

# Identification and Characterization of an *S*-Adenosyl-L-methionine: $\Delta^{24}$ -Sterol-C-methyltransferase cDNA from Soybean\*

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In plants, the dominant sterols are 24-alkyl sterols, which play multiple roles in plant growth and development, *i.e.* as membrane constituents and as precursors to steroid growth regulators such as brassinosteroids. The initial step in the conversion of the phytosterol intermediate cycloartenol to the 24-alkyl sterols is catalyzed by *S*-adenosyl-L-methionine: $\Delta^{24}$ -sterol-C-methyltransferase (SMT), a rate-limiting enzyme for phytosterol biosynthesis. A cDNA clone (SMT1) encoding soybean SMT was isolated from an etiolated hypocotyl cDNA library by immunoscreening using an anti-(plasma membrane) serum. The deduced amino acid sequence of the SMT1 cDNA contained three conserved regions found in *S*-adenosyl-L-methionine-dependent methyltransferases. The overall structure of the polypeptide encoded by the SMT1 cDNA is most similar to the predicted amino acid sequence of the yeast *ERG6* gene, the putative SMT structural gene. The polypeptide encoded by the SMT1 cDNA was expressed as a fusion protein in *Escherichia coli* and shown to possess SMT activity. The growing soybean vegetative tissues had higher levels of SMT transcript than mature vegetative tissues. Young pods and immature seeds had very low levels of the SMT transcript. The SMT transcript was highly expressed in flowers. The expression of SMT transcript was suppressed in soybean cell suspension cultures treated with yeast elicitor. The transcriptional regulation of SMT in phytosterol biosynthesis is discussed.

In plants, the dominant sterols are 24-alkyl sterols. Sterols such as cholesterol which are nonmethylated at C-24 are only present in low amounts. It has been suggested that sterols may have two distinct functions in higher plants (Haughan *et al.*, 1988), as has been found for fungi (Ramgopal and Bloch, 1983; Rodriguez and Parks, 1983; Nes and Heupel, 1986). The first is as a structural component of membranes, a function which requires relatively large amounts of sterols. The second function is related to cell proliferation; trace amounts of 24-alkyl sterols such as stigmasterol are required for cell growth to proceed (Haughan *et al.*, 1988). 24-Alkyl sterols also serve as precursors to steroid growth regulators such as brassinosteroids (Ikekawa, 1991). Biosynthesis of 24-alkyl phytosterols can be divided into two stages: conversion of acetate to cycloartenol and transformation of cycloartenol to 24-alkyl sterols. The transformation of cycloartenol to 24-alkyl sterols includes four steps: alkylation of the  $\Delta^{24}$ -bond in the side chain, removal

of methyl groups at C-4 and C-14, isomerization of the cyclopropyl group into a  $\Delta^{8(9)}$ -double bond, and rearrangement of the  $\Delta^{8(9)}$  to  $\Delta^5$  (Benveniste, 1986). The transmethylation of the  $\Delta^{24}$ -double bond in the cycloartenol side chain is catalyzed by *S*-adenosyl-L-methionine: $\Delta^{24}$ -sterol-C-methyltransferase (SMT).<sup>1</sup> Although several outcomes are possible for the methylation reaction, 24(28)-methylene cycloartenol appears to be the kinetically favored product in most, if not all, higher plants (Misso and Goad, 1983; Scheid *et al.*, 1982; Nes *et al.*, 1993; Guo *et al.*, 1995) (Fig. 1). The C-24 methylation step has been implicated as a rate-limiting step in sterol transformations (Guo *et al.*, 1995; Fang and Baisted, 1975; Parker and Nes, 1992; Nes *et al.*, 1991a, 1991b; Chappell *et al.*, 1995). In animals, instead of a C-24 methylation in the side chain of cycloartenol, a reduction of the  $\Delta^{24}$ -double bond in the side chain of lanosterol takes place, leading to cholesterol biosynthesis.

The SMT is a membrane-bound enzyme and has been localized in the endoplasmic reticulum in plant cells (Hartmann-Bouillon and Benveniste, 1978). The enzyme recognizes the sterols with a free  $3\beta$ -hydroxyl group, a  $\Delta^{24}$ -double bond, and the flat conformation that mimics the three-dimensional shape of cycloartenol and catalyzes their methylation at C-24 position in *in vitro* assay (Nes *et al.*, 1991a; Rahier *et al.*, 1984). So far, no SMT has been purified to homogeneity. Our knowledge on this enzyme is based on *in vivo* studies on plants fed with radiotracers and stable isotopes (Guo *et al.*, 1995), and *in vitro* studies using microsomal preparations as enzyme source (Misso and Goad, 1983; Scheid *et al.*, 1982; Nes *et al.*, 1991a). Cloning of the plant SMT genes and characterization of the gene products would provide an alternative approach to address some of the important questions on SMT such as the C-24 methylation mechanism and developmental regulation of the enzyme.

Earlier studies indicated that the yeast *ERG6* gene probably encodes an SMT (McCammon *et al.*, 1984; Gaber *et al.*, 1989). Mutations in *ERG6* locus, arising from mutagenesis or gene replacement, partially or totally abolished SMT activity and 24-alkyl sterol production in yeast cells. However, SMT activity of the *ERG6* gene product has not been documented. No plant SMT gene has been cloned previously. Reported here is the isolation of a cDNA encoding SMT from soybean. The polypeptide encoded by the soybean cDNA was expressed in *Escherichia coli* and shown to be an active SMT enzyme. The expression of the SMT transcript was found to be developmentally regulated, and suppressed following elicitor treatment. Transcriptional regulation of SMT in sterol biosynthesis is discussed.

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<sup>1</sup> The abbreviations used are: SMT, *S*-adenosyl-L-methionine: $\Delta^{24}$ -sterol-C-methyltransferase; AdoMet, *S*-adenosylmethionine; bp, base pair(s); GC-MS, gas chromatography-mass spectroscopy.

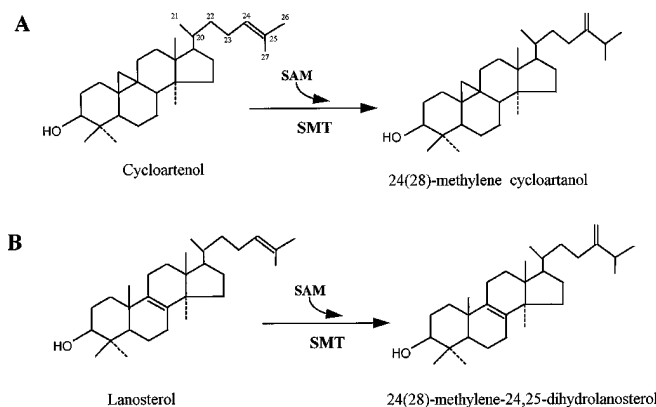


FIG. 1. Kinetically favored C-24 methylation of cycloartenol catalyzed by SMT in higher plants (A). SMT can also catalyze the C-24 methylation of lanosterol to produce 24(28)-methylene-24,25-dihydrolanosterol *in vitro* (B). SAM, S-adenosyl-L-methionine.

#### EXPERIMENTAL PROCEDURES

**Chemicals**—Lanosterol and desmosterol were obtained from Sigma. The lanosterol (catalog number L-5768) was purified by TLC before use in the SMT assay. [*methyl*- $^3\text{H}$ ]-S-Adenosyl-L-methionine (AdoMet) (83.3 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO).

**Cell Cultures and Elicitor Treatment**—Cell suspension cultures of soybean, cultivar Harosoy, were maintained in SH medium (Schenk and Hildebrandt, 1972) and subcultured weekly. After 40 h of subculture, yeast elicitor was added at 50  $\mu\text{g}$  of Glu equivalent of yeast cell wall hydrolysate per milliliter of soybean cell cultures. The cells were harvested by filtration, frozen in liquid nitrogen, and stored at  $-70^\circ\text{C}$ . Yeast elicitor was prepared as described previously (Schumacher *et al.*, 1987).

**Expression of SMT1 in *Escherichia coli***—A cDNA clone, Spm482, has been isolated from a soybean cDNA library in a previous study (Shi *et al.*, 1995). To identify this clone, the coding region was modified by insertion of a 24-bp fragment, which encodes the FLAG epitope (Kodak/IBI), preceding the ATG start codon. The chimeric cDNA was generated by the polymerase chain reaction using a T7 primer and a primer which contained an *Nhe*I site, the FLAG sequence (underlined), and a 28-bp matched sequence (5'-GTATGGCTAGCGGATCCGACTACAAGGAC-GACGATGACAAGATGCAAAAAAAAAAAAAAAAAATCGAAACG-3'). The polymerase chain reaction product was digested with *Nhe*I and the *Nhe*I fragment (including the FLAG sequence and the first 24-amino acid coding sequence of the soybean cDNA) was used to replace the corresponding region of the cDNA coding sequence. The resultant FLAG-tagged cDNA was inserted into the *E. coli* expression vector pRSET (Invitrogen) at its *Nhe*I and *Kpn*I sites downstream of the phage T7 promoter, and the resultant plasmid was used to transform *E. coli* strain JM109. Expression of the recombinant gene in *E. coli* was induced by infection with the recombinant M13 strain expressing the T7 polymerase (referred to as M13/T7 phage) in the presence of 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside. Cells (30 ml culture) were harvested 4 h after M13/T7 phage infection and the cell pellets were resuspended in 3 ml of buffer containing 50 mM Tris, pH 7.5, 2 mM  $\text{MgCl}_2$ , 1 mM phenylmethylsulfonyl fluoride, and 2 mM  $\beta$ -mercaptoethanol and then sonicated three times each for 20 s. After centrifugation at  $12,000 \times g$  for 20 min at  $4^\circ\text{C}$ , the supernatant was collected as a crude extract, divided into 200- $\mu\text{l}$  aliquots (2  $\mu\text{g}$  of protein/ $\mu\text{l}$ ), frozen in liquid nitrogen, and stored at  $-70^\circ\text{C}$ . Transformed *E. coli* cells that were neither induced by isopropyl-1-thio- $\beta$ -D-galactopyranoside nor infected by M13/T7 served as a negative control for expression of the fusion protein. Expression of the FLAG-tagged soybean polypeptide was examined by immunoblot analysis using anti-FLAG M2 monoclonal antibody (Kodak/IBI).

**Assay of S-Adenosyl-L-methionine: $\Delta^{24}$ -Sterol-C-methyltransferase**—SMT activity was assayed as described previously (Scheid *et al.*, 1982; Nes *et al.*, 1991a). The reaction mixture (220  $\mu\text{l}$ ) contained 200  $\mu\text{l}$  of crude extract prepared from transformed *E. coli* cells, 80  $\mu\text{M}$  [*methyl*- $^3\text{H}$ ]-AdoMet (diluted with unlabeled AdoMet to make  $3 \times 10^5$  dpm/reaction), 100  $\mu\text{M}$  lanosterol, and 0.1% (w/v) Tween 80. Incubation was conducted at  $30^\circ\text{C}$  for 60 min and stopped by adding 1 volume of 6% KOH in ethanol. The mixture was extracted with hexane ( $3 \times 3$  ml). After evaporation of the solvent under nitrogen gas, the radioactivity of

the residue was measured by liquid scintillation counting. The sterol content of the hexane extracts were analyzed by thin layer chromatography on Silica Gel G plates (250  $\mu\text{m}$ ; J. T. Baker) and by gas chromatography-mass spectrometry (GC-MS) with a HP-5890 mass detector equipped with a 25- $\mu\text{m}$  HP-1701 capillary column. The GC was operated from  $130^\circ\text{C}$  to  $280^\circ\text{C}$  ( $15^\circ\text{C min}^{-1}$ ), then held at  $280^\circ\text{C}$  for another 15 min.

**DNA and RNA Gel Blot Analysis**—Genomic DNA was isolated from etiolated soybean seedlings (White and Kaper, 1989). Total RNA was isolated from various soybean tissues (Logemann *et al.*, 1987) and cultured cells (Chomczynski and Sacchi, 1987). DNA and RNA gel blot hybridizations were conducted at  $65^\circ\text{C}$  as described by Church and Gilbert (1984). Probe was labeled with [ $\alpha$ - $^{32}\text{P}$ ]dATP using the Prime-a-gene system (Promega).

**DNA Sequencing and Analysis**—DNA was sequenced by the dideoxy sequencing method. A Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems) was used according to the manufacturer's instruction. The products were separated electrophoretically and the data were processed by an ABI373A automated DNA sequencer (Applied Biosystems). DNA and deduced amino acid sequences were analyzed using the PC/Gene programs (Intelligenetics, Inc., CA).

#### RESULTS

**Isolation and Sequence Analysis of the cDNA Encoding SMT1**—In a previous study, a cDNA expression library in  $\lambda$  Uni-ZAPII, constructed from poly(A)<sup>+</sup> RNA of etiolated soybean hypocotyls, was screened using an anti-(plasma membrane) serum produced against purified soybean plasma membranes. Positive clones obtained in the immunoscreening were classified into 40 groups based on partial sequence analysis and data base search for homology (Shi *et al.*, 1995). One group, including one clone Spm482, showed high similarity to the *Saccharomyces cerevisiae* ERG6 gene at its 3'-terminal.

The cDNA clone Spm482 contained a 1.5-kilobase insert (Fig. 2). A 1101-bp open reading frame encoded a polypeptide of 367 amino acids with a calculated molecular mass of 41.5 kDa. The DNA sequence upstream of the proposed ATG translation initiation codon contains four stop codons within the translation frame. Hydrophathy analysis of the deduced amino acid sequence showed no potential membrane-spanning sequence. The polypeptide was classified as a peripheral protein (with a P:I odds value 580.7) using the PC/Gene program SOAP (Klein *et al.*, 1985).

A data base search revealed similarity of the polypeptide encoded by the soybean cDNA to several known methyltransferases, such as *Herpetosiphon giganteus* cytosine-specific DNA-methyltransferase (P25265), *Rhodobacter sphaeroides* phosphatidylethanolamine N-methyltransferase (Q05197), *Streptomyces* sp. aklanonic acid methyltransferase (L35154), and *Rattus norvegicus* dihydroxypolyprenylbenzoate methyltransferase (L20427). Further sequence comparison revealed that the deduced amino acid sequence contained three methyltransferase sequence motifs identified in diverse S-adenosyl-L-methionine-dependent methyltransferases (Fig. 3). The three motifs were arranged in the same order on the soybean polypeptide chain as on other methyltransferases and the amino acid sequences separating the three motifs had comparable length as those in other methyltransferases. Motif I was highly conserved in the soybean protein. The motif II in the soybean protein contained the invariant central aspartate residue. The conserved aromatic amino acid phenylalanine and tyrosine were found at positions  $-1$  and  $+3$  with respect to the central aspartate, as in other methyltransferases. The motif III was located at an interval of 19 residues C-terminal to motif II. The first half of this region was well conserved and the central glycine residue was present. However, the other half of the region did not match with the consensus described by Kagan and Clarke (1994). The presence of the three sequence motifs of S-adenosyl-L-methionine-dependent methyltransferases suggests that the soybean cDNA may encode an S-adenosyl-L-



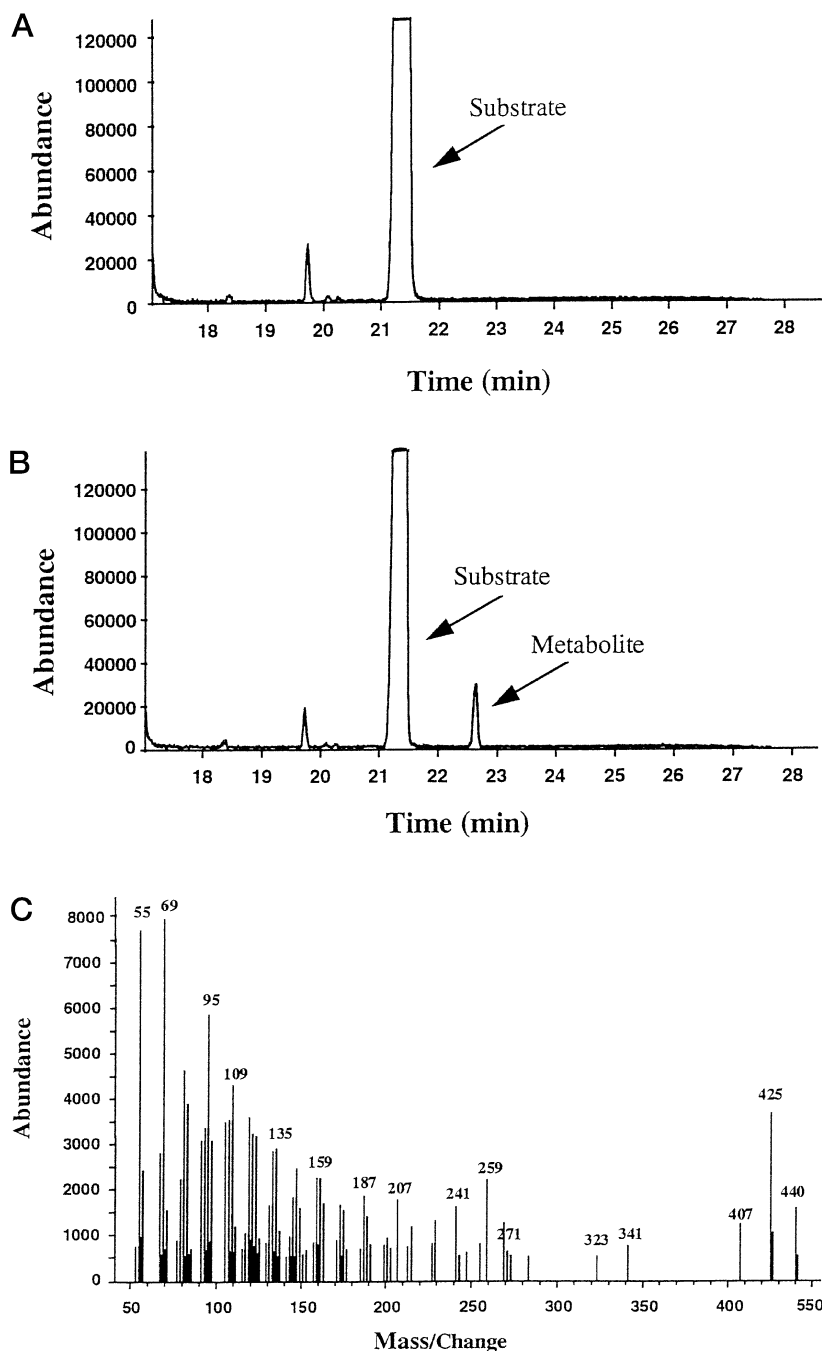


FIG. 5. GC-MS analysis of the metabolite from the incubation of lanosterol and *S*-adenosyl-*L*-methionine with cell lysates prepared from *E. coli* cells transformed with the plasmid carrying the soybean SMT1 cDNA. *A*, GC chromatogram of the sterol from control incubation (soybean SMT1 not expressed). *B*, GC chromatogram of the sterol from SMT incubation. *C*, the mass spectrum of the metabolite recorded on a GC-MS HP tabletop 5890 mass detector. The GC (capillary, 30 m:HP-1701) was operated from 130 to 280 °C (15 °C min<sup>-1</sup>), then held at 280 °C for another 15 min.

the two compounds were identical except for two minor peaks at  $m/z$  384 and  $m/z$  399 atomic mass units that were missing in the mass spectrum of the metabolite, possibly due to low levels of signals. Although we cannot identify the double bond location in the side chain of the methylated lanosterol based on mass spectrum, methylation at the C-24 position in the lanosterol side chain is demonstrated. Therefore, it was concluded that the soybean cDNA SMT1 encodes the *S*-adenosyl-*L*-methionine: $\Delta^{24}$ -sterol-*C*-methyltransferase.

**DNA Gel Blot Analysis**—To estimate the number of SMT genes in the soybean genome, total genomic DNA was digested with a variety of restriction enzymes and analyzed by DNA gel blot hybridization. Using a probe consisting of the entire SMT1 cDNA, multiple bands of hybridization were obtained at high stringency, as shown in Fig. 6. The restriction enzymes *Eco*RI, *Eco*RV, *Hind*III, and *Xho*I do not cut the SMT1 cDNA. *Bgl*II cuts once at nucleotide 993. The genomic DNA was also hybrid-

ized with probes derived from the 5'-end (nucleotide 15–207, including the 147-bp noncoding sequence and 45-bp coding sequence) and 3'-end (nucleotide 1198–1504, including the 243-bp noncoding sequence and 63-bp coding sequence) of the SMT1 cDNA (Fig. 6, *B* and *C*). The pattern of hybridization suggested that the soybean genome contains additional sequence(s) which shares similarity to the SMT1 cDNA.

**Expression Patterns of SMT Transcript**—The tissue distribution of SMT transcripts was examined through RNA gel blot analysis. As shown in Fig. 7, transcript levels of SMT in vegetative tissues were higher in young seedlings than in mature plants; the difference being more pronounced in roots. In plants with pods, growing leaves had higher levels of expression than old leaves. Flowers had the highest levels of expression. Expression of SMT transcripts was very low in immature seeds and young pods.

**Suppression of SMT Transcript in Response to Elicitor**—

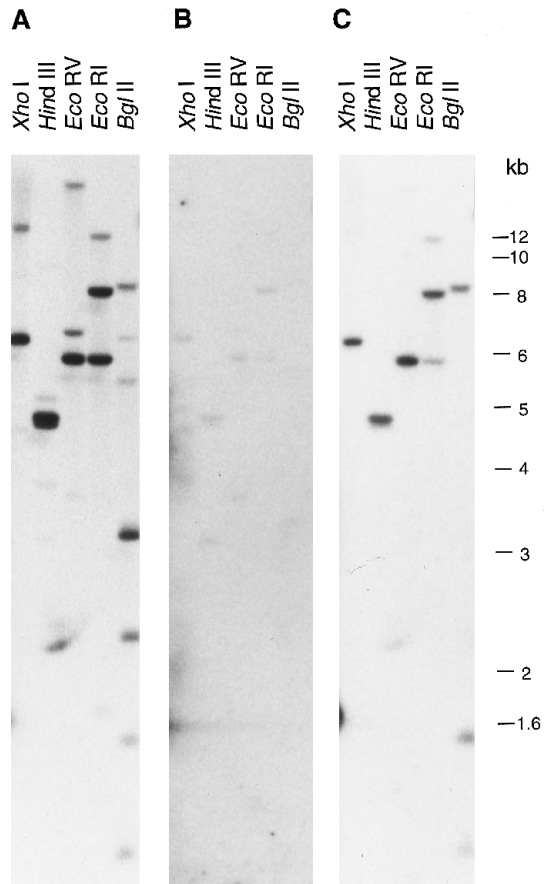


FIG. 6. Gel blot analysis of soybean genomic DNA. Soybean DNA (10  $\mu$ g) digested with the indicated restriction enzymes was hybridized with the entire SMT1 cDNA sequence (A), 5'-terminal region (192 bp) (B) and 3'-terminal region (306 bp) (C). DNA length markers are given at right in kilobases.

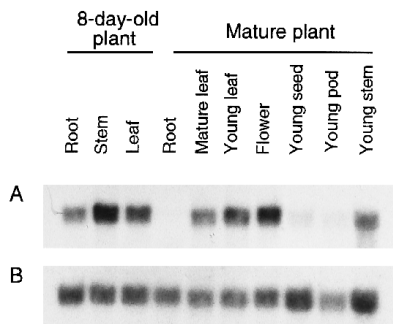


FIG. 7. Gel blot analysis of total RNA extracted from various soybean tissues. A, total RNA (10  $\mu$ g) was hybridized with the entire SMT1 cDNA sequence. B, the same blot was probed with 18 S ribosomal cDNA. The mature plants have flowered and podded.

Elicitors prepared from fungal and plant cell walls induce a variety of responses in plants such as activation of defense genes, accumulation of phytoalexins, and lignification of cell walls (Dixon and Lamb, 1990). It has been reported that elicitor treatment suppresses sterol biosynthesis in parsley, potato, tobacco, and *Tabernaemontana divaricata* (Vögeli and Chappell, 1988; Haudenschild and Hartmann, 1995; Brindle *et al.*, 1988; van der Heijden *et al.*, 1989). The possible effect of elicitor treatment on sterol biosynthesis in soybean cell suspension cultures was examined by monitoring the steady state SMT transcript levels. Elicitor treatment resulted in a suppression of SMT transcript detectable within 2 h, with the lowest levels of transcript occurring 6 h after treatment (Fig. 8). The sup-

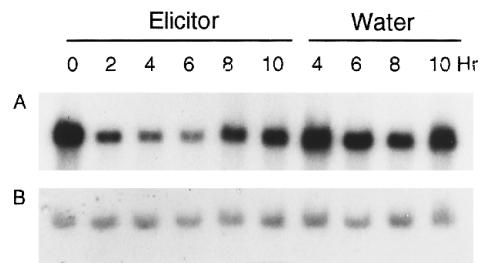


FIG. 8. RNA gel blot analysis of SMT expression in elicitor-treated soybean cell suspension cultures. A, total RNA (10  $\mu$ g) from cells at various times after treatment was hybridized with SMT cDNA. B, the same blot was probed with 18 S ribosomal cDNA.

pression was transient and transcript expression gradually increased 6 h after treatment.

#### DISCUSSION

Identification of the soybean SMT1 cDNA was accomplished using both molecular and biochemical criteria. Analysis of the deduced amino acid sequence of the SMT1 cDNA revealed three conserved regions found in *S*-adenosyl-L-methionine-dependent methyltransferases. The methyltransferase motifs may contribute to the binding of the substrate *S*-adenosyl-L-methionine and/or the product *S*-adenosyl-L-homocysteine (Kagan and Clarke, 1994). The high identity of deduced amino acid sequences in the entire length between the soybean cDNA and the putative yeast SMT structural gene *ERG6* suggested that the soybean cDNA might also encode SMT. This supposition was confirmed by expression of the cDNA in *E. coli*. Extracts from transformed bacteria had C-24 methylation activity on lanosterol. Although the natural substrate of plant SMT is cycloartenol, SMT can use lanosterol and other related sterols as methyl group acceptors in *in vitro* assays (Scheid *et al.*, 1982; Nes *et al.*, 1991a). Therefore, showing SMT activity on lanosterol provides biochemical evidence for positive identification of the soybean cDNA.

Genomic DNA hybridization analysis indicates that the soybean genome may contain additional sequence(s) with similarity to the cloned SMT1 cDNA. Soybean is believed to be a diploidized tetraploid generated from an allotetraploid ancestor (Hymowitz and Singh, 1987). Duplicated DNA sequences occur widely in the soybean genome (Zhu *et al.*, 1994). However, whether the additional genomic sequence(s) encode an active SMT enzyme remains to be studied. Alternatively, these sequences may represent unrelated gene segments.

The soybean SMT1 was cloned by immunoscreening a cDNA expression library using an anti-(plasma membrane) serum. It has been shown that plant SMT is localized in endoplasmic reticulum membranes (Hartmann-Bouillon and Benveniste, 1978) and yeast SMT is associated with lipid particles (Zinser *et al.*, 1993). However, recognition of the soybean SMT by anti-(plasma membrane) serum may not necessarily indicate the association of SMT with plasma membranes. It is more likely that the plasma membrane preparation used for raising antibodies contained some contaminating endoplasmic reticulum membranes, since the plasma membranes prepared by aqueous-polymer two-phase partitioning only have a purity of about 95% (Shi *et al.*, 1995 and Refs. contained therein).

Transcript levels of the soybean SMT were found to be regulated developmentally. Young roots, leaves, and stems had higher levels of steady state SMT transcripts as compared with those of mature tissues such as old leaves and roots of the plants with pods, reflecting a high rate of sterol biosynthesis in the growing vegetative tissues. These results agree with previous reports that the growing soybean vegetative tissues (shoots and roots) have very high sterol content on the basis of

dry weight (Fenner *et al.*, 1986). These results are also consistent with the observation that old leaves accumulate much more cycloartenol than young leaves do in transgenic tobacco plants overexpressing hydroxymethylglutaryl-CoA reductase (Chappell *et al.*, 1995). High expression of SMT transcript was detected in soybean flowers, indicating active synthesis of sterols in flowers. Young pods and immature seeds had very low levels of SMT transcripts. It has been reported that soybean seeds have the highest sterol concentration on the basis of dry weight and total lipids shortly after pollination and sterol concentration then steadily decreases during seed development and maturation (Katayama and Katoh, 1973; Kajimoto *et al.*, 1982). The low levels of SMT transcripts in immature seeds found in this study may indicate a low rate of sterol biosynthesis in soybean seeds during maturation.

Biochemical and *in vivo* radiolabeling studies have suggested that SMT is a rate-limiting enzyme and may regulate the biosynthesis of 24-alkyl sterols (Guo *et al.*, 1995; Fang and Baisted, 1975; Parker and Nes, 1992; Nes *et al.*, 1991a, 1991b). Recently, it has been shown that transgenic tobacco plants overexpressing the hydroxymethylglutaryl-CoA reductase accumulate a large amount of cycloartenol, providing further evidence for the role of SMT in the regulation of phytosterol biosynthesis (Chappell *et al.*, 1995). In this study, analysis of SMT transcript implicates the possibility of transcriptional control of SMT in regulating sterol biosynthesis. The transcript of SMT was highly expressed in the growing soybean vegetative tissues where sterol biosynthesis is very active (Fenner *et al.*, 1986). Low levels of SMT transcript expression was associated with low levels of sterol content on the basis of dry weight in immature soybean seeds (Katayama and Katoh, 1973; Kajimoto *et al.*, 1982). Furthermore, suppression of SMT transcripts was observed in soybean cell suspension cultures treated with elicitor. Suppression of sterol biosynthesis and alteration of sterol composition caused by elicitor treatment and pathogen infection have been demonstrated in various plants such as maize, parsley, potato, tobacco, and *T. divaricata* (Vögeli and Chappell, 1988; Haudenschild and Hartmann, 1995; Brindle *et al.*, 1988; van der Heijden *et al.*, 1989; Jennings *et al.*, 1970).

The  $\Delta^{24}$ -double bond transmethylation catalyzed by the SMT could produce several products with a double bond at various positions. The kinetically favored outcome of the reaction appears to be a 24(28)-sterol in maize and sunflower (Misso and Goad, 1983; Scheid *et al.*, 1982; Nes *et al.*, 1993; Guo *et al.*, 1995). It has been proposed that a single SMT may be responsible for the production of three C-24-methylated cycloartenol [24(28)-methylene cycloartanol,  $\Delta^{23}$ -24-methyl cycloartenol, and  $\Delta^{25}$ -24 $\beta$ -methyl cycloartenol] in the C-24 methylation reaction, and the product distribution may result from kinetic, thermodynamic, and allosteric control mechanisms acting on the development of the enzyme-substrate complex and subsequent catalysis (Janssen and Nes, 1992). At the present time, mechanism of the C-24 methylation is poorly understood, partially due to lack of knowledge of SMT protein structure. The

availability of SMT cDNA and active SMT enzyme expressed in *E. coli* will permit the establishment of the SMT protein structure. This might prove useful in elucidating the interactions of enzyme, AdoMet, and sterol substrate, and the C-24 methylation process.

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