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Molecular Characterization of Tobacco Squalene Synthase and Regulation in Response to Fungal Elicitor[1, 2]

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The enzyme squalene synthase (SS) represents the first commitment of carbon from the general isoprenoid pathway toward sterol biosynthesis and is a potential point for regulation of sterol biosynthesis. The isolation and characterization of tobacco (*Nicotiana tabacum*) squalene synthase (TSS) cDNA and genomic DNA clones, as well as determination of the steady state level of TSS mRNA in response to elicitor treatment, were investigated. cDNA clones for TSS were isolated from poly (A)$^+$ RNA using a reverse transcription/polymerase chain reaction (RT/PCR) method. A 1233-bp cDNA clone was generated that contained an open reading frame of 411 amino acids giving a predicted molecular mass of 46.9 kDa. Comparison of the TSS deduced amino acid sequence with currently described SS from different species showed the highest identity with *Nicotiana benthamiana* (97%), followed by *Glycyrrhiza glabra* (81%), *Arabidopsis thaliana* (74%), rat (40%), and yeast (37%). Expression of a soluble form of the TSS enzyme with enzymatic activity in *Escherichia coli* was achieved by truncating 24 hydrophobic amino acids at the carboxy terminus. Characterization of genomic TSS (gTSS) revealed a gene of 7.086 kb with a complex organization of small exons and large introns not typical of plant genes. Southern blot hybridization indicated only two copies of the SS gene in the tobacco genome. Treatment of tobacco cell suspension cultures with a fungal elicitor dramatically reduced TSS enzymatic activity, lowering it to zero within 24 h. Analysis of TSS mRNA levels, by RNA blot hybridization and primer extension assays, in elicitor-treated cells indicated that the transcript level remained largely unchanged over this 24-h period. These results suggest that the suppression of TSS enzyme activity in elicitor-treated cells may result from a posttranscriptional modification of TSS.

***Key Words: Nicotiana tabacum;* squalene synthase; sterol regulation.**

The isoprenoid pathway produces a diverse group of compounds that are found throughout nature. While structural identification of isoprenoid compounds has received a great deal of attention in the past, our understanding of the biochemistry and regulation of the isoprenoid pathway in plants is quite limited. In comparison, regulation of the isoprenoid pathway in mammals, and especially sterol biosynthesis, has been extensively investigated (1). The primary site of regulation in the production of sterols in mammals is 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR)[7] which is regulated in response to the availability of sterols and low-density lipoprotein (LDL) (1). Chin *et al.* (2) previously demonstrated that hamster HMGR mRNA levels and enzyme activities were drastically reduced in response to exogenous LDL and sterol, suggesting transcriptional

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[7] Abbreviations used: FPP, farnesyl diphosphate; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL, low-density lipoprotein; RT/PCR, reverse transcriptase/polymerase chain reaction; SS, squalene synthase; HSS, human SS; TSS, tobacco squalene synthase; gTSS, tobacco squalene synthase genomic DNA; ER, endoplasmic reticulum; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; RACE, rapid amplification of cDNA ends; IPTG, isopropyl β-D-thiogalactoside; NTR, nontranslated region.

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and posttranscriptional regulation of HMGR. Competitive inhibitors of HMGR enzyme activity, such as compactin and lovastatin, have also been used to study HMGR regulation. A Chinese hamster cell line selected for growth in the presence of compactin resulted in a 200-fold increase in HMGR protein levels due to an 8-fold increase in HMGR mRNA levels, a 5-fold increase in mRNA translation, and a 5-fold decrease in protein degradation (3). This was taken as evidence for feedback regulation since HMGR enzyme activity in the compactin-treated cells was inhibited, and sterols thus were not synthesized. The enhanced expression of the HMGR gene and decrease in protein degradation are therefore considered a compensatory mechanism activated in response to the decline in sterol levels (1).

Squalene synthase (SS) represents the first enzymatic step committing carbon exclusively to sterol production. Along with HMGR, SS is a potential site for the regulation of sterol production. Studies of mammalian SS have shown coordinate regulation with HMGR. In the presence of an exogenous source of sterol, rat (4) and human (5) SS mRNA levels decrease 60 to 95%, respectively. The HMGR enzyme activity inhibitor lovastatin has also been used to demonstrate an increase in SS mRNA levels in rat (4), human (5), and yeast (6). These results suggest that SS, like HMGR, is regulated by a sterol feedback mechanism. This regulation of SS, however, seems to be secondary to that of HMGR in terms of absolute changes in enzyme activities or mRNA levels (4, 5, 7, 8).

Regulation of the isoprenoid pathway in plant systems has not received as much attention as that in mammalian systems. Preliminary studies on the regulation of SS in tobacco cell suspension cultures have been carried out by correlating SS enzyme activity with sterol biosynthesis and accumulation (9–11). During a growth cycle of a tobacco cell suspension culture, SS activity was maximal when the cells were in their rapid growth phase (4 days after subculturing). Changes in the incorporation of [^{14}C]acetate and [^{3}H]mevalonate into sterols occurred in parallel to the changes in SS activity (9). When fungal elicitors were added to the cell cultures in their rapid growth phase, sterol accumulation ceased, and extracellular sesquiterpenes accumulated instead. The induced accumulation of sesquiterpenes was correlated with a preferential incorporation of radiolabeled acetate and mevalonate into sesquiterpenes, and the induction of sesquiterpene cyclase enzyme activity. The suppression of sterol accumulation was likewise correlated with a reduced incorporation of radiolabeled precursors into sterols and a decline in SS enzyme activity. Since SS and sesquiterpene cyclase are positioned at a putative branch point competing for farnesyl diphosphate (FPP), the induction of one enzyme and the suppression of the other are interpreted as one important regulatory mechanism controlling end-product formation.

SS is an endoplasmic reticulum (ER) associated enzyme with its carboxy terminus predicted to tether it to the ER membrane and the amino terminus containing the active site in the cytosol (12). It is the hydrophobic ER association that has hampered purification of SS. Isolation of solubilized SS has been accomplished in yeast (13–16) with the use of detergents, but enzyme activity was not stable and recoveries were low. Soluble, enzymatically active SS has been isolated from rat (17) when the hydrophilic amino terminus had been proteolytically released from the membrane bound protein. Expression of a full-length yeast SS gene in *Escherichia coli* with enzymatic activity has been achieved (18). Production of soluble, enzymatically active yeast SS protein in large enough quantities to be purified has also been accomplished by overexpressing a carboxy-terminal truncated yeast SS gene in *E. coli* (19, 20). A human SS (HSS) cDNA has been successfully expressed in *E. coli* and substantial amounts of SS enzyme purified from SF-9 insect cells infected with a recombinant baculovirus containing the HSS cDNA (21). Purification of SS from plants has shown difficulties similar to those of mammalian SS. Using a two-step detergent solubilization procedure, tobacco squalene synthase (TSS) has only been partially purified from cell suspension cultures (22). Recent studies of plant SS have not dealt with the purification of the protein, but rather the characterization of the cDNA and protein from *Arabidopsis thaliana* (23) and *Nicotiana benthamiana* (24) by expressing full-length cDNAs in *E. coli.*

In order to further study the regulation of TSS enzyme activity in plant cell cultures, molecular tools for correlating changes in TSS gene expression with SS enzyme activity were sought in the current study. We now report the molecular cloning of TSS, identification of the cDNA by expression of TSS activity in *E. coli,* isolation and characterization of the genomic TSS, and regulation of TSS mRNA levels in suspension cell cultures in response to fungal elicitor.

MATERIALS AND METHODS

Cell culture and elicitor treatment. Cell suspension cultures of *Nicotiana tabacum,* cultivar Kentucky 14, were maintained in Murashige-Skoog medium with 0.2 mg/liter 2,4-dichlorophenoxyacetic acid on a weekly subculturing system (25). Cultures in the rapid phase of growth (approximately 4 days after subculturing) were elicited by the addition of 0.5 μg/ml cellulase.

Cloning strategies for tobacco SS cDNAs. A cDNA clone of SS mRNA was isolated using reverse transcription/polymerase chain reaction (RT/PCR) strategies based on sequence comparisons of the rat (26) and yeast (18) genes. A degenerate forward 17-bp primer (5′-TA(T/C) TG(T/C) CA(T/C) TA(T/C) GT(T/C/A/G) GC-3′) was synthesized based on the amino acid sequence YCHYVA and a degenerate reverse 20-bp primer (5′-AC(T/C) TG(A/G/T/C) GG(T/G/A) AT(G/A/C/

T) GC(G/A) CA(G/A) AA-3′) based on the amino acid sequence FCA-IPQV. Both sequences are consensus sequences found in rat and yeast SS, and subsequently HSS. The forward sequence (YCHYVA) is located within domain III of the SS protein and the reverse sequence (FCAIPQV) is located within domain V of the SS protein according to the nomenclature proposed by Robinson *et al.* (6). Total RNA was isolated from tobacco cell suspension cultures in their rapid growth phase using a guanidine isothiocyanate/CsCl method (27), followed by isolation of poly $(A)^+$ RNA using an oligo(dT) cellulose chromatography column (Gibco Life Technologies, Gaithersburg, MD). First-strand cDNA was prepared using an oligo(dT) primer and reverse transcriptase (cDNA cycle kit, Invitrogen, San Diego, CA) in a PCR method previously described (28). The two degenerate primers and the first-strand cDNA were used for PCR amplification in a standard PCR protocol (29). A 371-bp PCR product (TSS3) was amplified from the first strand cDNA using the degenerate primers and isolated from an agarose gel using DE-81 ion exchange chromatography paper (Whatman, Maidston, England). The PCR product was ligated into the *Eco*RV site of the plasmid pBluescript II KS + (Stratagene, La Jolla, CA) (pBKS-TSS3), transformed into *E. coli* strain TB1 according to a standard $CaCl_2$ transformation procedure (30), and sequenced using the dideoxy nucleotide chain termination method according to the manufacturer (U.S. Biochemical, Cleveland, OH).

To obtain the 3′ end of the TSS mRNA, first-strand cDNA was prepared from poly $(A)^+$ RNA using a 32-bp oligo $(dT)_{14}$-adapter primer (5′-GACTCGAGTCGACATCGA-$(dT)_{14}$-3′) followed by PCR amplification of an 857-bp fragment (TSS8) using (5′-CCGTATGTT-CTGGCCCCG-3′) as the forward primer and the adapter primer as the reverse primer in a standard PCR reaction. The PCR product was subcloned into the *Eco*RV site of pBluescript II KS + and designated pBKS-TSS8. To obtain the 5′ end of the TSS mRNA, a RACE (rapid amplification of cDNA ends) strategy (31) was employed. First-strand cDNA was generated using a specific reverse primer (5′-CTTCTT-TCCCAGAGGCAT-3′) positioned within TSS8 and poly (A)+ RNA as the template. The product(s) was purified on a Sephadex G-25 column, and an extended adenosine tail was added to the 3′ terminus deoxynucleotidyltransferase and dATP, and then used as the template for PCR reactions with the 32-bp oligo$(dT)_{14}$-adapter primer as the forward primer and 5′-CTTCTTTCCCAGAGGCAT-3′ as the reverse primer (the same as used for first-strand cDNA synthesis). A 798-bp PCR product was recovered (TSS7), subcloned (pBKS-TSS7), and sequenced as above.

Construction of a 1233-bp tobacco SS cDNA. The entire TSS cDNA was amplified by PCR using a forward primer (5′-GCGGGAATT-CC**ATG**GGGA-3′) (*Eco*RI and *Nco*I restriction sites underlined and the translation start codon in bold) and a reverse primer (5′-CGG-CGGGATCC**CTA**AGATCGGTTTCCAGATAG-3′) harboring a *Bam*HI site (underlined) and a stop codon (bold), and first strand cDNA as the template (see above). The resulting 1258-bp PCR product (TSS1.2) was digested with *Nco*I and *Bam*HI, gel-purified, and ligated into the same restriction sites within the pET11d vector (Novagen, Madison, WI) and pGBT-T19 vector (Gold Biotechnology, St. Louis, MO). The construct, pET11d-TSS1.2, was transformed into *E. coli* strain BL21 (DE 3) according to the manufacturer's recommendations (Novagen).

Carboxy-terminal truncated TSS was generated using the forward primer harboring *Eco*RI and *Nco*I restriction sites (mentioned above), a reverse primer (5′-GCCGGATCC**TCA**GTAATTAGGCTCGCTCCT-GAT-3′) containing a *Bam*HI restriction site, and a TGA stop codon located 24 amino acids upstream of the native TAG stop codon in the pET11d-TSS1.2. The 1184-bp PCR product (TSS1.1) was subcloned into the pET11d vector (pET11d-TSS1.1) and transformed into *E. coli* strain BL21 (DE 3) as above.

Comparison of SS protein sequences. SS protein sequences were obtained from published reports for *N. benthamiana* (24), *Arabidopsis* (23), rat (26), and yeast (18). Sequences were analyzed for identity using the BLAST program and aligned using MacVector (Kodak, Rochester, NY).

Expression of TSS in E. coli. The TSS enzyme was expressed in *E. coli* by adding isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM to exponentially growing recombinant cells (OD_{600} = ~0.5) harboring the pET11d-TSS1.1 plasmid. One-milliliter aliquots of the culture were subsequently removed for enzyme assay and collected by centrifugation for 2 min, resuspended in 100 μl lysis buffer (25 mM potassium phosphate, pH 7.0, 1 mM DTT, and 2 mM PMSF), vortexed, and sonicated 3 times for 10 s each time. The sonicate was centrifuged for 10 min at 4°C and 5 μl of the supernatant was used for enzyme activity determinations as described below. Protein levels were determined by the Bradford method (Bio-Rad, Hercules, CA). For detection of TSS protein by SDS–PAGE, 100-μl aliquots of bacterial culture were harvested at the indicated times and centrifuged for 2 min. The pellet was resuspended with 100 μl of 50 mM Tris-HCl, pH 6.8, 10 mM DTT, 2% SDS, 0.01% bromophenol blue, and 10% glycerol. Thirty-five-microliter aliquots were separated on an 11.5% SDS–polyacrylamide gel and stained with Coomassie blue for protein detection.

Enzyme assays and protein detection. Assays for microsomal and soluble TSS activity were performed as previously reported (9, 10). This assay is based on the conversion of radiolabeled FPP to squalene, which can be quantified after a TLC separation of reaction products from substrate. Sesquiterpene cyclase activity was determined according to Vögeli and Chappell (10) and measures the conversion of radiolabeled FPP to hexane-extractable reaction products. Protein levels were determined by the Bradford method and both enzyme activities are expressed in nanomoles of reaction product formed per milligram protein per hour.

Isolation and sequencing of genomic SS. A genomic SS clone was isolated from a tobacco genomic library packaged in lambda EMBL 3 (Clontech, Palo Alto, CA) and overlapping subclones were generated by PCR using primers based on the TSS cDNA sequence. Subclones were approximately 1.0 to 1.8 kb in length and covered the entire region of the TSS gene from the translation start site to the polyadenylation site. The clones were blunt-end-ligated into the *Eco*RV site of pBluescript II KS + (Stratagene). DNA sequencing of the clones was carried out using the dideoxy nucleotide chain termination method according to the manufacturer (U.S. Biochemical).

Southern blot analysis. Genomic DNA was isolated from *N. tabacum* suspension culture cells using the cetyldimethylethylammonium bromide method (32). Genomic DNA (6 μg) was digested with the indicated restriction enzymes, size-fractionated on 0.8% agarose gels, and transferred to a nylon membrane (Zeta probe, Bio-Rad). DNA blots were hybridized with an [α-^{32}P]dCTP radiolabeled 371-bp internal fragment probe (TSS3) (prime-It kit, Stratagene) at 60°C in 5 ml of hybridization buffer (0.25 M sodium phosphate buffer, pH 7.2, 7% SDS, 1% bovine serum albumin, 1 mM EDTA). Blots were washed at 60°C twice with 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.01% SDS for 5 min and twice with 0.2× SSC, 0.01% SDS for 5 min at room temperature. Hybridization was detected using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and the digitized images were used to quantitate band intensity.

RNA blot analysis. Total RNA was isolated from 2 g of control and elicited cells by a phenol/chloroform extraction method (33). Five to 10 μg of total RNA was fractionated on a 1.0% agarose denaturing formaldehyde gel (30) and transferred to a nylon membrane (Zeta probe, Bio-Rad). Hybridization was carried out at 42°C in 5 ml of hybridization buffer (5× SSPE, 2× Denhardt's solution, 0.2% SDS, 20 μg/ml tRNA, 50% formamide) with either a full-length TSS cDNA probe (TSS1.2) or a full-length sesquiterpene cyclase cDNA probe (28) radiolabeled with [α-^{32}P]dCTP by the same method as described above for the Southern blot probe. RNA blots were washed twice at room temperature for 5 min with 2× SSC, 0.1% SDS, and twice at room temperature for 30 min with 0.2× SSC, 0.01% SDS. Hybridization was detected using a phosphorimager (Molecular Dynamics) and

the digitized images were used to quantitate band intensity. Similar RNA hybridization results have been observed a minimum of three times using independently isolated RNA samples.

Primer extension analysis. The TSS transcription start site was determined by primer extension analysis (30). The 42-nucleotide primer 5′-CCTTATGAAGCATTGAGTAACAGAAGCCCCAATGTG-GAGACG-3′, located 97 nucleotides 3′ to the AUG translation initiation codon, was 5′ ^{32}P-labeled using T4-polynucleotide kinase (Gibco Life Technologies) and [γ-^{32}P]ATP. The labeled primer was purified from unincorpotated [γ-^{32}P]ATP on an 8% sequencing gel; 6×10^5 dpm of the ^{32}P-labeled primer and 30 μg of total RNA were used for the extension reaction. Hybridization was carried out at 65°C for 10 min followed by incubation at room temperature for 5 min. The extension reaction was then carried out at 45°C for 60 min with SuperScript RNase H$^-$ Reverse Transcriptase (400 units) (Gibco Life Technologies) and 10 mM dNTPs in a 20-μl reaction volume. The radioactive extension product was analyzed on an 8% sequencing gel using a known TSS DNA sequencing ladder for size determination.

RESULTS

cDNA cloning of tobacco SS. A TSS cDNA was isolated using a combination of RT/PCR strategies. The deduced SS amino acid sequences reported for the rat (26) and yeast (18) proteins were used initially to design degenerate primers for the amplification of cDNA sequences from poly (A)$^+$ RNA. The forward degenerate primer was based on a consensus sequence within domain III and the reverse degenerate primer was based on a consensus sequence within domain V (see Fig. 2). From the initial RT/PCR reaction, a 371-bp internal TSS fragment (TSS3) was cloned and sequenced. Observation of domain IV within this fragment confirmed initial identity of a SS sequence.

Two RACE strategies were employed to obtain sequence information necessary in isolating a full-length cDNA. First-strand cDNA derived from poly (A)$^+$ RNA was used as the template with an internal specific primer and an oligo(dT)-adapter primer to obtain the 3′ sequence information. A similar strategy was used to obtain the 5′ end sequences, except that a specific internal primer was used for the initial reverse transcription and a poly (A) tail was added to the 3′ terminus of the resulting first-strand cDNA. The 5′ sequences were then amplified using the specific internal primer and the oligo(dT) adapter primer. Sequence information derived from the 5′ and 3′ cDNAs was used to design additional PCR primers, and a full-length TSS cDNA was ultimately PCR amplified (Fig. 1).

Comparison of TSS with other SS sequences. The molecular mass of the TSS protein predicted from the deduced amino acid sequence is 46.9 kDa, similar to other reported values from plants (23, 24). In terms of amino acid sequence comparisons, TSS is 97% similar to that of the recently described SS gene isolated from *N. benthamiana* (24), 81% similar to *Glycyrrhiza glabra* (34), 74% to that of *Arabidopsis thaliana* (23), 40% to rat (26), and 37% to yeast (18). Six highly conserved peptide domains of 14–23 amino acids previously pointed out by Robinson *et al.* (6) were also discernible within the TSS amino acid sequence (Fig. 2). Three of these domains (III, IV, V) are highly conserved within the TSS sequence, while the corresponding sequences for domains I and II are much less conserved. Although the TSS domain VI exhibits a low level of sequence identity with the other SS enzymes, the corresponding TSS sequence is very hydrophobic, very similar to the other SS sequences, and consistent with the putative membrane anchoring function of this part of the protein.

Expression of TSS in E. coli. The entire coding region of the TSS cDNA (TSS1.2), as well as a truncated TSS cDNA (TSS1.1) consisting of a deletion corresponding to the last 24 amino acids of the carboxy terminus, were inserted into two commonly used expression vectors, pET11d and pGBT-T19. These plasmids were then transformed into *E. coli* and expression induced by the addition of 1 mM IPTG. In numerous attempts, no SS enzyme activity or polypeptides of expected molecular mass (46.9 kDa) were observed in *E. coli* containing the putative full-length SS cDNA (TSS1.2). In contrast, readily measurable levels of SS enzyme activity were observed in extracts of *E. coli* expressing the truncated cDNA-pET11d plasmid (pET11d-TSS1.1), and resulted in the appearance of a new polypeptide of expected molecular mass at approximately 43 kDa (Fig. 3). TSS enzyme activity and the relatively high level of expression of the truncated TSS protein were observed 3 h after the induction with 1 mM IPTG (Fig. 3).

Determination of TSS transcription initiation site. The 5′ transcription initiation site was determined by primer extension analysis using total RNA as a template. Using a 42-nucleotide primer located 97 nucleotides 3′ to the AUG translation initiator that was 5′-^{32}P-labeled, only one extension product of 235 nucleotides was observed (Fig. 4). The transcription initiation site was, therefore, calculated to be at the adenosine located 96 nucleotides 5′ to the adenosine of the AUG translation initiator start site (Fig. 1). The primer extension assays were also carried out with RNA from control and elicitor-treated cells. Only a single band of roughly equal intensity with the same molecular mobility was seen in both RNAs.

Genomic organization of the TSS gene. When tobacco genomic DNA, digested with *Eco*RI, *Hin*dIII, and *Xba*I, was probed with the 371-bp internal TSS cDNA fragment, two hybridizing fragments were seen for each digestion treatment (Fig. 5). Based on the restriction maps for the cDNA clones and the relative hybridization intensity, the DNA blot is consistent with two gene copies per genome. The 371-bp internal cDNA fragment was subsequently used to isolate a genomic clone, gTSS-1. The sequence of gTSS-1 revealed a gene of 7.086 kb in length from the translation start site to the polyadenylation site (Fig. 6). The 3′ nontranslated

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                                                                  ATATTTATTTAAGTATTTGTGTGTG   -72
ATTGCATTGTGGATCAGCGATTGATCAAAAACAGTATTTGAATTTTGTTGTGAACAGAAGGCTGAATAAGA    -1
ATG GGG AGT TTG AGG GCG ATT TTG AAG AAT CCA GAT GAT TTG TAT CCA TTG GTA    54
 M   G   S   L   R   A   I   L   K   N   P   D   D   L   Y   P   L   V
AAG CTG AAG CTA GCG GCT CGA CAC GCG GAG AAG CAG ATC CCG CCG TCT CCA CAT   108
 K   L   K   L   A   A   R   H   A   E   K   Q   I   P   P   S   P   H
TGG GGC TTC TGT TAC TCA ATG CTT CAT AAG GTT TCT CGT AGC TTT GCT CTC GTC   162
 W   G   F   C   Y   S   M   L   H   K   V   S   R   S   F   A   L   V
ATT CAA CAA CTT CCC GTC GAG CTT CGT GAC GCC GTG TGC ATT TTC TAT TTG GTT   216
 I   Q   Q   L   P   V   E   L   R   D   A   V   C   I   F   Y   L   V
CTT CGA GCA CTT GAC ACT GTT GAG GAT GAT ACC AGC ATT CCC ACC GAT GTT AAA   270
 L   R   A   L   D   T   V   E   D   D   T   S   I   P   T   D   V   K
GTA CCT ATT CTG ATC TCT TTT CAT CAG CAT GTT TAT GAT CGT GAA TGG CAT TTT   324
 V   P   I   L   I   S   F   H   Q   H   V   Y   D   R   E   W   H   F
TCA TGT GGT ACA AAA GAG TAC AAG GTT CTC ATG GAC CAG TTC CAT CAT GTT TCA   378
 S   C   G   T   K   E   Y   K   V   L   M   D   Q   F   H   H   V   S
ACT GCT TTT CTG GAG CTT AGG AAA CAT TAT CAG CAG GCA ATT GAG GAT ATT ACC   432
 T   A   F   L   E   L   R   K   H   Y   Q   Q   A   I   E   D   I   T
ATG AGG ATG GGT GCA GGA ATG GCA AAA TTC ATA TGC AAG GAG GTG GAA ACA ACT   486
 M   R   M   G   A   G   M   A   K   F   I   C   K   E   V   E   T   T
GAT GAT TAT GAC GAA TAT TGT CAC TAT GTA GCT GGG CTT GTT GGG CTA GGA TTG   540
 D   D   Y   D   E   Y   C   H   Y   V   A   G   L   V   G   L   G   L
TCA AAA CTG TTC CAT GCC TCT GGG AAA GAA GAT CTG GCT TCA GAT TCT CTC TCC   594
 S   K   L   F   H   A   S   G   K   E   D   L   A   S   D   S   L   S
AAC TCC ATG GGT TTA TTT CTT CAG AAA ACA AAC ATC ATT AGA GAT TAT TTG GAA   648
 N   S   M   G   L   F   L   Q   K   T   N   I   I   R   D   Y   L   E
GAC ATA AAT GAA GTA CCC AAG TGC CGT ATG TTC TGG CCC CGT GAA ATA TGG AGT   702
 D   I   N   E   V   P   K   C   R   M   F   W   P   R   E   I   W   S
AAA TAT GTT AAC AAG CTT GAG GAA TTA AAG TAC GAG GAT AAC TCG GCC AAA GCA   756
 K   Y   V   N   K   L   E   E   L   K   Y   E   D   N   S   A   K   A
GTG CAA TGT CTC AAT GAC ATG GTC ACT AAT GCT TTA TCA CAT GTA GAA GAT TGT   810
 V   Q   C   L   N   D   M   V   T   N   A   L   S   H   V   E   D   C
TTG ACT TAC ATG TCT GCT TTG CGT GAT CCT TCC ATC TTT CGA TTC TGT GCT ATT   864
 L   T   Y   M   S   A   L   R   D   P   S   I   F   R   F   C   A   I
CCA CAG GTC ATG GCA ATT GGG ACA TTA GCT ATG TGC TAC GAC AAC ATT GAA GTC   918
 P   Q   V   M   A   I   G   T   L   A   M   C   Y   D   N   I   E   V
TTC AGA GGA GTG GTA AAA ATG AGA CGT GGT CTG ACT GCT AAG GTC ATT GAC CAG   972
 F   R   G   V   V   K   M   R   R   G   L   T   A   K   V   I   D   Q
ACC AGG ACT ATT GCA GAT GTA TAT GGT GCT TTT TTT GAC TTT TCT TGT ATG CTG  1026
 T   R   T   I   A   D   V   Y   G   A   F   F   D   F   S   C   M   L
AAA TCC AAG GTT AAT AAT AAT GAT CCA AAT GCA ACA AAA ACT CTG AAG AGG CTC  1080
 K   S   K   V   N   N   N   D   P   N   A   T   K   T   L   K   R   L
GAA GCT ATC CTG AAA ACT TGC AGA GAT TCG GGA ACC TTG AAC AAA AGG AAA TCC  1134
 E   A   I   L   K   T   C   R   D   S   G   T   L   N   K   R   K   S
TAC ATA ATC AGG AGC GAG CCT AAT TAC AGT CCA GTT CTG ATT GTT GTC ATA TTC  1188
 Y   I   I   R   S   E   P   N   Y   S   P   V   L   I   V   V   I   F
ATC ATA CTG GCT ATT ATT CTT GCA CAG CTA TCT GGA AAC CGA TCT TAG ACGATAT  1243
 I   I   L   A   I   I   L   A   Q   L   S   G   N   R   S   *
TTTGGGTTACAAAAAAGAAGTCAGGTCAAGGAAGACAGCACAAGCTCTTGGCCAATTATGTGATTAGTGCA  1314
AATTTTGATGTTTGTAATTCTATGTTCATTAAGTGATAGTTGCACCTTTTAACCTGACATGATAATTACGA  1385
AAACCTATTTTTGGTGGTTTGTTGGGTATGTACTTGTTTGCAAGCTACGAAAGCAAATTCCAAGTGTTGTA  1456
GAGTCACTATGATGTAATAAACATGTCTTTTATAATAGTTTGTTCATTTTTGTGGTAAAAAAAAAAAAAAA  1527
AA                                                                        1529
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FIG. 1. Nucleotide and predicted amino acid sequences, in single-letter code, of the cDNA for the TSS gene. Nucleotides are numbered starting at the A in the ATG start codon (underlined). An open reading frame of 1233 bp encoding 411 amino acids is shown below the nucleotide sequence. The transcription initiation site was mapped (see Fig. 4) to the adenosine located 96 nucleotides 5′ to the adenosine of the AUG translation initiator start site. Genbank cDNA Accession No. U60057.

region (3′ NTR) of gTSS-1 was identical to the 3′ NTR of the TSS cDNA, indicating that the isolated TSS genomic gene corresponded to an expressed gene. Comparison of the full gTSS sequence with the coding region of the TSS cDNA indicated a complex organization of exons and introns. The gTSS-1 gene contains 13 ex-

ons ranging in size from 42 to 195 nucleotides (corresponding to 14 to 65 amino acids). Twelve introns separate these exons and range in size from 72 to 1737 nucleotides.

TSS mRNA level and enzyme activity in elicitor-treated cells. Previous work correlated the induction of sesquiterpene biosynthesis and suppression of sterol biosynthesis in elicitor-treated cell cultures with the regulation of pertinent branch point enzymes (10). The induction of sesquiterpene biosynthesis was correlated with an induction of sesquiterpene cyclase enzyme activity, while the decline in sterol biosynthesis was correlated with a suppression of SS enzyme activity. An induction of cyclase mRNA was subsequently demonstrated to precede the induction of cyclase enzyme activity (35). To determine if SS might be subject to similar levels of regulation, the level of SS mRNA was determined relative to changes in the SS enzyme activity, and these measurements were made relative to those for sesquiterpene cyclase.

Enzyme activities and mRNA levels were monitored over a 24-h period in which tobacco cell suspension cultures in their rapid growth phase were subjected to elicitor treatment. Sesquiterpene cyclase enzyme activity was not detected in control cells, but was detected by 4 h after initiation of the elicitor treatment, reached a maximum by 12 h, and decreased thereafter (Fig. 7A). RNA blot hybridization detected no sesquiterpene cyclase mRNA in control cell samples. Elicitor-treated cell samples contained sesquiterpene cyclase mRNA corresponding to 1.4 kb in size. The cyclase mRNA was readily detected within 4 h of elicitation, reached a maximum by 8 h, and had declined to near control levels by 24 h (Fig. 7A). TSS enzyme activity in control cells remained fairly constant over the first 8 h and then nearly doubled over the course of the next 16 h. In contrast, TSS enzyme activity in elicitor-treated cells was comparable to control levels up to 12 h when TSS enzyme activity began to decrease. By 24 h, the TSS enzyme activity in elicitor-treated cells had decreased to almost below detectable activity (Fig. 7B). RNA blot hybridization for TSS (Fig. 7B) detected one major transcript corresponding to 1.6 kb in control cells that increased approximately 1.2- to 1.5-fold within 24 h. The TSS mRNA levels in elicitor-treated cells remained fairly constant throughout the experiment, and decreased only marginally by 24 h after elicitor treatment (Fig. 7B).

DISCUSSION

The amino acid sequence of the *N. tabacum* SS enzyme deduced from the cDNA clones described in the current report is very similar to those previously characterized (18, 23, 24, 26), and alignment of the plant, mammalian, and fungal SS sequences support the suggestion by Robinson *et al.* (6) that several highly conserved domains might be important for catalytic/functional activity. Accordingly, domains III (amino acid residues approximately positioned at 168–186), IV (residues 202–217), and V (residues 285–298) consist of 15 to 20 amino acids and are nearly identical between the representative proteins from all three kingdoms. Although domains I and II constitute a continuum approximately from amino acids 58 to 90 and are less conserved than the other domains, an aspartate-rich motif within domain II is highly conserved. Similar acidic-rich motifs have been noted for other isoprenoid biosynthetic enzymes, and at least for FPP synthase (36) and trichodiene synthase (37), which utilize FPP as their substrate, point mutations within the aspartate-rich motif compromise the catalytic activity of the enzymes. These aspartate-rich motifs were initially proposed by Ashby *et al.* (38) to coordinate/facilitate FPP binding through a magnesium ion requirement. Like the other FPP utilizing enzymes, SS has a divalent cation requirement (39). Domain VI of the SS proteins (6, 17) is not distinguished by sequence conservation but by the hydrophobic nature of the sequence. Domain VI was therefore implicated as a membrane targeting signal for the SS enzyme. Consistent with this suggestion, carboxy-terminal deletion of residues 388 to 411 (Fig. 2) in the tobacco enzyme resulted in a functionally soluble SS activity (Fig. 3), much like that observed for the yeast (20) and rat (17) enzymes.

In experiments designed to map functional domains within a particular class of sesquiterpene synthases, Back and Chappell (28, 40) noted that several functional activities mapped to specific exons. Interestingly, other plant monoterpene and diterpene synthases have a nearly identical intron–exon organization (41, 42), further supportive of a relationship between particular exons and enzymatic function. The identification of conserved domains I–VI within the SS proteins may be indicative of domains important for catalysis, but these conserved domains do not map to discrete exons as was noted for the terpene cyclases. To the best of our knowledge, only the intron–exon organization of the SS genes

FIG. 2. Comparison of the TSS (*Nicotiana tabacum*) amino acid sequence to that of SS amino acid sequences from *Nicotiana benthamiana* (24), *Arabidopsis thaliana* (23), rat (26), and yeast (18). Numbers indicate the amino acid residues in the sequences. Gaps in the alignment are designated by dashes. Amino acid residues which are common to more than three polypeptides are considered a consensus. Overlines numbered with roman numerals indicate highly conserved regions according to the nomenclature of Robinson *et al.* (6). Arrows indicate exon/intron splice sites for *N. tabacum*.

```
N. tabacum      MG---SLRAI LKNPDDLYPL VKLKLAARHA EKQIPPSPHW GFCYSMLHKV SRSFALVIQQ   57
N. benthamiana  MG---SLARI LKNPEDLYPL VKLKLAARHA EKQIPPSPNW GFCYSMLHKV SRSFALVIQQ   57
A. thaliana     MG---SLGTM LRYPDDIYPL LKMKRAIEKA EKQIPPEPHW GFCYSMLHKV SRSFSLVIQQ   57
Rat             MEFVKCLGHP EEFYNLLRFR MGGRRNFIPK MDRNSLSNSL KTCYKYLDQT SRSFAAVIQA   60
Yeast           MGKLLQL-AL HPVEMKAALK LKFCRTPLFS IYDQSTSPYL LHCFELLNLT SRSFAAVIRE   59
Consensus       MG....L... .......... .K........ ......SP.. ..CY..L... SRSFA.VIQ.   60

                _______I_______ _____________II_______                  _
N. tabacum      LPVELRDAVC IFYLVLRALD TVEDDTSIPT DVKVPILISF HQHVYDREWH FSCG--TKEY  115
N. benthamiana  LPVELRDAVC IFYLVLRALD TVEDDTSIPT DVKVPILISF HQHVYDREWH FSCG--TKEY  115
A. thaliana     LNTELRNAVC VFYLVLRALD TVEDDTSIPT DEKVPILIAF HRHIYDTDWH YSCG--TKEY  115
Rat             LDGDIRHAVC VFYLILRAMD TVEDDMAISV EKKIPLLRNF HTFLYEPEWR FTES--KEKH  118
Yeast           LHPELRNCVT LFYLILRALD TIEDDMSIEH DLKIDLLRHF HEKLLLTKWS FDGNAPDVKD  119
Consensus       L..ELR.AVC .FYL.LRALD TVEDD.SI.. D.K.P.L..F H...Y...W. F...--....  120

                _________III________                  _____________IV________
N. tabacum      CHYVAGLVGL GLSKLFHASG KED-LASDSL --SNSMGLFL QKTNIIRDYL EDINEVPKCR  225
N. benthamiana  CHYVAGLVGL GLSKLFHASE KED-LASDSL --SNSMGLFL QKTNIIRDYL EDINEVPKCR  225
A. thaliana     CHYVAGLVGL GLSKLFLAAG SE-VLTPDWE AISNSMGLFL QKTNIIRDYL EDINEIPKSR  227
Rat             CHYVAGLVGI GLSRLFSASE FEDPIVGEDT ECANSMGLFL QKTNIIRDYL EDQQEGRQ--  229
Yeast           CHYVAGLVGD GLTRLIVIAK FANESLYSNQ QLYESMGLFL QKTNIIRDYN EDLVDGRS--  236
Consensus       CHYVAGLVG. GLS.LF.A.. .......... ...NSMGLFL QKTNIIRDYL EDI.E.....  240

                _______V_______
N. tabacum      MFWPREIWSK YVNKLEELKY EDNSAKAVQC LNDMVTNALS HVEDCLTYMS ALRDPSIFRF  285
N. benthamiana  MFWPREIWSK YVNKLEELKY EDNSAKAVQC LNDMVTNALP HVEDCLTYMS ALRDPSIFRF  285
A. thaliana     MFWPREIWGK YADKLEDLKY EENTNKSVQC LNEMVTNALM HIEDCLKYMV SLRDPSIFRF  287
Rat             -FWPQEVWGK YVKKLEDFVK PENVDVAVKC LNELITNALQ HIPDVITYlS RLRNQSVFNF  288
Yeast           -FWPKEIWSQ YAPQLKDFMK PENEQLGLDC INHLVLNALS HVIDLLTYLA SIHEQSTFQF  295
Consensus       .FWP.EIW.K Y..KLED... .EN....V.C .N..VTNAL. H..D.LTY.. .LR..S.F.F  300

N. tabacum      CAIPQVMAIG TLAMCYDNIE VFRGVVKMRR GLTAKVIDQT RTIADVYGAF FDFSCMLKSK  345
N. benthamiana  CAIPQVMAIG TLAMCYDNIE VFRGVVKMRR GLTAKVIDRT RTIADVYGAF FDFSCMLKSK  345
A. thaliana     CAIPQIMAIG TLAlCYNNEQ VFRGVVKlRR GLTAKVIDRT KTMADVYGAF YDFSCMLKTK  347
Rat             CAIPQVMAIA TLAACYNNHQ VFKGVVKIRK GQAVTLMMDA TNMPAVKAII YQYIEEIYHR  348
Yeast           CAIPQVMAIA TLALVFNNRE VLHGNVKIRK GTTCYLILKS RTLRGCVEIF DYYLRDIKSK  355
Consensus       CAIPQVMAI. TLA.CYNN.. VF.GVVKIR. G.T...I... .T...V...F .......K.K  360

N. tabacum      VNNNDPNATK TLKRLEAI-- --LKT--CRD SG--TLNKRK SYIIRSEPN- Y------SPV  390
N. benthamiana  VNNNDPNATK TLKRLEVI-- --LKT--CRD SG--TLNKRK SYIIRSEPN- Y------SPV  390
A. thaliana     VDKNDPNASK TLNRLEAV-- --QKL--CRD AG--VLQNRK SYV------N --DKGQPNSV  391
Rat             VPNSDPSASK AKQLISNIR- -TQSLPNCQL IS--RSHYSP IYL------S F-IMLLAALS  397
Yeast           LAVQDPNFLK LNIQISKIEQ FMEEMYQDKL PPNVKPNETP IFLKVKERSR YDDELVPTQQ  415
Consensus       V...DPNA.K .......I.- -......C.. ..--...... .Y........ .-........  420

                _______________VI_______________
N. tabacum      LIVVIFII-L AIILA----- -QLS-GNRS                                  411
N. benthamiana  LIVVIFII-L AIILA----- -QLS-GNRS                                  411
A. thaliana     FIIMVVIL-L AIVFA----- -YLR-AN--                                  410
Rat             WQ-YLSTL-S QVTED----- -YVQREH--                                  416
Yeast           EEEYKFNMVL SIILSVLLGF YYIYTLHRA                                  444
Consensus       ........-L .I...----- -...-....                                  449
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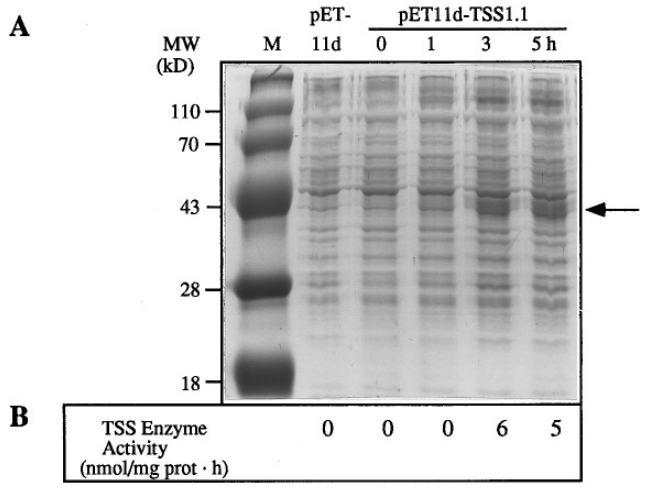

FIG. 3. Time course expression of truncated TSS gene (pET11d-TSS1.1) in *E. coli* strain BL21 (DE3). pET-TSS1.1 is a 24-amino-acid, carboxy-terminus deletion of the full-length TSS clone. Bacterial cells harboring this construct were grown at 37°C up to 5 h after the addition of 1 mM IPTG. Total protein extracts of the *E. coli* cells were used for detection of the total protein profiles generated by SDS–PAGE and stained with Coomassie blue. The arrow indicates the position predicted for the truncated TSS protein (A). Soluble protein extracts were used to determine TSS enzyme activity at the indicated times (B).

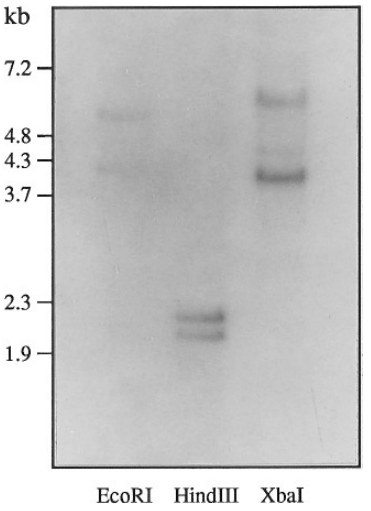

FIG. 5. DNA gel blot analysis of SS-like genes in the tobacco genome. Genomic DNA (5 μg) was digested with the indicated restriction enzymes, separated on 0.8% agarose gel, transferred to nylon membrane, and hybridized with a radiolabeled 371-bp internal TSS cDNA fragment (TSS3).

from *N. tabacum* and those recently obtained for two *Arabidopsis thaliana* genes (Devarenne and Chappell, unpublished, and Boronat *et al.*, personal communication) have been determined and found to be very similar.

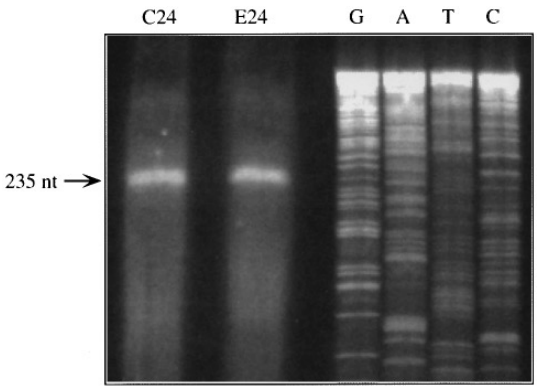

FIG. 4. Determination of the TSS transcription start initiation site. The 42-nucleotide primer 5′-CCTTATGAAGCATTGAGTAACAGA-AGCCCCAATGTGGAGACG-3′, located 97 nucleotides 3′ to the AUG translation initiator, was end-labeled using T4-polynucleotide kinase and [γ-^{32}P]ATP. Labeled primer and 30 μg of total RNA from control (C24) and elicitor-treated (E24) cells were hybridized at 65°C for 10 min followed by incubation at room temperature for 5 min. The RT reaction was then carried out at 45°C for 60 min and the radioactive extension product analyzed on an 8% sequencing gel. A known TSS DNA sequencing ladder was used for size determination of the extension product as noted by the arrow.

If exons of the SS gene do correspond to functional units that extend beyond the current conserved sequence alignments (domains I–VI), then conservation of the intron–exon organization among the plant, fungal, and mammalian genes would also be expected.

The structure of the *N. tabacum* SS gene with 13 exons and 12 introns is atypical for a plant gene. Plant genes are generally organized with large exons separated by a few small introns (43). In contrast, mammalian genes typically have small exons surrounded by large introns (43). Small intronic regions of 65 to 70 bp in plant genes are not unusual, with two-thirds being shorter than 150 bp (44), but introns larger than 2 to 3 kb are rarely observed (45). A few plant genes of complexity equal to that of SS have been described, including the maize pyruvate, orthophosphate dikinase gene with 16 introns ranging in size from 4.6 kb to 77 bp and 17 exons ranging in size from 57 to 372 bp (46), and the 140-kDa subunit of the *Arabidopsis* RNA polymerase II gene with 24 introns (47). Relative to the vast majority of plant genes, the small exon/large intron organization of the *N. tabacum* SS gene more closely resembles a mammalian gene structure pattern than a plant's. The importance of this gene organization is not obvious as this time and may relate more to the evolutionary introduction of the SS progenitor gene than to functional attributes for the encoded enzyme.

SS is typically depicted as the first branch point enzyme of the general isoprenoid pathway responsible for the diversion of carbon specifically to sterol biosynthesis. As such, it has attracted considerable interest as a potential regulatory point controlling carbon flux into ste-

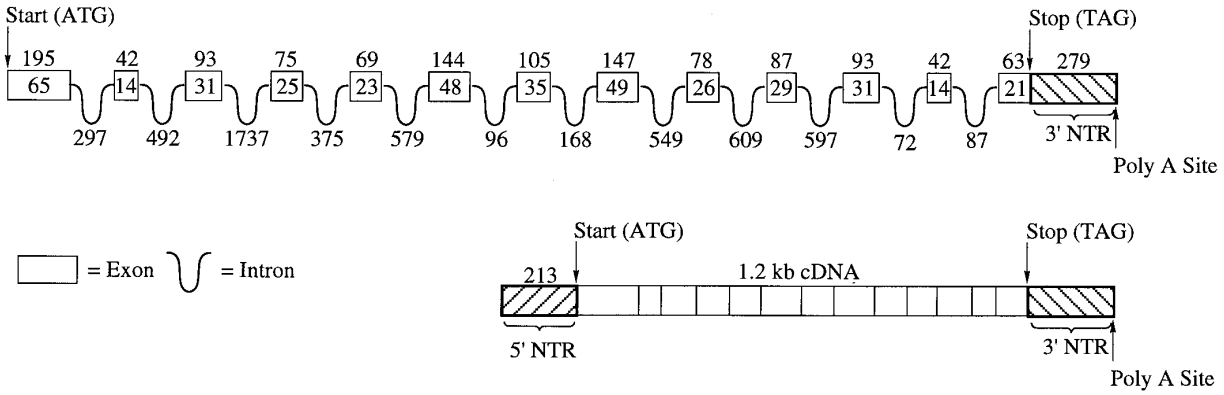

FIG. 6. Organization of a TSS genomic clone showing exon and intron localization as well as the 5′ and 3′ nontranslated regions (NTR). Numbers above exons indicate the number of base pairs in the exon. Numbers inside exons indicate the number of amino acids encoded by the exonic DNA. Numbers below introns indicate the number of base pairs in an intron. Start and stop codons, and polyadenylation site, are noted as such. Also shown is the comparison between the TSS genomic DNA and cDNA. The total size of the cDNA, from start site (ATG) to stop site (TAG), is indicated above the cDNA. Genbank gDNA Accession No. U59683.

rols. Regulation of SS enzyme activity in mammalian cells appears to occur predominately at the level of transcription. Faust *et al.* (7) previously demonstrated that SS activity was regulated in parallel with HMGR in fibroblast cells responding to the availability of exogenous sterols. For example, SS and HMGR enzyme activities were high in fibroblasts grown in the absence of exogenous sterols. Upon supplementing the growth media with sterols and LDL, both enzyme activities were suppressed. Subsequent studies have reported that the sterol-mediated regulation of SS was correlated with corresponding changes in the steady-state level of the SS mRNA (1, 2). That is, when cells were switched from sterol-depleted to sterol-supplemented media, SS mRNA levels decreased. These reports also described multiple SS mRNA species, but there is little evidence that these multiple forms are independently regulated. Transcriptional control as the principle means for regulating SS was recently corroborated by Guan *et al.* (48, 49) who reported in a promoter-deletion study of the human SS promoter the identification of cis-sequences important for sterol-regulated gene expression.

SS is apparently subject to a type of posttranscriptional regulation in plant cell cultures that has not been described previously. When fungal elicitors are added to rapidly growing tobacco cell suspension cultures, the cultures cease sterol production and, instead, synthesize and secrete antimicrobial sesquiterpenes (9, 10, 34, 25). The decline in sterol biosynthesis was previously correlated with a suppression of SS enzyme activity, while the induction of sesquiterpene biosynthesis was correlated with an induction of a sesquiterpene cyclase enzyme activity (9, 10). Using several different experimental techniques, including RNA blot hybridizations (Fig. 7A), the induction of the cyclase enzyme activity has been correlated with a transcriptional activation of the cyclase gene(s) (50). In contrast, the results presented in Figs. 4 and 7B provide independent evidence that the decline in SS enzyme activity in elicitor-treated cells is not preceded by a decline in the steady-state levels of the SS mRNA. An important caveat to this conclusion is the possibility that the two SS genes are differentially regulated in elicitor-treated cells. That is, one SS gene might be transcriptionally suppressed while the other gene could be induced, resulting in no obvious change in the steady-state levels of the SS mRNA. Even after preliminary characterization of the second tobacco SS gene (Devarenne and Chappell, unpublished observations), we cannot eliminate this possibility. The second SS gene predicts 5′ and 3′ NTRs in its derived mRNA highly homologous to the SS cDNA and genomic clone described here, but is distinguished from the first SS gene on the basis of an apparent 1-kb intron within the 3′ NTR and a significant number of nucleotide substitutions within the coding region of the gene. Hence, gene-specific probes to study expression of one SS gene or the other are not possible in this case. In addition, the current results are also consistent with SS being regulated posttranscriptionally. However, the results presented are not sufficient to distinguish between various posttranscriptional mechanisms, such as control mediated by the frequency of translation initiation, post-translational modifications affecting enzyme activity, or changes in the SS protein half-life. Such distinctions will require immunological techniques to measure more directly the level of the SS enzyme protein and modifications to the enzyme protein, as well as experiments designed to measure the percentage of SS mRNA associated with translationally active polysomes.

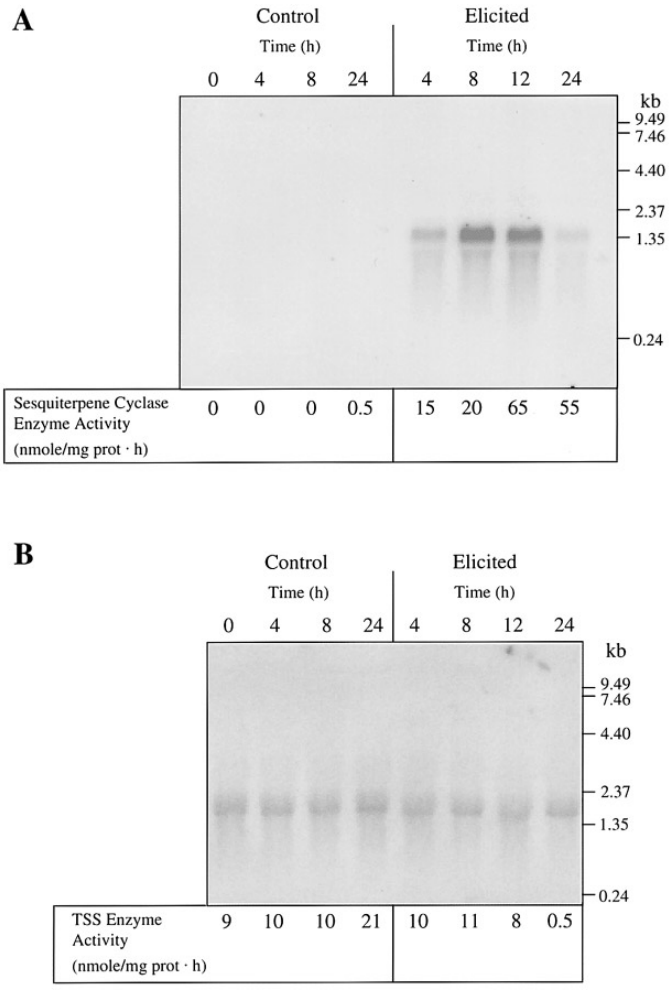

FIG. 7. Comparison of mRNA levels and enzyme activities for sesquiterpene cyclase (A) and SS (B) in control and elicitor-treated cells. Aliquots of cells collected at the indicated times were used for RNA extractions as well as determinations for enzyme activities. Samples of 5 μg (A) or 10 μg (B) of total RNA were size-separated on a 1% agarose gel, transferred to a nylon membrane, and probed with either a full-length sesquiterpene cyclase cDNA or SS cDNA.

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