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Isolation and characterization of cDNA encoding stilbene synthases from Chinese wild *Vitis pseudoreticulata*

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Summary

mRNA differential display was employed to study powdery mildew disease resistance gene expression in Chinese wild *Vitis pseudoreticulata* 'Baihe-35-1' inoculated with *Erysiphe necator* (syn. *Uncinula necator*) under natural field conditions. A cDNA fragment T_{11}AC/B0320-723 showing homology to stilbene synthase (STS) gene expressed more strongly at 1, 3, 5, 7 and 9 days after inoculation of leaves than in controls was found. The full cDNA length was cloned by rapid amplification of cDNA ends (RACE). Sequencing of the full length cDNA revealed cDNA sequences, sized 1288, 1411, 1468, 1492, 1506 and 1556 base pairs encoding 6 homologous polypeptides with 392 amino acid residues each, that were designated as VpSTS1, VpSTS2, VpSTS3, VpSTS4, VpSTS5 and VpSTS6 respectively. The deduced amino acid sequences shared identity of 65 %, 77 % and more than 94 % with *Pinus strobus* STS, *Vitis vinifera* chalcone synthase (CHS), and *Vitis riparia*, *Vitis labrusca*, *Parthenocissus henryana*, *Cissus rhombifolia*, *Parthenocissus quinquefolia* and *Vitis vinifera* STS, respectively.

Key words: *Vitis pseudoreticulata*; stilbene synthase; mRNA differential display; RACE.

Introduction

Resveratrol belongs to the class of phytoalexins that is involved in plant defence reactions (Hain *et al.* 1993). A positive correlation of resveratrol levels and resistance to the fungus *Botrytis cincerea* has been demonstrated (Stein and Blaich 1985). Adrian *et al*. (1997) reported that resveratrol reduced the germination of conidia of *Botrytis cincerea* and its mycelial growth. The production of resveratrol is regulated by the key enzyme stilbene synthase, which converts one molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA into 3,5,4'-trihydroxystilbene, commonly known as resveratrol (Schöppner and Kindl 1984). So far, stilbene synthase genes have been isolated from a very limited number of plants, including two cultivars of grapevine (*Vitis vinifera*) (Melchior and Kindl 1991; Sparvoli *et al*. 1994), *V. riparia* (Acc. No. AB046373), *V. labrusca* (Acc. No. AB046374), *Arachis hypogaea* (Schröder *et al*. 1988), *P. henryana* (Acc. No. AY094615), *C. rhombifolia* (Acc. No. AY094616), *P. quinquefolia* (Acc. No. AY094617) and *P. strobes* (Fliegmann *et al*. 1992; Schwekendiek *et al*. 1992; Raiber *et al*. 1995).

Worldwide powdery mildew, caused by *E. necator* Burr, is the economically most important fungal disease of grapes. Therefore, understanding the mechanisms of powdery mildew resistance and identifying key genes in resistant germplasm should provide valuable information and resources for the quick and efficient molecular breeding of highly resistant cultivars. In this research, mRNA differential display (DDRT-PCR) (Liang and Pardee 1992) was employed to study the differential expression of the genes of the resistance to the disease. STS genes that are specifically induced by the fungus infection were identified. Knowledge obtained from this research might be used to explore the resistance genes to the disease from Chinese wild grapes.

Materials and Methods

Plant material and treatments: For this study, grape material of the Chinese wild species *V. pseudoreticulata* 'Baihe-35-1' (highly resistant to powdery mildew) and *Vitis adstricta* Hance 'Taishan-2' (highly susceptible to powdery mildew) were used. These genotypes are maintained in the grape germplasm resources orchard of Northwest A&F University, Yangling Shaanxi, 712100, China. The powdery mildew inoculation was carried out under natural field conditions by pressing *E. necator* infected leaves of 'Taishan-2' against uninfected sterilized water pre-sprayed leaves of 'Baihe-35-1' in the morning from 8:00 am to 10:00 am on August 12, 2002.

Total RNA isolation and DDRT-PCR: Total RNA was isolated separately from the grape leaf samples 0, 1, 3, 5, 7 and 9 d after inoculation by a SDS/phenol method with few modifications (Zhang *et al*. 2003). mRNA differential display was performed as described by Liang and Pardee (1992) and primed with the oligonucleotide 5'-TTTTTTTTTTTAC-3' (T_{11}AC) in reverse transcription reactions. The reaction conditions for reverse transcription (20 µl) and PCR reactions (25 µl) were as described by Lin *et al.* (2006). The results of DDRT-PCR were analyzed on denaturing polyacrylamide gels by a silver-stain method (Bassam *et al*. 1991) with some modifications (Lin *et al*. 2006).

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3' RACE and 5' RACE and PCR amplification of the STS cDNA from *V. pseudoreticulata*: In order to obtain the full-length cDNA of the STS cDNA of the clone 'Baihe-35-1', a rapid amplification of 3'cDNA end (3' RACE) and 5'end PCR amplification were carried out. The gene specific primer GSP1: 5'- TTC GGA AGG TCC TCG GAA TGT AAC AGC -3' for 5' RACE and GSP2: 5'- CAG GTG GAA CTG TCC TTC GAA CCG C -3' for 3' RACE were designed based on the sequence of the differential cDNA fragment T_{11}AC/B0320-723. The distance between the two specific primers was 113 bp. Total RNA of 'Baihe-35-1' leaves after 7 d of inoculation with *E. necator* was isolated as described above and RACE was performed according to the manufacturer's instructions (BD SMART™ RACE cDNA Amplification Kit). The 5' and 3' RACE products were separated by 1.2 % agarose gel electrophoresis and purified with the UNIQ-5 columns DNA Gel Extraction Kit (Sangon, China). The 5' RACE and 3' RACE cDNA fragments were cloned into pGEM-T Easy Vector (Promega, USA), and sequenced by Takara Biotechnology Co. Lt.

Sequence analysis: Nucleotide and amino acid sequences of STS of 'Baihe-35-1' were compared with those in GenBank databases (http://www.ncbi.nlm.nih.gov) by using the BLAST (ALTSCHUL *et al.* 1990) analysis program. The alignment and phylogenetic reports were analyzed with DNAMAN (Lynnon Biosoft, Vaudreuil, Quebec, Canada).

Results

mRNA differential display: Total RNAs were extracted from leaves of *V. pseudoreticulata* 'Baihe-35-1' at various time points after *E. necator* inoculation, reverse-transcribed with anchor primer T_{11}AC, followed by PCR with 26 combinations of T_{11}AC and 26 random primers of 10 nucleotides. cDNA fragment T_{11}AC/B0320-700, amplified with the anchor primer T_{11}AC and the random primer B0320 5'-GAT CAA TCG C -3' was expressed more strongly in the leaves after 1, 3, 5, 7 and 9 d of inoculation than in control leaves of 'Baihe-35-1' from all primer combinations (Fig. 1). Sequence analysis revealed that this fragment from the 'Baihe-35-1' actually was 723 bp and its nucleotide sequence shared high identity with *V. vinifera*, *V. labrusca*, *V. riparia, P. henryana, C. rhombifolia, P. quinquefolia* and *P. strobus* STS genes released in GenBank databases, implying that it was probably a part of a STS gene. The nucleotide sequence of T_{11}AC/B0320 - 723 has been deposited in GenBank databases under the accession number DT725417.

3' RACE and 5' RACE: The 3' RACE and 5' RACE technique were employed to obtain the full-length cDNA sequence of the STS gene of 'Baihe-35-1'. The 3' RACE products were about 750 bp and 1000 bp, designated 3'-1 and 3'-2 (Fig. 2). The 3'-1 was cloned into pGEM-T Easy Vector, transformed into *E.coli* strain DH5α. The positive clones, characterized by blue / white screening and *Eco*R I digest (data not shown), were sequenced and actually resulted in 715 bp and 770 bp fragments, which were designated as 3'-1-1 and 3'-1-2. The 3'-2 actually contained five fragments of 839, 895, 919, 933 and 983 bp, which are designated as 3'-2-1, 3'-2-2, 3'-2-3, 3'-2-4 and 3'-2-5 respectively. Therefore, seven different fragments were obtained in 3' RACE products at the end. These 7 cDNA sequences were subjected to phylogenetic analysis using DNAMAN software and they were grouped into two clusters or families (Fig. 3). Family A contains three members: 3'-2-1, 3'-2-3 and 3'-2-5, whereas family B was composed of other four members: 3'-1-1, 3'-1-2, 3'-2-2 and 3'-2-4. Family A shares high identity (82 %) with family B. The 3'-1-2 cDNA sequence shares the highest identity 99 % with 3'-2-2. The 3'-1-2 and 3'-2-2 cDNA sequences share 95 % and 88 % identity, respectively, with 3'-2-4 and 3'-1-1. The 3'-2-1 cDNA sequence shares 92 % and 85 % identity with 3'-2-3 and 3'-2-5, respectively.

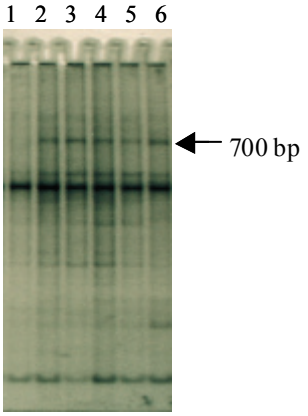

Fig. 1: Detection of DDRT-PCR of *V. pseudoreticulata* 'Baihe-35-1' 0, 1, 3, 5, 7 and 9 d after inoculation of *E. necator*. RT-PCR was performed with anchor primer 5'-T_{11}AC-3' and random primer B0320. Lane 1. 0, lane 2. 1, lane 3. 3, lane 4. 5, lane 5. 7 and lane 6. 9 d, respectively.

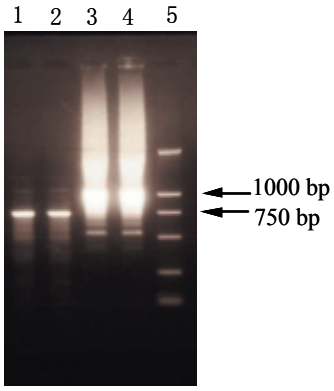

Fig. 2: 1.2 % Agarose gel electrophoresis of 5' RACE and 3' RACE cDNA of *V. pseudoreticulata* 'Baihe-35-1' STS. Lane 1 and lane 2. 5' RACE cDNA, lane 3 and lane 4. 3' RACE cDNA, lane 5. DNA Marker DGL 2000.

The 5' RACE product appeared as 750 bp (Fig. 2) and was actually 768 bp in length as revealed by its sequence analysis.

Gene cloning and sequence analysis of STS from 'Baihe-35-1': Seven cDNA fragments of 3' and 5' end fragments produced seven different cDNAs with full-length of 1288, 1343, 1411, 1468,

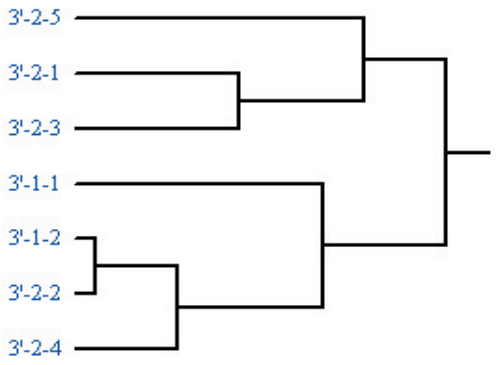

Fig. 3: Phylogenetic analysis of 3' end of the STS cDNA sequences from Chinese wild *V. pseudoreticulata* 'Baihe-35-1'.

1492, 1502 and 1556 bp because of 113 bp overlap. Each cDNA sequence contains a complete open reading frame encoding *V. pseudoreticulata* 'Baihe-35-1' STS, whose sizes of amino acid residues are 392. Among these cDNA sequences, amino acids coded by the 1343 bp and 1468 bp fragment are absolutely identical (data not shown), thus, six complete cDNA sequences of *V. pseudoreticulata* STS have been found. 1288, 1411, 1343 or 1468, 1492, 1506 and 1556 bp, which are designated as VpSTS1, VpSTS2, VpSTS3, VpSTS4, VpSTS5 and VpSTS6, respectively, have been deposited in GenBank (http://www.ncbi.nlm.nih.gov) under the accession numbers DQ445485, DQ445486, DQ445487, DQ445488, DQ445489 and DQ445490. VpSTS1, VpSTS2, VpSTS3, VpSTS4, VpSTS5 and VpSTS6 (all code for polypeptides with 392 amino acid residues, have a calculated molecular mass of 42.889 kDa, 42.757 kDa, 42.601 kDa, 42.711 kDa, 42.739 kDa and 42.767 kDa, respectively, and have a 77 bp non-coding region at the 5' end and an untranslated 3' end that includes the putative polyadenylation signal AATAAA and a polyA signal or tail (Fig. 4).

The comparison of deduced amino acid sequences showed that VpSTS3 and VpSTS5 share the highest identity (99 %) and five out of 392 amino acids were found to be different. These are Ala-182 corresponding to Val-182

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1     AACCCAGCTCCAAGAACGCTTCTCTTTCCTTCCTCAACTTAATCTTAAGCTTTCATTTCA
61    GTACGTAGCTGGCATCAATGGCTTCAGTTGAGGAAATTAGAAACGCTCAACGTGCCAAGG
                  M  A  S  V  E  E  I  R  N  A  Q  R  A  K  G  15
121   GTCCGGCCACCATCCTAGCCATTGGCACAGCTACTCCCGACCACTGTGTCTACCAGTCTG
        P  A  T  I  L  A  I  G  T  A  T  P  D  H  C  V  Y  Q  S  D  35
181   ATTATGCTGATTACTATTTCAGGGTCACTAAGAGCGAGCACATGACTGAGTTGAAGAAGA
        Y  A  D  Y  Y  F  R  V  T  K  S  E  H  M  T  E  L  K  K  K  55
241   AGTTCAATCGCATATGTGACAAATCAATGATAAAAAAGCGTTACATTCATTTGACTGAAG
        F  N  R  I  C  D  K  S  M  I  K  K  R  Y  I  H  L  T  E  E  75
301   AAATGCTTGAAGAACATCCAAACATTGGTGCTTATATGGCTCCATCTCTTAACATACGCC
        M  L  E  E  H  P  N  I  G  A  Y  M  A  P  S  L  N  I  R  Q  95
361   AAGAGATTATCACAGCTGAGGTACCTAAGCTTGGTAAGGAAGCAGCATTGAAGGCACTTA
        E  I  I  T  A  E  V  P  K  L  G  K  E  A  A  L  K  A  L  K  115
421   AAGAGTGGGGCCAGCCAAAGTCCAAGATCACCCACCTTGTATTTTGTACAACCTCTGGTG
        E  W  G  Q  P  K  S  K  I  T  H  L  V  F  C  T  T  S  G  V  135
481   TAGAAATGCCTGGTGCTGATTATAAACTTGCTAATCTCTTGGGTCTTGAAACTTCAGTTA
        E  M  P  G  A  D  Y  K  L  A  N  L  L  G  L  E  T  S  V  R  155
541   GAAGAGTTATGTTGTACCATCAAGGGTGCTATGCAGGTGGAACTGTCCTTCGAACCGCTA
        R  V  M  L  Y  H  Q  G  C  Y  A  G  G  T  V  L  R  T  A  K  175
601   AGGATCTTGCAGAGAATAATGCAGGAGCACGAGTTCTTGTGGTGTGCTCTGAGATCACTG
        D  L  A  E  N  N  A  G  A  R  V  L  V  V  C  S  E  I  T  V  195
661   TTGTTACATTTCGCGGCCCTTCCGAAGATGCTTTGGACTCTTTAGTTGGCCAAGCCCTTT
        V  T  F  R  G  P  S  E  D  A  L  D  S  L  V  G  Q  A  L  F  215
721   TTGGTGATGGGTCTGCAGCTGTAATCGTAGGATCAGATCCGGATATCTCAATTGAACGAC
        G  D  G  S  A  A  V  I  V  G  S  D  P  D  I  S  I  E  R  P  235
781   CACTCTTCCAGCTTGTCTCAGCAGCCCAAACATTTATTCCTAATTCTGCAGGTGCCATTG
        L  F  Q  L  V  S  A  A  Q  T  F  I  P  N  S  A  G  A  I  A  255
841   CAGGAAACTTACGTGAGGTGGGACTCACCTTTCATTTGTGGCCCAATGTGCCCACTTTAA
        G  N  L  R  E  V  G  L  T  F  H  L  W  P  N  V  P  T  L  I  275
901   TTTCTGAGAACATAGAGAAATGTTTGACTCAGGCTTTTGACCCACTTGGTATTAGCGATT
        S  E  N  I  E  K  C  L  T  Q  A  F  D  P  L  G  I  S  D  W  295
961   GGAACTCGTTATTTTGGATTGCTCACCCAGGTGGCCCTGCAATTCTTGATGCAGTTGAAG
        N  S  L  F  W  I  A  H  P  G  G  P  A  I  L  D  A  V  E  A  315
1021  CAAAACTCAAGTTAGATAAAAAGAAACTCGAAGCAACGAGGCATGTGCTAAGTGAGTATG
        K  L  K  L  D  K  K  K  L  E  A  T  R  H  V  L  S  E  Y  G  335
1081  GAAACATGTCAAGTGCATGTGTGTTGTTTATTTTGGATGAGATGAGAAAGAAATCCCTTA
        N  M  S  S  A  C  V  L  F  I  L  D  E  M  R  K  K  S  L  K  355
1141  AGGGGGAGAGGGCCACCACAGGTGAAGGATTGGATTGGGGAGTATTATTCGGTTTTGGAC
        G  E  R  A  T  T  G  E  G  L  D  W  G  V  L  F  G  F  G  P  375
1201  CAGGCTTGACTATTGAAACTGTTGTGTTGCATAGCATTCCTATGGTTACAAATTAAGTGA
        G  L  T  I  E  T  V  V  L  H  S  I  P  M  V  T  N  *        392
1261  AGGAAAAGAGAATGGTCCCTTCAATGTCCTATTATGTTGAATAGGAGTAAGGTATTTATC
1321  TCCGAAACTAAATTATACTCTTAAACTATTTTATTATTTTTTCTAAATTTAGATTGTAAT
1381  CTAGTGATTGTTAGGCCCTCTTGGTGAGCTCAAATTAAACGGTTGAGTTTCAAGTTCAGA
1441  CTGTTTTATTATCTTGAAGATTCCCCAAACATTGTAATGTTGTGTTCATATGAACATTGT
1501  TGAAAAGTAAATAAAAGAAATATTGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 4: The full length cDNA' sequence and deduced amino acid sequence of VpSTS6. Nucleotide positions are given on the left side of the sequence in the 5' to 3' orientation. The start codon ATG was underlined and the stop codon TAA was underlined and written in italics. The deduced amino acid sequence is shown beneath the nucleotide sequence and the amino acids are numbered on the right hand side of the sequence. 113 bp overlapping sequence of 5'RACE and 3'RACE are boxed, underlined sequences in the box are GSP1 and GSP2 respectively. Conserved amino acids (C164), essential for catalytic activity, are indicated by a box. The polyadenylation signal AATAAA is double-underlined. The cDNA sequence has been deposited in GenBank (Acc. No. DQ445490). Shaded amino acid sequences 247-257 and 368-378 are conserved motifs, respectively.

in VpSTS5, Arg-259 to His-259, Gln-322 to Arg-322, Gly-388 to Glu-388 and Thr-389 to Met-389 (Fig. 5). VpSTS2 also shares 99 % identity with VpSTS6 and five out of 392 amino acids were found to be different. These are Val-230 to Ile-230 in VpSTS6, Tyr-282 to Cys-282, Asn-318 to Lys-318, Lys-358 to Arg-358, Ser-391 to Thr-391 (Fig. 5). VpSTS6 and VpSTS2 are 98 % identical to VpSTS4; VpSTS2, VpSTS6 and VpSTS4 share 95 % identity with VpSTS3, VpSTS5 and VpSTS1 (data not shown). Alignment of the amino acid sequence of the stilbene synthases of *V. pseudoreticulata* 'Baihe-35-1' with that of other stilbene and chalcone synthase was done with DNAMAN software. The six proteins shared high identity of 65 % and 77 % with *P. strobus* STS and *V. vinifera* chalcone synthase gene (Fig. 5). VpSTS1, VpSTS3 and VpSTS5 shared 94 % with other plants STS. VpSTS2, VpSTS4 and VpSTS6 share 98 % and 96 % identity with *C. rhombifolia, P. henryana* and *P. quinquefolia* STS, and *V. riparia* and *V. labrusca* STS (Fig. 5), respectively.

The six STS cDNAs were deduced to encode stilbene synthase for the following reasons: First, the amino acid sequence deduced from the cDNAs exhibited significant homology (94 %-98 %) with that for the *V. riparia*, *V. labrusca*, *P. henryana*, *C. rhombifolia*, *P. quinquefolia* and *V. vinifera* STS genes except 77 % with *P. strobus* STS. Furthermore, an examination of the predicted protein sequence of the *V. pseudoreticulata* 'Baihe-35-1' gene showed that the sequence motif -GVLFGFGPGLT-, which is the family signature sequence for stilbene and chalcone synthases (FLIEGMANN *et al*. 1992; KODAN *et al*. 2001), was present. Additionally, detailed analysis of the sequence context around Ser-250 reveals –IPNSAGAIAGN- which fits to all stilbene synthase sequences (SCHRÖDER *et al*. 1988; MELCHIOR and KINDL 1991; SCHWEKENDIEK *et al*. 1992) and is distinct from –IPDSAGAIAGD- found in chalcone synthases (Fig. 4; Fig.5). Therefore, they had a conserved cysteine residue (amino acid position 164) located in the central section of these proteins (Fig. 4; Fig. 5). This residue is essential for the catalytic activity of both STS and CHS (LANZ *et al*. 1991; KODAN *et al*. 2001).

Discussion

Phytoalexins are believed to be involved in defense reactions of plants. Resveratrol is involved in the class of phytoalexins that are related to plant defense reactions (HAIN *et al*. 1993). Stilbene synthase, the key enzyme in the biosynthesis of resveratrol in grapevine has been described. In *Vitis*, expression of stilbene synthase can be induced by inoculation with pathogens such as *Botrytis cinerea* (LANGCAKE and PRYSE 1976; LISWIDOWATI *et al*. 1991). To get better understanding of the resistance mechanism of this disease in grape plants, DDRT-PCR was employed to study the differential expression of the powdery mildew diseases resistance genes. We obtained the cDNA fragment T_{11}AC/B0320-723 of the clone that was specifically induced by powdery mildew infection. 5' and 3' RACE were employed to isolate the corresponding STS gene of *V. pseudoreticulata* 'Baihe-35-1'. The 5' RACE produced only one fragment, but the 3' RACE led to the isolation of seven fragments. Sequence analysis of these seven cDNAs showed that they are all different in cDNA sequence in comparison to each other. The homology of the nucleotide sequence is between 82 % and 95 % (Fig. 5). Twelve STS cDNA clones were categorized into seven distinct subclasses according to 3'UTR sequences in Japanese red pine (KODAN *et al*. 2001). The 3' end sequence variation of the STS cDNA fragments highlights the structural diversity.

Stilbene synthase genes are known to belong to a multi-gene family in grapes, peanuts and a number of tree species (SCHRÖDER *et al*. 1988; SPARVOLI *et al*. 1994; WIESE *et al*. 1994). At least 7 stilbene synthase genes were found in *V. vinifera* 'Optima' (WIESE *et al*. 1994). Comparison of amino acid sequences of VpSTS1 (392 amino acid residues), VpSTS2, VpSTS3, VpSTS4, VpSTS5 and VpSTS6 shows only very few, mostly conservative, substitutions and the identity was between 95 % and 99 %. The stilbene synthase of *V. pseudoreticulata* was found very similar to those of the two species of *V. vinifera* and *V. riparia*. Unlike *V. vinifera, V. riparia* and *V. pseudoreticulata* are resistant to several diseases such as downy mildew, powdery mildew, anthracnose, white rot and ripe rot (HE *et al*. 1991). 'Baihe-35-1' is one of the clones of *V. pseudoreticulata* that shows highest level of resistance to powdery mildew and has been used as an important germplasm for grape breeding. Despite these differences, the genes are extremely similar, and it appears that the stilbene synthase gene has changed little from the ancestral grape species that evolved in these species.

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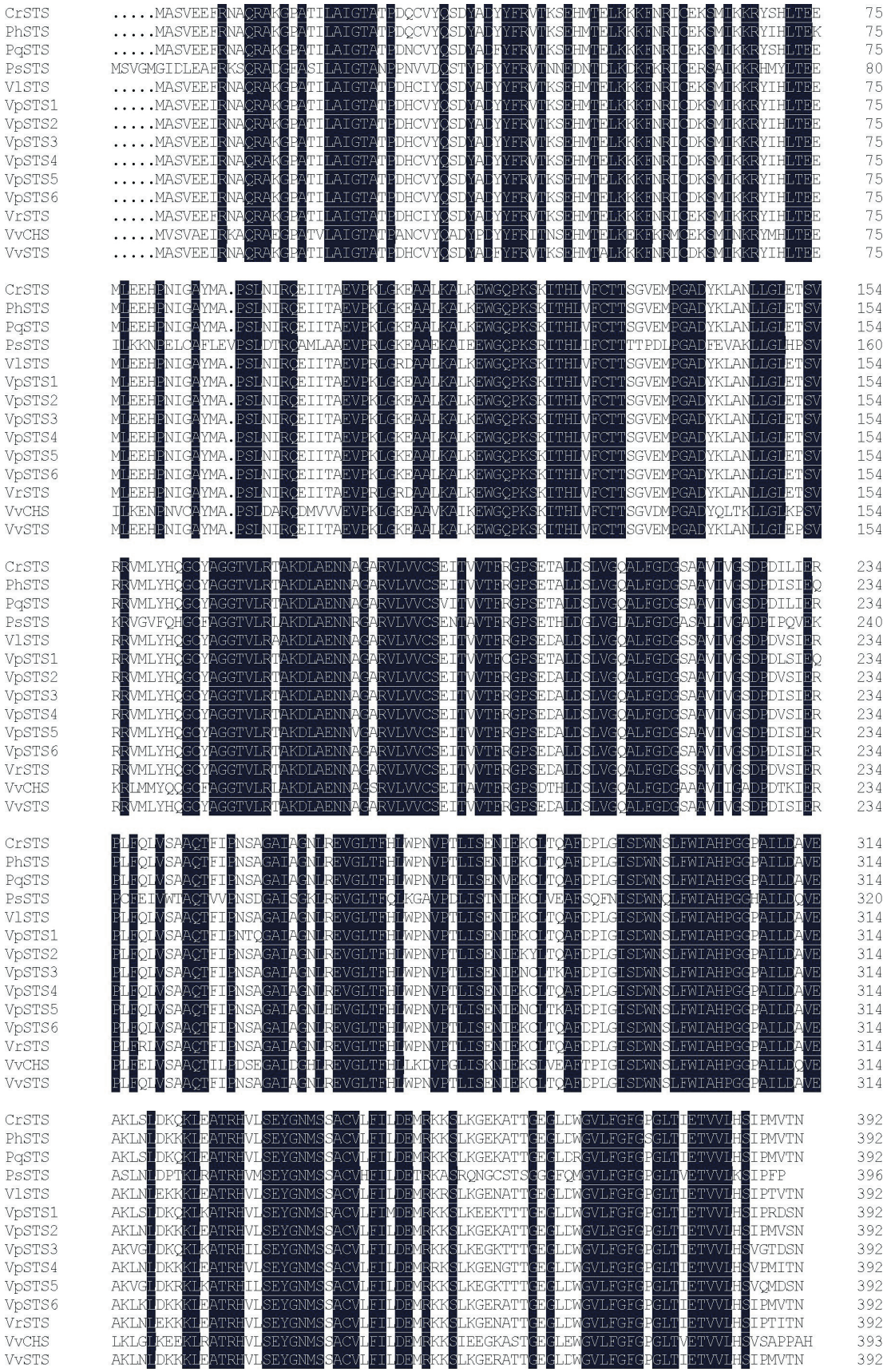

Fig. 5: Alignment of amino acid sequence of STSs. The predicted amino acid sequence of VpSTS was aligned with STS polypeptide sequences from *C. rhombifolia* (CrSTS, Acc. No. AY094616), *P. henryana* (PhSTS, Acc. No. AY094615), *P. quinquefolia* (PqSTS, ACC.No.AY094617), *P. strobus* (PsSTS, Acc.No. Z46914), *V. labrusca* (VlSTS, Acc. No. AB046374), *V. riparia* (VrSTS, Acc. No. AB046373), *V. vinifera* (VvSTS, Acc.No.X76892) and CHS polypeptide sequences from *V. vinifera* (VvCHS, Acc.No.AB015872), using the DNAMAN multiple alignment programme. Gaps to optimize alignments are designated by dots (···). The consensus amino acid identity among all organisms is given in black color. The amino acids are numbered on the right hand side of the sequence.

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