Grapevine stilbene synthase cDNA only slightly differing from chalcone synthase cDNA is expressed in *Escherichia coli* into a catalytically active enzyme

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Stilbene synthase is responsible for the formation of resveratrol and other stilbenes which function in grapevine as phytoalexins. A full-length stilbene synthase cDNA was prepared from grapevine mRNA and sequenced. The insert in pSV25 coding for a polypeptide with 392 amino acids was inserted into the vectors pKK233-2 and pDS12/RBSII-2, respectively. Expression of the cDNA in *Escherichia coli* yielded an enzymatically active dimer exhibiting solely stilbene synthase activity. The protein was characterized by enzyme activity and Western blot analysis.

Bacterial expression; Chalcone synthase; Stilbene synthase sequence; *Vitis*

1. INTRODUCTION

Stilbene synthase and chalcone synthase act on the same substrates, *p*-coumaroyl-CoA and malonyl-CoA (Fig. 1). While stilbene synthase is detectable only in a few plant species, chalcone synthase is present in most higher plants. Some stilbenes are phytoalexins and play in the mechanism of induced resistance an economically important role in three plant genera: peanut, grapevine and pine [1–3]. In these cases, the induction of the stilbene formation, and thus the expression of stilbene synthase genes, is a rapid process [4–6]. Resveratrol (3,4′,5-trihydroxystilbene) and its oligomeric derivatives have been implicated in the process of induced resistance in various tissues of grapevine [1–3]. In leaves of *Vitis* plants, resistance against *Plasmopara viticola* was positively correlated with the capacity to synthesize resveratrol and viniferin following induction by UV light [7]. In grape berries, the resistance against *Botrytis cinerea* was found to be proportional to the accumulation of pterostilbene, another resveratrol derivative [8]. In peanut (*Arachis hypogaea*), the gene encoding the key enzyme of resveratrol synthesis has been investigated [9]. In addition, changes in the rate of gene expression have been determined at the level of mRNA activity, protein synthesis, and the fully assembled enzyme [5].

We isolated and sequenced a full-length cDNA prepared from grapevine mRNA. The amino acid sequence comparison revealed an extremely high degree of homology of the putative stilbene synthase and chalcone synthase thus raising the question whether indeed a stilbene synthase clone was characterized earlier in peanut [9] or in the grapevine cDNA library used here. To exclude any wrong assignment, the expression of enzymatically active stilbene synthase in *E. coli* provided for the first time clear evidence that the amino acid sequence deduced from the nucleotide sequence is attributable to stilbene synthase and not to the very homologous chalcone synthase.

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2. MATERIALS AND METHODS

Poly(A)$^+$ RNA was isolated [5,10] from elicitor-treated cell suspension cultures of *Vitis* var. Optima [11] as described previously. A cDNA library in *E. coli* NM522 (Stratagene) using the vector pT7T3 was constructed following the supplier's recommendations (Pharmacia). A clone with pSV25 was identified by screening with oligonucleotides [10]. DNA sequence analysis was carried out by the dideoxy chain termination method [12] using the sequenase (US Biochemicals). The DNA sequence of the insert in pT7T3 was determined using two commercially available pT7T3 sequencing primers and 22-meric oligonucleotides synthesized according to sequence information obtained during sequencing.

A cDNA fragment of pSV25 was isolated by cutting at a *Nco*I site 1 bp upstream of the ATG and at a *Dra*I site in the 3′-noncoding region. The vector pKK233-2 (Pharmacia) was linearized with *Hin*dIII and the 5′-overhangs were filled in with Klenow enzyme to generate blunt ends. A 5′-overhang was formed at one end by cutting with *Nco*I. Finally, blunt and sticky ends of vector and insert were ligated. This construct called pKSV25 was introduced into competent cells of *E. coli* NM522. Transformants were analyzed by double digestion of the plasmids with *Eco*RI and *Pvu*I. Bacteria containing pKSV25 were grown in LB-medium at 37°C to OD_{600} = 0.7. For induction, IPTG was added to a final concentration of 1 mM, and the cells were incubated for 2 h. Cells from 1-ml culture were harvested,

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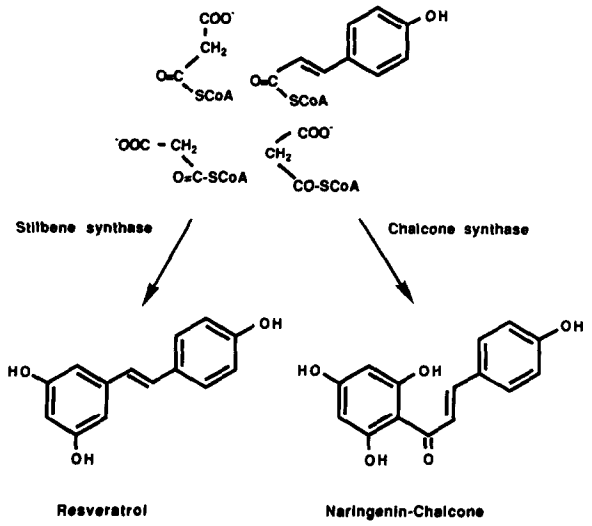

Fig. 1. Reaction sequences catalyzed by stilbene synthase and chalcone synthase.

washed, and resuspended in 300 μl of 50 mM Tris-HCl, pH 7.5, and 10% (w/v) sucrose. Following repeated cycles of freezing and thawing, 7.5 μl of 4 M NaCl and 15 μl of lysozyme (4 mg/ml) were added, and the mixture incubated 40 min at 0°C. The debris was sedimented at 10000 rpm for 15 min. After adjustment to 1 mM mercaptoethanol the enzyme tests were performed.

Alternatively, the *NcoI-DraI* fragment from pSV25 was inserted to the *PstI* site of the vector pDS12/RBSII-2 [13,14]. Blunt-end ligation of the modified fragment and the vector created a new *NcoI* site (Fig. 2). The recombinant plasmid (pDSV25) was transferred into *E. coli* W3110 *lac* I^Q (RB791) and *E. coli* NM522, respectively.

The determination of enzyme activities was as described earlier [15,16]. Western blot analysis was performed with anti-stilbene synthase serum, biotinylated second antibodies and peroxidase-labelled streptavidin according to the supplier's manual (Amersham).

3. RESULTS

3.1. *Isolation and characterization of clone pSV25 containing full-length stilbene synthase cDNA*

A 1.5 kb cDNA containing the complete coding sequence of stilbene synthase was isolated from a grapevine cDNA library. The sequence determined for grapevine stilbene synthase cDNA pSV25 (Fig. 3) shows 76% nucleotide sequence similarity to peanut stilbene synthase cDNA. Compared to peanut stilbene synthase pGSG11 and pGSG10 [9], the deduced amino acid sequence of grapevine stilbene synthase encompasses 3 additional amino acids at the C-terminus. The homology between the putative protein sequences of the two stilbene synthases is less than 68%. In contrast, the homology between grapevine stilbene synthase and chalcone synthase from parsley [17] is as much as 75%. Compared to grapevine stilbene synthase, chalcone synthase [17] contains at the N-terminus 5 additional amino acids and at the C-terminus 1 additional amino acid.

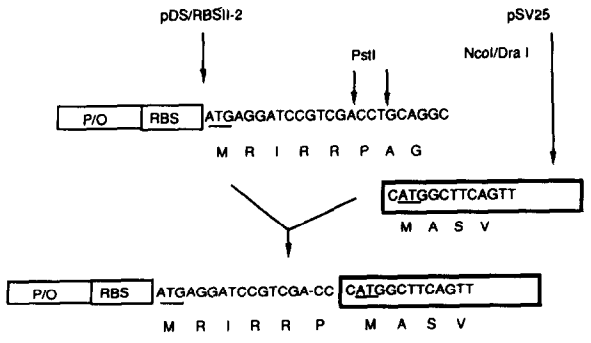

Fig. 2. Insertion in vector pDS12/RBSII-2 of cDNA coding for a modified stilbene synthase.

The mRNA used for the preparation of the cDNA library represented the gene expression in an elicitor-treated cell suspension culture. As stilbene synthase and chalcone synthase act upon the same substrates, and both stilbenes and flavonoids are constituents in grapevine, it was indispensable to know at which ratio the two enzymes were present in the induced cell cultures. Careful analysis of the reaction products in the enzyme assays revealed that the ratio of chalcone synthase to stilbene synthase was less than 1:20 at the level of enzyme activities.

3.2. *Expression of stilbene synthase in* Escherichia coli

A fragment of pSV25 obtained by digestion with *NcoI* and *DraI* was ligated into the linearized vector pKK233-2. Stilbene synthase cDNA was thus brought under the control of the *trp-lac* promotor. Cells of *E. coli* NM522 containing pKSV25 were grown in the presence or absence of IPTG as inducer. Extracts were prepared in a mercaptoethanol containing buffer and assayed using malonyl-CoA and *p*-coumaroyl-CoA as substrates.

In a second series of experiments, the cDNA in pSV25 was inserted to a different expression vector allowing modifications in the ribosome binding site and the N-terminal region of the stilbene synthase cDNA. This construct (Fig. 2) with an extended open reading frame under the control of the T5-derived promotor/*lac* operator (pDSV25) was transferred into *E. coli* W3110*lac* I^Q (RB791) and *E. coli* NM522. Bacteria cells were induced with IPTG. They synthesized a protein with an N-terminal extension of stilbene synthase with 6 amino acids (Fig. 2). The fusion protein differs from stilbene synthase by 3 additional positive charges and a size of 43601 instead of 42791.

The results of expression experiments are summarized in Table I. Resveratrol was the sole product; chalcone or naringenin were not detectable. An additional control showed that no product was formed in the absence of *p*-coumaroyl-CoA. Resveratrol formation was dependent on the induction of *E. coli* cells with IPTG (Expt. no. 2 and 3, 5 and 6). The control by the *trp-lac* promotor was leaky in *E. coli* NM522 while

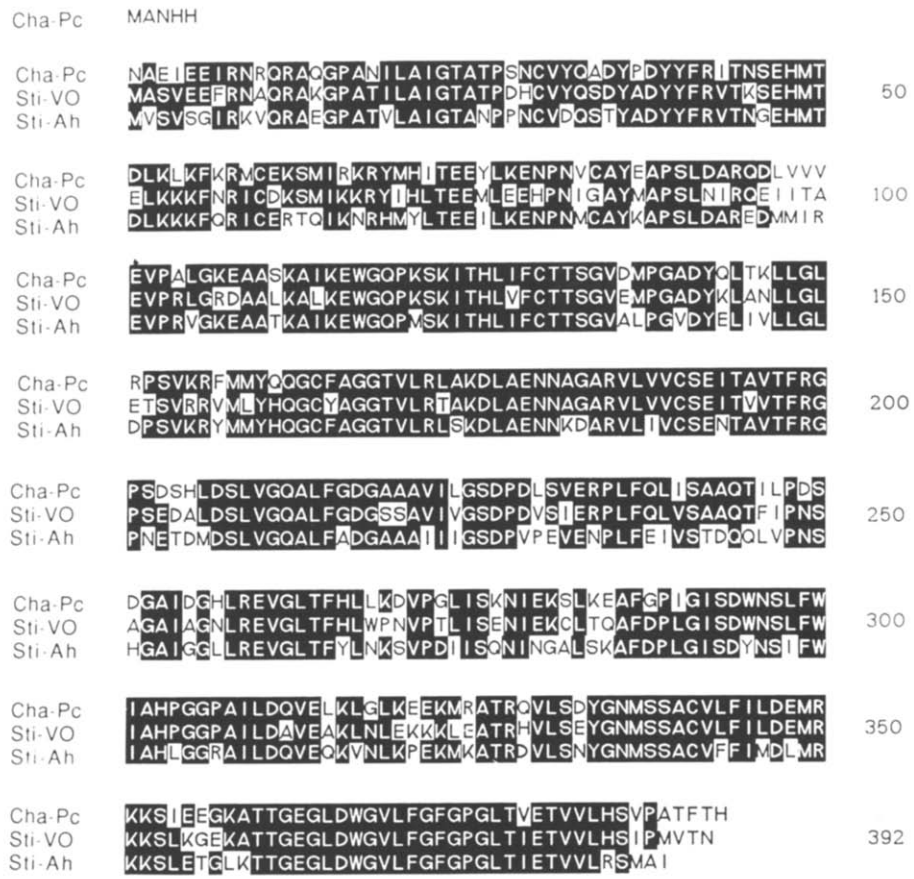

Fig. 3. Comparison of amino acid sequences derived for grapevine stilbene synthase (Sti-VO; *Vitis* var. Optima), peanut stilbene synthase (Sti-Ah; *Arachis hypogaea*), and chalcone synthase from parsley (Cha-Pc; *Petroselinum crispum*).

Table I

Enzyme activities (pkat) in extracts from *E. coli* containing or not containing pKSV25 or pDSV25

Expt. no.	Vector	Host	Induction	*p*-Coumaroyl – CoA	Products formed	
					Resveratrol (pkat/ml)	Naringenin (pkat/ml)
1	pKSV25	NM522	+	+	0.46	n.d.[a]
2	pKSV25	NM522	+	–	n.d.	n.d.
3	pKSV25	NM522	–	+	0.21	n.d.
4	pKK233	NM522	+	+	n.d.	n.d.
5	pDSV25	W3110	–	+	0.03	n.d.
6	pDSV25	W3110	+	+	2.30	n.d.
7	pDS12[b]	W3110	+	+	n.d.	n.d.
8	pDSV25	NM522	+	+	1.90	n.d.
9	pDSV25	NM522	–	+	1.38	n.d.
10	pDS12[b]	NM522	+	+	n.d.	n.d.

[a] n.d., not detectable

[b] pDS12-RBSII-2, the plasmid without any stilbene synthase cDNA insert

Two different strains of *E. coli* were used as host for the plasmids. 40 μl of extract from the bacteria grown in the presence or absence of IPTG as inductor were analyzed with radioactive malonyl-CoA and with or without *p*-coumaroyl-CoA as substrates

the expression was under more strict control of the *lac* operator of pDSV25 in *E. coli* W3110 *lac* I^Q.

Extracts from bacterial cells were analyzed by electrophoresis and Western blots (Fig. 4). The bacterial expression was highests if *E. coli* W3110 *lac* I^Q was transformed with pDSV25 (Fig. 4a). Using stilbene synthase cDNA in vector pKK233 to transform *E. coli* NM522 led to a comparatively high amount of other immunoreactive products. Besides intact stilbene synthase at 43000 (Fig. 4, b3) and the fusion protein at 44000 (a3), the blot shows immunoreactive bands at 34000 and 33000. Neither the band attributed to stilbene synthase nor the two smaller peptides are present in bacteria lacking the stilbene synthase cDNA.

Gel permeation chromatography was performed on Sephacryl G-200 (Pharmacia, Freiburg, FRG) using 10 mM phosphate, pH 7.5, as buffer. Following calibration with alcohol dehydrogenase, malate dehydrogenase and horseradish peroxidase as marker proteins, we estimated an apparent M_r 85000 for the stilbene synthase extracted from *E. coli* NM522 transformed with pKSV25 and induced for 2 h with IPTG.

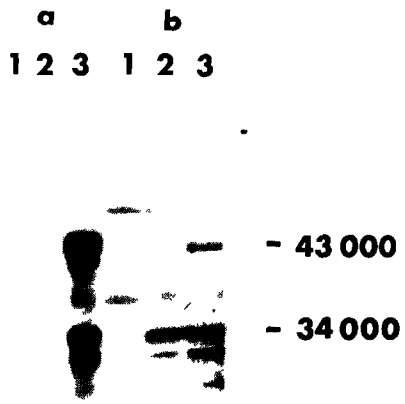

Fig. 4. Analysis of stilbene synthase proteins formed by bacterial expression. Aliquots from bacterial extracts were separated electrophoretically, blotted onto nitrocellulose and stained with anti-stilbene synthase antibodies, biotinylated second antibodies and peroxidase-labelled streptavidin. (a) shows 3 different extracts from *E. coli* W3110 *lac* I^Q, and (b) presents the extracts from *E. coli* NM522. Lane 1 shows the control without pSV25 insert. (Lane a1) vector pDS12/RBSII-2, + IPTG; (lane b1) pKK233-2, + IPTG. Lane 2 (without IPTG) vs lane 3 (with IPTG) indicates the degree of induction. (Lane a2 and 3) bacteria containing pDSV25; (lane b2 and 3) bacteria with pKSV25.

4. DISCUSSION

Clone pSV25 representing the first full-length sequence complementary to a stilbene synthase mRNA is to be used for probing the expression of resistance response genes in grapevine and for analysis of how phytoalexin formation [18] is controlled. However, prior to these experiments, additional evidence is required for attributing unequivocally the deduced sequences to stilbene synthase, as stilbene synthase and chalcone synthase are highly homologous. Grapevine stilbene synthase and peanut stilbene synthase exhibit 76% nucleotide sequence similarity. The protein of peanut stilbene synthase is 68% homologous to grapevine stilbene synthase while the homology between grapevine stilbene synthase and chalcone synthase from parsley [17] is as much as 75%. Thus, it is important to emphasize that our cDNA preparation via mRNA originated from cell extracts which contained virtually no chalcone synthase. Final evidence that we are dealing with stilbene synthase and unequivocal correlation is now being provided, for the first time, by the expression of the cDNA in *Escherichia coli* yielding an enzymatically active stilbene synthase. With the extracts from transformed and induced bacteria, no chalcone synthase activity was detectable besides the stilbene synthase activity. The formation of catalytically active, homodimeric stilbene synthase in *E. coli* indicates that spontaneous processes rather than mechanisms governed by the plant are responsible for the assembly of the enzyme.

It is noteworthy that the expression of stilbene synthase cDNA in the bacteria is highly dependent on the kind of vector and host. The introduction of 6 amino acids at the N-terminal site enhanced markedly the translation efficiency. On the Western blot (Fig. 4), immunoreactive bands at 34 kDa and 33 kDa appeared when stilbene synthase cDNA was transferred into the bacteria. The formation of a 34043 Da protein and a 32789 Da protein may be explained by a re-start of translation [19] at a Met-67 or Met-87 of stilbene synthase. A ribosome binding site can be found upstream of Met-87. It is unlikely that the formation of the proteins at 34 kDa and 33 kDa results from stilbene synthase by proteolysis. Prolonged incubation of bacterial extracts did not reveal a degradation of the stilbene synthase protein.

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