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Rapid Communication

Demonstration of a Senescence Component in the Regulation of the Mannopine Synthase Promoter

Virginia M. Ursin* and C. Kimball Shewmaker

Calgene Inc., 1920 Fifth Street, Davis, California 95616

Regulation of the mannopine synthase (*mas*) promoter during senescence in leaves and flowers of tobacco (*Nicotiana tabacum*) plants was investigated. In plants transformed with a *mas* 5′-β-glucuronidase (GUS)-*mas* 3′ transcriptional fusion, we observed that following the onset of senescence in either intact or excised leaves of the transgenic lines, GUS activity increased significantly, whereas in excised leaves in which the senescence process was inhibited, GUS activity increased only marginally. During flower petal senescence in the transgenic tobacco, GUS activity increased approximately 6-fold over preanthesis- and anthesis-stage flowers.

Senescence in plants has been defined as those processes that follow physiological maturity and lead to the death of the whole plant, organ, tissue, or cell (Watada et al., 1984), and it represents a highly controlled sequence of biochemical and physiological events. At the biochemical level, early events in the onset of senescence include a decrease in the expression levels of nuclear and plastid-encoded genes (Brady, 1988), the expression of new gene products (Meyer et al., 1991), proteolysis (Peoples and Dalling, 1988), membrane destabilization (Thompson, 1988; Borochov and Woodson, 1989), and fluctuations in hormone levels (Even-Chen et al., 1978). At the physiological level, senescence results in the yellowing of leaves due to Chl loss (for a review, see Thomas and Stoddard, 1980) and the curling and wilting of flower petals due to loss of turgor (Borochov and Woodson, 1989).

The ability to modify the onset or progression of senescence in plants requires both the identification of gene products capable of reversing or halting the cascade of events occurring during senescence and suitable promoters for expressing genes at appropriate points spatially and temporally during plant development. Promoters from the opine-producing T-DNA genes of *Agrobacterium tumefaciens* carry transcriptional control elements that confer regulatory properties that make them useful for expressing a variety of genes in plants (see Langridge et al., 1989; An et al., 1990; Comai et al., 1990; Saito et al., 1991, and refs. therein). Specifically, the *mas* promoter from the T_R-DNA region has been demonstrated to be under various levels of tissue-specific, hormonal, and stress-related regulation when stably integrated into the plant genome. In this study, we have utilized a *mas* 5′-GUS-*mas* 3′ transcriptional fusion to investigate the regulation of *mas* during the process of senescence in plants.

MATERIALS AND METHODS

Production of Transgenic Plants

Construction of pCGN7001, the binary plasmid containing the *mas* 5′-GUS-*mas* 3′ expression cassette, has been described previously (Comai et al., 1990). Briefly, the *mas* 5′ is from nucleotide 20,806 to nucleotide 20,128 and the *mas* 3′ is from nucleotide 19,239 to nucleotide 18,474 of the *mas* gene (Barker et al., 1983), and the GUS gene is from the *Bam*HI to *Sac*I fragment of pBI221 (Jefferson et al., 1987). Construction of pCGN7320, the binary plasmid containing double 35S-GUS-*mas* 3′, has been previously described (Comai et al., 1990). Tobacco (*Nicotiana tabacum*) transformation was performed as previously described (Ursin et al., 1991), and the resulting transgenic tobacco plants were screened for GUS expression (L. Comai, personal communication). Two GUS-positive lines, pCGN7001-1 and -4, were used for the analysis presented herein, and representative data are presented.

Analysis of GUS Activity in Transgenic Plants

Flurometric analysis of GUS activity was performed essentially as described by Jefferson et al. (1987). The leaf disc assay was performed as previously described (Ursin et al., 1991). Flower petals were harvested at the appropriate stage and immediately frozen in liquid N_2 or harvested at anthesis and placed in a beaker with distilled water until senescence was well advanced (approximately 4 d postanthesis, and frozen as above). Flowers in which pollen had been shed were washed extensively in sterile, distilled water before freezing to remove virtually all residual pollen grains. All samples were analyzed for GUS activity as previously described (Ursin et al., 1991). The experiment was repeated twice, and data from a representative experiment are shown.

Determination of Chl Content

The relative Chl content of leaf discs was determined as described (Osborne and McCalla, 1961). Individual leaf discs were incubated at 65°C in 80% ethanol until all of the green

* Corresponding author; fax 1–916–753–1510.

Abbreviations: GUS, β-glucuronidase; *mas*, mannopine synthase.

pigment was extracted (approximately 6 h). Aliquots of the extract were analyzed spectrophotometrically at 665 nm, the wavelength for maximum absorption of Chl *a*.

RESULTS AND DISCUSSION

From mature, preflowering tobacco plants containing the *mas*-GUS fusion plasmid, pCGN7001, a developmental series of leaves, ranging from young, unexpanded leaves near the plant apex to fully expanded and senescent leaves near the plant base, were analyzed. A basipetal gradient of GUS activity was observed, with approximately 30-fold more activity detected in the oldest leaves than in the youngest leaves (Fig. 1A). This pattern of GUS accumulation was in striking contrast to that observed when either the double-cauliflower mosaic virus 35S (Fig. 1B) or elongation factor-1α (Ursin et al., 1991) promoters were used to express GUS in developing tobacco leaves under identical conditions. This addresses the notion that the relative stability of the GUS protein in aging leaves, rather than transcriptional regulation, accounts for this accumulation, arguing strongly for the latter. These results are consistent with observations from previous studies utilizing several reporter genes, including GUS (Comai et al., 1990), *LuxA* and *LuxB* (Langridge et al., 1989), and *NptII* (Sanger et al., 1990), where *mas* was found to be expressed at higher levels in older tissues than in younger tissues.

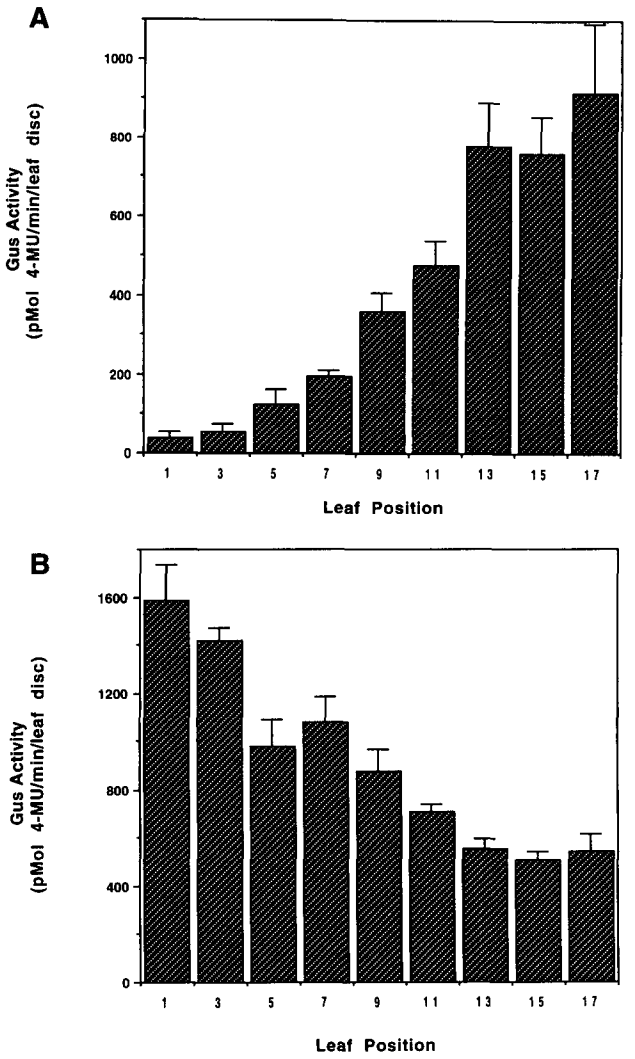

Figure 1. GUS activity in developing leaves of transgenic tobacco. Leaf discs from growth chamber-grown plants at the 20-leaf stage were analyzed for GUS activity. Leaves are numbered sequentially beginning at the plant apex. Each data point represents the average of four replicate samples per leaf assayed. Error bars represent the SE. A, *mas*-GUS; B, double-cauliflower mosaic virus 35S-GUS.

The above data indicate that there is an age- or position-dependent component to the regulation of the *mas* promoter, but do not fully address whether the senescence process per se can signal increased expression of *mas*. To examine the regulation of *mas* independently from leaf position on the plant, fully expanded leaves from approximately the same node of several plants were excised and incubated either in the dark to accelerate senescence or in the light to inhibit senescence (Thomas and Stoddard, 1980), and sampled for GUS activity at intervals spanning 9 d. Chl levels, a convenient indicator of senescence (Mothes, 1964), declined significantly over the 9-d period in the dark-incubated leaves but declined only marginally in the leaves kept in the light (Fig. 2A). Visually, the leaves kept in the dark appeared bright yellow, indicative of Chl degradation (not shown). Concomitant with the depletion of Chl, GUS activity in the dark-incubated leaves rose steadily over the 9-d incubation period, reaching levels approximately 15-fold higher than the initial levels (Fig. 2B). In contrast, leaves that were incubated in the light showed only a slight increase in GUS activity, reaching levels less than half that of the GUS activity of the dark-treated leaves after the 9 d (Fig. 2B). These results indicate that, specifically during leaf senescence, expression of *mas* is significantly enhanced. The small increase in GUS activity observed in the light-incubated leaves may be attributable to a much reduced but nonetheless active senescence process occurring in the light following leaf detachment, to chronological aging of the leaf, or to wounding of the leaf tissue by the sampling process.

In addition to leaves, flower petals also undergo a programmed senescence that is evident by a wilting of the flower petals, which eventually become brown, dry, and brittle. We analyzed GUS activity in flower petals during four stages of flower development (Fig. 3A). We observed that, in general, the level of GUS activity in flower petals was lower than that in leaves. In the first three flower stages analyzed, during which flowers were 4 d preanthesis, 2 d preanthesis, and at anthesis, respectively, GUS activity levels were approximately equivalent, but following the onset of senescence, approximately 4 d postanthesis (stage 4), GUS activity in the flower petals increased approximately 6-fold (Fig. 3B). When flowers were excised from the plants either before or at anthesis and allowed to senesce, a similar increase in GUS activity was observed (data not shown).

Regulation of the *mas* promoter has been found to be both induced and repressed during different phases of vegetative and reproductive growth and inducible following wounding, stress, and hormone treatment (Langridge et al., 1989; Comai et al., 1990; Saito et al., 1991). This is the first report on the specific expression of *mas* during the process of senescence.

Biochemically, a clear relationship between stresses such as wounding and senescence has not been demonstrated. However, it appears that events occurring during the senescence process in leaves and flowers are markedly analogous to those that occur following wounding. For example, early in the process of leaf and flower senescence and following wounding, there is a progressive loss of membrane integrity, resulting in a release of fatty acids and an increase in lipoxygenase activity and lipid peroxidation (Kahl, 1982; Thompson, 1988; Borochov and Woodson, 1989). Thus, it is possible that there are common biochemical signals responsible for triggering the wound response and the senescence response of *mas*. The senescence regulation of the *mas* promoter may make this promoter useful in defining specific gene products or metabolites that are involved in the senescence process.

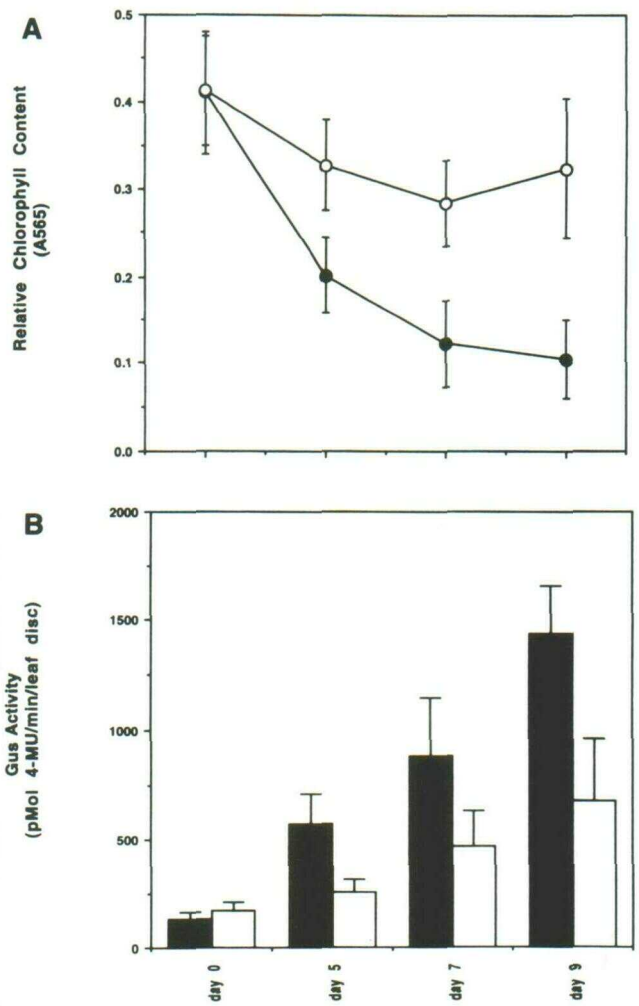

Figure 2. GUS activity during senescence of excised leaves from transgenic tobacco. A, Relative Chl content of leaf discs from leaves incubated in the light (O) or in the dark (●). Each data point represents the average of two leaf discs from each of 10 leaves sampled per time point. Error bars represent the SE. B, GUS activity levels in leaf discs from the above-described leaves. Leaves incubated in the light are indicated by open bars, leaves incubated in the dark are indicated by the closed bars. Each data point represents the average of two samples from each of 10 leaves per time point. Error bars represent the SE.

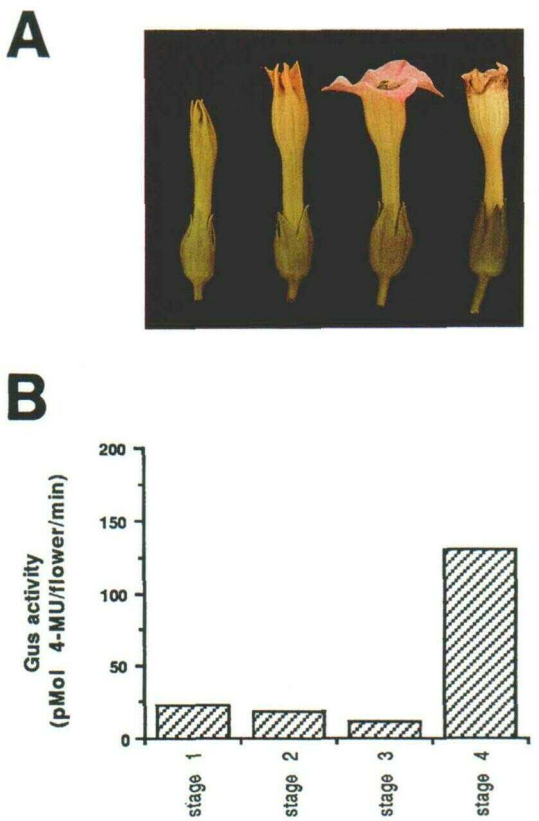

Figure 3. GUS activity during the development and senescence of transgenic tobacco flowers. A, Developing tobacco flowers at the four stages analyzed for GUS activity. At stage 1, flowers were approximately 4 d preanthesis; at stage 2, flowers were approximately 2 d preanthesis; at stage 3, flowers were anthesis-stage; at stage 4, flowers were approximately 4 d postanthesis. B, Gus activity levels in flower petals from the four stages indicated. Bars represent the average activity per flower petal from three to four flowers.

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