

# Differential Expression of the *S*-Adenosyl-*L*-Methionine Synthase Genes during Pea Development<sup>1</sup>

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Two genes coding for *S*-adenosyl-*L*-methionine synthase (SAMS, EC 2.5.1.6) were previously isolated from pea (*Pisum sativum*) ovaries. Both SAMS genes were highly homologous throughout their coding regions but showed a certain degree of sequence divergence within the 5' and the 3' untranslated regions. These regions have been used as gene-specific probes to analyze the differential expression of *SAMS1* and *SAMS2* genes in pea plants. The ribonuclease protection assay revealed different expression patterns for each individual gene. *SAMS1* was strongly expressed in nearly all tissues, especially in roots. *SAMS2* expression was weaker, reaching its highest level at the apex. Following pollination, *SAMS1* was specifically up-regulated, whereas *SAMS2* was expressed constitutively. The up-regulation of *SAMS1* during ovary development was also observed in unpollinated ovaries treated with auxins. In unpollinated ovaries an increase in *SAMS1* expression was observed as a consequence of ethylene production associated with the emasculating process. In senescing ovaries both *SAMS1* and *SAMS2* genes showed increased expression. Ethylene treatment of unpollinated ovaries led to an increase in the *SAMS1* mRNA level. However, *SAMS2* expression remained unchangeable after ethylene treatment, indicating that *SAMS2* induction during ovary senescence was not ethylene dependent. *SAMS* mRNAs were localized by *in situ* hybridization at the endocarp of developing fruits and in the ovules of senescing ovaries. Our results indicate that the transcriptional regulation of *SAMS* genes is developmentally controlled in a specific way for each gene.

SAMS (EC 2.5.1.6) is a key enzyme in plant metabolism, catalyzing the biosynthesis of SAM from Met and ATP. SAM is a precursor for the biosynthesis of ethylene (Yang and Hoffman, 1984) and polyamines (Heby and Persson, 1990) and is involved in methylation reactions (Tabor and Tabor, 1984). *SAMS* cDNA clones have been isolated from several plants (Izhaki et al., 1995). Recently, two genes from pea (*Pisum sativum*), *SAMS1* and *SAMS2*, were cloned in our laboratory (Gómez-Gómez and Carrasco, 1996). In all of the characterized systems, a multigene family encodes SAMS enzymes. The existence of different *SAMS* genes has generally been ascribed to the metabolic importance of

SAM (Thomas and Surdin-Kerjan, 1987; Peleman et al., 1989b). Moreover, it has been suggested that some of the *SAMS* genes would be expressed constitutively, whereas others would be specifically regulated by developmental and/or environmental factors strictly controlled according to the requirement for SAM (Boerjan et al., 1994).

Marked variations in the levels of *SAMS* mRNA have been observed at different stages of plant development (Woodson et al., 1992; Boerjan et al., 1994; Izhaki et al., 1995) and in different plant tissues (Peleman et al., 1989a; Dekeyser et al., 1990). *SAMS* expression is also increased by a variety of environmental factors, including salt stress (Espartero et al., 1994; Van Breusegem et al., 1994), fungal and bacterial elicitors (Somssich et al., 1989; Gowri et al., 1991; Kawalleck et al., 1992), ozone exposure (Tuomainen et al., 1996), and mechanical stimuli (Espartero et al., 1994; Kim et al., 1994), all of which are also known to induce ethylene biosynthesis (Abeles et al., 1992). Hormonal regulation of *SAMS* has also been reported in wheat embryos (Mathur et al., 1992) and dwarf mutants of pea (Mathur et al., 1993), in which treatment with GA<sub>3</sub> induced two additional isoenzymes of *SAMS*, and in tomato roots, in which treatment with ABA induced the accumulation of *SAMS* transcripts (Espartero et al., 1994).

In this paper we report a detailed study of the individual expression of *SAMS* genes, their developmental regulation, and the patterns of their response to auxins and ethylene in unpollinated pea ovaries, a convenient system with which to study both the regulation of senescence and the induction of fruit set by plant-growth regulators. Removing stamens 2 d before anthesis avoids self-pollination. Intact, unpollinated ovaries start to senesce naturally about 3 DPA, but treatment of unpollinated ovaries with different plant-growth regulators on the equivalent to the day of anthesis (d 0) prevents senescence and promotes fruit development (García-Martínez and Carbonell, 1980). The pea *SAMS* gene family consists of two genes. Analysis of the expression of the *SAMS* genes during ovary senescence and fruit development showed that *SAMS* transcript levels were up-regulated by auxins during fruit setting and by ethylene during ovary senescence (Gómez-Gómez and Carrasco, 1996). However, the high degree of identity made it impossible to differentiate the expression of each individual *SAMS* gene.

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Abbreviations: DPA, days postanthesis; RPA, RNase protection assay; SAM, *S*-adenosyl-*L*-Met; SAMS, *S*-adenosyl-*L*-Met synthase.

Using RPA and gene-specific probes, we now show that the *SAMS1* gene is highly expressed during fruit development and senescence and is also expressed in pea ovaries following treatment with ethylene. Furthermore, we report that *SAMS2* gene expression is reduced during auxin-induced parthenocarpic fruit development and that its expression during ovary senescence is not induced by ethylene. Spatial localization by *in situ* hybridization showed that *SAMS* mRNAs are differentially accumulated in the endocarp of developing ovaries, suggesting a role for SAM in pod wall development. We conclude that *SAMS* genes in pea are differentially regulated during ovary development and senescence.

## MATERIALS AND METHODS

Pea (*Pisum sativum* L. cv Alaska) plants were grown as previously described (Carbonell and García-Martínez, 1985). Unpollinated ovaries were obtained by removing petals and stamens from flowers 2 d before anthesis. Only the first and second flowers of each plant were used for the experiments. Samples were obtained from (a) pollinated ovaries; (b) untreated, unpollinated ovaries from emasculated flowers (including presenescent and senescent ovaries); (c) parthenocarpic ovaries, which were unpollinated and derived from emasculated flowers treated on d 0 with a 2,4-D solution (20  $\mu\text{L}$  of 100  $\mu\text{g mL}^{-1}$  2,4-D in 0.1% Tween-80); and (d) unpollinated ovaries from emasculated flowers treated on d 0 with 10  $\mu\text{L L}^{-1}$  ethylene. After the plants were harvested, samples to be used for RNA isolation were frozen in liquid N and stored at  $-80^\circ\text{C}$  until used.

Ethylene production was measured by placing ovaries at different stages of development in 5-mL gas-tight containers for 0.5 h, after which time the headspace gas was assayed for ethylene by GC. The gas chromatograph (model GC-14BPFSC, Shimadzu, Columbia, MD) was equipped with an activated alumina column and a flame-ionization detector. An ethylene standard was used for calibration of concentration and retention time.

### RNA Isolation and RPA

RNA was prepared from frozen plant material following the method of Prescott and Martin (1987) and quantified by measuring the  $A_{260}$ . The integrity of rRNA was verified by electrophoresis through 1% (w/v) agarose gels containing 2.2 M formaldehyde. RPA (Lee and Costlow, 1987) was performed using [ $\alpha$ - $^{32}\text{P}$ ]UTP strand-specific radiolabeled probes generated from a 124-bp fragment of the 5' portion of the *SAMS1* cDNA and a 423-bp fragment containing the 3' end of the *SAMS2* cDNA (Gómez-Gómez and Carrasco, 1996). The sequences selected to generate the probes were different enough in both pea *SAMS* genes to ensure probe specificity. To synthesize the *SAMS1* and *SAMS2* antisense RNA probes, both *SAMS* cDNAs were cloned in pGEM-1 (Promega), and the plasmids were linearized with *Pst*I and *Pvu*II and transcribed *in vitro* with T7 and Sp6 RNA polymerase, respectively. Ten micrograms of total RNA was

hybridized with 55,000 dpm, 760 and 250 attomoles of *SAMS1*- and *SAMS2*-labeled riboprobes, respectively.

Following hybridization, digestion buffer was added and unhybridized RNA was removed by digestion with a mixture of RNase-A and RNase-T1 at  $30^\circ\text{C}$  for 30 min following the directions of the manufacturer (Boehringer Mannheim). Adding 20% SDS and proteinase K and incubating at  $37^\circ\text{C}$  for 15 min stopped the digestion. After phenol extraction and ethanol precipitation, RNAs were separated on a 6% polyacrylamide-sequencing gel. An aliquot of undigested RNA probe was run as a marker for the size of the transcript. A quantitative measure of transcript abundance was obtained using a radioanalytical imaging system (InstantImager 2024, Packard Instruments, Meriden, CT). Radioactivity was directly measured on the gel. The data were corrected for the number of uracil residues within each probe, and the specific activity of the probes was calculated to determine the attomoles of mRNA according to the following equation:

$$\text{mol} = \frac{\text{cpm}^*}{E \times S \times N}$$

where  $\text{cpm}^*$  is the counts per minute counted by the imager and corrected according to the reference date of the [ $^{32}\text{P}$ ]UTP,  $E$  is the efficiency of the counting,  $S$  is the specific activity of the [ $\alpha$ - $^{32}\text{P}$ ]UTP (800 Ci  $\text{mmol}^{-1}$ ), and  $N$  is the number of uracil residues in the probe. All values are the means of three independent experiments. Autoradiography of the gel was independently obtained on radiographic film (XAR-5, Kodak) using an intensifying screen at  $-80^\circ\text{C}$ .

### In Situ RNA Hybridization

Ovaries were harvested from flowers at various stages of development, fixed, embedded in paraffin, and tissue-sectioned as described previously (Vercher et al., 1984). *In situ* hybridization was carried out essentially as described by Duck (1994). Tissue samples were cut into 10- $\mu\text{m}$  sections and placed on replicated slides coated with poly-L-Lys. The sections were dewaxed, hydrated, and treated with HCl, proteinase K, and acetic anhydride. Digoxigenin-labeled sense and antisense riboprobes were generated by *in vitro* transcription of a pGEM-1 template containing a 400-bp insert with the 5' region of the *SAMS1* cDNA (Gómez-Gómez and Carrasco, 1996). Plasmids were linearized, and digoxigenin-11-UTP was incorporated using either T7 or SP6 polymerase according to the instructions of the manufacturer (Boehringer Mannheim).

Slides were hybridized for 30 h at  $45^\circ\text{C}$  in 50% formamide, 30 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 $\times$  Denhardt's solution, 10% dextran sulfate, 100 mM DTT, 500  $\mu\text{g mL}^{-1}$  denatured salmon-sperm DNA, 150  $\mu\text{g mL}^{-1}$  yeast tRNA, and 0.2  $\mu\text{g mL}^{-1}$  digoxigenin-labeled probe. Following hybridization, slides were treated with RNase-A to remove nonhybridized probe and washed once at room temperature in 2 $\times$  SSC for 30 min, once at room temperature in 1 $\times$  SSC for 30 min, and once at  $37^\circ\text{C}$  in 0.5 $\times$  SSC for 30 min. Hybridization of the riboprobes was detected with anti-digoxigenin antibody conjugated to alkaline phosphatase.

tase and visualized by color development following the manufacturer's directions (Boehringer Mannheim).

## RESULTS

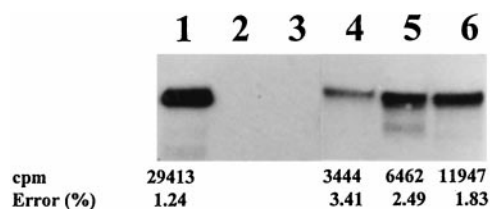
### Differential Expression of *SAMS* Genes in Pea Plants

Previous work in our laboratory showed that *SAMS* transcripts were present in vegetative and reproductive tissues. *SAMS* mRNA was detected at higher levels in roots and stems than in leaves when the *SAMS1*-coding sequence was used as a probe for northern analysis (Gómez-Gómez and Carrasco, 1996). To determine whether both *SAMS* genes contributed to this expression pattern, RPA (Lee and Costlow, 1987) was performed with RNA samples from different tissues. RPA was carried out using 10  $\mu$ g of total RNA, as described in "Materials and Methods." Antisense RNA probes of 124 and 436 bp, respectively, were synthesized from cDNA clones for *SAMS1* and *SAMS2* and labeled with [ $\alpha$ - $^{32}$ P]UTP. No RNase protection was observed after hybridizing the *SAMS* gene-specific probes to yeast tRNA or human RNA (Fig. 1).

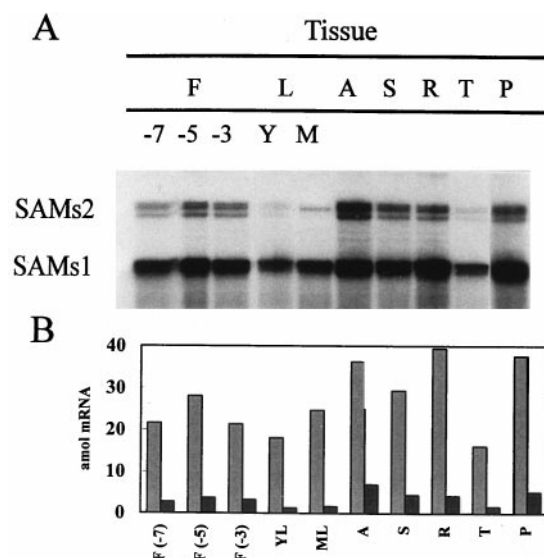
Quantification of RPA of increasing RNA amounts showed the linearity of the method (Fig. 1). As shown in Figure 2, expression of *SAMS1* mRNA was strong in most tissues, especially in roots, apices, and petals; a little lower in stems, flower buds, and mature leaves; and lowest in young leaves and tendrils. By contrast, *SAMS2* was expressed preferentially in the apex and was almost absent from vegetative tissues such as young and mature leaves and tendrils (Fig. 2). In general, changes in *SAMS1* mRNA level were more significant than changes in *SAMS2* mRNA. RPA of *SAMS2* transcripts gave rise to two bands, because the local denaturation of the 3' end of the probe located at the poly(A<sup>+</sup>) tail of the mRNA. Quantification of the counts and correction by the specific activity of the probes showed that in all tissues *SAMS2* transcripts were expressed at lower levels than *SAMS1* transcripts (Fig. 2B).

### Expression of *SAMS* Genes during Fruit Development

Pea ovaries ranging from 2 d before to 6 DPA were used to analyze the differential expression of the *SAMS* genes



**Figure 1.** RPA of *SAMS1* transcripts. Control lanes for the *SAMS1* RNA probe are: lane 1, undigested probe; lane 2, *SAMS1* RNA probe hybridized to yeast tRNA; lane 3, *SAMS1* RNA probe hybridized to total human RNA; and lanes 4 to 6, RNA (5, 10, and 15  $\mu$ g, respectively) from pollinated pea ovaries 5 DPA. All were hybridized to the *SAMS1* gene-specific RNA probe, and RPA was performed as described in "Materials and Methods." The counts per minute detected in the samples are shown under each band. Radioactivity was measured using a radioanalytical imaging system (see "Materials and Methods").



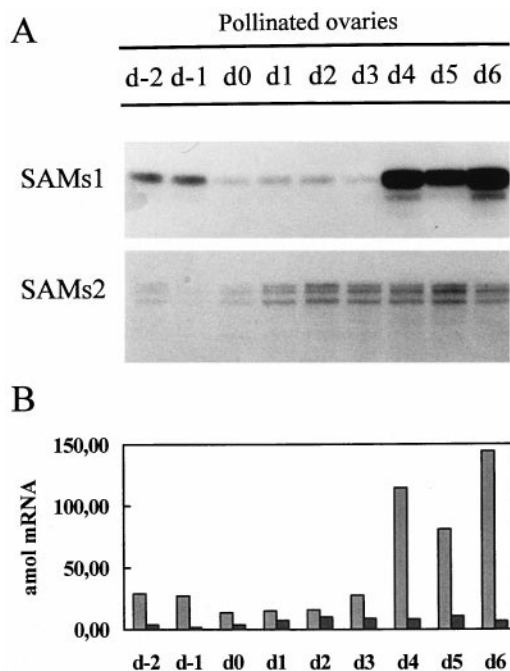
**Figure 2.** RPA of *SAMS* transcripts in pea tissues. A, Ten micrograms of total RNA extracted from different tissues of pea plants was hybridized to *SAMS* gene-specific RNA probes, and RPA was performed. F, Floral buds (-7, -5, and -3, d 7, 5, and 3 before anthesis, respectively); L, leaves (Y, young; M, mature); A, apex; S, stem; R, root; T, tendrils; and P, petals. B, Quantification of the *SAMS* mRNAs at the stages shown in A. *SAMS* transcript levels were quantified using a radioanalytical imaging system. Light gray bars, *SAMS1*; dark gray bars, *SAMS2*. The data were corrected for the number of uracil residues within each probe.

during fruit development. RPA analysis showed different expression patterns for *SAMS1* and *SAMS2* genes during fruit development (Fig. 3). Before d 0, *SAMS1* was expressed at a higher level than *SAMS2* mRNA, which was almost undetectable (Fig. 3). Immediately after pollination, *SAMS1* transcript level decreased and remained invariable up to 3 DPA, then increased suddenly on 4 DPA, and stayed high through 6 DPA (Fig. 3). On the contrary, *SAMS2* level increased after anthesis and reached its maximum after 24 h, which was maintained for the following 3 d (Fig. 3).

Analysis of *SAMS* expression in pods and seeds during fruit development showed opposite gradients of *SAMS1* mRNA expression. *SAMS1* mRNA accumulated during seed development (Fig. 4). On the contrary, *SAMS1* mRNA level in the pod decreased from 4 to 10 DPA, when the pod began to dry (Fig. 4). In the meantime, *SAMS2* levels varied in pods in a way similar to *SAMS1* but were present at almost undetectable levels in seeds during fruit development (Fig. 4).

Treatment of emasculated ovaries with auxins induced parthenocarpic development, generating fruits without seeds. Previous work in our laboratory showed that *SAMS* transcripts accumulate only in parthenocarpic ovaries generated by auxin application in a way similar to that produced by pollination (Gómez-Gómez and Carrasco, 1996). We analyzed the expression of the individual *SAMS* genes during this process. RPA using *SAMS1* and *SAMS2* antisense RNA probes showed a differential expression pattern for both genes during auxin-induced parthenocarpy (Fig.



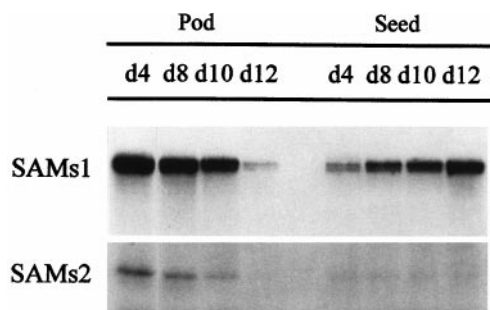


**Figure 3.** Temporal expression of *SAMS* genes during fruit setting. A, Ten micrograms of total RNA extracted from pea ovaries at different stages of development were hybridized to *SAMS* gene-specific RNA probes, and RPA was performed. d-2 to d6, Days with respect to anthesis. B, Quantification of the *SAMS* mRNAs at the stages shown in A. *SAMS* transcript levels were quantified using a radioanalytical imaging system. Light gray bars, *SAMS1*; dark gray bars, *SAMS2*. The data were corrected for the number of uracil residues within each probe.

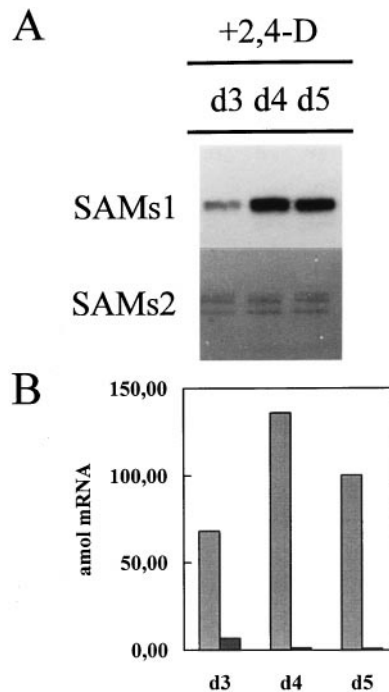
5). In parthenocarpic ovaries, *SAMS1* mRNA levels accumulate after 3 DPA (Fig. 5) in a way similar to that observed after pollination (Fig. 3). On the contrary, *SAMS2* mRNA levels were lower than those observed in pollinated ovaries (Fig. 5).

**Expression of *SAMS* Genes in Senescing Pea Ovaries**

Since ethylene levels are related to the senescence of plant organs (Davies and Grierson, 1989) and because SAM



**Figure 4.** Analysis of *SAMS* transcripts in pea pods and ovules during fruit development. Ten micrograms of total RNA extracted from pea pods and seeds from pollinated pea ovaries at different stages of development was hybridized to *SAMS* gene-specific RNA probes, and RPA was performed. d4 to d12, Days with respect to anthesis.

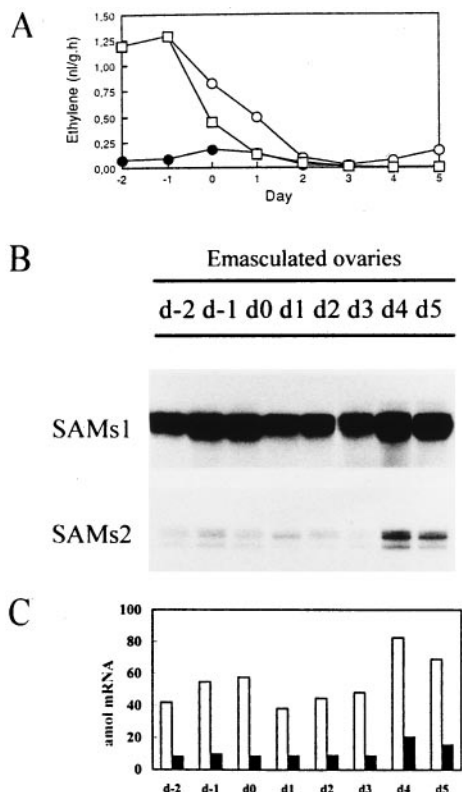


**Figure 5.** Effect of 2,4-D treatment on d 0 on *SAMS* expression in unpollinated pea ovaries. A, Ten micrograms of total RNA extracted from 2,4-D-treated pea ovaries was hybridized to *SAMS* gene-specific RNA probes, and RPA was performed. d3 to d5, Days with respect to anthesis. B, Quantification of the *SAMS* mRNAs at the stages shown in A. *SAMS* transcript levels were quantified using a radioanalytical imaging system. Light gray bars, *SAMS1*; dark gray bars, *SAMS2*. The data were corrected for the number of uracil residues within each probe.

is involved in ethylene biosynthesis, we determined ethylene production in pea ovaries (Fig. 6A) and compared it with *SAMS* expression in ovaries that had been pollinated (Fig. 3), 2,4-D-treated (Fig. 5), or emasculated (Fig. 6B). To induce senescence, flowers were emasculated 2 d before anthesis. Unpollinated ovaries grow slightly until the equivalent of 3 DPA, and then enter a degenerative process that ends with the death of the organ. A strong increase in ethylene production was immediately detected in ovaries emasculated 2 d before anthesis (Fig. 6A).

Because of the wounding caused by the emasculation process, this ethylene increase was accompanied by an increase in the level of *SAMS1* mRNA with no change in the expression of *SAMS2* (Fig. 6B). Later, on the equivalent of 4 DPA, there was a slight increase in ethylene production that could be associated with ovary senescence (Fig. 6A). There was a transcript accumulation of *SAMS1* mRNA at 4 DPA that was associated with the onset of senescence (Fig. 6, B and C). Thereafter, a decline in *SAMS1* mRNA abundance was associated with the advanced stages of ovary senescence. The *SAMS2* mRNA level remained almost unchanged in emasculated ovaries but was slightly induced at the onset of ovary senescence (Fig. 6B).

We examined the effect of exogenous ethylene on *SAMS1* and *SAMS2* gene expression in emasculated pea ovaries on d 0. As shown in Figure 7, the levels of *SAMS1* mRNA



**Figure 6.** Ethylene levels and temporal expression of *SAMS* genes during ovary senescence. A, Ethylene levels were determined in emasculated ovaries from 2 d before anthesis (at the time of emasculatation) to 6 DPA. ●, Ovaries allowed to be pollinated; ○, emasculated ovaries; and □, emasculated ovaries treated with 2,4-D on d 0. d-2 to d5, Days with respect to anthesis. B, Ten micrograms of total RNA extracted from the same samples used to measure ethylene was hybridized to *SAMS* gene-specific RNA probes, and RPA was performed. C, Quantification of the *SAMS* mRNAs at the stages shown in A. *SAMS* transcript levels were quantified using a radioanalytical imaging system. White bars, *SAMS1*; black bars, *SAMS2*. The data were corrected for the number of uracil residues within each probe.

increased in the ovaries in response to ethylene treatment. The level of *SAMS1* mRNA after 24 h of treatment was the same as the level in senescing ovaries (Figs. 5 and 6). In contrast to *SAMS1* mRNA, *SAMS2* mRNA remained almost constant, which is consistent with the lack of stimulation of this gene during the emasculatation process.

#### Spatial Distribution of *SAMS* Genes in Pea Ovaries

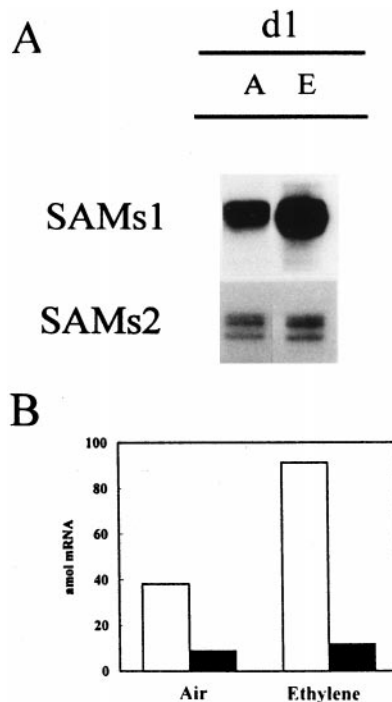
Previous reports have described the expression of *SAMS-GUS* reporter genes in roots, stems, and leaves of *Arabidopsis* (Peleman et al., 1989a) and tobacco (Peleman et al., 1989b) and in leaves of rice (Dekeyesen et al., 1990). We determined the spatial distribution of *SAMS* genes at different stages of ovary development and senescence. In situ hybridization was performed using *SAMS1* (Gómez-Gómez and Carrasco, 1996) sense and antisense RNA probes. Hybridization of longitudinal (Fig. 8c) and transverse (Fig. 8d) sections of pollinated ovaries on d 0 with an antisense *SAMS* probe revealed a general pattern of expres-

sion in all living cells, especially in vascular tissue, the ovule, and ovary epidermal cells. Control for cell density, which was accomplished by staining a longitudinal section with toluidine blue, showed that higher *SAMS* expression was not correlated with greater cell density (Fig. 8a).

In pollinated ovaries on 3 DPA, *SAMS* was expressed mostly in the endocarp and vascular bundles (Fig. 8f). This pattern of expression was reinforced 5 DPA (Fig. 8, h-j), when the transcripts were present in cells associated with the vascular tissue of developing seed and pod mesocarp. Moreover, at this stage of development, *SAMS* mRNAs were particularly abundant in endocarp cells. In senescing, unpollinated ovaries 4 DPA, *SAMS* mRNAs were localized in vascular tissues and also in the aborted ovules (Fig. 8l). No signal was detected when a sense probe was used to hybridize sections at different stages (Fig. 8, b, e, g, and k).

#### DISCUSSION

*SAMS* genes have been considered to be "housekeeping genes" that are expected to be expressed constitutively in all tissues. Previous work showed that *SAMS* transcripts were induced in developing pea ovaries in response to pollination and auxins and also during ovary senescence



**Figure 7.** Effect of ethylene treatment on *SAMS* expression in unpollinated pea ovaries. Treatment was made on d 0, and ovaries were collected 24 h later. A, Ten micrograms of total RNA extracted from ethylene-treated pea ovaries was hybridized to *SAMS* gene-specific RNA probes, and RPA was performed. Lane A, Air; lane E, after ethylene treatment. B, Quantification of the *SAMS* mRNAs at the stages shown in A. *SAMS* transcript levels were quantified using a radioanalytical imaging system. White bars, *SAMS1*; black bars, *SAMS2*. The data were corrected for the number of uracil residues within each probe.



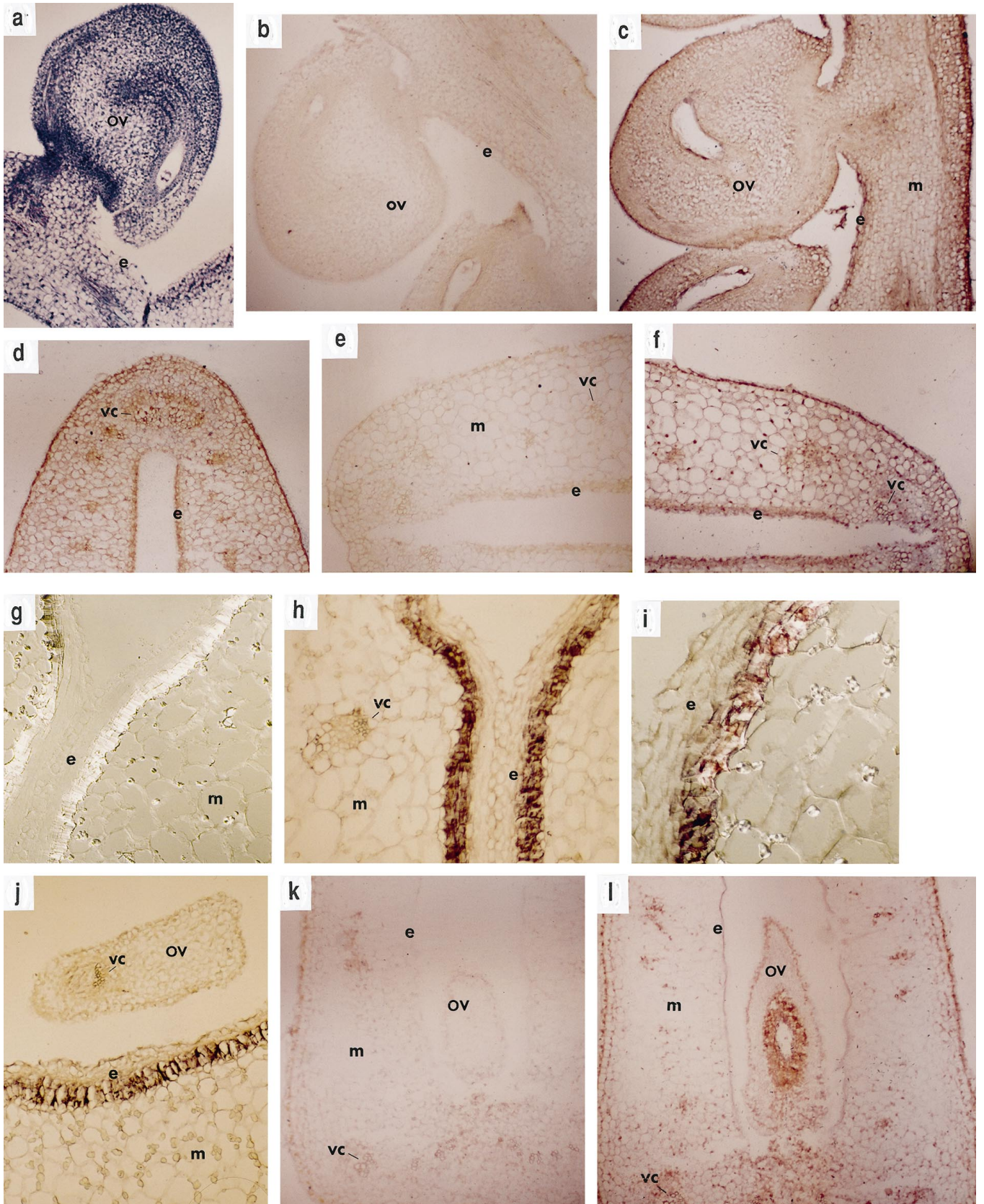


Figure 8. (Legend appears on facing page.)

(Gómez-Gómez and Carrasco, 1996). However, northern analysis was not able to determine whether the genes were biologically equivalent or whether they were differentially regulated. In this work we used RPA with gene-specific probes to distinguish the expression pattern of each *SAMS* gene. The steady-state levels of mRNA, corresponding to both *SAMS1* and *SAMS2* genes, were monitored for different pea tissues at different developmental stages and in response to different plant hormones.

Our studies demonstrate that *SAMS1* and *SAMS2* genes are individually regulated, with their transcripts accumulating to varying degrees among different tissues and at different stages of ovary development or senescence. *SAMS1* mRNA was found to be expressed at higher levels than *SAMS2* in all tissues. In fact, the *SAMS1* expression pattern was found to be the same as that obtained by northern analysis using a probe able to hybridize both *SAMS1* and *SAMS2* genes (Gómez-Gómez and Carrasco, 1996). Nonetheless, the expression pattern for *SAMS2* was quite different, although its contribution to the pool of *SAMS* mRNAs was much lower. A similar relationship between *SAMS* genes has been found in tomato, in which transcripts corresponding to the *sam1* gene were the most abundant, although the expression pattern for each gene was different in each tissue (Espartero et al., 1994).

In contrast, the two Arabidopsis *SAMS* genes show a similar expression pattern (Peleman et al., 1989b): *SAMS1* was highly expressed in roots, apex, and petals and also accumulated to significant levels in the other tissues examined; preferential expression of *SAMS2* was found in the apex. The expression of both genes in this tissue could be due to polyamine biosynthesis, which is required for cell proliferation in dividing tissues (Evans and Malmberg, 1989), and to the biosynthesis of macromolecules associated with the meristematic activity of this tissue.

In the apex SAM could be decarboxylated and used for the biosynthesis of spermidine and spermine. This would agree with the high levels of Arg decarboxylase mRNA and enzymatic activity detected in the pea apex (Pérez-Amador and Carbonell, 1995; Pérez-Amador et al., 1995). In floral buds both mRNAs change their levels coordinately according to the developmental stage of the organ. A developmental stage pattern has also been observed during corolla development in petunia (Izhaki et al., 1995) and has been related to the rapid growth of the tissue (Weiss and Halevy, 1989).

### Expression of *SAMS* Genes in Fruits Is Developmentally Regulated

Before the day of anthesis, coinciding with a period of elevated cell division rate, *SAMS1* was highly expressed, whereas *SAMS2* mRNA was almost undetectable. At the time of anthesis, when cell division was about to conclude, the relationship between *SAMS1* and *SAMS2* expression changed. *SAMS1* mRNA expression decreased, and *SAMS2* mRNA expression increased slightly 24 h after anthesis and remained almost constant through 6 DPA. The analysis of *SAMS2* expression in pods and seeds showed that the expression detected in fruits was almost completely restricted to the pod. In pollinated ovaries, the most dramatic increase in *SAMS1* expression was detected 4 DPA. *SAMS1* expression was mainly due to the level of *SAMS1* mRNA in the pod and was localized primarily to cells of the presclerenchyma zone of the endocarp. At this time the differentiation of the endocarp cells into sclerenchyma fibers starts (Vercher et al., 1984).

The accumulation of *SAMS1* mRNA in cells under lignification would suggest that *SAMS1* is involved in the biosynthesis of the secondary cell wall. During this process SAM is needed for methylation of lignin monomers before polymerization (Higuchi, 1981). At 8 DPA, when the pod reached its maximum length, *SAMS1* expression decreased. Then, as the pod began to dry out, the amount of dry matter declined. Simultaneously, the seeds enlarged rapidly and increased in fresh weight by accumulation of dry matter, partly at the expense of the pod wall (Eeuwens and Schwabe, 1975). During pea seed development the synthesis and amount of storage and metabolic proteins increases (Bewley and Black, 1994). *SAMS1* mRNA also increased, suggesting the participation of SAM in macromolecule biosynthesis as a methyl group donor. Moreover, the presence of stored *SAMS* mRNA in wheat embryos (Mathur et al., 1991) indicates that *SAMS1* mRNA could remain in the dry seed and then be reutilized upon subsequent hydration. mRNAs coding for enzymes essential for intermediary metabolism and proteins essential for the successful completion of germination have been found in the stored or conserved messages in dry seeds (Kermade, 1990).

### *SAMS* Genes Are Differentially Regulated in Parthenocarpic Developing Ovaries

The expression of *SAMS1* and *SAMS2* genes was investigated in parthenocarpic pea ovaries induced by auxin

**Figure 8.** (Figure appears on facing page.)

In situ localization of *SAMS* mRNAs in pea ovaries. Pollinated and unpollinated ovaries at different stages of development were sectioned and hybridized to an antisense *SAMS1* probe. a, b, and c, Longitudinal sections of a pollinated pea ovary at anthesis stained with toluidine blue (a) or hybridized with a *SAMS* sense probe (b) or a *SAMS* antisense probe (c). d to h, Transverse sections of a pollinated pea ovary at anthesis hybridized with a *SAMS* antisense probe (d), at 3 DPA hybridized with a *SAMS* sense probe (e) or a *SAMS* antisense probe (f), and at 5 DPA hybridized with a *SAMS* sense probe (g) or a *SAMS* antisense probe (h). i, Detail of a transverse section of a pollinated pea ovary 5 DPA hybridized with a *SAMS* antisense probe. j, Transverse section of a pollinated pea ovary at 5 DPA, including an ovule, hybridized with a *SAMS* antisense probe. k and l, Transverse sections of an emasculated pea ovary at the equivalent to 4 DPA hybridized with a *SAMS* sense probe (k) and a *SAMS* antisense probe (l). Bar in i = 200  $\mu\text{m}$ ; bars in all other panels = 100  $\mu\text{m}$ . e, Endocarp; m, mesocarp; ov, ovule; and vc, vascular cell.



treatment. The weight of these ovaries is lower than that of the pollinated ovaries, but at the histological and physiological levels the developmental pattern of the ovaries is the same (Vercher and Carbonell, 1991). Northern analysis showed that in pollinated and auxin-induced parthenocarpic ovaries the expression pattern of *SAMS* genes was the same (Gómez-Gómez and Carrasco, 1996). However, the individual analysis of *SAMS* transcripts showed that *SAMS1* mRNA was induced in auxin-treated unpollinated ovaries as well as in pollinated ovaries, which suggests that similar mechanisms are involved in the transcriptional control of the *SAMS1* gene.

On the contrary, *SAMS2* expression was dramatically reduced in parthenocarpic fruits. This could be due to the lack of pollination and/or fertilization in the parthenocarpic ovaries that may provide the stimulus for *SAMS2* mRNA induction, which began at anthesis in pollinated ovaries. In addition, since auxins induce the parthenocarpic development of the ovaries, generating fruits without seeds, the lack of induction of *SAMS2* mRNA could be associated with factors originating in the seeds and translocated to the pod, where they induce *SAMS2* transcription.

#### **SAMS Gene Expression during Ovary Senescence**

The involvement of ethylene in pea ovary senescence has already been reported (Gómez-Gómez and Carrasco, 1996). During senescence, the increase in *SAMS* gene expression was dependent on ethylene production. In the present study we showed that ethylene increases in unpollinated pea ovaries as a consequence of the emasculation process and during senescence. Both of these increases in ethylene production are accompanied by increases in *SAMS* expression. In both cases *SAMS1* was expressed at higher levels, whereas the *SAMS2* transcript accumulated only at the onset of ovary senescence and was less abundant than *SAMS1* mRNA. Because only the *SAMS1* gene was induced by ethylene, accumulation of *SAMS1* mRNA after emasculation and during ovary senescence could be associated with an increase in ethylene biosynthesis.

Increases in *SAMS* gene expression in response to mechanical stimuli have been reported in wounded Arabidopsis leaves (Kim et al., 1994) and tomato roots, in which only one of the three *SAMS* genes was induced (Espartero et al., 1994). However, induction of *SAMS2* mRNA at the onset of senescence was ethylene independent and could have been age related. Leaf senescence in Arabidopsis (Grbic and Bleecker, 1995) and leaf senescence and ripening in tomato (Picton et al., 1993) are developmental processes that are regulated by the interplay between putative age-related factors and ethylene. In unpollinated pea ovaries, these age-related factors could determine the sensitivity of ovary tissue to ethylene and the lack of sensitivity of ovary tissue to plant-growth substances 5 d after emasculation (García-Martínez and Carbonell, 1980).

The expression of *SAMS* genes in unpollinated pea ovaries at the equivalent of 4 DPA was localized specifically in cells of the embryo sac and in vascular cells, which were differentiating at this time (Vercher et al., 1989) and in

which SAM is necessary for lignin biosynthesis. Expression of other genes related to the senescence process has also been observed during the differentiation of vascular cells (Granell et al., 1992; Nadeau et al., 1993; Tang et al., 1994), a process related to senescence (Jones and Dangl, 1996).

In conclusion, *SAMS1* and *SAMS2* mRNAs are differentially expressed during ovary development in pea. Ethylene induction of *SAMS1* mRNA and the lack of induction of *SAMS2* mRNA by ethylene-treated and parthenocarpic fruits suggest the existence of different factors controlling *SAMS* gene transcription. These findings raise the question of how this separate action is established and maintained. Future research will focus on the mechanism regulating *SAMS1* and *SAMS2* expression during ovary development in pea.

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