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Distinct Phenotypes Generated by Overexpression and Suppression of *S*-Adenosyl-L-Methionine Synthetase Reveal Developmental Patterns of Gene Silencing in Tobacco

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S-Adenosyl-L-methionine synthetase (SAM-S) catalyzes the conversion of L-methionine and ATP into S-adenosyl-L-methionine. Tobacco plants that were transformed with a construct allowing high transcription levels of an Arabidopsis *sam-s* gene could be grouped into two main classes based on their morphology. One class developed yellow-green leaves and had high SAM-S activity and transgene mRNA levels, whereas the other class was stunted and had leatherlike leaves, very low SAM-S activity, and suppressed mRNA level of the transgene. Because both overexpression and silencing of transgene expression led to distinct, abnormal phenotypes, the developmental pattern of transgene silencing was visualized. In the lower leaves, the suppressed phenotype was associated with the veins. In successive leaves, the area of the suppressed tissue increased until all newly developed leaves displayed the suppressed phenotype. In this study, a hypothesis is presented for this developmental gene silencing. Furthermore, transgenic plants with suppressed SAM-S activity had a characteristic smell, a consequence of the accumulation of L-methionine that is converted into the volatile methanethiol.

INTRODUCTION

S-Adenosyl-L-methionine synthetase (SAM-S; EC 2.5.1.6) catalyzes the conversion of ATP and L-methionine into *S*-adenosyl-L-methionine (SAM). SAM is the major methyl group donor for numerous transmethylation reactions and is second to ATP as the most abundant cofactor in metabolic reactions in both prokaryotes and eukaryotes (for a review, see Tabor and Tabor, 1984a). After decarboxylation, SAM serves as a propylamine group donor in the biosynthesis of polyamines (Tabor and Tabor, 1984b). Furthermore, in plants, SAM is a precursor in the biosynthesis of ethylene (Yang and Hoffman, 1984) and serves as an effector in the methionine biosynthesis by allosteric stimulation of threonine synthase (Madison and Thompson, 1976; Aarnes, 1978; Giovanelli et al., 1984) and by feedback inhibition of aspartate kinase (Frankard et al., 1991).

In our laboratory, two genes from Arabidopsis, *sam1* and *sam2*, encoding SAM-S have been cloned (Peleman et al., 1989a, 1989b). Both genes are highly expressed in callus, stems, and roots, but to a lesser extent in leaves. Analysis of a promoter–β-glucuronidase (*gus*) fusion in transgenic Arabidopsis and tobacco plants revealed that the *sam1* promoter confers expression preferentially in vascular tissue (Peleman et al., 1989a, 1989b). This result was unexpected because SAM-S is an important housekeeping enzyme. One hypothesis to explain this result is based on the assumption that the expression of the gene is strictly regulated according to the need for SAM. Vascular tissues undergoing lignification require considerable amounts of SAM for the biosynthesis of lignin monomers. To investigate the consequences of a constitutive overproduction of SAM-S in plants, tobacco was transformed with a chimeric construct containing the 35S promoter of the cauliflower mosaic virus (P35S) coupled to the coding sequence of the Arabidopsis *sam1* gene.

In a number of experiments, transgenes have been introduced in the sense orientation into plants, and it was observed that in some transgenic plants expression of both the transgene and the homologous endogenous gene was silenced. This phenomenon has been called cosuppression (reviewed by Jorgensen, 1991; Kooter and Mol, 1993; Flavell, 1994). In a number of cases, it has been demonstrated that cosuppression is developmentally regulated. Hart et al. (1992) showed that *Nicotiana sylvestris* plants, transformed with a P35S–chitinase gene, fell into three classes: those with uniformly high levels of chitinase in different leaves, those with uniformly low levels in different leaves, and those with variable levels in different leaves. In the latter class, the lower leaves contained high levels of chitinase, whereas the top leaves contained low levels. It has been shown by Smith et al. (1990) that expression of a transgenic polygalacturonase gene and the homologous endogenous gene was cosuppressed, but only in ripe fruit tissue where the endogenous polygalacturonase gene is highly expressed. de Carvalho et al. (1992) have observed high levels

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of β-1,3-glucanase transgene expression early in development, whereas expression of the transgene was silenced at later stages.

In the cases mentioned above, the spatial and temporal occurrence of the switch between the overexpression and the silenced state is difficult to study because no phenotypic differences have been observed between the wild-type, overexpressed, and silenced states. In the cases where petunia was transformed with a chalcone synthase or a dihydroflavonol reductase gene, the spatial distribution of both states was visualized, but only in flowers (Napoli et al., 1990; van der Krol et al., 1990). Cosuppression of chalcone synthase or dihydroflavonol reductase genes results in the development of white sectors on otherwise colored petals.

In this article, we show that tobacco plants, transformed with a chimeric gene designed to overproduce SAM-S, can be grouped into two main classes: those that have higher and those that have lower SAM-S activity compared to that of wild-type plants. Plants of the first class expressed the transgene at a high level, whereas expression of the transgene in plants of the second class was developmentally silenced. Importantly, because both classes displayed distinct, abnormal phenotypes, the developmental pattern of *sam-s* gene silencing was revealed in the second class. Moreover, plants with reduced SAM-S activity had a characteristic smell, a direct result of the accumulation of L-methionine.

RESULTS

Analysis of *sam1* mRNA Levels in the Primary Transformants

To investigate the consequence of overproducing SAM-S in plants, a T-DNA vector (pO35SSAM) was constructed to express the Arabidopsis *sam1* gene under control of the cauliflower mosaic virus (CaMV) 35S promoter (see Methods). Figure 1 presents the T-DNA structure of pO35SSAM. Tobacco leaf discs were transformed, and 23 putative primary transgenic tobacco lines (T1 plants), which were derived from two independent transformation experiments, were isolated.

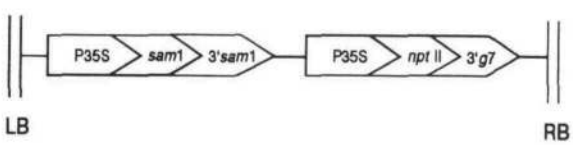

Figure 1. Schematic Presentation of the T-DNA Region of pO35SSAM.

The left and right border of the T-DNA are indicated by LB and RB, respectively. P35S is the 35S promoter of the cauliflower mosaic virus; *sam1*, coding sequence of the Arabidopsis *S*-adenosyl-L-methionine synthetase gene; *npt*II, coding sequence of the neomycin phosphotransferase II gene; 3′ *sam1* and 3′ *g7*, 3′ ends of the Arabidopsis *sam1* gene and the Agrobacterium gene 7, respectively.

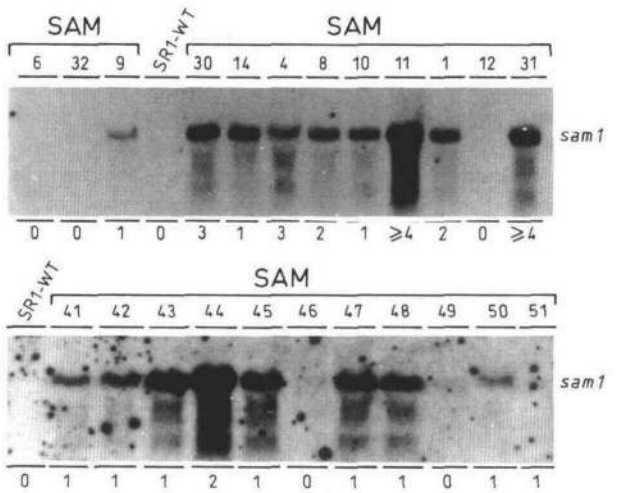

Figure 2. RNA Gel Blot Analyses of the Primary Regenerated Plants.

From each plant, a comparable mature leaf was taken for RNA extraction. Two autoradiographs are presented. RNA gel blot analyses of the first experiment (top) and a second, independent transformation experiment (bottom) are shown. Numbers above the autoradiographs indicate the plant lines, those below represent the numbers of independently segregating T-DNA loci. The 3′ end of the Arabidopsis *sam1* gene was used as a probe. Exposure time for the two blots was different. Therefore, SAM31 and SAM44 were compared in an independent RNA gel blot analysis and were found to be expressed approximately equally (data not shown). SR1-WT, wild type.

RNA gel blot analyses were performed to investigate whether the chimeric *sam1* gene was expressed in the plant. RNA was prepared from comparable leaves of all T1 plants grown in identical greenhouse conditions. The RNA was blotted and hybridized with a probe homologous to the 3′ end of the Arabidopsis *sam1* gene. This probe hybridizes specifically with the transgene. Figure 2 shows that the Arabidopsis *sam1* mRNA accumulates in most T1 plants. The wild type and the lines SAM6, SAM32, SAM12, SAM46, and SAM49 did not show any hybridization signal, and their progeny did not show resistance to kanamycin sulfate. The line SAM51 was transformed but did not express the *sam1* gene to detectable levels. For SAM31 and SAM44, *sam1* steady state mRNA levels were compared in an independent RNA gel blot analysis and were found to be approximately equal (data not shown). Segregation analysis indicated that the number of independently segregating T-DNA loci varied from one to more than four between the transgenic lines. There is no clear correlation between *sam1* expression levels and the number of T-DNA loci (Figure 2). Five transgenic lines, SAM11, SAM14, SAM30, SAM31, and SAM44, showing high *sam1* steady state mRNA levels were chosen for further analysis of their progeny (T2 generation).

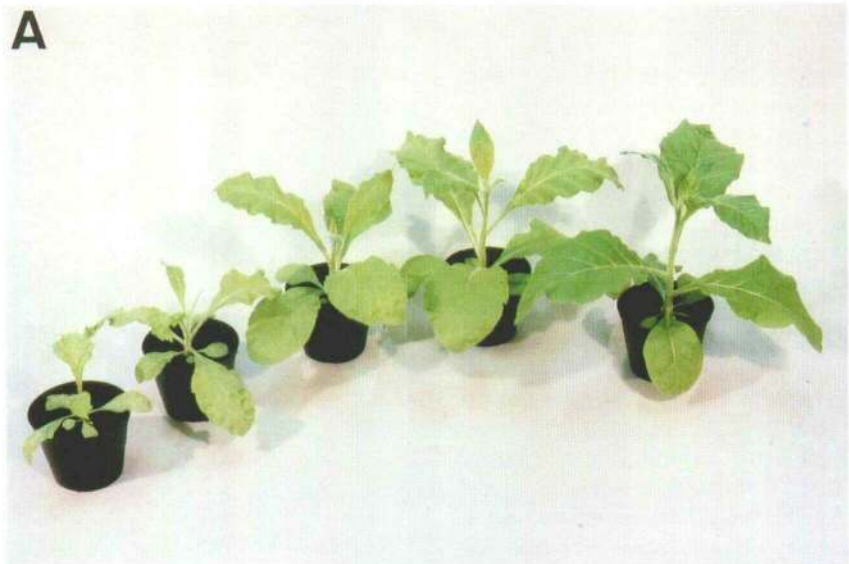

Figure 3. Phenotypic Characterization of T2 Plants.

Progeny of the five transgenic lines with high levels of *sam1* steady state mRNA were grown in soil and scored for phenotypic differences compared to wild-type plants.

(A) Overview of four progeny plants of the primary transformant SAM11 and a wild-type plant (right). Note the pale green color of the transformants.

(B) Flattened leaves with dark green veins and necrotic spots.

(C) Leaves with dark green veins and dents.

Phenotypic Analysis of T2 Plants

Progeny of these five transgenic lines (SAM11, SAM14, SAM30, SAM31, and SAM44) were selected on kanamycin sulfate, and 10 to 13 resistant plants from each transgenic line were transferred to soil. After 2 months of growth in the greenhouse, a phenotypic analysis was made. For all five transgenic lines, the progeny displayed variable degrees of abnormal phenotypes. Figure 3 reveals that the affected T2 plants were paler than wild-type plants and the veins appeared dark green, whereas the veins of wild-type plants were light green with a white shine. Plants with little pronounced abnormal phenotypes developed leaves with a flattened shape (Figure 3B), whereas normally the leaf tissue between the veins is curved. Eight of 13, five of 10, and two of 10 T2 plants of SAM31, SAM11, and SAM30, respectively, were significantly reduced in height (up to approximately one-fourth of the height of the control plants; Figure 3A). Later in development, UV-fluorescent necrotic lesions appeared on the leaves (data not shown). Severe lesions were also frequently detected at the petiole. The severity of these lesions was correlated with the height of the plant: at a given age, the smallest plants showed the most severe lesions that varied from little yellow spots (Figure 3B) to extensive cell death between the vascular bundles. Another phenotypic characteristic was the development of dents in the leaves (Figure 3C). Most plants were normal with respect to their flowering time and seed set. Plants with severe phenotypes (strongly reduced height) did not produce a main inflorescence, or if they did, the flower buds, flowers, or seed pods fell off at variable stages prior to seed pod ripening. However, these plants produced lateral shoots that flowered. The phenotype of the lateral shoots was generally less pronounced and sometimes almost similar to the wild type.

Because a distinct class of phenotypes will be described further in the text, the phenotypes described above will be referred to as "overexpression (OV)" phenotypes and the T2 plants showing these phenotypes as T2-OV plants.

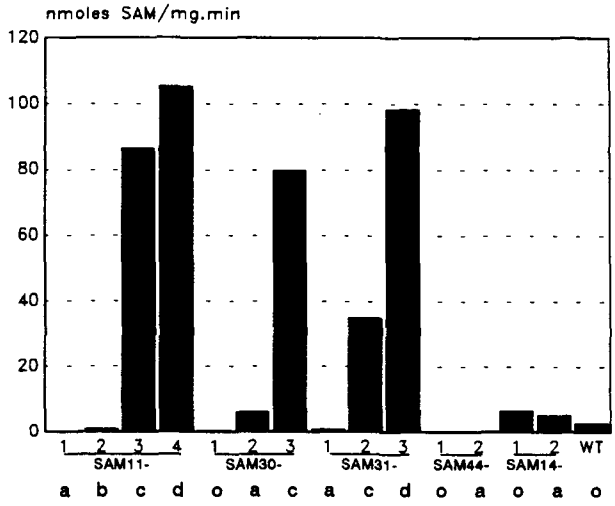

Figure 4. SAM-S Activities in Individual Progenies of the Five Transgenic Lines Having the Highest *sam1* Steady State mRNA Levels.

Individual T2 plants (indicated by numbers under the columns and above the corresponding transgenic lines) with different degrees of phenotypic abnormalities (indicated by lower case letters beneath the figure) were analyzed for their SAM-S activities: o represents plants with a wild-type phenotype; a, yellow-green plants with flat leaves; b, as given for a, but leaves with dark green veins and small necrotic lesions; c, as given for b, but plants are smaller with more lesions near the vascular bundles of the leaves and at the petiole and some of the leaves have dents; d, as given for c, but plants have a reduced height and have more severe lesions, abnormal leaf shape, and more dents in the leaves. For example, SAM11-1 is one particular T2 plant of primary transformant SAM11 and is phenotypically classified as "a." All plants were of the same age, and extracts were prepared from mesophyll tissue of the sixth leaf larger than 5 mm counted from the apex. These leaves did not show necrotic lesions. All values were corrected for background activities. Activities are expressed as nanomoles of SAM produced per milligram of protein per minute. WT, wild type.

Severity of the Phenotype Correlates with SAM-S Activity

To analyze whether the severity of the overexpression phenotype correlated with an enhanced SAM-S activity, individual T2-OV plants with different degrees of phenotypic abnormalities were selected from each population of progeny. All plants were of comparable age (~1 week before the development of the flower buds of the wild-type plant). SAM-S activity was measured for every sixth leaf larger than 5 mm counted from the apex. Figure 4 shows that a significant increase in SAM-S activity was only measured in plants presenting the most severe symptoms (SAM11-3, SAM11-4, SAM30-3, SAM31-2, and SAM31-3). However, in plants with less severe phenotypic abnormalities, SAM-S activity was barely detectable or comparable to wild-type levels (Figure 4; SAM11-1, SAM11-2, SAM30-1, SAM30-2, SAM31-1, SAM44-1, SAM44-2, SAM14-1, and SAM14-2). Nevertheless, these plants were transformed and most of them did show symptoms.

SAM-S Activity Is Translationally and/or Post-Translationally Regulated

To find out the reason for the unexpectedly low SAM-S activity levels in the transformed plants presenting little phenotypic abnormalities, the SAM11-1 plant and the control (Figure 4) were further investigated by analyzing the SAM-S activity of the first, fourth, and seventh leaf counted from the top. In addition, the first, third, fifth, and seventh leaf of SAM1-1, a transformed plant not presenting symptoms, and a comparable wild-type plant were analyzed. Figure 5 shows that SAM-S

activities in young leaves are higher than in mature leaves in both wild-type and transformed plants. In wild-type plants, a gradient in SAM-S activity of ~20-fold was observed between the first and the seventh leaf, while in the analyzed transgenic plants a 40-fold gradient was observed. Thus, transgenic plants presenting little but significant (SAM11-1) or no symptoms (SAM1-1) also have higher SAM-S activity, but maintain the gradient observed in wild-type plants and, as a consequence, have low SAM-S activities in the mature leaves. RNA was prepared from the same leaves of SAM1-1 as were used to measure SAM-S activities. As shown in Figure 6, the *sam1* steady state mRNA levels of the transgene in these four leaves did not correlate with the gradient in SAM-S activity, indicating that the expression of the *sam1* gene under the control of the P35S is translationally and/or post-translationally regulated.

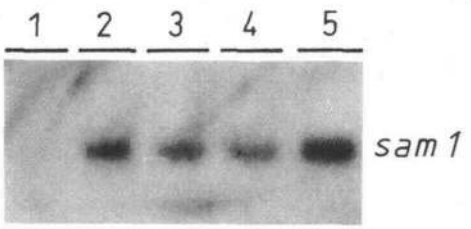

Figure 6. *sam1* mRNA Accumulation in Leaves of Different Ages.

RNA was prepared from the same leaves of the SAM1-1 plant that was used for SAM-S activity measurements (see Figure 5). Lane 1, untransformed; lane 2, first leaf; lane 3, third leaf; lane 4, fifth leaf; lane 5, seventh leaf.

Development of Dark Green Sectors

When further analyzing the phenotype of the T2-OV plants, we observed that some plants had leaves with one or more dark green sectors. As presented in Figure 7, some of these sectors were circular spots of 1 to 3 mm in diameter, and others were oval or irregular or appeared to be associated with the veins. Small circular and oval spots were regularly observed (Figure 7A). Larger sectors were observed in the lateral shoots of the T2-OV plants that were incapable of producing flowers on the primary shoot. On these lateral shoots, several leaves had multiple dark green sectors (Figures 7B and 7C).

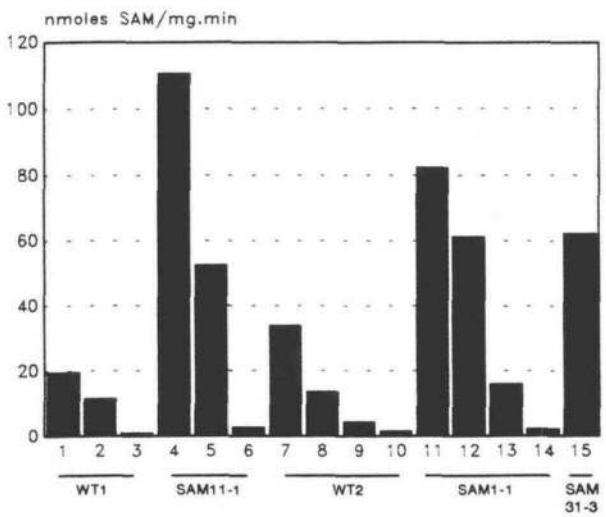

Figure 5. SAM-S Activities in Different Leaves of Wild-Type and Transformed Plants.

Plant SAM11-1 and wild type 1 (WT1) are the same plants as given in Figure 4, but they had developed flower buds at the time of the experiment. The transgenic plant SAM1-1 did not show phenotypic abnormalities and was grown in identical conditions as wild type 2 (WT2). Plants SAM11-1 and WT1 were grown and analyzed at a different time compared to plants SAM1-1 and WT2. Columns 1, 2, and 3 represent the first, fourth, and seventh leaf of WT1, respectively; columns 4, 5, and 6, first, fourth, and seventh leaf of SAM11-1, respectively; columns 7, 8, 9, and 10, first, third, fifth, and seventh leaf of WT2, respectively; lanes 11, 12, 13, and 14, first, third, fifth, and seventh leaf of SAM1-1, respectively; lane 15, seventh leaf of SAM31-3 (Figure 4). Activities are expressed as nanomoles of SAM produced per milligram of protein per minute.

Among the progeny of SAM31, SAM11, and SAM14, 1 to 15% (depending on the greenhouse conditions; see below) of the kanamycin sulfate–resistant T2 plants developed completely differently compared to their T2-OV siblings. These plants developed leaves from which the veins were bordered by a dark green zone (Figures 7D, 7F, 7G, and 7H). This pattern became visible at variable stages in development. The relative surface area of this dark green tissue increased in successive leaves (Figures 7F, 7G, and 7H). As represented schematically in Figure 8, plants showing this pattern can further develop in different ways. Part of them remained small as a result of a reduction of the internode length in the base to apex direction (Figure 7I). The leaves of these plants became dark green, thick, leatherlike, and asymmetric. Leaves that developed closer to the apex had a more yellow color and were extremely curved with tearing of the leaf tissue as a result (Figure 7I). No inflorescence developed on the main stem, but lateral shoots that were able to flower could emerge (data not shown). The leaves of the lateral shoots were generally less affected. The seed pods produced on these lateral shoots were small and shrivelled (Figure 7E), and a high number of seed were abortive; most seed were normal with respect to their size, but the embryo was very small and shrivelled (data not shown). This type of plant will be further referred to as a T2-S plant. Segregation analysis of the viable seed of these T2-S plants indicated that homozygosity for the transgene was not a prerequisite to obtain this particular phenotype (data not shown).

Alternatively, part of these T2 plants developed in a way that resembled wild-type plants, although the leaves were somewhat darker green compared to wild-type leaves. However, similar to the T2-S plants, the flowers were generally less intensely colored and had shrivelled seed pods and low seed

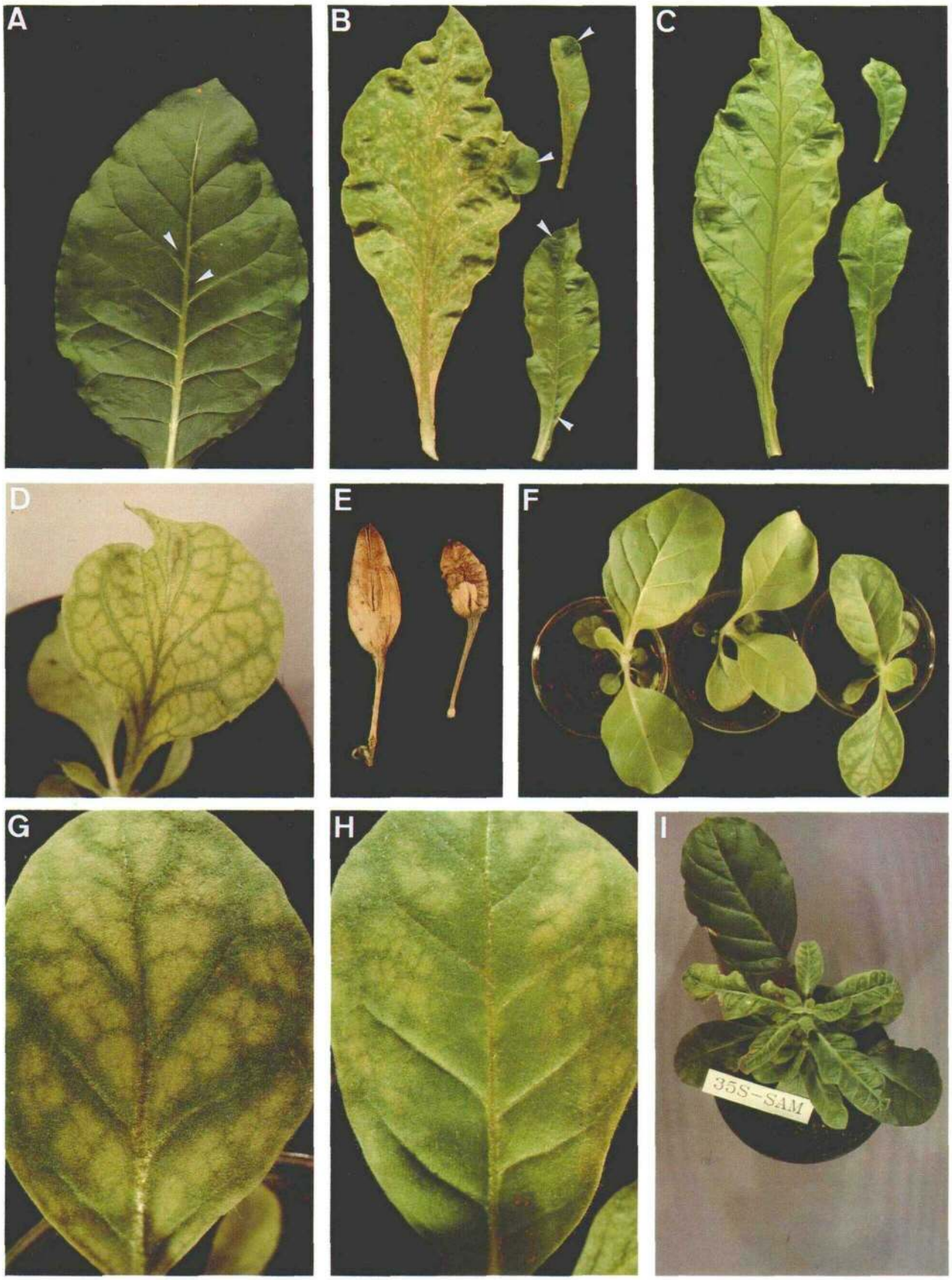

Figure 7. Patterns of Dark Green Tissue on P35S–*sam1*–Transformed Tobacco.

set. These plants are referred to as the T2-SN plants. Some plants showed the described symptoms only with respect to their upper leaves and the seed pods (intermediates). Most surprisingly, both the T2-S and T2-SN plants as well as plants with the intermediate phenotype smelled like cabbage.

SAM-S Activity Is Reduced in the Dark Green Tissue

The SAM-S activity of the dark green tissue, developed on the T2-OV plants, was compared to that of adjacent tissue on the same leaf displaying overexpression symptoms. The assays were performed on extracts from young leaves that had not yet developed necrotic spots. Table 1 shows that SAM-S activity was eight- to 18-fold lower in the dark green sectors.

Because the T2-S plants developed in a completely different way than their siblings developing overexpression phenotypes (T2-OV plants), we were interested in comparing SAM-S activities in both types. Table 1 shows that SAM-S activity was barely detectable in leaves of T2-S plants, even when young leaves, known from previous experiments to have the highest activity (Figure 5), were analyzed. Importantly, SAM-S activities of young leaves of the T2-S plants were up to 109-fold lower than those of young leaves of wild-type plants.

Table 1. SAM-S Activities of Dark Green Tissue Compared to Tissue Showing Overexpression Symptoms

Plant	nmol mg^{-1} min^{-1}	Reduction (Fold)
SAM31-7 overexpressing tissue	112.3	9.1
SAM31-7 dark green sector	12.3	
SAM31-8 overexpressing tissue	104.8	14.6
SAM31-8 dark green sector	7.2	
SAM31-9 overexpressing tissue	61.9	8.1
SAM31-9 dark green sector	7.6	
SAM31-10 overexpressing tissue	67.4	17.7
SAM31-10 dark green spots	3.8	
SAM31-11 young leaf	229.0	763
SAM31-12 young leaf	0.3	109
Wild-type young leaf	32.8	
SAM31-12 mature leaf	0.3	
SAM31-13 young leaf	0.4	
SAM31-14 young leaf	0.6	

All plants analyzed are progeny of SAM31. SAM31-7 to SAM31-11 belong to the T2-OV plants; SAM31-12, SAM31-13, and SAM31-14 belong to the T2-S plants. SAM-S activities are expressed as nanomoles of SAM produced per milligram of protein per minute.

Expression of the Transgene Is Silenced in Dark Green Tissue

To investigate whether the reduction in SAM-S activity in dark green tissue is a reflection of a reduced *sam1* steady state mRNA level, RNA gel blot analysis was performed. As shown in Figure 9, no *sam1* steady state mRNA was detected in dark green tissue of a lower leaf of a T2-SN plant (lane 2), whereas a high level was detected in the adjacent tissue of the same leaf showing overexpression symptoms (lane 1; also see Figure 8). No *sam1* steady state mRNA was detected in either young or mature leaves of a T2-S plant (lanes 4 and 5), whereas a high level was detected in young leaves of a control plant (i.e., a sibling showing overexpression phenotypes, T2-OV plant; lane 3). Lane 6 shows *sam1* steady state mRNA levels from a top leaf of a T2-OV progeny of SAM11 that developed non-severe overexpression phenotypes (classification "a" according to Figure 4); lanes 7 and 8 show *sam1* steady state mRNA levels from top leaves of the T2-SN plants that had an overexpression phenotype early in development but developed the dark green phenotype 6 and 10 weeks after germination, respectively. These data show that expression of the transgene is silenced in the dark green tissue (the dark green tissue will for convenience be further referred to as "suppressed" tissue).

Variability in Phenotype

We have grown T2 progenies of the transgenic line SAM14 in different greenhouses. Transgenic plants were selected on

Figure 7. (continued).

(A) Leaf of a T2-OV plant. The arrowheads show oval spots of dark green tissue.
(B) Dark green sectors. On the left is a mature leaf of a T2-OV plant. The yellow-green color and the necrotic lesions at the leaf blade and the petiole are the consequences of higher SAM-S activities. On the right are two leaves of a lateral shoot from a T2-OV plant that was unable to develop flowers on the main stem. The arrowheads indicate dark green sectors.
(C) Dark green tissue associated with the veins. Three leaves are shown that were derived from a lateral shoot of a T2-OV plant that was unable to develop flowers on the main stem. Note that the relative surface area of the dark green tissue becomes larger with successive leaves.
(D) Development of dark green tissue associated with the veins.
(E) At left, seed pod of a T2-OV plant showing a wild-type morphology; at right, seed pod of a T2-S plant showing a shrivelled morphology.
(F) At left, wild type; center, T2-OV plant; right, T2-S plant.
(G) Close-up of a basal leaf of the T2-S plant presented in **(F)**.
(H) Close-up of the leaf succeeding the one presented in **(G)**.
(I) A T2-S plant at a later stage of development.

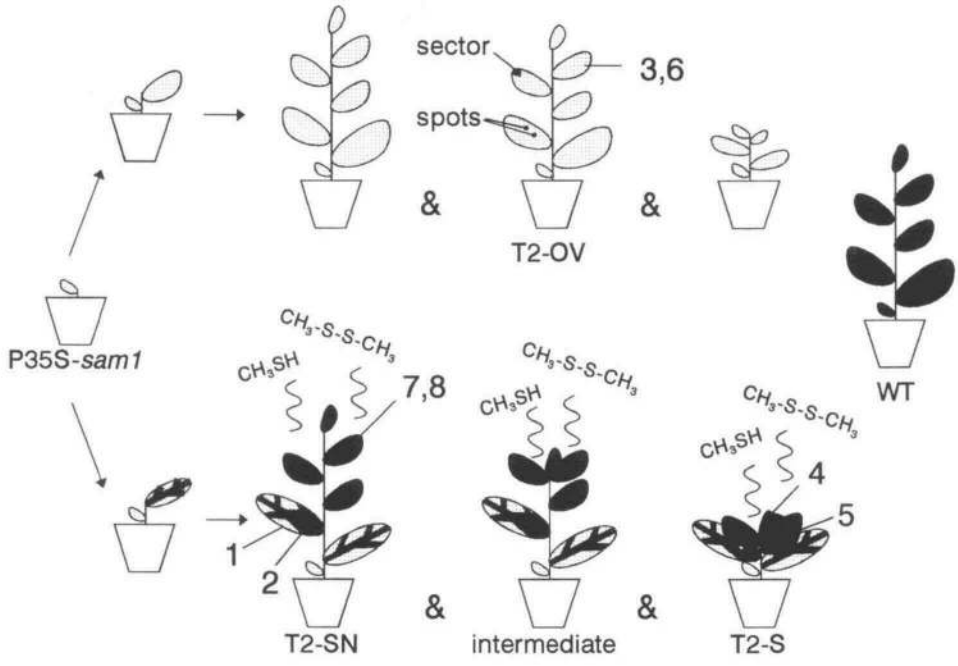

Figure 8. Simplified Presentation of the Different Ways of Development of T2 Tobacco Plants Transformed with the Chimeric P35S–*sam1* Gene.

The light leaf area indicates the overexpression phenotype; the dark area represents the suppressed phenotype. The T2-OV plants exhibit different degrees of overexpression phenotypes (normal-sized plants and smaller plants). Suppressed tissue appears on the T2-OV plants as sectors or small spots. T2 seedlings can develop into plants that have a partial overexpression phenotype and a partially suppressed phenotype. The suppressed phenotype is associated with the veins. These plants further develop into the T2-S plants that are stunted, into the T2-SN plants that exhibit a nearly wild-type phenotype, or into intermediates between T2-S and T2-SN plants. T2-S, T2-SN, and intermediate plants emit methanethiol and dimethyl disulfide as indicated by the wavy lines. The directional arrows connect successive developmental stages of the transformed plants. Numbers refer to tissue from which RNA was extracted for RNA gel blot analyses as given in the legend to Figure 9. Two numbers per one leaf indicate that RNA was prepared from analogous leaves of two independent plants with similar phenotypes. WT, wild type.

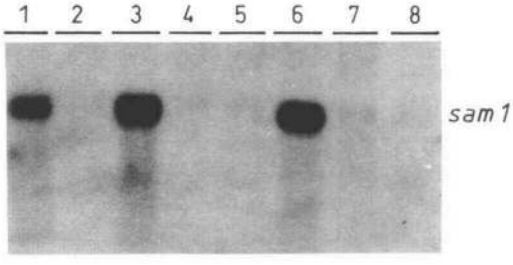

Figure 9. RNA Gel Blot Analysis of the Leaves of T2-OV, T2-S, and T2-SN plants.

For the designation of the lanes, see also Figure 8. Lane 1 contains RNA from a region of a lower leaf of a T2-SN plant showing overexpression symptoms; lane 2, RNA from adjacent tissue of the same leaf having the dark green phenotype; lane 3, RNA from a T2-OV plant; lane 4, RNA from a young leaf of a T2-S plant; lane 5, RNA from a mature leaf of a T2-S plant; lane 6, RNA from a young leaf of a T2-OV plant; lane 7, RNA from a young leaf of a T2-SN plant; lane 8, RNA from a young leaf of the same T2-SN plant as analyzed in lanes 1 and 2. The wild-type control is shown in lane 1 of Figure 6 (same RNA gel blot analysis).

sterile media containing 100 mg/L kanamycin sulfate. In parallel, control wild-type plants were grown on kanamycin sulfate-free medium. After 3 weeks to 1 month of growth, the plants were transferred to soil and incubated in the greenhouse. We observed that in one greenhouse, the frequency of suppressed plants (i.e., T2-S, T2-SN, and intermediate plants) was 1%, whereas in another greenhouse, this frequency was 15% (in both cases, counted on a total of 300 plants). The conditions of the greenhouse in which high incidence of silencing (15%) occurred were as follows: a 16-hr light/8-hr dark cycle, 23°C, 48% relative humidity, light intensities of 55, 68, and 81 μmol m^{-2} sec^{-1} at 0, 20, and 40 cm above the soil level, respectively (Son-T-Agro HqI-TD incandescent lamps; Philips, Eindhoven, The Netherlands), and shielding from incident light. Plants were transferred to soil in January. The conditions of the greenhouse in which a lower incidence of silencing (1%) occurred were as follows: a 16-hr light/8-hr dark cycle, 23°C, 40% relative humidity, light intensities (MBFR/U 400W incandescent lamps; Philips) of 45, 65, and 120 μmol m^{-2} sec^{-1} at 0, 20, and 40 cm above the soil level, respectively, without shielding from incident light. Plants were transferred to soil

in October. These data indicated that environmental conditions played a major role in the development of the different phenotypes.

Tobacco Plants with the Suppressed Phenotype Emit Methanethiol

To determine what chemical compound caused the cabbage-like smell that was produced by the T2-S, T2-SN, and intermediate plants, gas chromatography–mass spectrometry analyses were performed. Three different samples were analyzed: the headspace of a closed beaker containing three shoots (length ~120 mm) derived from plants that smelled like cabbage (i.e., T2-S, T2-SN, or intermediate plants) in 100 mL of water, the headspace of a closed beaker containing three shoots derived from wild-type plants in 100 mL of water, and the headspace of a vial containing L-methionine. The latter sample was included because we had noticed that pure L-methionine and L-methionine solutions had an odor similar to that of suppressed plants. Both the suppressed plants and L-methionine produced the volatile methanethiol as well as its oxidation product, dimethyl disulfide. These compounds were not detected in the headspace of the vial containing wild-type shoots (data not shown). Moreover, the smell of cabbage is typical for methanethiol (Merck Index, 1989).

The Level of Free L-Methionine Is at Least Partially Controlled by SAM-S Activity

It has been shown that methanethiol is emitted from pumpkin leaf discs when floated on L-methionine (Schmidt et al., 1985). Because the suppressed plants emit methanethiol, a compound that is also emitted by pure L-methionine, we investigated whether the intracellular concentrations of free L-methionine were elevated in these plants. Two series of quantifications were done. In a first series, the free L-methionine content was determined from the top leaf of a wild-type plant, a T2-OV plant, and a T2-S plant. In a second series, the free L-methionine content of the top leaf of a wild-type plant, a T2-OV plant, and a T2-SN plant was analyzed. Plants of the first series were grown and analyzed at a different time compared to plants analyzed in the second series. Table 2 shows that leaves of T2-S and T2-SN plants accumulate high levels of free L-methionine.

Table 2. Analysis of Free L-Methionine Levels in Top Leaves of Wild-Type Plants, T2-OV Plants, and Smelling Plants

Plant	nmol g^{-1}	%
Wild type 1	12.41	0.57
T2-OV-1	30.41	0.81
T2-S	5263.40	25.13
Wild type 2	ND	–
T2-OV-2	5.97	0.04
T2-SN	2300.86	8.09

Amino acid analysis was performed as described in Methods. Absolute levels of L-methionine are expressed as nanomoles per gram of fresh tissue. Relative amounts are expressed as percentages and were calculated as described in Methods. ND, could not be detected; –, not available. Plants wild type 1, T2-OV-1, and T2-S were grown at a different time compared to wild type 2, T2-OV-2, and T2-SN.

DISCUSSION

In this work, the consequences of overproducing SAM-S were investigated in transgenic tobacco containing the Arabidopsis *sam1* gene under control of the CaMV P35S. The transgenic plants could be grouped roughly into two classes: those that have higher (T2-OV) and those that have lower (T2-S) SAM-S activity compared to that of wild-type plants. It was found that the level of SAM-S activity correlated with the degree of phenotypic abnormalities displayed by the plants. Moderately elevated SAM-S activities led to plants with yellow-green and flat leaves, whereas highly elevated levels also led to reduced plant height and necrotic lesions. On the other hand, highly reduced SAM-S activities led to stunted plants with thick, leatherlike, asymmetric, and torn leaves. Moreover, plants with reduced SAM-S activity had a disagreeable odor.

In the T2-S and T2-SN plants, expression of the transgene was silenced (Figure 9). In the T2-S plants, SAM-S activities were barely detectable in young leaf tissue (109-fold lower than in wild-type tissue) (Table 1), suggesting cosuppression of the endogenous *sam-s* genes. The T2-SN plants developed in a nearly wild-type fashion but still emitted methanethiol, suggesting only a partial cosuppression of the endogenous *sam-s* genes. Moreover, although SAM-S activity is reduced in suppressed tissue of the T2-OV plants, considerable activity remained (Table 1). Thus, our results suggest that the endogenous tobacco *sam-s* genes are cosuppressed to different degrees. Cloning of the endogenous *sam-s* genes from tobacco and analysis of their expression levels in the T2-S and T2-SN plants will have to be performed to investigate whether the expression of all tobacco *sam-s* genes is reduced in T2-SN plants or whether a subset is completely silenced. Hart et al. (1992) demonstrated that in *N. sylvestris* transformed with a P35S–chitinase gene, the amount of residual chitinase present in the silenced plants varied from plant to plant. These researchers also suggest that cosuppression is not an all-or-nothing effect. Moreover, in tomato plants transformed with sense constructs containing sequences homologous to the endogenous polygalacturonase gene, cosuppression led only to a reduced level and not to a complete absence of polygalacturonase steady state mRNA in ripe fruit of the transgenic tomato plants (Smith et al., 1990; Seymour et al., 1993).

In a number of cases, gene silencing has been demonstrated to be developmentally regulated. de Carvalho et al. (1992) showed that the expression of a transgenic β-1,3-glucanase was silenced in a homozygous transgenic tobacco line (T17),

but not in the hemizygous line T17. However, the transgenic β-1,3-glucanase mRNA was detected at high levels in the homozygous plant during the first 4 weeks of development. After 4 weeks, the mRNA level decreased gradually. In some *N. sylvestris* plants transformed with a P35S–chitinase gene the lower leaves showed a high chitinase content, whereas the upper leaves, formed later in development, showed low chitinase content and cosuppression of both the transgenic and the endogenous chitinase gene (Hart et al., 1992). Our results are similar to those of Hart et al. (1992) in the sense that in the lower leaves, generated early in development, expression of the transgene is high, whereas expression of the transgene is suppressed in leaves that developed at a later stage. In the cases mentioned above, it is not easy to follow the spatial and temporal development of the suppressed state at the cellular level, because neither the overexpression nor the suppressed state results in visible phenotypes. Because overexpression and suppression of SAM-S lead to phenotypes with distinct characteristics, the developmental pattern of the suppressed state can be visualized. We observed that in the leaves generated early in development the suppressed tissue was associated with the veins and that the area of suppressed tissue increased in successive leaves until all newly developed leaves displayed the suppressed phenotype. How can this developmental pattern be explained?

We propose a hypothetical model that is based on two assumptions. First, high levels of *sam-s* gene expression can trigger *sam-s* gene silencing, and second, silenced cells act as a sink for a molecule that is involved in negative regulation of the endogenous *sam-s* genes.

This hypothesis is based on the following observations. First, early in development the suppressed tissue is associated with the veins (Figures 7D, 7F, 7G, and 7H). Interestingly, analysis of a *Psam1–gus* fusion in transgenic Arabidopsis and tobacco plants has shown that the Arabidopsis *sam1* promoter confers high expression in vascular tissue and low expression in mesophyll tissue (Peleman et al., 1989a, 1989b). Assuming a similar expression pattern of the endogenous tobacco *sam-s* genes, it is quite possible that the extra-high expression level in the vascular tissue, obtained by introduction of the transgene, would trigger the switch from the overexpression to the suppressed state. This hypothesis is in agreement with the observation that suppression of a transgenic polygalacturonase gene in tomato occurred only in ripe fruit; this is the tissue where the endogenous polygalacturonase gene is highly expressed in wild-type plants (Smith et al., 1990). Furthermore, it was observed by de Carvalho et al. (1992) that suppression of β-1,3-glucanase transgene expression occurred only in homozygous progeny of the primary transformant that had the highest expression level of the transgene.

Second, it is observed that (1) the suppressed tissue is not restricted to the veins, but covers the area bordering the veins to different degrees; (2) the area of suppressed tissue increases in successive leaves; and (3) suppression can also be displayed as small circular or oval spots or sectors in interveinal tissue. If one assumes the presence of a molecule that is involved in a negative feedback regulation of the expression of the endogenous *sam-s* genes according to the need for SAM, it follows that suppression of *sam-s* in the veins would create a sink for this factor.

The existence of such a factor, perhaps SAM itself or a metabolite thereof, is not unfounded. It has been shown that the presence of SAM in the growth medium represses the expression of one of the *sam-s* genes in yeast and that SAM is a corepressor in the biosynthesis of methionine in *Escherichia coli* (Saint-Girons et al., 1986; Thomas et al., 1988; Rafferty et al., 1989). A reduced level of this factor in neighboring cells would upregulate the expression of the endogenous *sam-s* genes to the putative threshold level necessary to elaborate the switch to the suppressed state. This would allow the sink to enlarge and the process to proceed to neighboring cells, implying that an originally patterned leaf will gradually become completely suppressed. However, this does not occur. Lower leaves of suppressed plants stay phenotypically patterned, and the transgene is still expressed in the areas that have the overexpression phenotype (Figures 8 and 9). This suggests that *sam-s* gene expression in neighboring cells can become silenced only when they are susceptible to becoming silenced.

This susceptibility could again be a result of the expression level of the endogenous *sam-s* genes because the expression level in the mesophyll cells might decrease when the leaf matures. This is evidenced by the fact that the SAM-S activity decreases when the leaf mesophyll matures (Figure 5) and that no GUS activity is detected in the mesophyll of mature leaves of tobacco plants transformed with a *Psam1–gus* fusion (Peleman et al., 1989b). There will thus be a stage at which the putative threshold expression level of *sam-s* gene expression is not reached; hence, at this stage in development, the tissue remains overexpressing *sam-s*.

On the other hand, the area of suppressed tissues increases with successive leaves. This can be explained by the fact that at the time new leaves develop the sink is already present in older leaves, which allows the silencing process to proceed through more cell layers at the stage they are still susceptible to becoming silent, that is, when they are still young and highly expressing the *sam-s* genes. Because the advance of the silencing process in one particular leaf is not visually apparent, it is expected that this silencing process occurs during the early stages of leaf development.

The pattern of leaf development could largely contribute to the development of the silencing pattern: in a young leaf primordium, the primary lateral veins make up the majority of tissue in the leaf blade. Later in development, interveinal regions expand considerably so that in a mature leaf lateral veins occupy only a small fraction of the leaf (Poethig and Sussex, 1985). This suggests that if *sam-s* gene expression is silenced in young leaves, the percentage of interveinal tissue that will eventually become silenced will be larger than when the silencing process was initiated in older leaves. The observation that in successive leaves the basal part of the leaf consists of more suppressed tissue compared to the more distal part (Figures 7G and 7H) is in agreement with the fact that growth first stops

at the tip of the leaf and then progressively in more basal regions of the axis (Poethig and Sussex, 1985).

The observation of small circular and oval spots (Figure 7A) and, less frequently, sectors (Figure 7B) that were not associated with the veins is in agreement with the described hypothesis. It seems reasonable to assume that the small circular and oval spots are derived from occasional situations of high *sam-s* gene expression. Because *sam-s* genes are generally not expressed in mesophyll tissue of mature leaves, it would be unlikely that the cells that constitute the spot would become independently silenced at a certain stage of development. Rather a gradient in the concentration of a certain factor originally emanating from the cell that was first silenced could be involved.

In maize, somatically heritable switches in *Mutator* (*Mu*) transposon activity can be monitored with suppressible mutants. The pattern of these suppressed mutant phenotypes resembles the one obtained by suppression of SAM-S activity in that at a certain stage of development, and from then on with each successive leaf, the mutant phenotype is progressively suppressed. In maize, this suppression correlates with *Mu* inactivity and methylation of *Mu* (Martienssen et al., 1990; Martienssen and Baron, 1994). These authors interpret this pattern by assuming that the longer a given cell lineage spends in the meristematic condition, the more likely it is to adopt an inactive phase (and thus methylation and suppression of the mutant phenotype). Our observations suggest that the longer a cell spends in a tissue that expresses the *sam-s* genes to a high level (i.e., young tissue and vascular tissue), the more likely it is to become silenced by the sink generated by suppression of SAM-S in the neighboring cells earlier in development. The main difference with the work published by Martienssen et al. (1990) and Martienssen and Baron (1994) is that in their case the pattern is most likely to be clonally inherited, whereas in the case of SAM-S silencing, the developmental pattern is not likely to be fully clonally developed. We observe a pattern of silencing that, early in its development, is associated with the veins. Veins in leaves are believed to be differentiated from preexisting nonclonal cells (Sachs, 1981; Steeves and Sussex, 1989). Moreover, the patterns we have observed were not described in the clonal analysis of tobacco leaf development (Poethig and Sussex, 1985).

Thus, the developmental pattern of gene silencing could largely be explained by the combination of the level of *sam-s* gene expression (endogenous plus transgene derived expression) with the normal development of the plant; that is, leaves at different developmental stages (younger versus older) will respond differently (patterned or increasing) to a silencing phenomenon that has started at a certain stage in development. Environmental influences affecting growth dynamics and levels of gene expression are thus expected to influence the incidence and timing of the switch between the overexpression and the silenced state.

The T2-S and T2-SN plants have strongly reduced SAM-S activities. As a result, free L-methionine accumulates to high levels (Table 2). Mutants of yeast and *E. coli* with a defect in SAM-S have also been reported to overproduce L-methionine (Greene et al., 1970, 1973; Cherest et al., 1973; Markham et al., 1984; Thomas et al., 1988). The elevated L-methionine level in our plants might be caused by the blockage of the conversion of L-methionine to SAM, but possibly also to an increased flux toward L-methionine, resulting in an accumulation of this amino acid. SAM plays a regulatory role in L-methionine biosynthesis by allosterically stimulating threonine synthase (Madison and Thompson, 1976; Aarnes, 1978; Rognes et al., 1980; Giovanelli et al., 1984, 1989; Frankard et al., 1991) and by negatively regulating the activity of the lysine-sensitive aspartate kinase. An alteration in feedback inhibition of aspartate kinase can result in L-methionine overproduction (Hibberd et al., 1980). Consequently, the presumed reduction in the SAM level in plants with reduced SAM-S activity would lead to an increased flux toward L-methionine. As yet, we are not able to elucidate the relative contribution of these two possibilities to the observed elevation in L-methionine levels.

It has been shown that pumpkin leaves have an L-methionine–inducible enzymatic system, capable of converting L-methionine into methanethiol (Schmidt et al., 1985). It is therefore likely that the emission of methanethiol in the smelling plants is a direct consequence of the accumulation of L-methionine.

It will be of great interest to investigate whether silencing of SAM-S correlates with increased methylation of the *sam-s* genes, in particular because the activity of SAM-S, the enzyme responsible for the synthesis of the methyl donor SAM, has been drastically reduced in the suppressed plants.

The possibility of identifying plants with a patterned phenotype, which is caused by an as yet unresolved mechanism of gene silencing, will not only allow us to study the different aspects of gene silencing in detail, but opens up new ways to study physiological processes that are usually hampered by hardly controllable parameters.

METHODS

Plasmid Construction

Plasmid pO35SSAM was constructed by digesting pATC9A1-13N (Peleman et al., 1989a) with NcoI and SalI. The 840-bp fragment containing the 5′ terminal part of the coding sequence of the *Arabidopsis thaliana* *S*-adenosyl-L-methionine synthetase (*sam1*) gene was cloned into the cauliflower mosaic virus (CaMV) 35S promoter containing plasmid pDE9 (Plant Genetic Systems N.V., Gent, Belgium), which was digested in the polylinker with NcoI and SalI, yielding the plasmid pDE9-13N. pATC9A1-10 consists of the 2.15-kb EcoRI-AvaI fragment containing the *sam1* gene cloned into the EcoRI-AvaI site of pSP65 (Peleman et al., 1989a). This clone was digested with SalI and PstI. The 622-bp fragment containing the 3′ terminal part of the *sam1* coding sequence and the 3′ end of the *sam1* gene was cloned into pDE9-13N, which

was digested with SalI and PstI, yielding p35SSAM. This plasmid was digested with PstI and EcoRI. The fragment containing the CaMV 35S promoter, the coding sequence, and the 3′ end of the *sam1* gene was cloned into the 13-kb EcoRI-NsiI fragment of the binary vector pWBATS1 (W. Boerjan, W. Dewitte, T. Beeckman, M. Cervera, H. Van Onckelen, M. Van Montagu, and D. Inzé, manuscript in preparation), yielding the plasmid pO35SSAM.

pATC9A1-15 was constructed by cloning the 280-bp HindIII-PstI fragment of pATC9A1-10, containing the 3′ end of the *sam1* gene, into the HindIII-PstI linearized vector pGEM2.

Plant Transformation

Plasmid pO35SSAM was mobilized by the helper plasmid pRK2013 to *Agrobacterium tumefaciens* C58C1Rif^R containing the plasmid pGV2260 (Deblaere et al., 1985) by triparental mating (Van Haute et al., 1983) and used for transformation of *Nicotiana tabacum* cv Petit Havana SR1 by leaf disc transformation (Horsch et al., 1985).

RNA Gel Blot Analysis

Tobacco RNA was prepared according to the method described by Logemann et al. (1987). The ^{32}P-labeled riboprobe, complementary to the 3′ end of the *sam1* gene, was prepared from the plasmid pATC9A1-15 by in vitro transcription using the kit supplied by Boehringer Mannheim.

For RNA gel blots, 6 μg of total RNA was denatured and run on formaldehyde–agarose (1.2%) gels and then transferred to nylon membranes. Prehybridizations and hybridizations were performed in 5 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 50% formamide, 0.5% SDS, 10% dextran sulfate, and 20 μg/mL denatured herring sperm DNA at 65°C using ^{32}P-labeled riboprobes. The final wash was performed in 1 × SSC at 68°C. RNA quantities were measured twice photometrically, and prior to blotting, the RNA gel was stained with ethidium bromide to exclude loading artifacts and problems as a result of RNA degradation.

SAM-S Assays

Protein extracts were prepared by grinding 250 mg of leaf mesophyll tissue in 0.5 mL of extraction buffer (100 mM Tris, pH 7.5, 2 mM EDTA, 20% glycerol, 20 mM β-mercaptoethanol, 1 mM DTT). After centrifugation in an Eppendorf centrifuge (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) at 13,000 rpm for 10 min, the protein concentration of the supernatant was determined by the method of Bradford (1976) using the kit supplied by the Bio-Rad laboratories.

The SAM-S assays were done essentially as described by Mudd et al. (1965) and Peleman et al. (1989a). Fifty micrograms of total proteins was incubated in 0.25 mL of a reaction mixture containing 100 mM Tris, pH 8.0, 30 mM $MgSO_4$, 10 mM KCl, 20 mM ATP, and 5 mM ^{35}S-methionine (15 μCi). Control reactions contained all reagents except ATP. After incubation for 1 hr at 25°C, the reaction was terminated by adding 2 mL of ice-cold water, and the mixtures were then loaded onto 0.67 × 30 mm Dowex 50W-X2 cation-exchange columns (100- to 200-μm mesh; NH_4^+ form; Bio-Rad) prepared in Pasteur pipettes. Unreacted methionine and ATP were eluted by washing with 10 mL of ice-cold water. Adsorbed SAM was eluted with 5 mL of NH_4OH (32%). The NH_4OH was almost completely evaporated; scintillation liquid (ReadySafe; Beckman Instruments Inc., Fullerton, CA) was added, and the sample was counted by scintillation spectrometry.

Alternatively, the reaction mix was further treated essentially as described by Mathur et al. (1991). After incubation of the reaction mix for 1 hr at 25°C, a 50-μL aliquot was spotted on an ~2-cm^2 phosphocellulose filter disc (P81; Whatman International Ltd., Maidstone, UK) and dried under an infrared lamp. The filters were washed with ice-cold water to remove the unincorporated ^{35}S-methionine and transferred to scintillation vials containing 1 mL of ammonium hydroxide (1.5 M). After 5 min, scintillation liquid (ReadySafe) was added, and the sample was counted by scintillation spectrometry. The latter assay is the easiest and is as accurate as the former. For both assays, SAM-S activity measurements in successive analyses of the same protein extracts did not vary by more than 15%.

Gas Chromatography–Mass Spectrometry

Gas chromatography–mass spectrometry analyses were performed with a Finnegan-Mat type 4000 EI (70 electron volt), INCOS data system (Finnegan, Veenendaal, The Netherlands), and a self-manufactured polydimethylsilane column (30 m, 0.53 mm i.d., 1.2 μm phase). One milliliter of headspace was injected splitless at room temperature (28°C). A temperature gradient from 40°C to 120°C with an increase of 5°C per minute was applied.

Amino Acid Extraction and Analysis

Amino acid extraction was mainly performed as described by Vernaillen and Verbruggen (1991) with minor modifications. Between 0.5 and 1 g of leaf tissue was ground in liquid nitrogen to a fine powder. Norleucine was added as a standard to correct for losses during extraction and manipulation. Free amino acids were extracted by adding 1 mL of extraction liquid (MeOH, $CHCl_3$, and H_2O; 12:5:2). After centrifugation in an Eppendorf centrifuge for 3 min, 12,000 rpm, the pellet was re-extracted twice. The supernatants were pooled, and 2 mL of $CHCl_3$ was added. The liquid phase was filtered through 0.5-μm HV filters (Millipore Corp., Bedford, MA). Subsequently, the filtered aqueous phase was lyophilized and redissolved in 200 μL of EtOH–H_2O (1:1) supplemented with 50 μL of wash solution (EtOH–H_2O–triethylamine, 2:2:1). After drying, the amino acids were derivatized with phenylisothiocyanate (PITC). The extracts were dissolved in 100 μL of EtOH–H_2O (1:1), and 50 μL of reaction solution (EtOH–H_2O–TEA–PITC, 7:1:1:1) was added. The reaction was performed at 37°C for 30 min. The samples were lyophilized for 12 hr to remove excess PITC. The phenylisothiocarbamyl(PTC)-derivatized amino acids were dissolved in the appropriate amount of buffer (5% CH_3CN, 0.05 M NaAc, pH 7.2). PTC-amino acid analysis was performed as described by Bidlingmeyer et al. (1984) using a HP1050Ti high-pressure liquid chromatographer (Hewlett Packard, Palo Alto, CA). Relative amounts of free L-methionine were calculated as percentages of the total amount of Asp, Glu, Asn, Ser, Gln, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe, Trp, and Lys. Analysis of free amino acid levels in individual samples of the same leaf half but harvested from another area of the leaf generally did not differ by more than 25%.

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REFERENCES

Aarnes, H. (1978). Regulation of threonine biosynthesis in barley seedlings (*Hordeum vulgare* L.). Planta **140,** 185–192.

Bidlingmeyer, B.A., Cohen, S.A., and Tarvin, T.L. (1984). Rapid analysis of amino acids using pre-column derivatization. J. Chromatogr. **336,** 93–104.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Cherest, H., Surdin-Kerjan, Y., Antoniewski, J., and de Robichon-Szulmajster, H. (1973). Effect of regulatory mutations upon methionine biosynthesis in *Saccharomyces cerevisiae*: Loci *eth2-eth3-eth10.* J. Bacteriol. **115,** 1084–1093.

Deblaere, R., Bytebier, B., De Greve, H., Deboeck, F., Schell, J., Van Montagu, M., and Leemans, J. (1985). Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. Nucl. Acids Res. **13,** 4777–4788.

de Carvalho, F., Gheysen, G., Kushnir, S., Van Montagu, M., Inzé, D., and Castresana, C. (1992). Suppression of β-1,3-glucanase transgene expression in homozygous plants. EMBO J. **11,** 2595–2602.

Flavell, R.B. (1994). Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA **91,** 3490–3496.

Frankard, V., Ghislain, M., Negrutiu, I., and Jacobs, M. (1991). High threonine producer mutant of *Nicotiana sylvestris* (Spegg. and Comes). Theor. Appl. Genet. **82,** 273–282.

Giovanelli, J., Veluthambi, K., Thompson, G.A., Mudd, S.H., and Datko, A.H. (1984). Threonine synthase of *Lemna paucicostata* Hegelm. 6746. Plant Physiol. **76,** 285–292.

Giovanelli, J., Mudd, S.H., and Datko, A.H. (1989). Regulatory structure of the biosynthetic pathway for the aspartate family of amino acids in *Lemna paucicostata* Hegelm. 6746, with special reference to the role of aspartokinase. Plant Physiol. **90,** 1584–1599.

Greene, R.C., Su, C.-H., and Holloway, C.T. (1970). *S*-Adenosylmethionine synthetase deficient mutants of *Escherichia coli* K-12 with impaired control of methionine biosynthesis. Biochem. Biophys. Res. Commun. **38,** 1120–1126.

Greene, R.C., Hunter, J.S.V., and Coch, E.H. (1973). Properties of *metK* mutants of *Escherichia coli* K-12. J. Bacteriol. **115,** 57–67.

Hart, C.M., Fischer, B., Neuhaus, J.-M., and Meins, F., Jr. (1992). Regulated inactivation of homologous gene expression in transgenic *Nicotiana sylvestris* plants containing a defense-related tobacco chitinase gene. Mol. Gen. Genet. **235,** 179–188.

Hibberd, K.A., Walter, T., Green, C.E., and Gengenbach, B.G. (1980). Selection and characterization of a feedback-insensitive tissue culture of maize. Planta **148,** 183–187.

Horsch, R.B., Fry, J.E., Hoffman, N.L., Eichholtz, D., Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science **227,** 1229–1231.

Jorgensen, R. (1991). Silencing of plant genes by homologous transgenes. AgBiotech **4,** 265N–273N.

Kooter, J.M., and Mol, J.N.M. (1993). *trans*-Inactivation of gene expression in plants. Curr. Opin. Biotechnol. **4,** 166–171.

Logemann, J., Schell, J., and Willmitzer, L. (1987). Improved method for the isolation of RNA from tissues. Anal. Biochem. **163,** 16–20.

Madison, J.T., and Thompson, J.F. (1976). Threonine synthase from higher plants: Stimulation by *S*-adenosylmethionine and inhibition by cysteine. Biochem. Biophys. Res. Comm. **71,** 684–691.

Markham, G.D., DeParasis, J., and Gatmaitan, J. (1984). The sequence of *metK*, the structural gene for *S*-adenosylmethionine synthetase in *Escherichia coli.* J. Biol. Chem. **259,** 14505–14507.

Martienssen, R., and Baron, A. (1994). Coordinate suppression of mutations caused by Robertson's *Mutator* transposons in maize. Genetics **136,** 1157–1170.

Martienssen, R., Barkan, A., Taylor, W.C., and Freeling, M. (1990). Somatically heritable switches in the DNA modification of *Mu* transposable elements monitored with a suppressible mutant in maize. Genes Dev. **4,** 331–343.

Mathur, M., Saluja, D., and Sachar, R.C. (1991). Post-transcriptional regulation of *S*-adenosylmethionine synthetase from its stored mRNA in germinated wheat embryos. Biochim. Biophys. Acta **1078,** 161–170.

The Merck Index, an Encyclopedia of Chemicals, Drugs, and Biologicals, 11th ed. (1989). (Rahway, NJ: Merck and Co).

Mudd, S.H., Finkelstein, J.D., Irreverre, F., and Laster, L. (1965). Transsulfuration in mammals: Microassays and tissue distributions of three enzymes of the pathway. J. Biol. Chem. **240,** 4382–4392.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans.* Plant Cell **2,** 279–289.

Peleman, J., Boerjan, W., Engler, G., Seurinck, J., Botterman, J., Alliotte, T., Van Montagu, M., and Inzé, D. (1989a). Strong cellular preference in the expression of a housekeeping gene of *Arabidopsis thaliana* encoding *S*-adenosylmethionine synthetase. Plant Cell **1,** 81–93.

Peleman, J., Saito, K., Cottyn, B., Engler, G., Seurinck, J., Van Montagu, M., and Inzé, D. (1989b). Structure and expression of the *S*-adenosylmethionine synthetase gene family in *Arabidopsis thaliana.* Gene **84,** 359–369.

Poethig, R.S., and Sussex, I.M. (1985). The developmental morphology and growth dynamics of the tobacco leaf. Planta **165,** 158–169.

Rafferty, J.B., Somers, W.S., Saint-Girons, I., and Phillips, S.E.V. (1989). Three-dimensional crystal structures of *Escherichia coli met* repressor with and without corepressor. Nature **341,** 705–710.

Rognes, S.E., Lea, P.J., and Miflin, B.J. (1980). *S*-Adenosylmethionine—a novel regulator of aspartate kinase. Nature **287,** 357–359.

Sachs, T. (1981). The control of the patterned differentiation of vascular tissues. Adv. Bot. Res. **9,** 151–262.

Saint-Girons, I., Belfaiza, J., Guillou, Y., Perrin, D., Guiso, N., Bârzu, O., and Cohen, G.N. (1986). Interactions of the *Escherichia coli* methionine repressor with the *metF* operator and with its corepressor, *S*-adenosylmethionine. J. Biol. Chem. **261,** 10936–10940.

Schmidt, A., Rennenberg, H., Wilson, L.G., and Filner, P. (1985). Formation of methanethiol from methionine by leaf tissue. Phytochemistry **24,** 1181–1185.

Seymour, G.B., Fray, R.G., Hill, P. and Tucker, G.A. (1993). Down-regulation of two non-homologous endogenous tomato genes with a single chimaeric sense gene construct. Plant Mol. Biol. **23,** 1–9.

Smith, C.J.S., Watson, C.F., Bird, C.R., Ray, J., Schuch, W., and Grierson, D. (1990). Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. Mol. Gen. Genet. **224,** 477–481.

Steeves, T.A., and Sussex, I.M. (1989). Patterns in Plant Development, 2nd ed. (Cambridge, UK: Cambridge University Press).

Tabor, C.W., and Tabor, H. (1984a). Methionine adenosyltransferase (*S*-adenosylmethionine synthetase) and *S*-adenosylmethionine decarboxylase. Adv. Enzymol. **56,** 251–282.

Tabor, C.W., and Tabor, H. (1984b). Polyamines. Annu. Rev. Biochem. **53,** 749–790.

Thomas, D., Rothstein, R., Rosenberg, N., and Surdin-Kerjan, Y. (1988). *SAM2* encodes the second methionine *S*-adenosyl transferase in *Saccharomyces cerevisiae*: Physiology and regulation of both enzymes. Mol. Cell. Biol. **8,** 5132–5139.

van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N.M., and Stuitje, A.R. (1990). Flavonoid genes in petunia: Addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2,** 291–299.

Van Haute, E., Joos, H., Maes, M., Warren, G., Van Montagu, M., and Schell, J. (1983). Intergeneric transfer and exchange recombination of restriction fragments cloned in pBR322: A novel strategy for the reversed genetics of Ti plasmids of *Agrobacterium tumefaciens.* EMBO J. **2,** 411–418.

Vernaillen, S., and Verbruggen, I. (1991). Extraction of amino acids from plant samples and their analysis using ion-exchange chromatography. In A Laboratory Guide for Cellular and Molecular Plant Biology (BioMethods, Vol. 4), I. Negrutiu and G.B. Gharti-Chhetri, eds (Basel: Birkhäuser Verlag), pp. 184–190.

Yang, S.F., and Hoffman, N.E. (1984). Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. **35,** 155–189.