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# Leaf Senescence Is Delayed in Tobacco Plants Expressing the Maize Homeobox Gene *knotted1* under the Control of a Senescence-Activated Promoter

**Naomi Ori,[a] Michelle T. Juarez,[a] David Jackson,[b] Judy Yamaguchi,[a,c] Gary M. Banowetz,[d] and Sarah Hake[a,c,1]**

[a] Department of Plant and Microbial Biology, University of California, Berkeley, California 94720
[b] Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724
[c] Plant Gene Expression Center, U.S. Department of Agriculture–Agricultural Research Service (USDA-ARS), 800 Buchanan Street, Albany, California 94710
[d] USDA-ARS, 3450 S.W. Campus Way, Corvallis, Oregon 97331

**Leaf senescence is an active process involving remobilization of nutrients from senescing leaves to other parts of the plant. Whereas senescence is accompanied by a decline in leaf cytokinin content, supplemental cytokinin delays senescence. Plants that overexpress isopentenyl transferase (*ipt*), a cytokinin-producing gene, or *knotted1* (*kn1*), a homeobox gene, have many phenotypes in common. Many of these phenotypes are characteristic of altered cytokinin physiology. The effect of *kn1* on leaf senescence was tested by driving its expression using the promoter of the senescence-associated gene *SAG12*. *SAG*:*kn1* tobacco plants showed a marked delay in leaf senescence but otherwise developed normally. The delay in senescence was revealed by an increase in chlorophyll content in *SAG*:*kn1* leaves relative to leaves of the control plants and by a decrease in the number of dead leaves. Senescence was also delayed in detached leaves of *SAG*:*kn1* plants. Delayed senescence was accompanied by increased leaf cytokinin content in older leaves expressing *kn1*. These experiments extend the current understanding of *kn1* function and suggest that in addition to mediating meristem maintenance, *kn1* is capable of regulating the onset of senescence in leaves.**

## INTRODUCTION

Senescence is considered the final stage in leaf development. It is an active, ordered process that involves mobilization of nutrients from the senescing leaves to other parts of the plant, leading to the eventual death of the leaf (Buchanan-Wollaston, 1997; Gan and Amasino, 1997; Nam, 1997; Nooden et al., 1997; Bleecker, 1998). A number of genes, referred to as senescence-associated genes (*SAG*s), are upregulated in leaves before or during senescence in different species. Some *SAG*s share sequence similarity with genes expected to be involved in the breakdown and mobilization of nutrients, such as proteases, RNases, and glutamine synthetases (Smart, 1994; Buchanan-Wollaston, 1997; Buchanan-Wollaston and Ainsworth, 1997; Gan and Amasino, 1997; Nam, 1997; Weaver et al., 1997, 1998; Lers et al., 1998; Park et al., 1998). The function of other *SAG*s has yet to be determined. The senescence process is likely to be tightly regulated to prevent it from occurring prematurely or in inappropriate tissues, such as meristems and young leaves. Common regulatory elements, however, have not been identified among the promoters of *SAG*s (Buchanan-Wollaston, 1997; Weaver et al., 1997; Bleecker, 1998), and single mutants with impaired senescence have not been isolated (Buchanan-Wollaston, 1997), which implies complex and multiroute control of senescence.

Senescence is delayed when cytokinins are applied exogenously (van Staden et al., 1988) or overproduced in transgenic plants by use of an Agrobacterium cytokinin biosynthesis gene, isopentenyl transferase (*ipt*) (Estruch et al., 1991; Smart et al., 1991; Li et al., 1992). A direct effect of cytokinin on senescence was shown by expression of the *ipt* gene under control of a senescence-specific promoter that directs expression of the Arabidopsis cysteine protease, SAG12 (Gan and Amasino, 1995). This construct was designed to express *ipt* only at the onset of senescence. The resulting transgenic tobacco plants had delayed senescence and produced more flowers with no apparent effect on morphology. Whereas plants expressing a β-glucuronidase (*gus*) construct driven by the *SAG12* promoter accumulated GUS during senescence, GUS did not accumulate in plants expressing both *SAG*:*gus* and *SAG*:*ipt* (Gan and Amasino, 1995), confirming the senescence-specific effect of *SAG*:*ipt*.

[1] To whom correspondence should be addressed. E-mail maizesh@nature.berkeley.edu; fax 510-559-5678.

In addition to the delay in senescence, a number of phenotypes are characteristic of plants expressing *ipt.* Many of these phenotypes are similar to those observed in plants overexpressing the maize homeobox gene *knotted1* (*kn1*), including alterations of leaf shape, loss of apical dominance, and the production of ectopic meristems on leaves (Medford et al., 1989; Estruch et al., 1991; Li et al., 1992; Kano-Murakami et al., 1993; Sinha et al., 1993; Lincoln et al., 1994; Chuck et al., 1996; Hareven et al., 1996; Tamaoki et al., 1997). Other phenotypes resulting from cytokinin overproduction, such as root growth inhibition, have not been reported in plants that overexpress homeobox genes. *kn1* and its homologs from other plant species normally are expressed in shoot meristems (Smith et al., 1992; Kano-Murakami et al., 1993; Jackson et al., 1994; Lincoln et al., 1994; Muller et al., 1995; Hareven et al., 1996; Long et al., 1996) and are essential for meristem maintenance and/or initiation (Endrizzi et al., 1996; Long et al., 1996; Kerstetter et al., 1997).

The wide range of phenotypes characteristic of plants that either overexpress homeobox genes or overproduce cytokinin complicates our interpretation of any correlation between the two, due to the problems associated with distinguishing primary and secondary effects. Here, we studied the similarity of cytokinin and *kn1* responses with respect to their direct effect on leaf senescence in tobacco by driving *kn1* expression via the senescence-specific promoter of the *SAG12* gene (Lohman et al., 1994; Gan and Amasino, 1995; Weaver et al., 1998). The resulting plants exhibited a specific delay in senescence with no other developmental abnormalities. Cytokinin levels were significantly increased in leaves that expressed *kn1.*

## RESULTS

### *SAG*:*kn1* Plants Develop Normally but Are Delayed in Senescence

Tobacco plants overexpressing the *kn1* gene show a wide range of phenotypes (Figure 1), including a marked delay in leaf senescence (data not shown). To study the effect of *kn1* on senescence apart from its other effects, we expressed *kn1* under the control of the senescence-specific promoter of the *SAG12* gene (Lohman et al., 1994; Gan and Amasino, 1995). Nineteen independent transgenic tobacco plants (cv Wisconsin 38; W38) containing the *SAG*:*kn1* construct were generated. Eighteen of the 19 $T_1$ families appeared morphologically normal, except that they showed various degrees of delay in senescence compared with nontransgenic siblings. One plant had additional phenotypes that were nonheritable. $T_2$ plants of two representative homozygous *SAG*:*kn1* lines, lines 16 and 18, were selected for detailed analysis and compared with wild-type (W38) and *SAG*:*ipt* plants. The overall morphology of *SAG*:*kn1* plants, including leaf development and flowering time, appeared similar to that of wild-type and *SAG*:*ipt* plants (data not shown).

*SAG*:*kn1* plants showed a marked delay in senescence. Older leaves of a 5-month-old *SAG*:*kn1* plant are compared in Figure 2 with a control W38 plant of the same chronological age. When the older leaves of the control plant began to senesce, similarly positioned leaves of *SAG*:*kn1* plants were still green. To quantify this effect, we counted the number of dead leaves on 3- to 5-month-old *SAG*:*kn1* and control plants every 1 to 3 weeks. For comparison, we also counted the number of dead leaves of *SAG*:*ipt* plants grown under the same conditions. Figure 3 shows the number of dead leaves for control W38, plant lines 16 and 18 carrying SAG:*kn1*, and *SAG*:*ipt* plants. Leaves of both *SAG*:*kn1* and *SAG*:*ipt* plants senesced at a lower rate than did those of the controls.

To further characterize the delayed senescence of *SAG*:*kn1* plants, we performed a leaf-detachment assay with control and *SAG*:*kn1* plants. Green healthy leaves of comparable age were detached from the plants, and their petioles were placed in water. Figures 2C and 2D provide a comparison of mature leaves from control W38 and plant line 18 carrying *SAG*:*kn1* 2 and 5 weeks after detachment. Starting 2 to 10 weeks after detachment, depending on the age of the leaves when detached, control leaves gradually senesced until complete necrosis occurred. *SAG*:*kn1* leaves,

**Figure 1.** Overexpression of *kn1* in Tobacco.

35S:*kn1* plants are characterized by a reduction in leaf and plant size, altered leaf shape, loss of apical dominance, delay in senescence, and formation of ectopic meristems. At left is a leaf from a nontransformed tobacco plant; at right is a 35S:*kn1* branch.

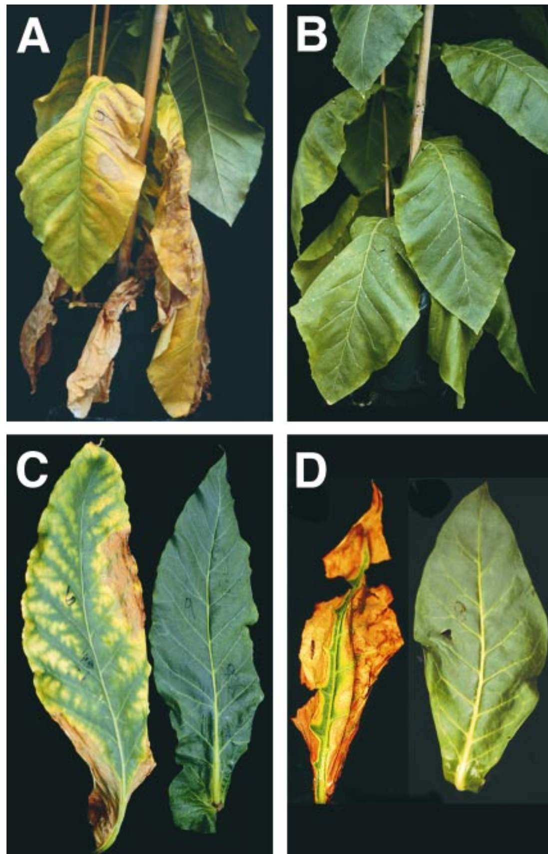


**Figure 2.** Delay in Senescence of Intact and Detached Leaves of *SAG*:*kn1*–Transformed Tobacco Plants.

**(A)** The lower portion of a control W38 plant at 5 months.
**(B)** The lower portion of a *SAG*:*kn1* plant from line 16 at 5 months.
**(C)** Detached leaves of a control plant (left) and a *SAG*:*kn1* plant from line 18 2 weeks after detachment.
**(D)** Detached leaves of a control plant (left) and a *SAG*:*kn1* plant from line 18 5 weeks after detachment.
**(C)** and **(D)** show results from different experiments, and the leaves were detached from different plants. Fully expanded green healthy leaves were used in both cases.

however, maintained their green color and turgor for at least 10 weeks longer than did those of the controls.

As a measure of the photosynthetic capacity of the plants, we measured the chlorophyll content in successive leaves of *SAG*:*kn1*, *SAG*:*ipt*, and control plants. Figure 4 shows the increase in chlorophyll in a representative individual from plant line 18, carrying *SAG*:*kn1*, compared with a control. The total chlorophyll content (chlorophyll *a* plus chlorophyll *b*) is similar between control and *SAG*:*kn1* young leaves at the top of the plant (leaf 19). However, in older leaves (leaves 1 to 17), the chlorophyll content is much lower in control leaves than in *SAG*:*kn1* leaves. Similar results were obtained with two repeats each of plant lines 16 and 18 carrying *SAG*:*kn1* as well as with *SAG*:*ipt* plants (data not shown). These data further establish that the delay in senescence is the result of *kn1* or *ipt* expression.

## Levels of *kn1* Transcript in *SAG*:*kn1* Plants

To verify that the expression of *kn1* under control of the *SAG12* promoter was restricted to leaves that were programmed to senesce, we performed RNA gel blot analysis by using the *kn1* cDNA as a hybridization probe. The developmental stages used are schematically illustrated in Figure 5A. In this figure, the terms "old," "mature," and "young" designate developmental stages. No transcript was detected in leaves of any developmental stage from control plants or in *SAG*:*kn1* leaves at developmental stages in which the control leaves were still green (mature and young), as shown in Figure 5B. As expected for the *SAG12* promoter (Gan and Amasino, 1995), the *kn1* transcript was only detected in leaves of *SAG*:*kn1* plants at the chronological age during which leaves on control plants had begun to senesce (old). Because *SAG*:*kn1* expression blocked senescence, these leaves were still green.

## Increased Levels of Cytokinin in *SAG*:*kn1* Plants

The similarity between the effects of *SAG*:*kn1* and *SAG*:*ipt* on senescence raised the possibility that *SAG*:*kn1* delayed senescence by altering cytokinin physiology. To test this possibility, we compared the cytokinin content of *SAG*:*kn1* and control plants at two stages, old and young (see Figure 5). Figure 6 shows that "old" leaves, expressing *kn1* from the

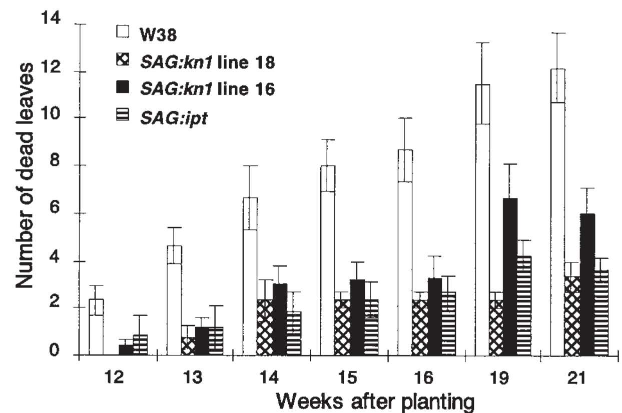


**Figure 3.** Effect of *SAG*:*kn1* and *SAG*:*ipt* on Leaf Senescence.

The number of dead leaves from four to six plants was counted at different times after germination. Plant lines 16 and 18 carrying *SAG*:*kn1* were compared with *SAG*:*ipt* and the control, W38.

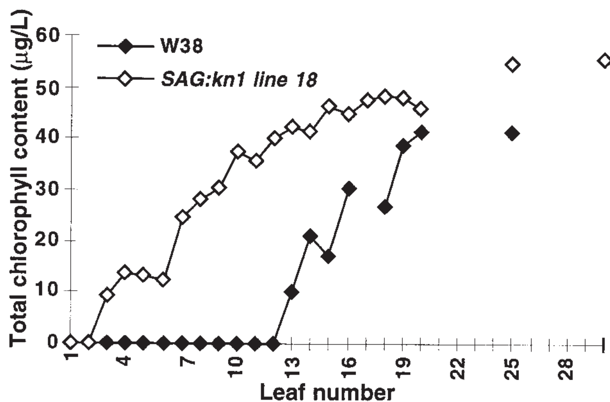


**Figure 4.** Higher Chlorophyll Content in *SAG:kn1* Plants.

Total chlorophyll (chlorophyll *a* plus chlorophyll *b*) was measured for five 1.2-inch-diameter leaf disks in control (W38) and *SAG:kn1* plants from line 18. Leaves are labeled such that leaf 1 is the oldest.

*SAG12* promoter, had ~15-fold higher cytokinin levels relative to leaves from control plants. The increase was primarily from zeatin riboside–type cytokinins (zeatin, dehydrozeatin, and their respective 9-ribosides), with some contribution from isopentenyl adenine and adenosine (Table 1). Cytokinin levels were ~1.5-fold higher in younger leaves of *SAG:kn1* plants before the accumulation of *kn1* transcript.

## DISCUSSION

We have used a senescence-activated promoter (Gan and Amasino, 1995) to demonstrate a specific inhibiting effect of the homeobox gene *kn1* on leaf senescence. Tobacco plants harboring the *SAG:kn1* construct developed normal morphology but had delayed senescence in both intact and detached leaves. Cytokinin levels were significantly raised in leaves of *SAG:kn1* plants that expressed *kn1*, suggesting that the delay in leaf senescence may be mediated through changes in cytokinin metabolism.

### Relationship of *kn1* to Senescence

*SAG*s have been isolated from several plant species. These *SAG*s have been grouped by their timing of expression relative to the onset of senescence or by their functions (Buchanan-Wollaston, 1997; Nam, 1997; Weaver et al., 1997). Some *SAG*s are similar to genes expected to be directly involved in senescence, such as genes coding for proteases and RNases, but none appears to be a transcriptional regulator. It is striking, therefore, that *kn1*, presumably a transcription factor, specifically delays senescence. Is *kn1* naturally involved in the negative regulation of senescence? *kn1* and members of this large gene family (Kerstetter et al., 1994; Burglin, 1997) are expressed to high levels in meristems and unexpanded stems but have not been detected in leaves. Therefore, if *kn1* negatively regulates senescence in leaves, very low levels of *kn1* are required.

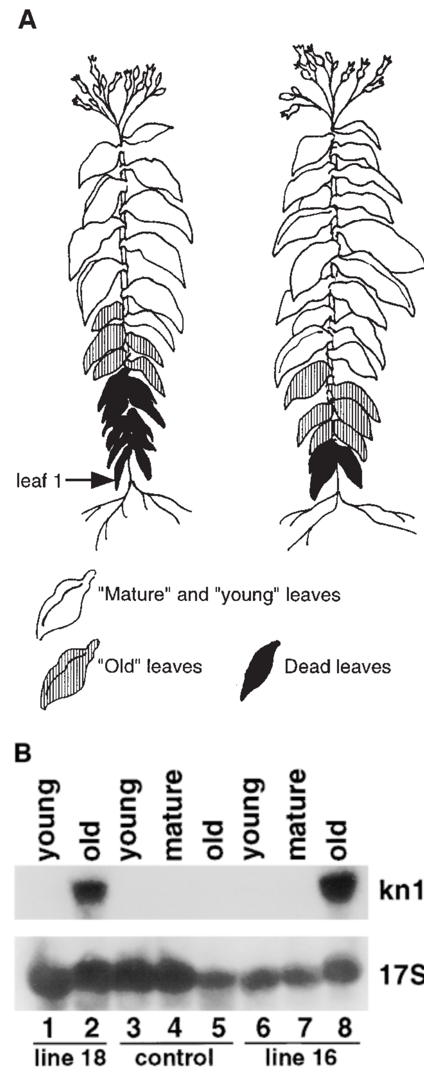


**Figure 5.** Accumulation of *kn1* RNA in Leaves Induced to Senesce in *SAG:kn1* Plants.

**(A)** Diagram of control versus *SAG:kn1* tobacco plants illustrating representative leaf developmental stages. The terms "old," "mature," and "young" refer to the developmental status of the leaf rather than to its chronological age. "Young" denotes green young leaves; "mature," mature green and healthy leaves; "old," oldest green leaves in controls and oldest leaves in *SAG:kn1*.

**(B)** RNA gel blot. Ten micrograms of total RNA from leaves of developmental stages young (lanes 1, 3, and 6), mature (lanes 4 and 7), and old (lanes 2, 5, and 8) of control plants (lanes 3 to 5) and *SAG:kn1* lines 18 (lanes 1 and 2) and 16 (lanes 6 to 8) was loaded. The blot was hybridized with the *kn1* probe and with an rRNA (17S) probe.

**Figure 6.** Cytokinin Content Is Increased in *SAG*:*kn1* Plants.

Total cytokinins (see Table 1) are presented as nanograms per gram dry weight (DW) for control plants and two independent *SAG*:*kn1* transgenic lines for two different developmental stages, old and young (Figure 5A). *SAG*:*kn1* old leaves were at the stage during which *kn1* transcripts accumulated. Control old leaves were the oldest green leaves. Young leaves of control and *SAG*:*kn1* plants were the same age.

Is the mechanism by which *kn1* delays senescence similar to its normal function in the meristem? Both gain- and loss-of-function phenotypes in maize support a role for *kn1* in repressing differentiation. *kn1* loss-of-function mutants in the B73 inbred background have fewer branch meristems and extra determinate organs (Kerstetter et al., 1997). In other inbred backgrounds, *kn1* recessive mutants fail to progress beyond the production of the cotyledon (E. Vollbrecht and S. Hake, unpublished data). These phenotypes suggest that one of the roles of *kn1* is to maintain populations of indeterminate cells in which differentiation is delayed or inhibited. In maize leaves harboring dominant *Kn1* mutations, the ectopic expression of *kn1* causes cells of the leaf blade, the flat, distal portion of the leaf, to adopt leaf sheath cell fates. Because the proximal sheath portion of the leaf differentiates after the blade portion, the blade-to-sheath conversion is thought to result from a delay in differentiation (Freeling and Hake, 1985; Hake et al., 1995). Given that senescence is considered the final stage of leaf development (Buchanan-Wollaston, 1997; Gan and Amasino, 1997) and the inhibition of differentiation by *kn1*, it is possible that the delay in senescence and meristem maintenance are mediated by similar mechanisms. In other words, *kn1* expression blocks developmental progression.

The effect of *kn1* on leaf cells clearly depends on their developmental stage. Cells in a leaf primordium respond to ectopic *kn1* expression by changing cell fate and rates of division, resulting in striking morphological alterations to leaf form (Lincoln et al., 1994; Sinha and Hake, 1994; Hake et al., 1995). In contrast, we show here that cells in a fully mature leaf respond to *kn1* by a delay in senescence with no visible morphological change. Alternate fates, depending on timing of expression, have also been demonstrated for overexpression of the Antirrhinum transcription factor MIXTA (Glover et al., 1998). Expression of MIXTA in cells that were actively dividing resulted in an excess of trichomes, whereas expression in cells after cell cycle arrest resulted in a change in cell shape.

Certain *SAG*s show differential expression between naturally occurring and artificially induced senescence (Becker and Apel, 1993; Park et al., 1998; Weaver et al., 1998). *SAG12* is strongly upregulated by natural senescence at a stage at which the leaf begins to yellow. The promoter is weakly upregulated in leaves that are detached from the plant (Weaver et al., 1998). In the experiments reported here, we tested natural senescence as well as senescence induced by detachment of leaves in the light. *SAG*:*kn1* delays senescence in both systems. Interestingly, the senescence-delaying effect of *SAG*:*kn1* plants was more striking in the detached leaf assay than in naturally senescing intact plants. In view of the weaker induction of the *SAG12* promoter by leaf detachment, lower levels of *kn1* may act more efficiently to delay senescence than do higher levels. Alternatively, detachment per se may increase the effectiveness of *kn1* by reducing inhibitory activities that are specific for *kn1.*

**Table 1.** Cytokinin Content[a] in Control (W38) and *SAG*:*kn1* Plants

| Leaf Age | Genotype[b] | Total ZR[c] | P[d] | Total IPA[c] | P[d] | ZR+IPA[e] | P[d] |
|---|---|---|---|---|---|---|---|
| Old[f] | W38 | 1.14 ± 0.43 | | 1.07 ± 0.28 | | 2.21 ± 0.6 | |
| | *SAG*:*kn1* line 16 | 25.07 ± 0.96 | $4.8 \times 10^{-7}$ | 9.51 ± 2.31 | 0.01 | 34.58 ± 2.69 | $2.3 \times 10^{-5}$ |
| | *SAG*:*kn1* line 18 | 23.67 ± 1.45 | $3.4 \times 10^{-5}$ | 4.81 ± 2.64 | 0.09 | 30.34 ± 2.23 | $6.7 \times 10^{-5}$ |
| Young[f] | W38 | 5.22 ± 0.82 | | 0.83 ± 0.32 | | 6.05 ± 0.60 | |
| | *SAG*:*kn1* line 16 | 9.93 ± 0.86 | 0.008 | 1.22 ± 0.35 | 0.45 | 11.14 ± 0.88 | 0.003 |
| | *SAG*:*kn1* line 18 | 7.46 ± 2.08 | 0.28 | 1.98 ± 1.98 | 0.43 | 9.44 ± 4.06 | 0.27 |

[a] Cytokinin content in nanograms per gram dry weight of tissue.
[b] The number of samples was four for W38 and for *SAG*:*kn1* plant lines 16 and 18.
[c] Cytokinin content is expressed as zeatin riboside (ZR) or isopentenyl adenosine (IPA) equivalents based upon reactivity of HPLC-purified fractions with anti-ZR and anti-IPA monoclonal antibodies in an immunoassay.
[d] P is ($T \leqslant t$) for two-tail *t* test, assuming equal variances.
[e] ZR, zeatin riboside-type; IPA, isopentenyl adenine and adenosine.
[f] See text for definition of "old" and "young" leaves.

### *kn1* and Cytokinin

The similar effect of *SAG*:*ipt* and *SAG*:*kn1* on senescence adds to previous observations showing that misexpression of both *kn1* and *ipt* early in development can reduce leaf size, alter leaf shape, reduce apical dominance, and promote ectopic meristem formation (Figure 1; Estruch et al., 1991; Li et al., 1992; Sinha et al., 1993; Chuck et al., 1996; Williams-Carrier et al., 1997). A possible interpretation of these similarities is that *kn1* induces cytokinin production. Ectopic expression of *kn1* homologs from tobacco and rice has been shown to alter the levels of several plant hormones, including an elevation in cytokinin (Tamaoki et al., 1997; Kusaba et al., 1998). These changes in hormone levels, however, could be secondary; therefore, they do not imply a direct effect of *kn1* on cytokinin production.

In this study, *SAG*:*kn1* plants contained higher cytokinin levels than did control plants. Cytokinin levels were 15-fold higher than controls in older leaves, in which *kn1* transcripts accumulated, and 1.5-fold higher in young green leaves, in which *kn1* transcripts could not be detected. Notably, the cytokinin levels in old leaves of *SAG*:*kn1* plants, which were expressing *kn1*, were higher than in young leaves, whereas in control plants, the cytokinin levels were higher in young leaves than in old leaves. The striking increase in cytokinin content in old leaves that accumulate *kn1* transcripts suggests that *kn1* affects senescence through a specific effect on cytokinin accumulation.

We also explored the possibility that elevated cytokinin levels can induce *kn1* transcription by isolating a probe for *NTH15*, a *kn1* homolog in tobacco that has an expression pattern similar to that of *kn1* (Tamaoki et al., 1997). Using both reverse transcription–polymerase chain reaction and RNA gel blot analysis, we were unable to detect *NTH15* expression in *SAG*:*ipt* plants upon leaf senescence in detached leaves, but we were able to detect it in tobacco apices (data not shown). This finding does not rule out cytokinin-mediated induction of *kn1* homologs that play other biological roles. Because *kn1* mRNA and cytokinin normally accumulate in shoot apices (Smith et al., 1992; Jackson et al., 1994; Letham, 1994), a cause and effect relationship between the presence of *kn1* and cytokinin has been difficult to definitively determine. Only by placing *kn1* expression in this novel context—the senescing leaf—can such a correlation be made. Additional experiments are required to determine the extent to which this effect is direct; nonetheless, it is significant that expression of a transcription factor that functions in the meristem results in a specific accumulation of cytokinin.

The accumulation of cytokinin in younger leaves is clearly secondary to the expression of *kn1* and could represent cytokinin transport from older to younger leaves. Gan and Amasino (1995), however, did not observe a delay in senescence in control plants that were grafted to *SAG*:*ipt* plants. Alternatively, a signal from leaves that are expressing *SAG*:*kn1* may result in the higher cytokinin content observed in younger leaves. Measurement of leaf cytokinin content in *SAG*:*kn1* plants before the induction of the *SAG* promoter would help address this possibility. It is also possible that young *SAG*:*kn1* leaves express low levels of *kn1*, which were not detected in the RNA gel blots but were sufficient to induce some cytokinin accumulation in young leaves.

Although we have demonstrated that *kn1* expression causes an elevation of cytokinin in *SAG*:*kn1* plants, differences in the effects of overproduction of *kn1* and cytokinin demonstrate that these regulators are also controlling independent processes. For example, *SAG*:*ipt* inflorescence branches were thicker in diameter than were the branches of *SAG*:*kn1* or control plants (data not shown). In addition, neither 35S:*kn1*–expressing Arabidopsis plants nor 35S:*kn1*–expressing tobacco plants have obvious root inhibition phenotypes (data not shown), whereas excessive cytokinin prevents roots from forming.

Identification of transcriptional targets of *kn1* may help investigations of the intriguing and complex relationship between *kn1* and cytokinin. Recent progress in the identification of putative cytokinin receptors, and of genes that are rapidly induced by cytokinin (Kakimoto, 1996; Brandstatter and Kieber, 1998), will also provide important tools. Equally important will be the use of tissue-specific and inducible promoters to separate primary and secondary effects.

## METHODS

### Nucleic Acid Experiments

An NcoI (partial)-XbaI fragment containing the *knotted1* (*kn1*) cDNA (Vollbrecht et al., 1991), starting at the first ATG, was cloned downstream of a 2.18-kb fragment of the *SAG12* promoter (Gan and Amasino, 1995), followed by a nopaline synthase terminator. The *SAG12* promoter and *SAG*:*ipt* seed were kindly provided by R. Amasino (University of Wisconsin, Madison). The *SAG*:*kn1* plasmid was transformed into tobacco plants (*Nicotiana tabacum* cv Wisconsin 38) by *Agrobacterium tumefaciens*–mediated transformation, using the Agrobacterium strain LBA4404.

An EcoRI fragment of the *kn1* cDNA clone (Vollbrecht et al., 1991) was used to probe the RNA gel blots. A rice 17S rDNA probe, kindly provided by A. Theologis (Plant Gene Expression Center), was used as a loading control for RNA gel blots. Reverse transcription–polymerase chain reaction was performed with 5 $\mu$g of total RNA from tobacco (cv Wisconsin 38) plants to obtain the *NTH15* probe (Tamaoki et al., 1997). The polymerase chain reaction product was cloned into the T.A. cloning vector (Invitrogen, Carlsbad, CA) and used to probe RNA gel blots.

### Plant Material and Senescence Measurements

Tobacco plants were grown in the greenhouse, and leaves were labeled from oldest (number 1) to youngest. At 1- to 3-week intervals, the number of dead leaves was counted on four to six individual homozygous $T_2$ progeny of each independent transformant and averaged. A leaf was considered dead when it was brown throughout and

brittle. Chlorophyll levels were measured (Moran and Porath, 1980) and calculated (Inskeep and Bloom, 1985) on 1.2-cm-diameter leaf discs collected into 2 mL of *N,N*-dimethylformamide. Two repeats were averaged from each leaf sampled. Similar results were obtained with two different individual $T_2$ progeny of each original transformant and with controls.

### Leaf Detachment

Fully expanded, green, healthy leaves were detached and placed in a beaker with only the petiole in the water. The leaves were maintained in a growth room with 16-hr-light and 8-hr-dark periods at 24°C.

### Cytokinin Measurements

Leaves were removed from the plants, frozen, and lyophilized. Leaf tissue (∼100 mg per sample) was ground in liquid nitrogen, and cytokinins were extracted by using 100% methanol for 30 min. After 9 volumes of 40 mM ammonium acetate, pH 6.5, were added to the extracts, cytokinins were isolated on C18 SepPaks (Waters Associates, Bedford, MA), purified, and quantified using a previously described combined HPLC–immunoassay method (Banowetz, 1992). The immunoassay utilized monoclonal antibodies tZR3 (Trione et al., 1985) and IPA3 (Trione et al., 1987) that were prepared against the ribosides of zeatin and isopentenyl adenosine, respectively. Triplicate measurements of each HPLC-purified cytokinin were performed, and cytokinins were quantified in independent replicate samples of young and old leaves from each genotype. These antibodies are reactive with free bases, 9-glucoside and 9-ribosides of the respective cytokinin (and in the case of tZR3, the dihydro-derivatives). The 9-glucoside contents are not reported here because they are relatively inactive in bioassays (Letham et al., 1983). All cytokinin quantities are expressed as zeatin riboside or isopentenyl adenosine equivalents to reflect the fact that the antibodies were prepared against the cytokinin ribosides.

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