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Structural organization and differential expression of three stilbene synthase genes located on a 13 kb grapevine DNA fragment

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Abstract

A 13 kb DNA fragment was isolated from a grapevine (*Vitis* var. Optima) genomic library by hybridizing with elicitor-induced stilbene synthase cDNA as a probe. After fragmentation with *Eco* RI, subcloning and sequencing, two full-size stilbene synthase genes (*Vst1* and *Vst2*) and the 3′ end of a third stilbene synthase gene (Vst3) were located within the 13 kb fragment. *Vst1* and *Vst2*, differing only slightly in the coding region, are distinguished in the intron size and in the structure of the promoter region. The 5′ flanking region of gene *Vst1* contains a TATAA box at nucleotide −48. The substantial structural differences found for the promoters of the two genes are paralleled by a striking difference in the expression of the two genes in elicitor-treated cells. Moreover, the accumulation upon elicitation of six different stilbene synthase mRNAs was studied and found to differ by two orders of magnitude.

Introduction

Stilbene synthase (EC 2.3.1.95) is a polyketide synthase catalyzing the synthesis of 3,4′,5-trihydroxystilbene (resveratrol) from *p*-coumaroyl-CoA and three molecules malonyl-CoA [9, 24]. This cytosolic enzyme is confined to a few plant genera. In intact and unchallenged cells, the enzyme is very weakly expressed, but it is rapidly synthesized *de novo* upon attack by pathogens. Pathogen-derived elicitors turn on the synthesis of stilbene phytoalexins which are part of the process of induced resistance in these plants [8, 29]. In grapevine, resveratrol and other hydroxystilbenes with fungicidal potential are the dominating phenols produced under biotic stress [10, 11, 13, 16]. Recently, it has been shown that resveratrol indeed acts as a phytoalexin and thus enhances disease resistance of plants whose wild type is not capable of synthesizing stilbenes but receives this capacity after transformation with a stilbene synthase gene [3].

Genes of peanut stilbene synthase (resveratrol forming) have been studied and found to constitute a multigene family [12, 25]. Using grapevine cDNAs, we investigated the sequence and expression of four different stilbene synthase mRNAs [15]. Studies on structures of stilbene synthase genes including 5′-upstream sequences have so far not been reported. As stilbene syn-

thase activity is strongly induced during fungal attack [7, 19, 28] it was of interest to investigate whether all genes are activated in the same manner. It seemed likely that expression of the genes amenable to activation proceeds differentially. Two types of genes contributing to disease resistance genes are envisaged, one characterized by a quick response and another showing a more delayed response towards fungal elicitors. Such kind of gene regulation enables the plant to adapt to a fungal attack by modulating the transient response for different periods of time. From the profile of total mRNA obtained after induction of grapevine suspension cultures [15] it was argued that the occurrence of two maxima, an early (3–5 h) and a late (11–16 h) response following the onset of elicitation, may be due to the consecutive expression of at least two types of genes. The type of genes characterized by a quick and intense activation and a rapid degradation of the mRNA was detected previously. Presumably, the other kind of genes is slowly expressed upon elicitation but provides a more stabile mRNA.

We describe here a genomic DNA fragment containing the entire structure of two stilbene synthase genes in opposite position and the 3′ part of a third stilbene synthase gene. Furthermore, we demonstrate that, under conditions of biotic stress, the expression of these three genes and of the other stilbene synthase genes differed by factors of 2–100.

Materials and methods

Plant material

Cell suspension cultures of grapevine (*Vitis* var. Optima) were established and maintained as described previously [13, 15]. For the preparation of DNA from untreated cells and RNA of induced cells, cells at the stationary phase were used, which were harvested 9 days after transfer to new medium [13]. As elicitor for activating phytoalexin formation, we used crude cell wall preparations of *Phytophthora cambivora* obtained after exhaustive extraction as described [29].

Preparation and analysis of a grapevine genomic library

Crude nuclei were prepared from cultures cells as described [23]. After lysis of nuclei, DNA was extracted and purified by centrifugation on a CsCl gradient. The DNA was partially digested with

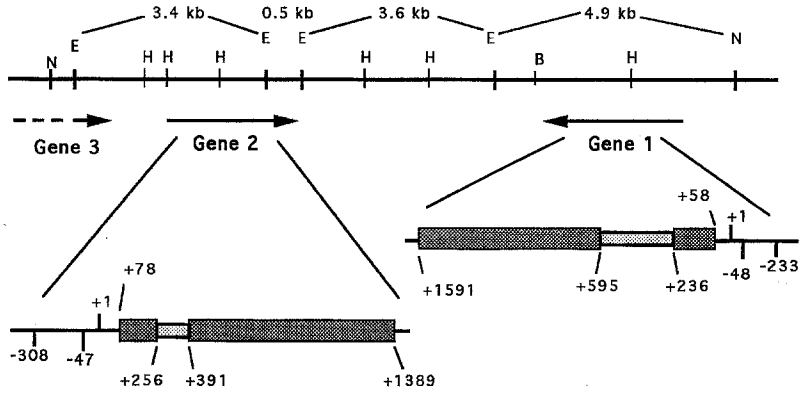

Fig. 1. Physical map of the 13 kb fragment containing three linked stilbene synthase genes. On the restriction endonuclease site map, sites for *Bam* HI (B), *Eco* RI (E), *Nde* II (N) and *Hind* III (H) are indicated. Below the map, the sequenced regions of *Vst2* and *Vst1* shown in Fig. 2 are indicated. + 1 indicates the transcription start (determined by nuclease S1 mapping in the case of *Vst1*). For *Vst2*, the attribution of the transcription start is preliminary and based on sequence homology to *Vst1*. The TATAA box (−48 for *Vst1* and −47 for *Vst2*) and the 5′ non-coding regions shown in Fig. 4 are indicated. With + 78 and + 1389 (*Vst2*) and + 58 and + 1591 (*Vst1*) the first and last nucleotides in the coding regions are designated. The dashed area presents the intron, the numbers indicating the first and last nucleotide of the intron.

Nde II, and the 10–18 kb fragments were fractionated by centrifugation on a 10–30% potassium acetate gradient as described by Sambrook *et al.* [21]. Size-fractionated DNA (0.5 μg) was ligated to *Bam* HI-digested phage lambda EMBL4 [1]. Freeze thaw lysate and sonication extract were prepared from induced *Escherichia coli* BHB2688 and BHB2690 cells, respectively, according to Sambrook *et al.* [21]. Packaging *in vitro* using the two extracts was performed with varying amounts of DNA. Using host strain *Escherichia coli* BHB2600 we determined the plaque-forming units and the packaging efficiencies by well described methods [6, 21]. For the screening of recombinant bacteriophage (5×10^5), the phage particles were transferred to nitrocellulose filters. Subsequent to denaturation and neutralization, the filters were gently but carefully freed from remants of bacteria and agarose.

Grapevine stilbene synthase cDNA clone SV368 [15] was used to screen the genomic library. Restriction mapping of long inserts in phage EMBL4 was performed according to [17]. Synthetic oligonucleotides complementary to the 5′ end and the 3′ end, respectively, of the coding region of stilbene synthase cDNAs were applied to detect distinct fragments on Southern blots. Lambda-DNA was digested with *Eco* RI, and fragments were separated and subcloned in pT7T3. Subcloned *Eco* RI fragments were sequenced on both strands by the dideoxy nucleotide chain termination technique [22].

Probes used for hybridization analysis

Identification and characterisation of the stilbene synthase cDNA clones have been reported previously [15]. Clones pSV21, pSV25, and pSV368 were complementary to the mRNAs present in elicitor-challenged cells. The following oligonucleotides were synthesized chemically and applied as specific probes: 5′-GGATGCTAGATACGTAATGA-3′ (for pSV25), TACTCAAATTAAAGCCTA-3′ (for pSV21), 5′-TTCCTTCCCAAGTTTG-3′ (for pSV368), 5′-GAAGCTGAAGATTGAGGA-3′ (for *Vst1*), 5′-AGTAGGATGACTGCTGAA-3′ (for *Vst2*), and 5′-GAATGGTTTTTCCGATCA-3′ (for *Vst3*).

For RNA blot hybridization, RNA was denatured by formaldehyde, fractionated on 1.1% agarose gels containing 2 M formaldehyde and transferred to nylon membranes [18, 21]. Identical amounts of total RNA (10 μg) were applied

```
Vst1     MASIEEIRNAQRAKGPATILAIGTATPDHC
Vst2        V              V         N
Vit2-5      V  F
                                      60
Vst1     VYQSDYADYYFRVTKSEHMTELKKKFNRIC
Vst2     L    F
Vit2-5

Vst1     DKSMIKKRYIHLTEEMLEEHPNIGAYMAPS
Vst2              S      I
Vit2-5
                                     120
Vst1     LNIRQEIITAEVPKLGKEAALKALKEWGQP
Vst2
Vit2-5                    D

Vst1     KSKITHLVFCTTSGVEMPGAYYKLANLLGL
Vst2                 L       D
Vit2-5                       D
                                     180
Vst1     ETSVRRVMLYHQGCYAGGTVLRTAKDLAEN
Vst2
Vit2-5

Vst1     NAGARVLVVCSEITVVTFRGPSEDALDSLV
Vst2                              T
Vit2-5
                                     240
Vst1     GQALFGDGSAAVIVGSDPDVSIERPLFQLV
Vst2                        L
Vit2-5            S

Vst1     SAAQTFIPNSAGAIAGNLREVGLTFHLWPN
Vst2              TQ
Vit2-5
                                     300
Vst1     VPTLISENIEKCLNQAFDPLGISDWNSLFW
Vst2                  T     I  N
Vst3                                NSLFW
Vit2-5                T

Vst1     IAHPGGPAILDAVEAKLNLEKKKLEATRHV
Vst2                      SPD Q
Vst3                      S D Q       I
Vit2-5
                                     360
Vst1     LSEYGNMSSACVLFILDEMRKKSLKGEKAT
Vst2                    M          EQ T
Vst3                               EG T
Vit2-5

Vst1     TGDGLDWGVLFGFGPGLTIETVVLHSVPMV
Vst2       E                        I RD
Vst3       E                        Q D
Vit2-5     E                          I

Vst1     TN
Vst2     SN
Vst3     SN
Vit2-5   TN
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Fig. 2. Amino acid sequence deduced from the nucleotide sequences of the genes *Vst1*, *Vst2*, *Vst3* (C-terminal section), and cDNA pSV25 [15]. Except for *Vst1*, only amino acid residues differing from *Vst1* are shown. The sequences consist of 392 amino acid residues while the part of *Vst3* included here starts from amino acid residue 296 (see underlined letters) onwards.

onto all lanes. Oligonucleotides were labelled in 5′ position using polynucleotide kinase from phage T4 and [γ-^{32}P]ATP. Hybridizations with random primer-labelled cDNA probes were carried out in 50% (v/v) formamide at 42 °C. Hundred ml of the hybridization mixture contained 0.2 g of Ficoll, 0.2 g of polyvinylpyrrolidone, 0.2 g of bovine serum albumin, 0.1 g SDS, 10 mg salmon sperm DNA, 0.5 mmol EDTA, 75 mmol sodium chloride, 56.5 g formamide, and 50 mmol sodium phosphate, pH 7.4. Filters were exposed to Kodak XAR-5 films with an intensifying screen at −80 °C.

For mapping of the 5′ terminus of *Vst1* mRNA, a probe consisting of a 5′-labelled 143 bp DNA fragment was prepared as follows. Using the 4.9 kb *Eco* RI/*Nde* II fragment with gene *Vst1* as template and the two primers 5′-TGGATGAGAATTCGTGAGACAC-3′ and 5′-CGCTTTCTGAATTCCTCAAT-3′, we prepared a 164 bp dsDNA. This DNA contained a portion of the genomic sequence of *Vst1*, 144 bp upstream of ATG and 20 bp corresponding to the first 7 amino acid residues at the N-terminus, except that exchanges were introduced at nucleotides 10, 13, 154, 156 thus creating two terminal *Eco* RI sites. After digestion with *Eco* RI, the fragment (see Fig. 4) was purified by electrophoresis and labelled at the 5′ ends using polynucleotide kinase from phage T4 and [γ-^{32}P]ATP. Mung bean nuclease mapping was basically performed according to Green and Roeder [2]: 10 μg of RNA from elicited grapevine cells were co-precipitated with the DNA probe. After resuspension in 30 μl of hybridization buffer, the nucleic acids were denatured at 85 °C for 10 min, and then allowed to anneal for 16 h at 48 °C. The hybridization buffer consisted of 40 mM Pipes-NaOH pH 6.4, 1 mM EDTA, 400 mM NaCl and 80% formamide. Annealed samples were dissolved in 300 μl buffer containing 30 mM sodium acetate pH 5.0, 100 mM NaCl, 1 mM $ZnCl_2$, 10% glycerol, calf thymus DNA (500 μg/ml) and 300 U of mung bean nuclease. After incubation for 60 min at 30 °C, the nucleic acids were extracted with phenol and precipitated with ethanol. Electrophoresis was performed on a 6% acrylamide sequencing gel.

Results

Isolation of a genomic stilbene synthase clone

A genomic library was prepared from DNA of grapevine suspension cultures. The library representing 10–20 kb fragments was prepared in the phage EMBL4 and screened for stilbene synthase sequences using the grapevine stilbene synthase pSV368 cDNA as a probe [15]. Forty-eight positive clones were found among 5×10^5 recombinant phage. Clones were analysed and selected according to their ability to hybridize with cDNA probes, oligonucleotides representing 5′-coding

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1  GTAAGTATATATATTCATGCATTAATTCTTACATTCACAACATTTCTATACATATACGAGTGTGCT
2  GTAAGTCT-----------------------------------------------------------
c  GTAAGTAT

1  ATTAAGTGAGGGTCACCTCCAAGTGAATGAATGTTTCAAGCTTAGAGAATAGCTTTTAGCTAAATT
2  ------------------------------------------------------------------

1  ACTTTAGGAAACTTGAAAATCATTTTACATCAG-TAACCGATATTCCTTTCATTTGATTGTAAGGG
2  ---------AATTTTAACATCCTTTTCATACATATAATTGTGTATCCATATACTCACTTGAACA--

1  CTTGAAGAGCTGTTCTTTGAATCATGTAGCATTGCTAGCTATAATTAAGAATAACCTTTTATAATT
2  ------------------------------------------------------------------

1  TCTTCAATGTTAAATGCATGTTGATCATCTTGAACAATATACTATATGACTAGTCGATTGGTAAAA
2  -----------------ATGTTGATCATCTTGAACAATCAAACTGGTTCCTTGAGCTAACATATTA

1  CTAATGTGTTCATGTTATTTCTTTTACAG
2  GTATTATTAATAATTTA-----TATACAG
c                        TTTGCAG
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Fig. 3. Comparison of intron nucleotide sequences of *Vst1* and *Vst2*. The alignment emphasizes the splice sites and a stretch of 21 nucleotides identical in both introns. The putative context sequence around branch point adenosine [5] is underlined.

sequences and oligonucleotides representing 5′-flanking sequences, respectively. Finally, a clone containing a 13 kb fragment between two *Nde* II sites was chosen for further analysis.

Organization of the stilbene synthase genes within the 13 kb DNA fragment

A physical map of the genomic 13 kb fragment was constructed by Southern blot analysis of DNA digested with various restriction endonucleases. In addition, we subjected the end-labelled genomic fragment to partial digestion with *Eco* RI, *Bam* HI and *Hin*d III. Thus, the consecutive sequence of endonuclease cleavage sites extending from the 5′ end could be determined. As a control, we used oligonucleotides and cDNA fragments as probes to detect specific regions on fragments of the 13 kb DNA. According to the physical map (Fig. 1), the 13 kb DNA was cleaved with *Eco* RI, and four *Eco* RI fragments were prepared and subcloned prior to further analysis. The genomic fragments were identified by sequence analysis. Two of the subclones contained full-length stilbene synthase genes (*Vst1*, *Vst2*) while one clone contained a 3′ portion of a third stilbene synthase gene (*Vst3*). *Vst3* and *Vst2* have the *Eco* RI site at identical positions, corresponding to Asn-296 of the amino acid sequence.

Comparison of coding regions, promoter regions and introns

Both *Vst1* and *Vst2* code for polypeptides with 392 amino acid residues which have a calculated molecular mass of 41453 Da and 42936 Da, respectively. The amino acid sequence comparison (Fig. 2) showed that the protein-encoding parts of the two full-length genes were 93% identical. Detailed analysis of the sequence context around Ser-250 reveals –IPNSAGAIAGN– which fits to all stilbene synthase sequences [15, 25, 26] and is distinct from –IPDSAGAIAGD– found in chalcone synthases.

Introns were found in *Vst1* and *Vst2* at a position typical of stilbene synthase DNA. Each of

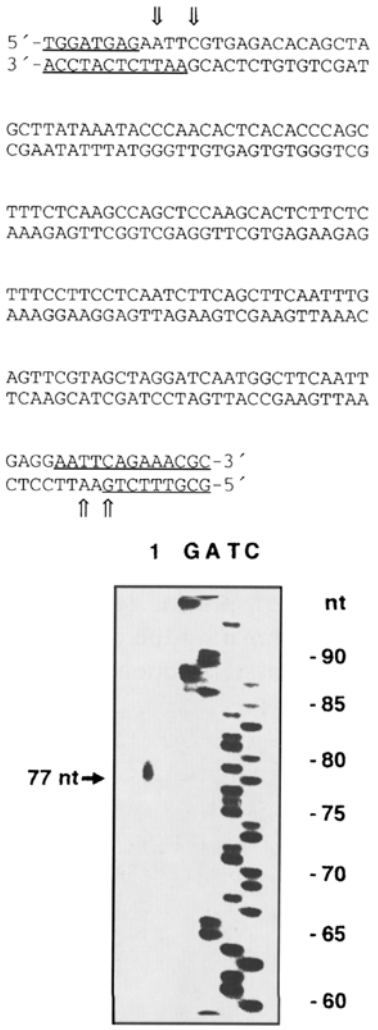

Fig. 4. Determination of the size of a labelled oligonucleotide prepared from RNA. DNA hybrid by nuclease S1 degradation. For mapping of the 5′ terminus of mRNA, a 5′-labelled DNA fragment from *Vst1* was used as probe (upper part). The arrows indicate where exchanges were introduced (see Materials and methods) in order to create *Eco* RI sites. Underlined are the fragments removed by *Eco* RI digestion. Lower part: electrophoretic analysis. After hybridization and digestion with nuclease S1, the size of the labelled DNA fragment (lane 1) was determined by comparison with the molecular weight markers run on the same gel (GATC). Fragment sizes of the sequencing ladder are indicated in nucleotides (nt). According to the probe prepared and the sequencing primers used, the fragments obtained in the sequencing gel contained 9 additional nucleotides.

the introns extended from nucleotide 179 relative to the A of the start codon [12]. However, the size of the introns differed markedly, i.e. 136 nucleotides in *Vst2* versus 358 nucleotides in *Vst1*. Comparison of the introns of the two genes (Fig. 3) revealed a stretch of 21 identical nucleotides.

The transcriptional start site of *Vst1* was determined by nuclease S1 mapping. From the size of the protected fragment (Fig. 4), it was deduced that an A at position 57 upstream of the ATG, and flanked by Cs, corresponded to the cap site. Parts of the promoters of *Vst1* and *Vst2* were included in a sequence comparison. Thus, the upstream regions in *Vst1* (starting at −233) and the upstream regions in *Vst2* (starting at −308) were aligned (Fig. 5). This alignment was also of interest in view of the dramatic differences observed for the elicitor-mediated expression of these two genes (see below). Included in the alignment (Fig. 5) was also the non-coding region of mRNAs analysed previously, i.e. pSV21 and pSV25 [15]. Comparing the 5′ region upstream of the translation start codon of *Vst1* and *Vst2*, stretches with marked similarities or even identities as well as regions characterized by deletions were observed. These dissimilarities may be sufficient to cause substantial differences in expression. However, comparing 5′-upstream untranslated regions, *Vst1* and *Vst2* seem to differ mainly in the number and length of inserted nucleotide stretches found in *Vst2* but not in *Vst1*.

Elicitor-induced expression of various genes of the family

The three genes located on the DNA fragment studied here differ not only in their nucleotide sequence. More remarkable is the drastic difference in their expression in response to the addition of fungal wall preparations to cultured cells. It was our intention to find out which of the members of the multigene family were activated rapidly and intensely. Thus, northern blot analyses were done with RNA isolated from cultured cells challenged with fungal cell wall for different periods of time (Fig. 6). Sequence-specific oligo-

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                                                                        60
Vst1   ATTGCTCTCTCCTTCCTGTGGGTTGCATTCATCAT-ATATGGGTAGGTG-----------
Vst2   CTTCCAACAACCTTAC-GTGGGCTAAAGACAAAAGGATTAGAGTTATAGAGAAAGATCTA

                                                                       120
Vst1   AAGAACAGAGAGCAAAA--------------------CAAATACATTAACACATGCATAC
Vst2   AAGAATGAAAACCGAAGCCTACCAAGTTGGAAATGATCAAAACCCATGTCAC-TGCAGCA

                                                                       180
Vst1   ATGTCTGAAAATCCAAAAATAAAATCACGC--AC--ACA---AA---------CTTTGAA
Vst2   -TGGAT--TATTC-------AAATGCACGCCTACCTACATGCAAGTCCCTGCCCTTTCAT

                                                                       240
Vst1   GCCACCTGAT--------CATTGACT--GCCGA------------------------TG
Vst2   GTATCATCATCAAGTATCCATTGACTGGGCAGACAAATCAATTTCAATAGCGTCCAAGTG

                                                                       300
Vst1   GATGAGAGTTGGTGAGACACAGC-TAGC------TTATAAATACCCAACACTCACACCCA
Vst2   GATGAGAGTTGGTGAAACACAGCATTCCTTATGATTATAAATACC-AACCTCAAGACACA

                              ↓                                        360
Vst1   GCTTTCTCAAGCCAGCTCCAAGCACT-------------------CTTCTCTTTCCTTCC
Vst2   ACTTTTTCCAGCCAGCTCCAAGCACTCTGTGCTCCGAAACATTCACTTCTCTTTCCTTCT
SV25           AGCTTTCT-CAAGCCAACTCCAAGCACTTGAGTTCTCTTTTCTT-CCTCAA
SV21                                        CTGAGTTCTCTTTCCTTCCTTCAA

Vst1   TCAA-TCTTCAGCTTCAATTTGAGTTCGTA-GCTAGGATCAATG
Vst2   TCAACTTTTAAGCTTCGATTTCAGCAGTCATCCTACTATCAATG
SV25   CTTAATCTTAAGCTTCAATTTCATTACGTATCTAGCCATCCATG
SV21   CTTAATCTTAGGCTTTAATTTGAGTACGTA-GCTGGGATCAATG
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Fig. 5. Comparison of the promoter regions of *Vst1* and *Vst2*. 5′ Portions of the transcribed regions of the cDNAs of pSV25 and pSV21 are included for comparative purpose. TATAA boxes and a putative consensus element are underlined. An arrow indicates the transcription start determined for Vst1 by nuclease S1 mapping (see Fig. 4).

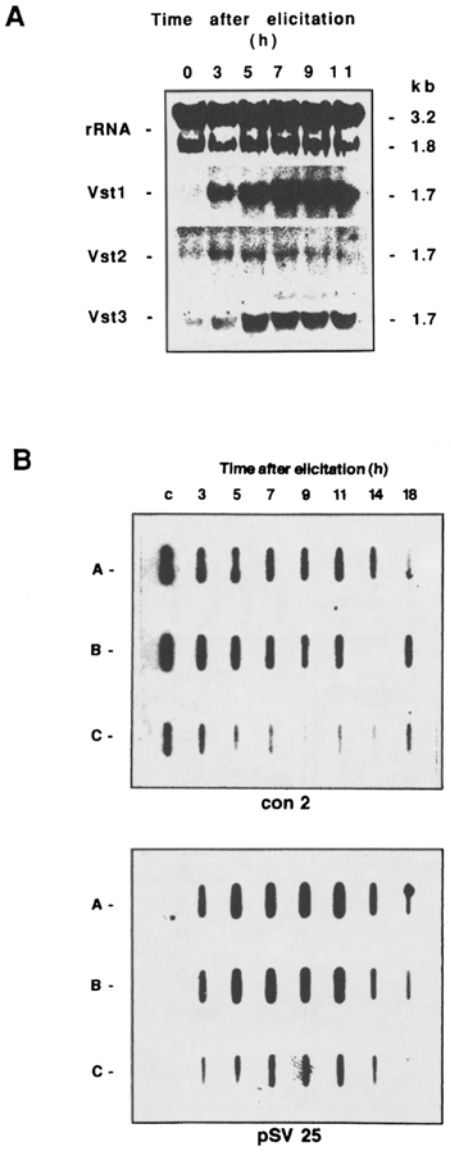

Fig. 6. Elicitor-induced expression of the genes located on the 13 kb fragment. Part A of the figure presents four rows taken from a northern blot analysis of total RNA. For each row, an internal marker (kb) is given on the right margin. For comparison, the amounts of rRNA loaded onto the lanes are shown in the uppermost row as ethidium bromide stain. The other rows (*Vst1*, *Vst2*, *Vst3*) show the hybridization with sequence-specific oligonucleotides (see Fig. 7). The labels above the lanes indicate the time (h) after addition of elicitor. In part B, on slot blots the time course of the expression of pSV25 is shown. Three different concentrations of labelled oligonucleotide probe were applied: 8 μg (series A), 5 μg (series B), and 2.5 μg (series C). As a control, a cDNA probe of clone con2 was used. In parsley cells [27], this probe hybridized with a constitutively expressed RNA.

nucleotides were first tested (Fig. 7) for their specificity by applying them on Southern blots. We succeeded in selecting nucleotide sequences for oligonucleotide probes which were virtually specific for the mRNAs corresponding to *Vst1*, *Vst2*, *Vst3*, pSV368 and pSV25 (Fig. 7, part B).

The interest was to see differences in expression during the early time of response to elicitors. Figures 6 and 7 show that steady state levels of all stilbene synthase mRNAs are increased by elicitor treatment. Figure 6 includes controls of rRNA patterns demonstrating that the amount of total RNA remained roughly constant during the course of the experiment. In addition, it becomes evident that the kinetics of the elicitor-dependent gene activation differs for all analyzed genes. Moreover, the extent of gene activation differs enormously considering that the time of exposure for the film was 40 times longer for *Vst2* than for *Vst1*. As a survey, Fig. 7 summarizes the results of a great number of experiments with all stilbene synthase mRNAs presently known. By using probes exhibiting almost identical specific radioactivities and calculating the duration of film exposure a semiquantitative determination of gene expression was achieved. The expression of *Vst1* and the expression of *Vst2* differed by a factor of 10 (after 5 h induction) or by a factor of 100 (after 12 h induction), respectively.

Discussion

The genomic DNA sequences for grapevine stilbene synthases presented in this paper extend our knowledge on stilbene synthase sequences in a particular plant. As already four different cDNA sequences have been reported [15], we can now state that the stilbene synthase multi-gene family consists of at least seven genes. Sequence comparison revealed that none of the mRNA sequences determined earlier [15] matched the sequences found on the 13 kb fragment. In addition, Fig. 4 shows that *Vst1*, *Vst2*, pSV25, and pSV21 differ from each other also in the 5′ noncoding region of the mRNAs. Applying sequence-specific probes (Fig. 7, part B), we were able to

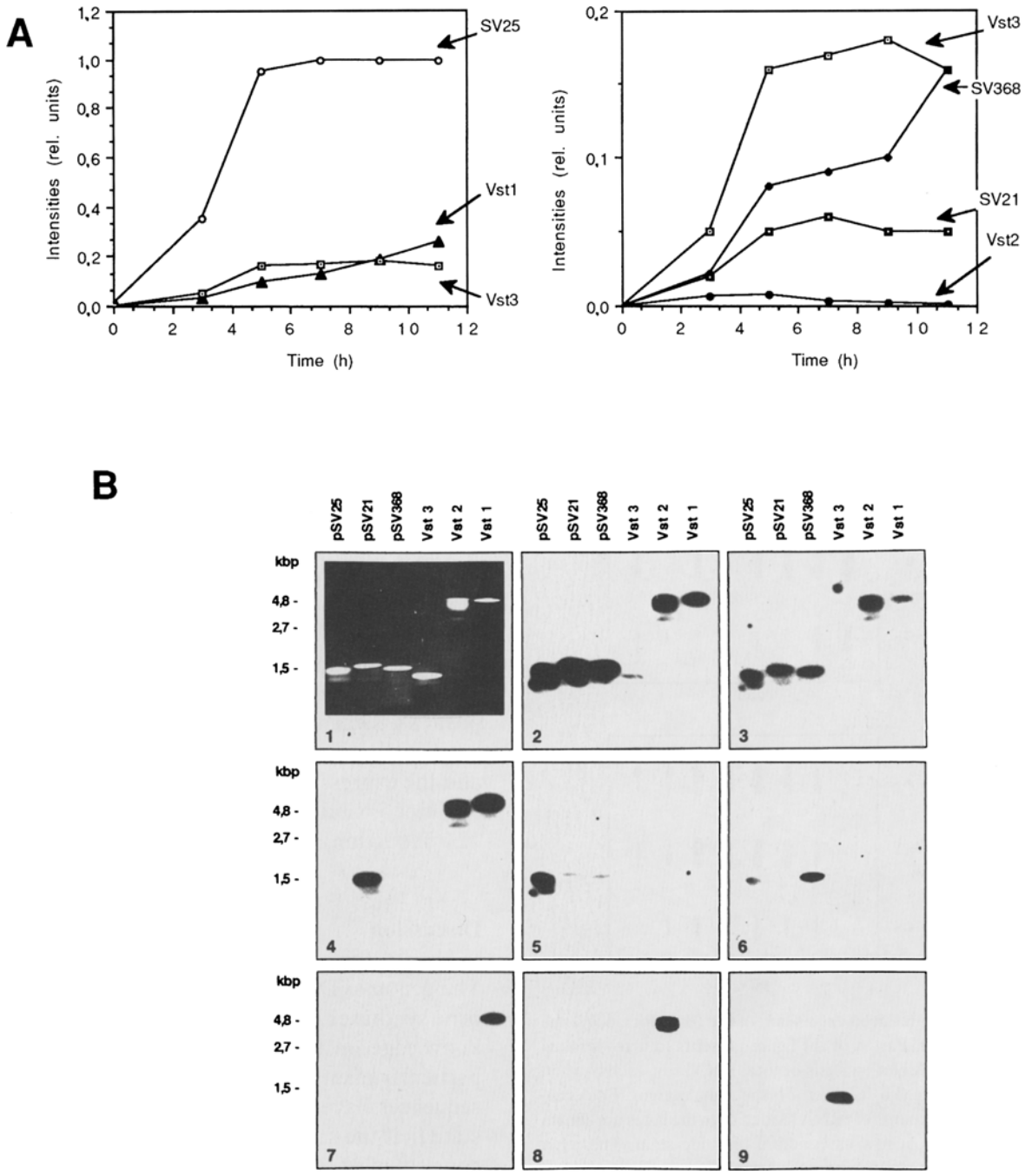

Fig. 7. Profiles of the accumulation of individual stilbene synthase mRNAs in elicitor-induced grapevine cells. A. Survey of the expression of three genes (described here) and the levels of three mRNAs characterized previously as cDNA clones (pSV25, pSV21, pSV368). For technical reasons, the level of SV25 mRNA as determined 11 h after onset of elicitation was set arbitrarily to 1. For better comparison, the profiles of the mRNAs expressed at high and low levels were delineated on two graphics drawn at different scales. Care was taken to apply labelled probes exhibiting almost identical specific radioactivities. B. Controls of the probes used for assaying the expression of particular stilbene synthase genes. cDNA inserts or fragments of genomic DNAs encoding single stilbene synthases were analyzed on Southern blots using sequence specific probes (see Materials and methods). On each of the 9 gels presented, 6 different DNAs were run. For detection on the blots, the following probes were applied for

compare the elicitor-dependent expression of the seven genes in relation to other genes. It was our intention to see how the expression of *Vst1* and *Vst2* compares to that of pSV25 which is, according to preliminary studies, among the highest expressed stilbene synthase genes. Most remarkable are the findings that the extent of elicitor-mediated activation within the gene family differs by more than two orders of magnitude, as examplified by probing with oligonucleotides specific for pSV25 and for *Vst2* (Fig. 7). Even the elicitor-activated expression of the two genes *Vst1* and *Vst2* located closely together on the genomic fragment differed by a factor 10 (for the values after 5 h induction) or by a factor 100 (for the values after 12 h induction), respectively. It seems that all individual members of the stilbene synthase gene family are elicitor-responsive. Despite the fact that we are now able to analyse the expression of a large number of stilbene synthase genes we still need to look for other highly expressed genes characterized by slower responses and thus explaining the findings that the transient expression of total stilbene synthase mRNAs always shows two maxima [15]. Although the absolute time point of the maxima in the profiles may vary according to the kind and stage of cell culture the basic pattern of mRNA profiles showing two peaks is characteristic of grapevine [15].

Like the two clustered chalcone synthase genes in soybean [20, 30] separated by only 2 kb, stilbene synthase genes may be organized in clusters. In the case of the 13 kb fragment studied here, two tandem-like genes, *Vst3* and *Vst2*, are positioned in opposite direction to a third stilbene synthase gene (*Vst1*). Two genes transcribed in opposite direction may be 2 kb apart, as found for chalcone synthase in a few instances [20].

Vst1 and *Vst2*, which did not differ markedly in their coding region, are substantially distinct as far as the size of the introns and the structure of the promoters are concerned. The sequence context of the initiating ATG (namely ATCAATGGC) matches in five out of six positions the consensus sequence context for plant initiation codons (AACAATGGC). The nucleotide sequence of both genes shows in-frame stop codons shortly upstream from the predicted initiation codon. The size of the enzyme subunit, i.e. 43 kDa [13], is not very different from the sizes calculated from the sequences, i.e. 41453 Da (*Vst1*) and 42936 Da (*Vst2*). Comparison of amino acid sequences of *Vst1* (392 amino acid residues), *Vst2* (392 amino acid residues) and part of *Vst3* (from amino acid residue 296 onwards) shows only very few, mostly conservative, substitutions. As far as the known sequence of *Vst3* allows the comparison, *Vst3* is very similar to *Vst2*, the sequence identity being 93%. Comparing full-length sequences, *Vst1* has 93% identity with *Vst2* and 98% identity with pSV25.

In view of the substantial differences in the extent of activation of *Vst1* and *Vst2* upon elicitation it is not surprising that also the promoters of *Vst1* and *Vst2* are markedly different. Putative promoter elements were located in the 5′-upstream sequences of gene 1 and gene 2. However, only *Vst1* possesses a motif TCTCTCCTTCC which matches in nine out of eleven positions the consensus sequence TCTCACCTACC in the promoters of several genes involved in phenylpropanoid metabolism [14].

Introns of plant genes have been characterized by their size, splice sites and branch point encompassing the adenine [4]. They have also been grouped into 3 classes [5] by their pyrimidine content at the 3′ splice site. In this respect, the *Vst1* intron belongs to class I, and *Vst2* intron to class II. All stilbene synthase genes and chalcone synthase genes so far investigated possess a single intron located within the triplet for Cys-60. The intron of *Vst1*, 358 bp in length, and the intron in

hybridization: gel 1: ethidium bromide; gel 2: near full-length stilbene synthase cDNA, pSV368; gel 3: oligonucleotide derived from a highly homologous region on the stilbene synthase DNA; gel 4: 143 bp fragment representing the 5′ noncoding region of *Vst1* as used for nuclease S1 mapping (see Fig. 4). As pSV25 does not hybridize with this probe we infer that promotors in *Vst1* and pSV25 differ significantly; gel 5: oligonucleotide specific for pSV25; gel 6: oligonucleotide specific for pSV386; gel 7: oligonucleotide specific for *Vst1*; gel 8: oligonucleotide specific for *Vst2*; gel 9: oligonucleotide specific for *Vst3*.

Vst2, 136 bp in length, display AG/GT consensus at both splice sites. The introns of the two genes contain, beyond the homology in the splice sites, a stretch of 21 identical nucleotides (shown in bold letters in Fig. 3). The putative branch points selected by the procedure of Harris and Senepathy [5] are indicated in Fig. 3 by underlined letters. The distance between the putative branch point adenine nucleotide and the 3′ splice site is 25 nucleotides in the case of gene 1 and 30 nucleotides in gene 2.

As the genomic fragment analysed here is being transferred to other plants it will be interesting to see how the activity of *Vst1* and *Vst2* is affected in the new environment.

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