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Expression of genes related to flavonoid and stilbene synthesis as affected by signaling chemicals and *Botrytis cinerea* in grapevines

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Abstract

Recent studies have shown that the expression of genes related to the synthesis of flavonoids, such as the phenylalanine-ammonia lyase (*PAL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), and stilbene synthase (*STS*) genes were induced in response to different signaling molecules and *Botrytis cinerea* inoculation in grapevine leaves. Therefore, in the present study, the nucleotide and deduced amino acid sequences of *STS* genes from cultivars Campbell Early and Kyoho were compared. The deduced amino acid sequences of VlKSTS12, VlKSTS11, VICESTS13, and VlKSTS1 showed 100 % homology to VlCESTS12, VlCESTS11, VICESTS24, and VlKSTS13, respectively, in Campbell Early and Kyoho. In addition, the maximum transcription was observed 6 h after chemical treatments. In Campbell Early, the transcription of *PAL*, *CHS*, and *CHI* was higher in leaves treated with ethephon than in those treated with hydrogen peroxide, methyl jasmonate, and salicylic acid. The *PAL, CHS,* and *CHI* genes were more induced in Campbell Early than in Kyoho. The mRNA content of *STS* genes started to increase at 6 h and peaked at 48 h after the treatments. In Kyoho leaves, the expression of *STSs* was highly up-regulated at 1 h and peaked at 6 h after the treatments. The expression of the *STS* genes was induced in both the cultivars in leaves inoculated with *B. cinerea*. *STS11* and *STS12* showed differential expression patterns from *STS1*, *STS24*, and *STS13* in Campbell Early leaves inoculated with *B. cinerea.*

Additional key words: amino acid sequence, differentially expressed gene, multigene family, resveratrol content.

Introduction

Phenylpropanoid compounds are a large family of secondary metabolites involved in plant responses to various biotic and abiotic stresses, and stress-induced phenylpropanoids are classified as phytoalexins. Among the phenylpropanoid antimicrobial compounds, flavonoids and stilbene synthase (STS) are induced in response to wounding (Hahlbrock and Scheel 1989), UV (Adrian *et al.* 2000), low temperature (Christie *et al.* 1994), and pathogen attack (Dercks and Creasy 1989). Accordingly, their roles in defense-related responses in plants, such as signaling defense responses, protection against UV radiation, and increases in bioavailability of recalcitrant nutrients, have been investigated (Dixon and Paiva 1995, Chong *et al.* 2009).

Stilbenes, including resveratrol, represent a small class of plant secondary metabolites derived from the phenylpropanoid pathway (Langcake and Pryce 1977, Chong *et al.* 2009). Stilbene synthase (*STS*) genes belong to a large multigene family consisting of 20 - 40 copies per haploid genome (Sparvoli *et al.* 1994, Richter *et al.* 2006, Jaillon *et al.* 2007). A draft grape genome analysis has shown that most of these copies are clustered in LG 16 (Velasco *et al.* 2007). During the defense response of grapevine to the infection with *Plasmopara viticola*, more than 20 *STS* genes were expressed (Richter *et al.* 2006). *STS* is closely related to chalcone synthase (*CHS*) which is important in the flavonoid biosynthesis (Halbrock and Grisebach 1979) and these two enzymes are known to compete for the same substrates in the branching point of the phenylpropanoid pathway leading

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Abbreviations: CHI - chalcone isomerase; CHS - chalcone synthase; ET - ethephon; FLS - flavonol synthase; GABA - γ-amino-butyric acid; MeJA - methyl jasmonate; PAL - phenylalanineammonia lyase; PR - pathogenesis-related; SA - salicylic acid; STS - stilbene synthase.
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to the productions of chalcone and resveratrol, respectively (Richter *et al.* 2006). The first enzyme of phenylpropanoid pathway, phenylalanineammonia lyase (*PAL*), catalyzes the mono-oxidative deamination of phenylalanine to produce cynnamate (Kiselev *et al.* 2009). Flavonoids have been reported to be involved in many physiological functions including flower coloration, protection from UV, and defense against pathogens and pests (Chen *et al.* 2012). In the flavonoid biosynthesis, chalcone isomerase (*CHI*) which catalyzes the conversion of chalcones to flavanones plays a key role in the cyclization reaction (Nishihara *et al.* 2005, Wang *et al.* 2012).

According to Wiese *et al.* (1994), three *STS* mRNAs show drastic differences in their expression in response to an elicitor in grapevine cells. Using grapevine cDNAs, Melchior and Kindl (1991) investigated the expressions of four different *STS* and *PAL* mRNA sequences and found that their expressions are differentially induced reaching a maximum at different times after the fungal elicitation.

Treatment with elicitors has been reported to increase antimicrobial compounds and the expression of pathogenesis-related (PR) proteins (Fritzmeier and Kindl 1981, Liswidowati *et al.* 1991). We previously reported that treatments with salicylic acid (SA), methyl jasmonate (MeJA), ethephon (ET), and hydrogen peroxide induced the production of stilbene compounds, γ-aminobutyric acid (GABA), and pathogenesis related (PR) proteins, and retarded the development of grey mold in grapevine leaves (Ahn *et al.* 2013).

This study investigated the enhancement of expressions of *PAL*, *CHI*, *CHS*, and *STS* genes involved in the phenylpropanoid pathway after signaling molecules treatments and infection with *B. cinerea*, and analyzed nucleotide sequences and differential expressions of *STS* genes important for the production of resveratrol.

Materials and methods

Cuttings from grape vine (*Vitis vinifera* L.) cvs. Campbell Early and Kyoho, which show a different stilbene accumulation after the grey mold infection (Ahn *et al.* 2013), were grown in a glasshouse at a 16-h photoperiod, an irradiance of 800 - 1 000 μmol m^{-2} s^{-1}, day/night temperatures of 25/20 °C, and a relative humidity of 50 - 70 %. Plants with 15 - 20 developed leaves were sprayed with ethephon (ET, 7 mM), H_2O_2, (10 mM), methyl jasmonate (MeJA, 0.5 mM), or salicylic acid (SA, 1 mM) dissolved in distilled water until droplets appeared. Control leaves were sprayed with water. Leaves were collected at different time points (0, 1, 6, 24, 48, and 72 h) after treatments, immediately frozen in liquid nitrogen, and stored at -80 °C for future use.

The transcriptome data of Campbell Early and Kyoho were identified from *de novo* assembly which was carried out using *Velvet* (*v. 1.2.07*) and *Oases* (*v. 0.2.08*) with a k-mer value of 65, a minimum contig length of 200, and an insert length of 300. The nucleotide and amino acid sequences of *STSs* were searched in transcriptome data, and primer pairs for real-time PCR were designed from these sequences. Nucleotide and amino acid sequence data of grapevine *STS1, STS24, STS11, STS12,* and *STS13* were deposited in the National Agricultural Biotechnology Information Center (NABIC) database under the accession numbers NS-0314-000001, NS-0317-000001, NS-0319-000001, NS-0320-000001, and NS-0321-000001, respectively, in Campbell Early, and NS-0322-000001, NS-0325-000001, NS-0326-000001, NS-0327-000001, and NS-0328-000001, respectively, in Kyoho (Table 1). The nucleotide and amino acid sequences of the *STS* genes of Campbell Early and Kyoho were compared using *CLC Main Workbench* (*v. 4.0, CLC Bio,* Aarhus, Denmark).

Botrytis cinerea was grown in Petri dishes on potato dextrose agar (potato starch 4 g, dextrose 20 g, and agar 15 g dm^{-3}, *PDA; Difco,* Sparks, MD, USA) under a 12-h photoperiod, an irradiance of 150 μmol m^{-2} s^{-1}, and a temperature of 25 °C. Spores of *B. cinerea* were collected from the plates and suspended in a 0.24 % (m/v) potato dextrose broth at a concentration of 10^6 spores per cubic centimeter (after centrifugation at 3 000 *g* for 5 min to remove debris). The upper fourth or fifth leaves from the shoot apex were detached and injured by slightly scratching the abaxial epidermis with a pencil tip. The injured leaves were inoculated with 20 mm^3 of a spore suspension on both wounded and non-wounded areas. The leaves inoculated with the pathogen were placed on two layers of a moist paper towel in a closed box and kept in the dark at 25 °C for 4 d, and the diameter of the lesions was then measured. Ten leaves were used for each treatment, and the experiment was repeated twice.

Frozen leaves (1 g) were ground in liquid nitrogen using a mortar and pestle. RNA was then isolated with the modified pine tree method (Chang *et al*. 1993). The quality of total RNA was measured according to absorbances at 230, 260, and 280 nm using a spectro-photometer *ND-1000* (*Nano Drop Technologies,* Delaware, USA). First-strand cDNA was then synthesized from the total RNA (500 ng) using the *GoScript*™ reverse transcription system (*Promega,* Madison, USA) and subsequently used as template for PCR. Real-time PCR was performed on a *C1000*™ thermal cycler (*CFX96*™ *Real-Time System, BioRad,* Hercules, USA) using S*YBR Premix Ex Taq* (*TaKaRa,* Osaka, Japan) as fluorescent dye. The amplification was at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Transcript amounts were calculated

using the standard-curve method and normalized against a grapevine *actin* gene (AB372563) as internal control and non-treated leaves (at time zero) as reference sample, and melting curves of the amplified products were recorded. For each gene, the reference sample was defined as expression = 1, and results were expressed as the fold increase over the reference sample. All reactions were performed in triplicate for experiment to ensure consistency of the results. Gene specific primer pairs were designed using the *Primer3* (http://frodo.wi.mit.edu/primer3) software and used for real-time PCR (Table 2).

Table 1. Information of *STS* genes.

Gene	*V. vinifera* ID	Campbell Early NABIC ID	length [bp]	Kyoho NABIC ID	length [bp]
Resveratrol synthase (*STS1*)	GSVIVT01010584001	NS-0314-000001	1856	NS-0322-000001	1389
Stilbene synthase 1-like (*STS24*)	GSVIVT01010589001	NS-0317-000001	2000	NS-0325-000001	2000
STS1a-1 (*STS11*)	GSVIVT01000521001	NS-0319-000001	2000	NS-0326-000001	2000
STS1a-2 (*STS12*)	GSVIVT01032968001	NS-0320-000001	1412	NS-0327-000001	1611
STS1a-3 (*STS13*)	GSVIVT01010557001	NS-0321-000001	1021	NS-0328-000001	2000

Table 2. Gene names, accession numbers, and sequences of forward and reverse primers used for RT-qPCR analysis.

Name	Acc. No.	Primer sequences
Phenylalanine ammonia-lyase (*PAL*)	X75967.1	5'-TGAACAATGGCGAAAGTGAGAA-3' 5'-TCTCTTGCGCTCTCAACCTCTT-3'
Chalcone synthase (*CHS*)	EF192464.1	5'-AGTTCAAGCGCATGTGTGAAAA-3' 5'-CTTCAACCACCACCATGTCTTG-3'
Chalcone isomerase (*CHI*)	XM002282072.2	5'-TACACTGACGCAGAAGCCAAAG-3' 5'-TACACTGACGCAGAAGCCAAAG-3'
Resveratrol synthase (*STS1*)	NS-0314-000001	5'-GCCATATAAGCCCCAATGTTTG-3' 5'-CACATGACCGAGCTCAAGAAGA-3'
Stilbene synthase 1-like (*STS24*)	NS-0317-000001	5'-ATGAAAATGCCAAGGTTCCTGA-3' 5'-TCCAAACCTTATCTTGGGCAGA-3'
STS1a-1 (*STS11*)	NS-0319-000001	5'-AAGCCTGAGAAGTTACGCTCCA-3' 5'-GACTTCCTCCTCATCTCGTCCA-3'
STS1a-2 (*STS12*)	NS-0320-000001	5'-GAGGTGGTGCAGAAGACAAGGT-3' 5'-CAAGACATGGTGGTGGTTGAAG-3'
STS1a-3 (*STS13*)	NS-0321-000001	5'-GAATAACGCAGGAGCACGAGTT-3' 5'-AGGGCTTGGCCAACTAAAGAGT-3'
VACT	AB372563	5'-ACGAGAAATCGTGAGGGATG-3' 5'-ATTCTGCCTTTGCAATCCAC-3'

Results

The deduced amino acid sequences (Fig. 1) of VlKSTS12, VlKSTS11, VICESTS13, and VlKSTS1 showed 100 % homology to VlCESTS12, VlCESTS11, VICESTS24, and VlKSTS13, respectively (Table 3). The deduced amino acid sequences of each VlCESTS11 and VlKSTS11 showed 90.00 % similarity with VlCESTS12 and VlKSTS12, respectively. The nucleotide sequences of VlKSTS11, VlKSTS12, and VlKSTS24, showed 90.76, 85.16, and 73.95 % similarity with VlCESTS11, VlCESTS12, and VlCESTS24, respectively (Table 4). Based on these results, primers could be designed for specific regions to detect different expression of each *STS* gene.

The expressions of *PAL, CHS,* and *CHI* were induced differentially and depended on signaling molecules in both the cultivars (Fig. 2). The tested genes showed the maximum amount of transcripts at 6 h after the chemical treatments except the treatment with H_2O_2. The expressions of *PAL, CHS,* and *CHI* were higher in ET-treated leaves than in leaves treated with H_2O_2, MeJA, and SA. *PAL, CHS,* and *CHI* transcripts were only slightly induced by the SA treatment compared to the control leaves. In ET-treated leaves, the expressions of *PAL, CHS,* and *CHI* genes were rapidly up-regulated, peaking at 6 h (the expressions 20-, 8-, and 6-fold higher than in the control leaves, respectively; Fig. 2*A*). The

expressions of *CHS* were highly induced in Campbell Early leaves treated with MeJA, but it was barely induced in Kyoho leaves. The inductions of *PAL, CHS,* and *CHI* genes were higher in Campbell Early than in Kyoho. In Kyoho, the *PAL* transcription in ET-treated leaves increased from 1 to 6 h after the treatment up to being 12-fold higher than in the control leaves (Fig. 2*B*).

The *STS* genes expression was also studied after the same treatments (Figs. 3 and 4). The amount of mRNA of the *STS* genes increased, reaching peak values at 6 and 48 h after the treatments. In Campbell Early, the amounts of *STS24*, *STS12* and *STS13* transcripts weres higher than of *STS1* and *STS11* transcripts (Fig. 3). In H_2O_2-, and MeJA-treated leaves, the mRNA content of the *STS* genes was up-regulated, reaching its first peak value at 1 h after the treatment. The total amount of transcripts of *STS* genes were higher in ET-treated than in SA-, MeJA-, or H_2O_2-treated Campbell Early. The expression of *STSs* was highly up-regulated from 1 h and peaked at 6 h after the treatments in Kyoho (Fig. 4). The expressions of the six tested genes decreased from 6 to 72 h after the treatments except the treatment with SA. The expressions of *STS1*, *STS24*, and *STS13* genes were more up-regulated when compared to *STS11* and *STS12*.

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                                      150                                 170
                                       |                                   |
VICESTS12 TTSGVDMPGA DYQLTKLLGL KPSVKRLMMY QQGCFAGGTV 40
 VIKSTS12 TTSGVDMPGA DYQLTKLLGL KPSVKRLMMY QQGCFAGGTV 40
VICESTS11 TTSGVDMPGA DYQLTKLLGL RPSVKRFMMY QQGCFAGGTV 40
 VIKSTS11 TTSGVDMPGA DYQLTKLLGL RPSVKRFMMY QQGCFAGGTV 40
VICESTS24 ---------- ---------- -------MLY HQGCYAGGTV 13
VICESTS13 ---------- ---------- -------MLY HQGCYAGGTV 13
 VIKSTS24 TTSGVEMPGA DYKLANLLGL DNSVRRVMLY HQGCHAGGTV 40
 VIKSTS13 TTSGVEMPGA DYKLANLLGL ETSVRRVMLY HQGCYAGGTV 40
 VICESTS1 TTSGVEMPGA DYKLANLLGL ETSVRRVMLY HQGCYAGGTV 40
  VIKSTS1 TTSGVEMPGA DYKLANLLGL ETSVRRVMLY HQGCYAGGTV 40
                                      190                                 210
                                       |                                   |
VICESTS12 LRLAKDLAEN NAGSRVLVVC SEITAVTFRG PSDTHLDSLV 80
 VIKSTS12 LRLAKDLAEN NAGSRVLVVC SEITAVTFRG PSDTHLDSLV 80
VICESTS11 LRLAKDLAEN NKGARVLVVC SEITAVTFRG PSDTHLDSLV 80
 VIKSTS11 LRLAKDLAEN NKGARVLVVC SEITAVTFRG PSDTHLDSLV 80
VICESTS24 LRTAKDLAEN NAGARVLVVC SEITVVTFRG PSESALDSLV 53
VICESTS13 LRTAKDLAEN NAGARVLVVC SEITVVTFRG PSESALDSLV 53
 VIKSTS24 LRTAKDLAEN NAGARVLGVL ---------- ---------- 60
 VIKSTS13 LRTAKDLAEN NAGARVLVVC SEITVVTFRG PSEDALDSLV 80
 VICESTS1 LRTAKDLADN NAGARVLVVC SEITVVTFRG PSETALDSLV 80
  VIKSTS1 LRTAKDLAEN NAGARVLVVC SEITVVTFRG PSEDALDSLV 80
                                      230                                 250
                                       |                                   |
VICESTS12 GQALFGDGAA AVIIGADPDT KIERPLFELV SAAQTILPDS 120
 VIKSTS12 GQALFGDGAA AVIIGADPDT KIERPLFELV SAAQTILPDS 120
VICESTS11 GQALFGDGAA AVIVGSDPIP GVEKPMFELV SAAQTILPDS 120
 VIKSTS11 GQALFGDGAA AVIVGSDPIP GVEKPMFELV SAAQTILPDS 120
VICESTS24 GQALFGDGSA AVIIGSDPDI SIERPLFQLV SAAQTFIPNT 93
VICESTS13 GQALFGDGSA AVIIGSDPDI SIERPLFQLV SAAQTFIPNT 93
 VIKSTS24 ---------- ---------- ---------- ---------- 60
 VIKSTS13 GQALFGDGSA AVIIGSDPDI SIERPLFQLV SAAQTFIPNS 120
 VICESTS1 GQALFGDGSA AVIVGSDPDL SIERPLFQLV SAAQTFIPNT 120
  VIKSTS1 GQALFGDGSA AVIIGSDPDI SIERPLFQLV SAAQTFIPNS 120
                                      270                                 290
                                       |                                   |
VICESTS12 EGAIDGHLRE VGLTFHLLKD VPGLISKNIE KSLVEAFTPI 160
 VIKSTS12 EGAIDGHLRE VGLTFHLLKD VPGLISKNIE KSLVEAFTPI 160
VICESTS11 DGAIDGHLRE VGLTFHLLKD VPGLISKNIE KSLNEAFQPL 160
 VIKSTS11 DGAIDGHLRE VGLTFHLLKD VPGLISKNIE KSLNEAFQPL 160
VICESTS24 QGAIAGNLRE VGLTFHLWPN VPTLISENVE KCLTQAFDPL 133
VICESTS13 QGAIAGNLRE VGLTFHLWPN VPTLISENVE KCLTQAFDPL 133
 VIKSTS24 ---------- ---------- ---------- ---------- 60
 VIKSTS13 AGAIAGNLRE VGLTFHLWPN VPTLISENIE KCLTQAFEPT 160
 VICESTS1 QGAIAGNLRE VGLTFHLWPN VPTLISENIE KCLTQAFDPI 160
  VIKSTS1 AGAIAGNLRE VGLTFHLWPN VPTLISENIE KCLTQAFEPT 160
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Fig. 1. The comparison of the amino acid sequence deduced from the transcript nucleotide sequence of stilbene synthase (STS) using *CLC Main Workbench* (*Ver. 4.0, CLC Bio,* Aarhus, Denmark). STS proteins and accession numbers from *Vitis labruscana* cv. Campbell Early: VlCESTS1 (NS-0314-000001), VlCESTS24 (NS-0317-000001), VlCESTS11 (NS-0319-000001), VlCESTS12 (NS-0320-000001), and VlCESTS13 (NS-0321-000001). STS proteins and accession numbers from *Vitis labruscana* cv. Kyoho: VlKSTS1 (NS-0322-000001), VlKSTS24 (NS-0325-000001), VlKSTS11 (NS-0326-000001), VlKSTS12 (NS-0327-000001), and VlKSTS13 (NS-0328-000001).

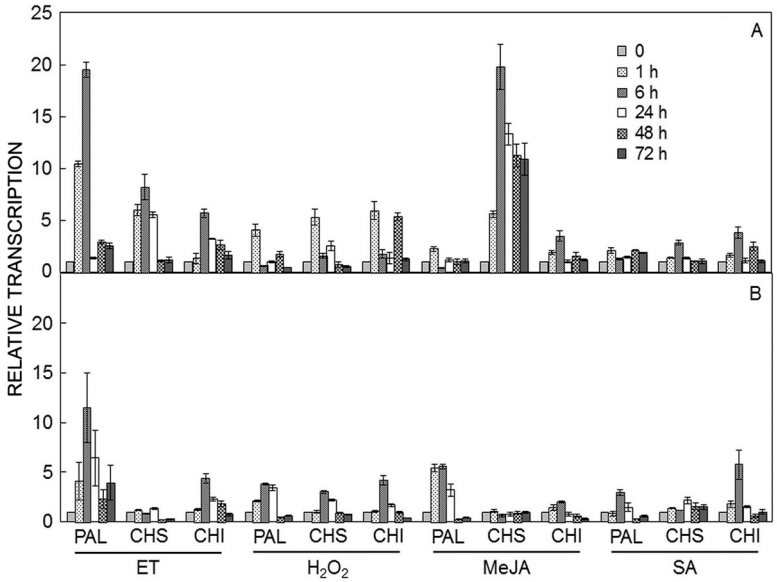

Fig. 2. Expressions of genes coding enzymes involved in the flavonoid synthesis (*PAL* - phenylalanine ammonia-lyase, *CHS* - chalcone synthase, *CHI* - chalcone isomerase) determined by RT-qPCR analysis in cv. Campbell Early (*A*) and cv. Kyoho (*B*) treated with different signaling molecules (ET - ethephon, H_2O_2 - hydrogen peroxide, MeJA - methyl jasmonate, and SA - salicylic acid). The transcription was calculated by using the standard curve method from triplicate data with a grapevine *actin* gene as internal control and non-treated leaves (at time zero) as reference sample. Results represent the mean fold increase in mRNA over non-treated leaves referred to 1. Results are means of triplicate data of three experiments. *Bars* indicate SD.

Table 3. Identities [%] of the deduced amino acids from alignment comparisons among *STS* genes (*VlCESTS1*, *VlCESTS24*, *VlCESTS11*, *VlCESTS12*, and *VlCESTS13* from Campbell Early, and *VlKSTS1*, *VlKSTS24*, *VlKSTS11*, *VlKSTS12*, and *VlKSTS13* from Kyoho).

	VlCESTS12	*VlKSTS12*	*VlCESTS11*	*VlKSTS11*	*VlCESTS24*	*VlCESTS13*	*VlKSTS24*	*VlKSTS13*	*VlCESTS1*	*VlKSTS1*
VlCESTS12		100	90.00	90.00	63.75	63.75	28.12	76.25	75.62	76.25
VlKSTS12	100		90.00	90.00	63.75	63.75	28.12	76.25	75.62	76.25
VlCESTS11	90.00	90.00		100	61.88	61.88	28.12	73.75	73.75	73.75
VlKSTS11	90.00	90.00	100		61.88	61.88	28.12	73.75	73.75	73.75
VlCESTS24	63.75	63.75	61.88	61.88		100	20.00	80.62	80.62	80.62
VlCESTS13	63.75	63.75	61.88	61.88	100		20.00	80.62	80.62	80.62
VlKSTS24	28.12	28.12	28.12	28.12	20.00	20.00		34.38	33.75	34.38
VlKSTS13	76.25	76.25	73.75	73.75	80.62	80.62	34.38		95.00	100
VlCESTS1	75.62	75.62	73.75	73.75	80.62	80.62	33.75	95.00		95.00
VlKSTS1	76.25	76.25	73.75	73.75	80.62	80.62	34.38	100	95.00	

Table 4. Alignment comparisons [%] of the nucleotide sequences of *STS* genes (*VlCESTS1*, *VlCESTS24*, *VlCESTS11*, *VlCESTS12*, and *VlCESTS13* from Campbell Early, and *VlKSTS1*, *VlKSTS24*, *VlKSTS11*, *VlKSTS12*, and *VlKSTS13* from Kyoho).

	VlCESTS12	*VlKSTS12*	*VlCESTS11*	*VlKSTS11*	*VlCESTS24*	*VlCESTS13*	*VlKSTS24*	*VlKSTS13*	*VlCESTS1*	*VlKSTS1*
VlCESTS12		61.31	23.58	37.64	45.35	43.22	13.42	43.10	19.76	20.26
VlKSTS12	61.31		28.57	39.80	56.83	51.38	15.77	15.35	26.36	15.89
VlCESTS11	23.58	28.57		73.95	20.86	23.69	24.80	25.69	10.27	12.59
VlKSTS11	37.64	39.80	73.95		28.38	31.27	23.63	23.76	12.28	17.46
VlCESTS24	45.35	56.83	20.86	28.38		85.16	14.10	13.88	23.37	15.32
VlCESTS13	43.22	51.38	23.69	31.27	85.16		16.00	15.78	20.50	17.02
VlKSTS24	13.42	15.77	24.80	23.63	14.10	16.00		91.76	24.44	24.57
VlKSTS13	13.10	15.35	25.69	23.76	13.88	15.78	91.76		24.04	24.44
VlCESTS1	19.76	26.36	10.27	12.28	23.37	20.50	24.44	24.04		28.68
VlKSTS1	20.26	15.89	12.59	17.46	15.32	17.02	24.57	24.44	28.68	

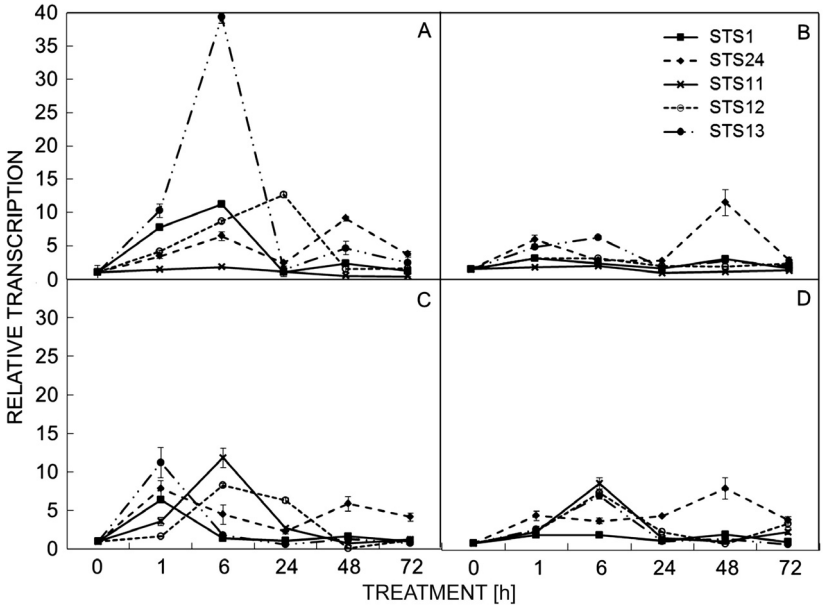

Fig. 3. Expressions of *STS* genes determined by RT-qPCR analysis in cv. Campbell Early leaves treated with different signaling molecules (*A* - ethephon, *B* - H_2O_2, *C* - methyl jasmonate, and *D* - salicylic acid). The transcription was calculated by using the standard curve method from triplicate data with a grapevine *actin* gene as internal control and non-treated leaves (at time zero) as reference sample. Results represent the mean fold increase in mRNA over non-treated leaves referred to 1. Results are means of triplicate data of three experiments. *Bars* indicate SD.

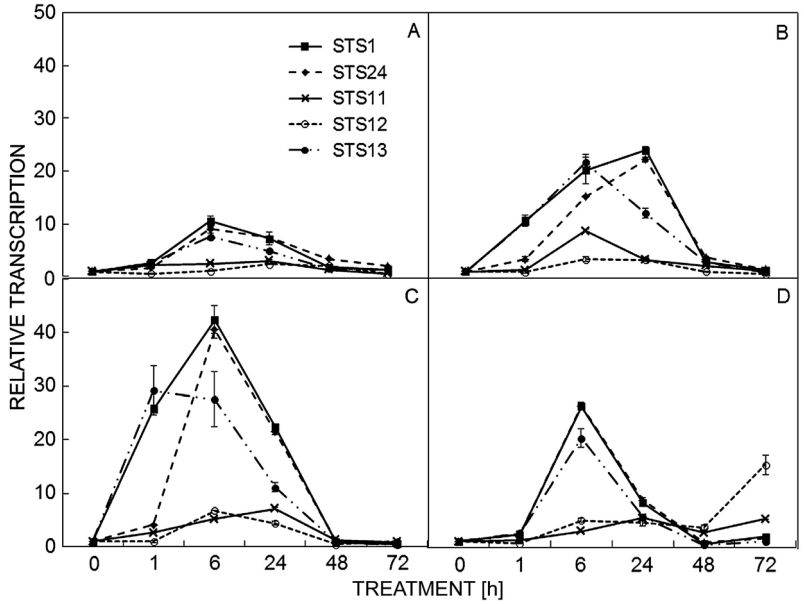

Fig. 4. Expressions of *STS* genes determined by RT-qPCR analysis in cv. Kyoho leaves treated with different signaling molecules (*A* - ethephon, *B* - H_2O_2, *C* - methyl jasmonate, and *D* - salicylic acid). The transcription was calculated by using the standard curve method from triplicate data with a grapevine *actin* gene as internal control and non-treated leaves (at time zero) as reference sample. Results represent the mean fold increase in mRNA over non-treated leaves referred to 1. Results are means of triplicate data of three experiments. *Bars* indicate SD.

Development of grey mold symptoms was detected on grapevine leaves 4 d after the inoculation with *B. cinerea* (Fig. 5). The expressions of the *STS* genes were induced in both the cultivars in response to the infection with *B. cinerea* (Fig. 6). In agreement with results from signaling molecule-treated Campbell Early leaves, the transcription peaked at 6 h and 48 h after the *B. cinerea* inoculation. The transcriptions of *STS1*, *STS24*, and *STS13* were up-regulated, peaking at 6 h, decreasing at 24 h, and being induced again 48 h after the inoculation.

STS11 and *STS12* showed different expression patterns from *STS1*, *STS24*, and *STS13* (Fig. 6*A*). In Kyoho, the transcriptions of *STS1*, *STS24*, *STS12*, and *STS13* peaked at 24 h after the inoculation. Otherwise, the expression of *STS11* was observed at 6 h after the *B. cinerea* inoculation in Kyoho (Fig. 6*B*).

Discussion

Stilbene synthase genes are known to belong to a multigene family in grapes (Schröder *et al*. 1988, Sparvoli *et al*. 1994), and at least seven *STS* genes were found in *V. vinifera* cv. Optima (Wiese *et al*. 1994). In the present study, the comparison of deduced amino acid sequences of VlKSTS12, VlKSTS11, VICESTS13, and VlKSTS1 showed 100 % homology with VlCESTS12, VlCESTS11, VICESTS24, and VlKSTS13, respectively, in two grapevine cultivars.

Richter *et al*. (2006) identified approximately 25 *STS* genes from the sequence alignment and qPCR of genomic DNA. A cluster tree was derived by aligning *Vitis CHS* and *STS* gene family consensus sequences which exhibit 17 main branches with allelic variations. The homology among *STS* sequences in the coding region ranged from 98 to 83 %. The sequenced fragments of *STS* genes of *V. amurensis* exhibit significant homology (92 % amino acid sequence identity) with *STS*s from other *Vitaceae* species (Kiselev *et al*. 2009). Among them, *VaSTS1* shares 98 - 99 % amino acid sequence homology with *Vitis* stilbene synthase 1 (*Vst1*) and *V. riparia* stilbene synthase 1 (*Ripst1*).

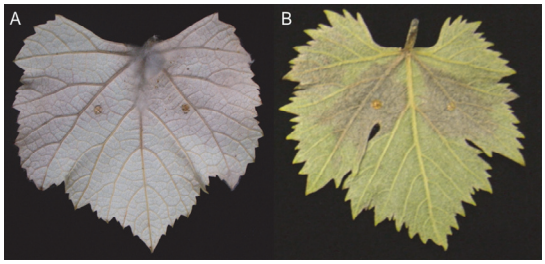

Fig. 5. Symptoms on Campbell Early (*A*) and Kyoho (*B*) grapevine leaves 4 d after the inoculation with *Botrytis cinerea*.

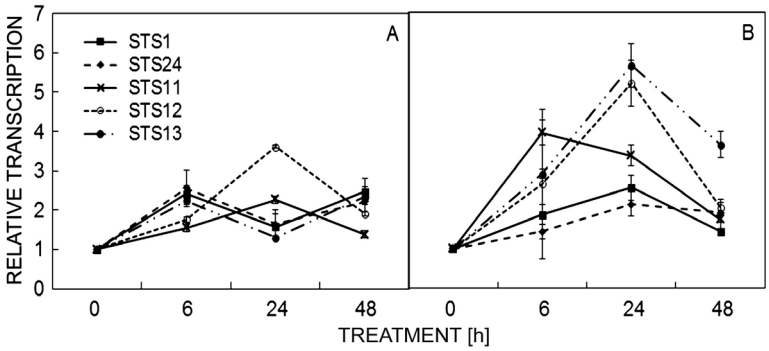

Fig. 6. Expressions of *STS* genes in cv. Campbell Early (*A*) and cv. Kyoho (*B*) in response to *Botrytis cinerea*. The transcription was calculated by using the standard curve method from triplicate data with a grapevine actin gene as internal control and non-treated leaves (at time zero) as reference sample. Results represent the mean fold increase in mRNA over non-treated leaves referred to 1. Results are means of triplicate data of three experiments. *Bars* indicate SD.

To investigate the differential expression patterns of the *STS* gene family members, primers specific to unique regions in each gene were obtained by the alignment of the family member sequences. Real-time PCR analysis shows that the signaling molecule treatments resulted in an increased the expression of genes involved in the flavonoid and stilbene syntheses in both the cultivars. The gene expression peaked at 6 h, being 2- to 40- and 1- to 43-fold higher than in the untreated leaves of Campbell Early and Kyoho, respectively.

In the present study, the application of signaling molecules increased the expressions of *PAL, CHS, CHI*, and *STS*. The *Botrytis* infection also induced the expression of *STS* genes, and according to Bézier *et al*. (2002) also the syntheses of stilbenes. The enhanced expression of these genes is correlated with the accumulation of resveratrol (Repka, 2001, Belhadj *et al*. 2006, 2008b, Kiselev *et al*. 2009, Ahn *et al*. 2013) which is synthesized from phenylalanine *via* the phenyl-propanoid pathway. The results of HPLC analysis showed that elicitor JA, SA, and ET treatments increase the concentration of two major resveratrol derivatives, 3-O-glucosyl-resveratrol and 4-(3,5-dihydroxyphenyl)-phenol in *V. vinifera* cell suspensions (Riedel *et al*. 2012). In ethephon- and MeJA-treated grapevine leaves, the expressions of *PAL* and *STS* are up-regulated which lead to the accumulation of resveratrol and its derivatives, piceid, viniferins, and pterostilbene (Belhadj *et al*. 2006, 2008a). The ET treatment also causes a several fold increase in *PAL* activity and thus a significant

accumulation of phenolic acids (Cvikrová *et al*. 2007). In case of *CHI*, the expression is dependent upon fruit developmental stage and modulated at both transcriptional and translational levels. It may contribute to the accumulation of total flavonoids in grape berry (Wang *et al.* 2012). The flavonoids can be used as antioxidant to extinguish ROS produced under biotic and abiotic stresses (Zhang *et al.* 2012). We also observed the transient differential up-regulation of *PAL, CHS, CHI*, and *STS* in ET, H_2O_2, JA, and SA-treated-leaves of the Campbell Early and Kyoho grapevine cultivars. Taken together, our results show that *PAL, CHS, CHI*, and *STS* genes were differentially regulated by ET, H_2O_2, JA, and SA.

UV-C irradiation induces the accumulation of *STS* mRNA and the respective protein with two peaks (Borie *et al*. 2004, Wang *et al*. 2010). According to Liswidowati *et al*. (1991) and Wiese *et al*. (1994), the *STS* gene family may be divided into two groups; those expressed early with a rapid degradation of the mRNA produced, and others that are expressed later, but are more stable. In addition, the presence of two peaks in the *STS* transcription is typical in grapevine cell culture indicating a modulated phytoalexin response that certainly enhances a defense potential. In many cases, close correlations between the phytoalexin production and the resistance to disease is observed (Langcake 1981, Adrian *et al*. 1997b, Bavaresco *et al*. 1997). Moreover, the distributions of resveratrol and *STS* are organ- and tissue-specific, and the accumulation of resveratrol is regulated by the transcription and translation of *STS* (Wang *et al*. 2010). Although the accumulation of *STS* mRNA induced by the elicitors presented two differential peaks at 6 h and 48 h in Campbell Early, this did not occur in Kyoho grapevine. It suggests that the modulation of transcription and translation of *STS* is also regulated dependently on cultivar in response to several elicitors in grapevines.

Kiselev *et al*. (2009) suggested that an increased resveratrol production occurs in response to the enhanced expression of specific genes of the *PAL* and *STS* families in *rolB* transgenic *V. amurensis*. *VaSTS1* is the predominant transcript among *STSs* in all cell cultures but *VaSTS*2, 3, 4, 5, and 7 also increase. In the present study, predominant genes among *STS*s showed a differential expression in the two investigated grape vine cultivars.

The expressions of *STS* genes in grapevines are induced upon infection with several fungal pathogens including *B. cinerea* (Bézier *et al*. 2002), *Erysiphe necator* (Fung *et al*. 2008), and *Plasmopara viticola* (Adrian *et al*. 1997a). Bavaresco *et al.* (1999) reported that disease-resistant grapevine shows a rapid and high accumulation of stilbene in leaves and fruit after *B. cinerea* infection, whereas the synthesis of stilbene is rather slow and limited in susceptible plants. Richter *et al*. (2006) reported differential patterns in the timing and level of the expression of *STS* gene members. Here, profiling *STS* transcripts in leaves treated with the signaling molecules showed different expression patterns from those observed in leaves infected with *B. cinerea* in both Campbell Early and Kyoho.

In this study, defense genes, such as *PAL, CHS, CHI,* and *STSs* showed the differential expression patterns in response to the different signaling molecules in two grapevine cultivars. Signaling molecule treatments that improve defense abilities of grapevine could allow the plant to accumulate stilbene or flavonoids and to keep down the spread of the disease caused by *B. cinerea*. In the present study, we elucidated nucleotide sequences specific for differentially expressed *STSs* and observed a differential expression of *STS* gene members and genes involved in the phenylpropanoid pathway in grapevines treated with ET, H_2O_2, MeJa, and SA, or inoculated with *B. cinerea*. These results can be useful for further characterization of grapevine defense responses at the molecular level and for the analysis of differential expression of individual gene members under various stimuli. These chemicals can also be used as elicitors to induce the accumulation of stilbene compounds, such as resveratrol, which is important for human health and can be used as alternatives to fungicides during grape production in greenhouses or vineyards.

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