence in detached transgenic leaves. A leaf from a P_{SAG12}-*IPT* plant (left) compared to a leaf from a wild-type plant (right) 30 days after detachment (*19*). (**F**) The pattern of senescence in plants resulting from grafts between P_{SAG12}-*IPT* and wild-type plants (*14*). Arrows indicate the grafting points. Left plant: wild-type scion (top) and P_{SAG12}-*IPT* stock (bottom); right plant: P_{SAG12}-*IPT* scion and wild-type stock.

ficient to affect leaf senescence in the wild-type regions.

The photosynthetic rates (*15*) were approximately equal in upper nonsenescent young leaves on both P_{SAG12}-*IPT* and wild-type plants. However, as photosynthesis declined to low levels in the senescing leaves of the wild-type plants, high rates of photosynthesis were maintained in age-matched leaves of P_{SAG12}-*IPT* plants (Fig. 3). Therefore, the leaves of the transgenic plants exhibit a prolonged photosynthetic life-span, which is likely to contribute to the 50% increase in dry weight and seed yield of P_{SAG12}-*IPT* plants compared to wild type (Table 1).

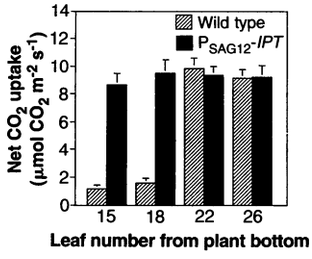

Fig. 3. Sustained photosynthesis in senescence-retarded leaves of P_{SAG12}-*IPT* transgenic plants (*15*). The leaves were numbered from the bottom of a plant. At the time of measurement, leaves 15 and 18 of wild type were senescing whereas leaves 22 and 26 were nonsenescent.

To assess the autoregulatory nature of P_{SAG12}-*IPT* expression, we determined whether *SAG12* promoter activity was attenuated in plants containing P_{SAG12}-*IPT*. To monitor promoter activity, the reporter gene β-glucuronidase (*GUS*) was joined to P_{SAG12} to create the chimeric gene P_{SAG12}-*GUS* (*12*). The level of P_{SAG12}-*GUS* expression was determined as a function of leaf age in plants containing both P_{SAG12}-*GUS* and P_{SAG12}-*IPT* and in plants containing only P_{SAG12}-*GUS*. Plants containing both P_{SAG12}-*GUS* and P_{SAG12}-*IPT* were the progeny of a cross between plants homozygous for a single locus of each transgene; these F_1 plants were therefore hemizygous for both transgene loci. Wild-type plants were also crossed to the same homozygous lines to create F_1 plants hemizygous for each locus. In leaves of plants containing only P_{SAG12}-*GUS*, GUS activity increased to extremely high levels with the progression of senescence until ~71 days after leaf emergence; at this point, the leaves of these plants had become desiccated (Fig. 4). The leaves of the P_{SAG12}-*GUS* plants had the same life-span as the wild-type controls. In contrast, leaves of plants containing both P_{SAG12}-*GUS* and P_{SAG12}-*IPT* survived for much longer periods, and the level of P_{SAG12}-*GUS* expression was over 1000 times lower in the presence of P_{SAG12}-*IPT*. Thus, expression of P_{SAG12}-*IPT* negatively regulates expression from the *SAG12* promoter, most likely as a result of cytokinin production, because we found that exogenous application of cytokinin delayed leaf senescence of P_{SAG12}-*GUS* plants and suppressed GUS expression as well (*11*).

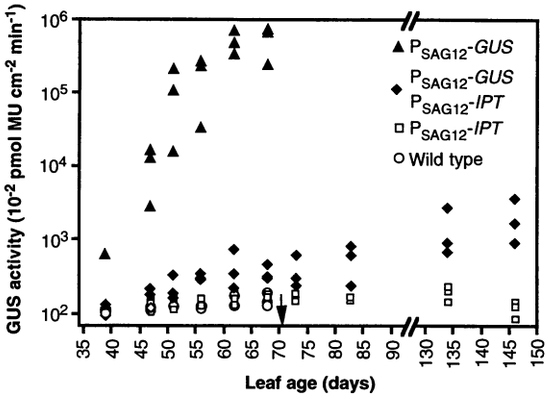

Fig. 4. Regulation of the *SAG12* promoter in P_{SAG12}-*IPT* transgenic plants. GUS activity (*20*) in leaves of plants hemizygous for both P_{SAG12}-*GUS* and P_{SAG12}-*IPT* was compared to that in plants hemizygous for P_{SAG12}-*GUS*. Fluorescence in wild type and plants hemizygous for P_{SAG12}-*IPT* was measured as a control.

These results demonstrate that joining a senescence-specific promoter to the *IPT* gene creates an autoregulatory system that inhibits senescence. The key features of this system that provide such a specific effect of *IPT* expression on only one aspect of development are (i) the use of a promoter that targets cytokinin production spatially and temporally and (ii) the autoregulatory nature of the expression, which ensures that cytokinin production is maintained at the minimum level capable of inhibiting senescence. This autoregulation is likely to explain the absence of phenotypic differences between plants that were hemizygous or homozygous for a transgene locus. Although leaf senescence is thought to be an evolutionary adaptation to recycle nutrients, this process may have negative effects on yields in an agricultural setting. For example, a delay of leaf senescence in some maize hybrids is associated with a significant increase in yields (*16*). In our tests with tobacco, plants that expressed the P_{SAG12}-*IPT* fusion had an increased yield of both biomass and seed as well as an enhanced postharvest longevity of leaves, and therefore this system may have applications in agriculture.

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12. A 2.18-kb P_{SAG12} fragment from the Eco RV site (at 2073 bp upstream of the transcription start site) to an Nco I site (created at the *SAG12* translation start codon by oligomutagenesis) was fused to the open reading frame of *GUS* and *IPT* (*8*) at their translation start codon, resulting in plasmids containing P_{SAG12}-*GUS* and P_{SAG12}-*IPT* chimeric genes, respectively. The P_{SAG12}-*IPT* fusion flanked by Spe I sites was recloned into a binary vector at an Xba I site, resulting in the plasmid pSG529 (Fig. 1). A Pst I–Xba I fragment containing P_{SAG12}-*GUS* was inserted into a binary vector to create pSG514.
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14. Shoot tips (a 25-mm region) from 4-week-old P_{SAG12}-*IPT* transgenic and wild-type tobacco plants were reciprocally grafted by the wedge grafting method [R. J. Garner, *The Grafter's Handbook* (Cassell, London, 1988)]. Twelve grafted plants were analyzed.
15. Leaves of 11-week-old transgenic and wild-type plants grown in a greenhouse with a 16-hour photoperiod at ~26°C were measured for net CO_2 uptake rate with a LI-6400 infrared monitor (Li-Cor). The internal light source was set at 1500 μmol m^{-2} s^{-1} and at a temperature of 26°C. Six plants of each genotype were measured.
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17. After 6 months growth the seed pods and the remainder of the above-soil parts were harvested separately and dried at 55°C until no further weight change was observed (7 to 10 days).
18. Seedlings were started in sterile culture and transplanted when the cotyledons had expanded into 3-liter clay pots containing Jiffy-Mix soil (Jiffy Products of America) saturated with Peters 20-20-20 fertilizer at the concentration of 473 parts per million of nitrogen (Peters Fertilizer Products, W. R. Grace & Co.) and grown in a greenhouse (*15*). The plants were sub-irrigated as needed with water.
19. Leaves that had just reached full expansion at the same positions of P_{SAG12}-*IPT* or wild-type plants were cut from the plants, immediately inserted into a jar filled with water, and maintained in a growth chamber at 23°C and 70% relative humidity under 120 μmol m^{-2} s^{-1} of continuous light. Four leaves for each genotype were tested.
20. At the indicated ages (expressed as days after the emerging leaf was 3 mm in length) a 1-cm^2 leaf disk was harvested with a cork borer from the distal part of leaf number 7 (counted from bottom). Three plants of each genotype were sampled each time. GUS activity in leaf disks was assayed with 4-methylumbelliferyl-β-D-glucuronide as substrate according to standard protocol [R. A. Jefferson, *Plant Mol. Biol. Rep.* **5**, 387 (1987)].
21. We thank A. Bleecker and M. Sussman for valuable discussions, T. Sharkey and M. Laporte for help in measurement of photosynthetic rates, and C. O. Miller for discovering the cytokinin class of phytohormones. Supported through the Consortium for Plant Biotechnology Research (DE-FC05-92OR22072) and CIBA-Geigy Corporation. S.G. was a recipient of a Rockefeller Foundation and a DOE/NSF/USDA *Arabidopsis* Training Grant predoctoral fellowship.

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Tobacco MAP Kinase: A Possible Mediator in Wound Signal Transduction Pathways

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A complementary DNA encoding a mitogen-activated protein (MAP) kinase homolog has been isolated from tobacco plants. Transcripts of the corresponding gene were not observed in healthy tobacco leaves but began to accumulate 1 minute after mechanical wounding. In tobacco plants transformed with the cloned complementary DNA, trans inactivation of the endogenous homologous gene occurred, and both production of wound-induced jasmonic acid and accumulation of wound-inducible gene transcripts were inhibited. In contrast, the levels of salicylic acid and transcripts for pathogen-inducible, acidic pathogenesis–related proteins were increased upon wounding. These results indicate that this MAP kinase is part of the initial response of higher plants to mechanical wounding.

One of the severest environmental stresses to which plants may be subjected is wounding, which may come about through such diverse causes as mechanical injury or pathogen or herbivore attack. To cope with such stresses, plants have developed multiple self-defense systems, activating a set of genes that are mostly involved in wound healing (*1*). Well-known examples are the genes encoding proteinase inhibitor (PI)-I and PI-II, which accumulate not only in wound sites but also in distal unwounded tissues to defend the plant body against, for example, insect proteases (*1*). The transcriptional activation, by wounding, of genes encoding PI is therefore systemic, and it is generally considered that the endogenous activator of these genes is jasmonic acid (JA) along with its methyl ester (MeJA) (*2*–*6*). The biosynthesis pathway of JA and MeJA has been proposed to involve a lipid-based signaling system including lipases, linolenic acids, lipoxygenases (*2*, *6*–*8*), and phosphorylation of proteins (*2*, *3*). However, the molecular mechanism responsible for activation of these pathways in response to wound stress remains unknown. Here, we report identification and characterization of a MAP kinase homolog that may function in the initial step of wound signal transduction pathways.

To identify genes that are involved in mechanical wounding or lesion formation after pathogen attack of tobacco plants, we isolated a particular complementary DNA (cDNA) clone (DS22) by differential hybridization (*9*). Northern (RNA) hybridization analyses revealed that DS22 transcripts of 1.8 kilobases (kb) in size are absent in healthy leaves of tobacco plants but accumulate upon mechanical wounding. Indeed, DS22 transcripts began to accumulate as early as 1 min after wounding, reaching a maximum level within an hour and rapidly declining thereafter (Fig. 1). The accumulation of DS22 transcripts, however, was not limited to wounded leaves, but rapidly expanded into unwounded adjacent leaves (Fig. 1). This observation was confirmed by experiments showing that DS22 transcripts reach nearly a maximum level in leaves 1 min after the stem was crosscut at the basal position. Almost the same amounts of DS22 transcripts were observed in lower leaves adjacent to the cut stem and in much more distant upper leaves (*10*). Crosscutting of petioles or leaf apex also induced a rapid accumulation of DS22 transcripts (*10*). Thus, the DS22 gene response to wounding is systemic.

The cDNA of DS22 contains a 1725–base pair open reading frame encoding a polypeptide of 375 amino acids with a relative molecular weight of 42,858 (Fig. 2A). The putative amino acid sequence is similar to those of MsERK1, a mitogen-activated protein (MAP) kinase homolog from alfalfa (71% similarity), ATMPK1 from *Arabidopsis* (54%), and ERK2 from rat (46%) (Fig. 2A). The similarity is especially high throughout the 11 conserved kinase domains, and amino acids TEY (Thr, Glu, and Tyr, residues 201 to 203) that are required for MAP kinase activation are well conserved (*11*), which indicates that DS22 is a member of the MAP kinase subfamily. That DS22 encodes a protein kinase was confirmed by autophosphorylation activity of the overexpressed products in a bacterial system (Fig. 2B). We therefore designated

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