Identification and Characterization of an S-Adenosyl-L-methionine: Δ24-Sterol-C-methyltransferase cDNA from Soybean*

(Received for publication, November 16, 1995, and in revised form, January 30, 1996)

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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:Δ24-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.

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Soybean S-Adenosyl-L-methionine-dependent 24-Sterol-C-methyltransferase

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-3H]-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 Hildick, 1972) and subcultured weekly. After 40 h of subculture, yeast elicitor was added at 50 µg 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 °C. Yeast elicitor was prepared as described previously (Schumacher et al., 1987).

Expression of SMT I 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/Invitrogen), before the T7 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 unique sequence, SAGCTATCGAAACGG-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 phase 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), which was isolated 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-β-D-galactopyranoside. Cells (30 ml culture) were harvested by filtration, frozen in liquid nitrogen, and stored at −70 °C. Yeast elicitor was prepared as described previously (Schumacher et al., 1987).

RESULTS

Isolation and Sequence Analysis of the cDNA Encoding SMT I—In a previous study, a cDNA expression library in λ Uni-ZAPII, constructed from poly(A)+ 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 database searches 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. Hydropathy 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/Geno program SOAP (Klein et al., 1985).

A database 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-methylethermethyltransferase (L35154), Streptomyces sp. aklanonic acid methyltransferase (L35154), and Rattus norvegicus dihydroxypropylbenzoate 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-
nitural gene encoding gested, but not yet proven, that the yeast sequences share 47.1% amino acid identity. It has been sug-

aldesyl-L-methionine-dependent methyltransferase. The cDNA was therefore desig-

rylated with CH₂Cl₂ (two runs), as expected. No SMT activity was observed when lanosterol was omitted from incubation mixtures. In reaction mixtures containing the extracts of uninduced cells, no SMT activity was detected. Extracts prepared from transformed E. coli cells that were not induced for expression of the soybean SMT1 protein did not show SMT activity. Incubation buffer only or boiled protein and the yeast 

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<th>Soybean S-Adenosyl-L-methionineΔ⁷-24-Sterol-C-methyltransferase</th>
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<td>The yeast ERG6 gene is the most closely related sequence to the soybean polypeptide in current protein data bases (Hardwick and Pelham, 1994). Overall, the two sequences share 47.1% amino acid identity. It has been suggested, but not yet proven, that the yeast ERG6 is the structural gene encoding S-adenosyl-L-methionineΔ⁷-24-sterol-C-methyltransferase (McCammon et al., 1984; Gaber et al., 1989). The high identity of amino acid sequence between the soybean protein and the yeast ERG6 gene product indicated that the soybean cDNA may encode an SMT1 cDNA and grown in the absence of isopropyl-1-thio-β-D-galacto-pyranoside and without M13/T7 phage infection. Lane 2, the transformed J M109 cells were grown in the presence of 1 mM isopropyl-1-thio-β-D-galactopyranoside and infected with M13/T7 phage.</td>
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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:D^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 EcoRI, EcoRV, HindIII, and XhoI do not cut the SMT1 cDNA. BglII cuts once at nucleotide 993. The genomic DNA was also hybridized 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 Transcripts**—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**—
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 (Vogeli and ChapPELL, 1988; Haudenschild and Hartmann, 1995; Brindle et al., 1988; vander 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).

**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 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...
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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., 1991, 1991b). Recently, it has been shown that 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.

Acknowledgments—We thank Valerie Graves and Ann Harris for assistance with DNA sequencing and oligonucleotide synthesis, David V. Huhman for help in GC-MS analysis, and Richard A. Dixon and Nancy L. Paiva for critical reading of the manuscript. We thank Nancy L. Paiva for providing yeast elicitor.

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