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ORIGINAL PAPER

Differential Expression Patterns Within the Grapevine Stilbene Synthase Gene Family Revealed Through Their Regulatory Regions

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Abstract

The analyses of the grapevine (*Vitis vinifera* L.) genome have revealed an unusually large and closely related stilbene synthase (*VvSTS*) gene family. Interestingly, despite the high sequence similarity among those genes, several studies have observed clear differences between their expression patterns. Here, we studied the transcriptional responses to different elicitors of several *VvSTSs* in cellular suspension cultures. Primarily, we performed the in silico analysis of the *VvSTS* regulatory sequences and found the presence of several putative *cis*-regulatory elements. Then, we evaluated the effect of three treatments—naphtalene acetic acid, methyl jasmonate (MeJA), and ethylene—over the gene expression and found that the genes follow expression patterns probably specific to their sequences. According to this, we focused our study on their regulatory regions and adopted a novel and efficient transient expression assay to determine the activity of these promoters. The results demonstrated that variation in gene expression could be assessed through the analysis of *VvSTS* regulatory sequences under the effect of different stimuli such as MeJA and cyclodextrins. Furthermore, taking advantage of the lower sequence identity at the promoter level, this strategy accomplished a more accurate alternative to differentiate the members of a large multi-gene family such as *STS*.

Keywords *Vitis vinifera* · Transcriptome · Promoter analysis · Expression profiles · Transient transformation · Resveratrol · *Cis*-regulatory elements

Abbreviations

BAP	6-Benzylaminopurine
CDs	Cyclodextrins
C4H	Cinnamate-4-hydroxylase
CHS	Chalcone synthase
4CL	Coumaroyl-CoA ligase
CREs	*Cis*-regulatory elements
HREs	Hormone response elements
IBA	Indole-3-butyric acid
Ja	Jasmonate
MeJA	Methyl jasmonate
NAA	Naphthalene acetic acid
PAL	Phenylalanine ammonia lyase
STS	Stilbene synthase
Un	Untreated

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Introduction

Grapevine (*Vitis vinifera* L.) is one of the oldest world's fruit crops. Native to the Mediterranean region, central Europe and Southwestern Asia, nowadays is widely cultivated in temperate areas all around the world (This et al. 2006). Its adaptability has become it in one of the largest world's fruit crops with several renowned varieties of commercial significance for wine and table grape production. It is also a natural source of functional compounds, including the well-known stilbene called resveratrol (Jeandet et al. 2002; Kiselev 2011; Hasan and Bae 2017).

Stilbenes are a small family of phenylpropanoids occurring in diverse plant families, including grape (Vitaceae), peanut (Fabaceae), sorghum (Poaceae), and pine (Pinaceae). As a class of plant secondary metabolites, stilbenes are synthesized inducibly (phytoalexin) or enhanced constitutively which confer selective advantage in the defense of plants against pathogens and environmental stresses (Jeandet et al. 2002; Kiselev 2011; Hasan and Bae 2017).

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is the most important stilbene phytoalexin produced naturally in grapevine due to physical damage or when the plant is under attack by pathogens such as fungi, bacteria, nematodes, or herbivores (Hain et al. 1990; Adrian and Jeandet 2012). However, the accumulation of resveratrol in seed, grape skin, leaves, and cell cultures has been studied to be triggered by various strategies (Li et al. 2006; Kiselev 2011; Hasan and Bae 2017). Resveratrol is synthesized in the last step of the well-characterized phenylpropanoids/malonate pathway (Langcake and Pryce 1977), by the action of the enzyme *stilbene synthase* (STS). Together with phenylalanine ammonia lyase (*PAL*), cinnamate-4-hydroxylase (*C4H*), and coumaroyl-CoA ligase (*4CL*) phenylpropanoid-related genes, *STS* are up-regulated in a positive manner with the elicited accumulation of resveratrol (Lijavetzky et al. 2008; Almagro et al. 2014).

In most plants that produce stilbenes, there are several *STSs* forming small gene families of closely related paralogs (Schröder et al. 1988; Preisig-Müller et al. 1999; Kodan et al. 2002). However, grapevine seems to constitute a noteworthy exception to the rule. The last release of the 12X genome sequence annotation (v2) of grapevine (Jaillon et al. 2007; Vitulo et al. 2014) allowed an accurate analysis of the *VvSTS* multigenic family. The identification and re-annotation of the *VvSTS* family returned 48 *VvSTSs*, of which at least 32–33 were potentially functional ones (Parage et al. 2012; Vannozzi et al. 2012).

As the *VvSTS* family exhibits a high level of conserved gene structure and 392-amino acid proteins (Vannozzi et al. 2012), several expression analyses like microarray and mRNA-seq (Vannozzi et al. 2012), semi-quantitative RT-PCR (Parage et al. 2012), RT-qPCR (Shi et al. 2014), and qPCR (Dai et al. 2012), in different tissues and grape cultivars or species and under diverse conditions, were designed to evaluate the transcriptional responses of the genes. In general, all these analyses revealed different patterns of transcript accumulation between the different *VvSTS* family members. Thus, the study of *VvSTSs* promoters becomes pivotal and genetic engineering provides a convenient approach for functional verification of targeted regulatory sequences.

Most *STSs* research, across many species, has focused on gene expression, substrate determination, stress resistance, developmental response, and transgenic engineering (Fan et al. 2008; Dai et al. 2012; Parage et al. 2012; Vannozzi et al. 2012; Shi et al. 2014; Tyunin et al. 2017). On the other hand, little is known about the regulation of *STSs* at translational level in response to different stimuli. Xu et al. (2010) derived a *VpSTS* promoter from Chinese wild *V. pseudoreticulata* and transformed plantlets of *V. vinifera* cv. Thompson Seedless for overexpressing the *VpSTS*. High-performance liquid chromatography (HPLC) revealed that the resveratrol concentration in the transgenic lines was 5.5 times higher than that in non-transformed control plants (Fan et al. 2008). Isolation and functional characterization of the promoter region confirmed this as a pathogen- and stress-inducible promoter. Further studies conducted a comparative analysis of the *STS* 5′-flanking region identified in Chinese wild *V. pseudoreticulata* with that of two susceptible cultivated grapevines, *V. vinifera* cvs. Carignane and Thompson Seedless (Xu et al. 2011). Consistently with the significant different structure of the *STS* promoter in *V. pseudoreticulata* compare to that found in the two *V. vinifera*, functional studies using *Alternaria alternata*, methyl jasmonate, and wounding for inducing the three promoter-driven GUS responses in transformed tobacco plants indicated that differential expression patterns were about the specific regulatory function of the structurally different *STS* promoter of *V. pseudoreticulata* (Xu et al. 2011).

Accordingly, it is of main interest to investigate how higher levels of resveratrol can be produced. Likewise, it is a challenge to increase disease resistance in grapevines without altering berry quality parameters. This focuses *STSs* as principal targets of this study. Particularly, in this work, we have analyzed the influence of different elicitors on the *VvSTSs* expression and their promoters' activation through a transient expression experiment.

Material and Methods

Promoter Identification, Phylogenetic Analysis, and *Cis*-Regulatory Element (CREs) Prediction

For the promoter isolation, the specific grapevine databases, Genoscope (http://www.genoscope.cns.fr) and CRIBI (The CRIBI Biotechnology Center, University of Padua; http://genomes.cribi.unipd.it/grape/) (Vitulo et al. 2014), were searched and the regulatory 5′ sequence of each *VvSTS* under study was identified in addition to two chalcone synthase (*VvCHS1*—VIT_14s0068g00930 and *VvCHS2*—VIT_14s0068g00920-) genes (1500 bp upstream from the translation start codon ATG) (Rombauts et al. 1999). With this information, a phylogenetic analysis was performed to determine the relationships existing between the abovementioned promoters and the STS proteins. For this purpose, we followed the recommendations of Vannozzi et al. (2012) and generated an unrooted phylogenetic tree with the neighbor-joining method (Saitou and Nei 1987) using MEGA 7

software (Kumar et al. 2016). Reliability of tree obtained was tested using bootstrapping with 1000 replicates. Additionally, the regulatory sequences were analyzed for CREs recorded in databases such as PLACE (Higo et al. 1999) (verified in January 2017) and PlantCARE (Lescot et al. 2002) (verified in January 2017). The CREs related to methyl jasmonate (MeJA), ethylene, and naphthalene acetic acid (NAA) were identified.

Establishment of Callus Culture and Cellular Suspension Cultures

The Cabernet Sauvignon callus culture of *V. vinifera L.* was established in 2012 as described previously (Keller et al. 1998; Keskin and Kunter 2008). The callus culture was cultivated with 21-day subculture intervals in the dark at 24–25 °C in flasks with 60 mL of medium. Grapevine cell suspensions were initiated by inoculating friable callus pieces (12 g FW) in 250-mL flasks containing 100 mL of Gamborg B5 basal medium supplemented with 250 mg/L casein hydrolysate, 20 g/L sucrose, 0.12 mg/L 6-benzylaminopurine (BAP), and 0.5 mg/L NAA without agar and rotary shaking (110 rpm) in the dark at 24 ± 1 °C. The pH value of the medium was set to 5.5 by adding KOH. Cell suspension lines were routinely maintained by periodical subculturing duplicating the culture flasks every 14 days.

Elicitation of Cell Suspension Cultures

Elicitation experiments were carried out on three replicates of 8-day-old grapevine cell suspensions (linear phase of growth). Ethylene was added at a final concentration of 20 μM. The solutions of MeJA and NAA, dissolved in 100% (*v*/*v*) ethanol and 2 N NAOH, respectively, were added both at a final concentration of 20 μM. The elicited cell suspensions were incubated with continuous orbital shaking at 100 rpm, in the dark at 24 ± 1 °C. Control cultures contained no additional ethylene, MeJA, or NAA. After elicitation, samples were collected in 2-mL Eppendorf tubes at 0, 5, 10, 24, 48, and 72 h and centrifuged to remove the supernatant in a cooled centrifuge. Immediately, they were frozen in liquid nitrogen and stored at − 80 °C until processing.

Reagents

Reagents for the bacteria, cell, and plant culture medium were purchased from Phytotechnology Laboratories (USA), Sigma-Aldrich (USA), Oxoid (Inglaterra), Biopack (Argentina), Anedra (Argentina), and Britania (Argentina). Ethylene (ETHREL) was purchased from Bayer CropScience (Alemania), MeJA from Sigma-Aldrich (USA), NAA from Phytotechnology Laboratories (USA), and cyclodextrins (CD) from Wacker Chemie (Germany).

RNA Isolation, cDNA Synthesis and Real-Time Quantitative PCR (RT-qPCR) Expression Analysis

For gene expression, total RNA was extracted from 500 μL samples through the use of the TriReagent from Sigma-Aldrich (USA) according to the manufacturer procedures. Final RNA purification and DNase digestion of contaminating DNA in the RNA samples were completed using the SV Total RNA Isolation System from Promega (USA) following standard protocols. Reactions for cDNA synthesis and RT-qPCR were performed according to Lijavetzky et al. (2008) using a StepOne Real-Time PCR System from Applied Biosystems (USA). Non-template controls were included for each primer pair, and each RT-qPCR reaction was completed in triplicates. Expression data were normalized against the grapevine *ACT1* gene (VIT_04s0044g00580). The normalization gene was chosen after the comparison of *ACT1*, *EFα1* (VIT_06s0004g03220), and *UBI* (VIT_16s0098g01190) genes using NormFinder software (Andersen et al. 2004). The comparison was performed using samples of grapevine cellular suspension cultures treated with NAA, MeJA, and Ethylene along six time points (a total of 144 data points). All three genes were previously tested for grapevine gene expression analysis (Reid et al. 2006). Relative quantification was performed by means of the ΔΔCt method using StepOne software v2.2.2 from Applied Biosystems (USA). Gene-specific primers were designed using the QuantPrime web tool (Arvidsson et al. 2008) and were tested by means of the PrimerBlast tool on NCBI website (Ye et al. 2012). The sequences were described in Table 1.

Statistical Methods

The generation of the heatmaps and the cluster analysis of the RT-qPCR expression data were performed with the help of the Genesis v1.7.7 software (Sturn et al. 2002). After loading the information into the software, the data were adjusted using the Normalize Gene option and then forced to the maximum contrast for the visualization of the experimental values.

Gene Constructs

The 35S:GUS vector used for testing the transient transformation procedure was obtained by sub-cloning a CaMV 35S promoter from pGWB2 (GenBank accession number AB289765.1) into a pENTR entry vector (Thermo Fisher Scientific) and subsequently recombined with the binary vector pGWB3 (GenBank accession number AB289766.1; Nakagawa et al. 2007).

V. vinifera cv. Malbec genomic DNA was extracted using a DNeasyTM Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The 5′ flanking sequences (approximately 1500 bp upstream from the

Table 1 Primer pairs used for RT-qPCR

Gene name[a]	Gene annotation[b]	Sequence for the forward and reverse primers (F: forward, R: reverse)	Primer length (pb)	Temperature (°C)	Expected fragment size (bp)
VvSTS6	VIT_10s0042g00930	F-CCCAAACATGGGTGCTTACATGGC	24	64.74	183
		R-TCTACACCGGGCATTTCTACTCCC	24	63.77	
VvSTS7	VIT_16s0100g000750	F-CAGTTAGTTTCAGCAACCCAAACG	24	61.02	68
		R-TCACGTAAGTTACCCGCAATGG	22	60.93	
VvSTS31	VIT_16s0100g01030	F-TGCAGAGAATAATGCAGGAGCAAG	24	61.46	74
		R-AAGGGCCACGAAATGTAACAACTG	24	62.05	
VvSTS36	VIT_16s0100g01100	F-TTGTTACATTCCGTGGGCCTTCC	23	63.25	143
		R-TGCTGAGATGAGCTGGAAGTGTGG	24	64.7	
VvSTS38	VIT_16s0100g01110	F-ACTCTTCCAGCTCATCTCAGCAGC	24	64.51	181
		R-CCAGTCGCTAATACCAAGTGGGTC	24	63	
VvSTS42	VIT_16s0100g01140	F-ACTGCTGAGGTACCCAAACTGG	22	62.2	131
		R-TAATCCGCACCGGGCATTTCAAC	23	64.13	
VvSTS45	VIT_16s0100g01160	F-AGCAGGAGCCATTGCTGGAAAC	22	63.69	189
		R-ACTGCATCGAGAATTGCAGGGC	22	63.77	
VvSTS46	VIT_16s0100g01170	F-TGGGACTCACCTTTCATTTGTGG	23	61	113
		R-TAACGAGTTCCAATCGCTAATGCC	24	61.5	

[a] Gene nomenclature according to Grimplet et al. (2014)

[b] Gene ID according to The CRIBI Biotechnology Center, University of Padua (http://genomes.cribi.unipd.it/grape/) (Vitulo et al. 2014)

translation start codon ATG) (Rombauts et al. 1999) of the subset of *VvSTSs* under study were isolated from the genomic DNA using conventional PCR. Promoter-specific primers were designed using the Oligo Explorer 1.2 software (Gene Link) according to the flanking sequence of the genes in *V. vinifera* PN40024 and were listed in Table 2. After the purification of the PCR products, the promoter sequences were cloned into the pENTR entry vector (Thermo Fisher Scientific) and then recombined to the expression vector pGWB504 (Nakagawa et al. 2007). This vector had its cloning site followed by the reporter gene *sGFP* (synthetic green fluorescent protein). Thus, the expression of *sGFP* was guided by the sequence that precedes it. The seven isolated promoter sequences cloned into the vector were checked by Sanger sequencing.

Plant Material

In vitro *V. vinifera* cultivars "Thompson Seedless" (GI and TS), "Chardonnay," "Pinot Meunier," "Carménere," Harmony, and Salt Creek plantlets were started from field plants using micro-cuttings from green axillary buds. Explants were washed with commercial non-ionic detergent and rinsed with tap water for about 30 min. Cleaned materials were sterilized by immersion in a solution containing 20% of sodium hypochlorite and 10 μL of Tween 20 for 20 min, followed by six washes in distilled sterile water. Bleached out tips and leaves were removed with a scalpel, and scions were introduced into culture flasks containing 30 mL of Murashige and Skoog (MS) solid medium (Murashige and Skoog 1962) supplemented with BAP 1 mg/L. Flasks were placed in a growth chamber at 21 ± 2 °C using a photoperiod of 16 h light/8 h darkness for 1 month. New shoots were transferred to a C2D medium (Chee and Pool 1987) supplemented with 0.1 mg/L indole-3-butyric acid (IBA) and 1 mg/L BAP for rooting. Rooted plants were obtained after 30 days. The population was propagated and maintained by 30 days sub-culture in the same C2D derivative medium.

Gene Transfer by Agro-infiltration

Previous procedures (Ahmad and Mirza 2005; Santos-Rosa et al. 2008; Zottini et al. 2008) were combined and modified for the use of complete in vitro plants in agro-infiltration experiments.

Rhizobium Suspensions

Each promoter vector was incorporated into *Rhizobium radiobacter* (updated scientific name of *Agrobacterium tumefaciens*) (Young et al. 2001) through electroporation following procedures described by Dower et al. (1988). Vector-specific bacterial were supplemented with 100 μg/μL spectinomycin. Vector-specific *Rhizobium* pre-cultures were prepared from single colony of cells harboring the corresponding binary vector and inoculation of 5 mL of

Table 2 Primer pairs used for the isolation of the *VvSTS* regulatory sequences

Gene name[a]	Gene annotation[b]	Sequence for the forward and reverse primers (F: forward, R: reverse)	Primer length (pb)	Temperature (°C	Expected fragment size (bp)
VvSTS6	VIT_10s0042g00930	F-TTAAGCAAGCGAGCACCT	18	56.88	1441
		R-CCCACAGAGCTTCAAAGC	18	55.99	
VvSTS7	VIT_16s0100g00750	F-CACCGTCTCAGATTTAGGCATAAG	27	66.8	1378
		R-TGCCAGCTACGTACTGAAATGA	22	60.3	
VvSTS31	VIT_16s0100g001030	F-CCAAGTACCATAATGGTTGACC	22	56.7	1469
		R-GCCAGCTACGTACTCAAATTG	21	57.3	
VvSTS36	VIT_16s0100g01100	F-TGACTAATGGCTAATAATGGATC	23	53.77	1418
		R-CGTACTCAAAATGAAGCTTAAG	22	53.58	
VvSTS38	VIT_16s0100g01110	F-GAAGGAGTTAACTTACCTATTGA	23	53.26	1459
		R-TGCTGCTACTCCAATTGG	18	54.56	
VvSTS45	VIT_16s0100g01160	F-ACTACAGTGGAATTCTCTCAG	21	54.41	1501
		R-GGATGTCAGATACGTACTGAA	21	54.62	
VvSTS46	VIT_16s0100g01170	F-CTGGTATAAAAACCATGAGGCCG	23	59.68	1456
		R-CGATGCCAGCTAGGTACTCA	20	58.97	

[a] Gene nomenclature according to Grimplet et al. (2014)

[b] Gene ID according to The CRIBI Biotechnology Center, University of Padua (http://genomes.cribi.unipd.it/grape/) (Vitulo et al. 2014)

LB medium (Bertani 1951) supplemented with the adequate selection antibiotic. Pre-cultures were incubated at 28 ± 1 °C for 16–18 h, 220 rpm in a Certomat-U incubator (B. Braun Biotech, Goettingen, Germany). Fresh cultures (250 mL) of vector-specific bacterial clones at $OD_{600} = 0.1$ were prepared adding aliquots from pre-cultures (usually between 100 and 300 µL) to LB medium with selection antibiotics. Cultures were grown for additional at 28 ± 1 °C at 220 rpm up to final $OD_{600} = 0.3$ (usually 2.5 h). Bacterial suspensions were prepared by centrifugation at 4500 rpm for 10 min, and the pellet was resuspended in 250 mL of freshly prepared induction buffer (50 mM MES pH = 5.6, 10 mM $MgCl_2$, 2% sucrose, 100 µM acetosyringone). Suspensions were incubated at room temperature for 1 h allowing for bacteria activation. Induction buffer was sterilized using 0.22 µm Millipore filters (Merck & Co., Kenilworth, New Jersey, USA).

Plant Infiltration and Recovery

In vitro plantlets were recovered from their maintenance flasks, and roots were trimmed and covered with sterile aluminum paper. Agro-infiltration assays were prepared by immersion of aerial plant tissues into glass vessels containing the vector-specific bacterial suspensions, keeping roots outside. Plantlets were placed into a desiccator and subjected to a double round of vacuum pulse (20 mmHg, 3 min) using an oil-pump (Model 5KCR38UN929HX; GE Motors, Boston, MA, USA) or until plantlets were complete infiltrated, judged as leaves presented a slight transparent aspect. The infiltrated plantlets were rinsed six times with sterile distilled water and transferred to solid C2D medium for recovery for 2 days and then evaluated. Both, distilled water and C2D medium were supplemented with 200 mg/L carbenicillin.

Elicitation and Promoter Activity Analysis

In vitro Thompson Seedless plantlets were elicited on the first day of the experiment (day 1, 0 h), using sterile solutions of the following chemical elicitors: 50 mM CD, 0.1 mM MeJA and the combination of both molecules (CDMeJA) (Vezzulli et al. 2007; Lijavetzky et al. 2008; Faurie et al. 2009; Belchí-Navarro et al. 2012; Almagro et al. 2014). For this purpose, working in sterile conditions, a volume of the elicitor solution was added to cover completely the plantlet. The sampling was performed by taking transformed leaves during 3 days, every 24 h. Leaves were removed with a clamp and immediately observed under a microscope. The experiment was performed in triplicates, for each treatment and at each sampling time (0, 24, and 48 h). As controls, we used untransformed plantlets, untreated untransformed plantlets (Un), and plantlets transformed with a constitutive promoter fused to the reporter gene (35S:sGFP—positive control) and with a non-inducible promoter fused to the reporter gene (agl11:sGFP—negative control) (Kooiker et al. 2005).

To evaluate the induction of *sGFP*, leaves of transformed plantlets were observed using a Zeiss Axio Scope Lab A.1 epifluorescence microscope equipped with two types of filters: filter 09 (BP 450–490 nm) and filter 38 (BP 470–

40 nm) (Zeiss, Germany). The light source was provided by an LED lamp at 470 nm. The images were captured with a Canon Rebel T3 camera using the EOS utility program (Canon Inc., Japan). The ISO was set to 800 and the exposure time to ½.

Histochemical Assay for GUS Enzyme Activity

GUS staining assay was carried out using 5-bromo-4-chloro-3-indolyl glucuronide according to Jefferson et al. (1987).

Results

VvSTS Promoter Sequences Had Lower Identity Scores Compare to That at Protein Level Sequences

The promoter sequences of 13 *VvSTSs* coding for a complete ORF were identified after the isolation of 1500 bp of DNA sequence upstream from the start codon of each gene. The assigned name for each *VvSTS* promoter (p*VvSTS*), its chromosome localization, orientation, and the size of the isolated fragments were shown in Table 3. The selection of these 13 *VvSTSs* was performed following the criteria of validating the expression data obtained from Almagro et al. (2014). These 13 *VvSTSs* were identified as completed ORFs having a response to MeJA, CDs, and the combination of CDMeJA in the Affymetrix GrapeGen GeneChip used for the analysis.

The initial phylogenetic analysis based on the VvSTS protein sequences showed the high identity scores of the phylogenetic relationships between them (Fig. 1a). However, in this study, we made a more focused analysis comparing the regulatory sequences of a set of 13 *VvSTSs* using as outgroup the promoter sequences of two chalcone synthase genes (p*VvCHSs*) (Fig. 1b). Considering the topology of both trees and the length of their branches, the last phylogenetic tree highlighted that the identity values of the regulatory sequences were lower than that of the protein sequences (Fig. 1a, b). Additionally, we identified four paired sequences with high identity scores and a high bootstrap support (≥ 90%) (p*VvSTS42* and p*VvSTS46*; p*VvSTS9* and p*VvSTS15*) (Fig. 1b). The pair corresponding to the promoter sequences p*VvSTS42* and p*VvSTS46* was also defined as a putative paralogous pair in the protein tree (Fig. 1a). So, the evaluation of the promoter sequences may constitute a more efficient strategy to find functional divergence between the *VvSTSs* and to take over their study.

Table 3 Genomic information about the promoter sequences of each *VvSTS* (p*VvSTS*) gene according to the *V. vinifera* cv. PN40024 12X v2 genome annotation (Grimplet et al. 2014)

Promoter name	Regulatory sequences	
	Chromosomic localization	Size (bp)
p*VvSTS48*	chr16: 16713319..16711819	− 1500
p*VvSTS46*	chr16: 16685765..16684265	− 1500
p*VvSTS45*	chr16: 16677025..16675525	− 1500
p*VvSTS42*	chr16: 16630592..16629092	− 1500
p*VvSTS39*	chr16: 16618759..16617259	− 1500
p*VvSTS38*	chr16: 16610231..16608731	− 1500
p*VvSTS36*	chr16: 16558936..16557436	− 1500
p*VvSTS31*	chr16: 16510980..16509480	− 1500
p*VvSTS19*	chr16: 16369911..16368411	− 1500
p*VvSTS15*	chr16: 16334196..16335696	+ 1500
p*VvSTS9*	chr16: 16267315..16268815	+ 1500
p*VvSTS7*	chr16: 16237527..16239027	+ 1500
p*VvSTS6*	chr10: 14303286..14304786	+ 1500

Promoter name, chromosomic localization, orientation, and size of the isolated fragments are given for each promoter

CREs Discovery on the p*VvSTS* for Ethylene, MeJA, and NAA Found a Specific Distribution for Each Promoter

In order to characterize the response divergence of each gene within the gene family and given the higher variability inherent to the promoters, the p*VvSTSs* were analyzed using the bioinformatic scanning tools of the CREs databases of plant regulatory sequences: PLACE (Higo et al. 1999) and PlantCARE (Lescot et al. 2002). CREs predictive analysis showed that the p*VvSTSs* contained several putative hormone response elements, stress-regulated elements, and defense response elements. Given this perspective, the evaluation of CREs was focused particularly in the hormone response elements (HREs). Accordingly, the presence of CREs for MeJA, ethylene, and NAA was checked (Table 4). We found that all genes except *VvSTS31* had at least one ethylene response element. Only the genes *VvSTS9*, *VvSTS15*, *VvSTS19*, *VvSTS31*, *VvSTS36*, and *VvSTS38* possessed at least one of the two response elements to MeJA. On the other hand, NAA, a synthetic plant hormone belonging to the auxin family, had several CREs for its putative regulation. All genes, except *VvSTS6*, had at least one type of auxin response element.

VvSTSs Responded Differentially to the Induction with NAA, MeJA, or Ethylene

To investigate the responses of the studied *VvSTSs* after the induction by different elicitors, mRNA accumulation in treated grapevine cellular suspension cultures cv. Cabernet Sauvignon was evaluated at six time points. Expression analyses via RT-qPCR were accomplished using specific primer pairs for eight different *VvSTSs*. It was worth to note that the high identity between *VvSTS* sequences implied a restriction to the possibility of unequivocally differentiates each of the

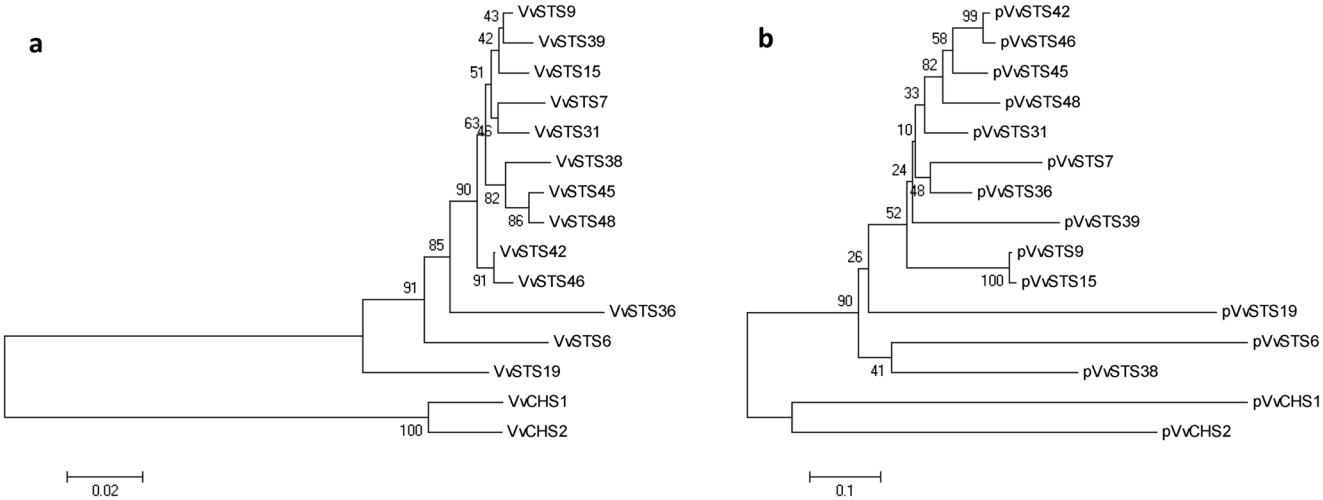

Fig. 1 Phylogenetic tree for the grapevine VvSTS protein sequences (**a**) and *VvSTS* promoter sequences (p*VvSTSs*) (**b**). Consensus phylogenetic tree generated after alignment of sequences using the Neighbor-Join algorithm. The regulatory sequences and protein sequences considered correspond to 13 *VvSTSs*. The regulatory sequences and protein sequences of *VvCHS1* and *VvCHS2* were included in each analysis as outgroups respectively. The confidence of the predicted tree was evaluated using a bootstrap with 1000 replicates

gene sequences. Therefore, five out of the original 13 primer pairs produced qPCR amplifications with undesired melting curves and/or low amplification efficiencies.

As elicitors, we selected the phytohormones NAA and ethylene and the signaling molecule MeJA. This choice meant to evaluate the effect of molecules for which we identified several CREs in the in silico analysis of the p*VvSTSs* (Table 4). To understand the differential gene responses, the results were showed as the fold change for each gene and treatment (Fig. 2). For a more global approach, the results were also exhibited as treatment responses in a matrix where all gene expression values were represented as colors (heatmaps) and similar responses were related with hierarchical joins (Figs. A1, A2, and A3).

According to Fig. 2, it was observed that MeJA constituted a strong inducer of gene expression that caused for most genes an early peak (5 h post-treatment) in the transcriptional response which then decreased with time (10 and 24 h post-treatment), except for the genes *VvSTS42*, *VvSTS45*, and *VvSTS46* as also described for the MeJA heatmap (Fig. A2). Particularly, genes *VvSTS45* and *VvSTS46* clustering together re-experienced an induction peak at 72 h, and *VvSTS42* also at 24 h. The expression pattern to MeJA was accompanied by the effect generated by ethylene, albeit to a much smaller extent. Similarly, ethylene exerted an early stimulation (0 h post-treatment to 10 h post-treatment) which then decreased with time, except for the genes *VvSTS45* and *VvSTS46* that showed an additional induction at 48 h which was maintained thorough 72 h post-treatment (Fig. 2). These results were also supported by the heatmap representation (Fig. A2) where these two genes were clustered together having an induction of expression at 10 h after treatment, which then was repressed at 24 h and induced again from 48 h. For the rest of the genes, grouped in closer clusters, induction was maintained stable up to 10 h post-treatment and then it was repressed. Apparently, the *VvSTSs* showed a similar pattern of response by the application of these two treatments.

On the other hand, NAA showed an expression-inducing effect that was variable in time (Fig. 2), with an early induction at 0 h, and additional peaks at medium and late times as also exhibited in the heatmap (Fig. A3). Here, genes *VvSTS36* and *VvSTS6* grouped distant from the rest of the clusters presenting a late response at 48 and 72 h. The rest of the genes were clustered in two closer groups. All of them had in common an early response with induction values at 0 and 10 h post-treatment. A subset of them, composed of the genes *VvSTS45* and *VvSTS46*, showed a later increase in expression at 72 h. On the other hand, while the gene *VvSTS38* did not modify its expression until the last time point (72 h), *VvSTS31*, *VvSTS7*, and *VvSTS42* genes showed an additional induction of the response at 48 h. It was interesting to highlight that the induction in almost all genes due to the effect of NAA did not exceed once the value of the expression presented in the control treatment, except for the genes *VvSTS31* and *VvSTS7*.

The Whole Plant Agro-infiltration Assay Efficiently Transformed Different Grapevine Cultivars

As a proof of concept of the transformation method, we infiltrated seven different grapevine cultivars with a bacterial suspension carrying a 35S:GUS construct. As showed in Fig. A4, all seven cultivars displayed an efficient transformation with the reporter gene compared to the control plantlets. According to this result, we proceed to use the technique for the analysis of the p*VvSTS* responses.

Table 4 List of putative CREs present in the *VvSTS* promoter sequences identify in the PLACE (Higo et al. 1999) and Plant CARE (Lescot et al. 2002) databases

Name of CRE	Consensus motif	Number of CREs (for each promoter)												Function
		VvSTS48	*VvSTS46*	*VvSTS45*	*VvSTS42*	*VvSTS39*	*VvSTS38*	*VvSTS36*	*VvSTS31*	*VvSTS19*	*VvSTS15*	*VvSTS9*	*VvSTS7*	*VvSTS6*	
TGACG-motif	TGACG	–	–	–	–	–	1	1	–	1	1	1	–	–	MeJA-responsiveness
CGTCA-motif	CGTCA	–	–	–	–	–	1	1	2	1	1	1	–	–	MeJA-responsiveness
ERE	AWTTCAAA	2	1	2	1	2	2	1		1	1	1	2	1	Ethylene-responsive element
ARFAT	TGTCTC	3	2	–	2	1	1	1	1	1	2	2	1	–	Auxin response factor binding site
ASF-1	TGACG	–	–	–	–	–	1	1	2	1	1	1	–	–	Auxin response factor binding site
NtBBF1	ACTTTA	3	2	2	2	2	1	2	1	2	3	2	–	–	Auxin-responsiveness
SAUR	CATATG	4	–	–	–	–	–	–	–	–	–	–	2	–	Auxin-responsiveness

In the table, the number of times the element is present in each promoter is indicated

Promoter Induction Analysis Reflected the Divergence Between the Different *VvSTSs* Expression Patterns

To detect activity variations between the different p*VvSTSs*, we used the methodology tested in "The Whole Plant Agro-infiltration Assay Efficiently Transformed Different Grapevine Cultivars" section. We examined the *sGFP* gene expression directed from the p*VvSTS* constructions of seven *VvSTSs* and two additional constructions, one for the positive and one for the negative control (Fig. 3). Due to technical difficulties, we failed in obtaining the promoter construction for *pVvSTS42*. The expression of *sGFP* was detected in leaves of agro-infiltrated plantlets of grapevine cv. Thompson Seedless under ultraviolet light after the elicitors' application. The molecules used as elicitors were MeJA, CD, and the combination of both compounds (CDMeJA). We used these treatments in order to correlate the results with the data obtained in previous gene expression studies (Lijavetzky et al. 2008; Almagro et al. 2014) where MeJA and CDs were the evaluated elicitors. As shown in Fig. 3, the induction of the promoters with the chemical elicitors at 0 h was imperceptible and equivalent to that observed for untreated (Un) transformed plantlets. Generalizing, at 24 h, higher fluorescent intensity of *sGFP* begun to be evident and was usually maintained up to 48 h post-treatment for the two elicitors and their combined effect. Also, it was observed that CD, and its combined effect with MeJA (CDMeJA), exerted a stronger effect on the induction with respect to MeJA alone. Being more precise, we could point some interesting behaviors: (i) some promoters responded slightly to MeJA as p*VvSTS31*, −36, −45, and −46 even they had a strong response to the combination of both molecules; (ii) while most genes responded to CD, p*VSTS7* showed little response to CD but a strong response to MeJA and the combination of CDMeJA; (iii) on the other hand, promoters like p*VvSTS38* showed responses to both elicitors individually but not to the combined treatment; (iv) differently to most of the promoters presenting inductions at 24 and 48 h, p*VvSTS38* did not show any expression at 48 h; (v) contrarily, p*VvSTS36* displayed strong induction at 48 h but almost no expression at 24 h. As a result of this, we could say that CD and CDMeJA were able to direct the transcription of the *VvSTSs*.

Discussion

Identification and characterization of individual members of the *STS* gene family in grapevine was a requirement to evaluate their differential expression patterns before the publication of the grapevine genome sequence back in 2007 (Jaillon et al. 2007; Velasco et al. 2007). Constraints like the high homology between members of the *STS* genes, in addition to minimal allelic variations were essential to be considered for

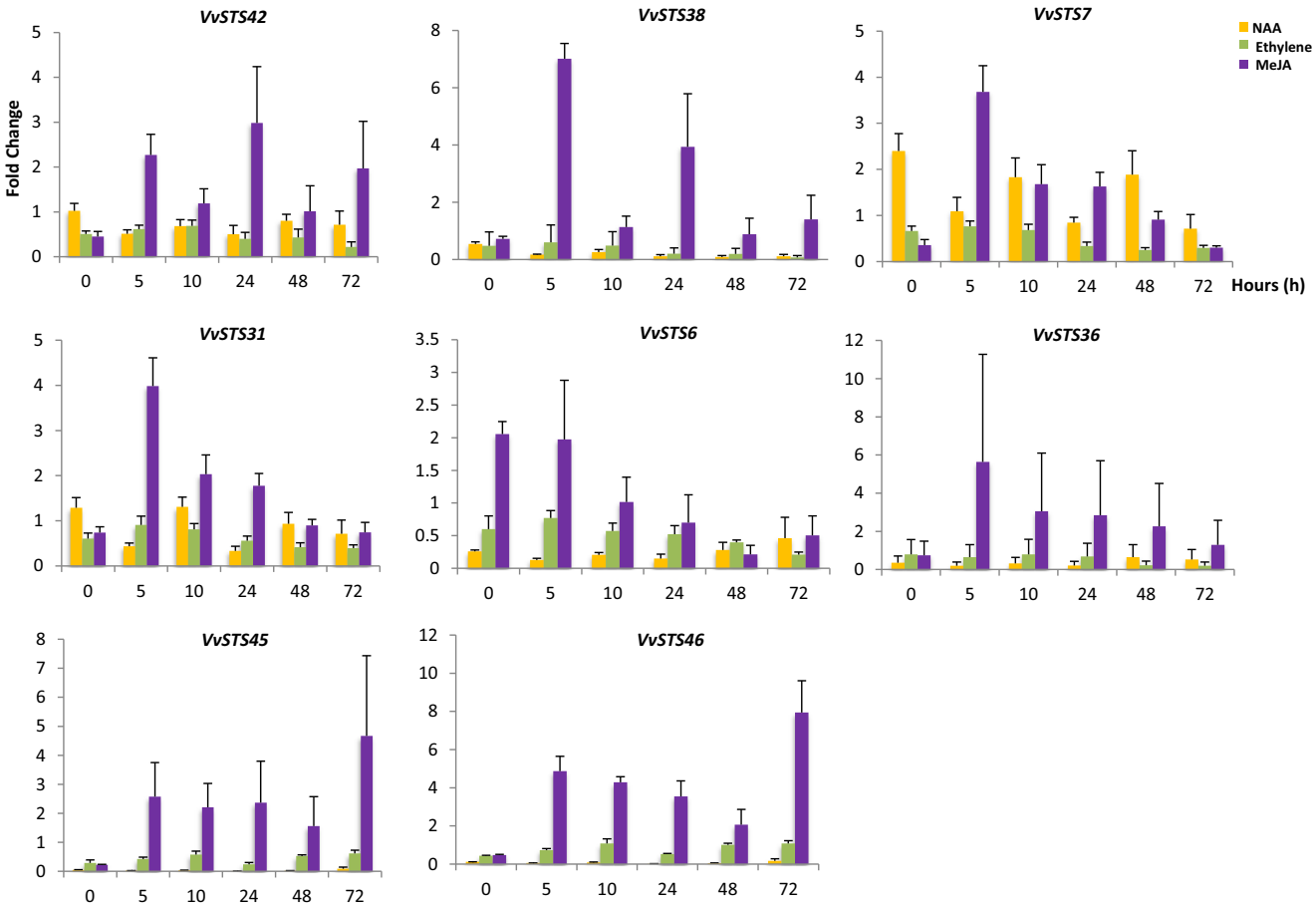

Fig. 2 Fold changes in expression of *VvSTSs* in response to NAA, ethylene and MeJA for grapevine cellular suspension cultures of cv. Cabernet Sauvignon. The fold changes were calculated by relativizing the expression values of the treated samples with those from the untreated samples at the same time point. Error bars represent standard deviation

the selection of primers in amplification processes or the design of short sequences in hybridization setups (Richter et al. 2006; Gatto et al. 2008). Later experiments using the *Vitis* GeneChip microarray (Affymetrix) had some issues too as it did not replicate each member of the entire family of *VvSTSs* that was annotated afterwards on the PN40024 genome. Moreover, the probe sets were not specific to identify individual *VvSTS* (Fung et al. 2008). Recent annotations found that the grapevine PN40024 genome (Jaillon et al. 2007) encoded 48 putative *VvSTSs*, of which 32–33 *VvSTSs* were complete genes (Parage et al. 2012; Vannozzi et al. 2012). The existence of multiple *VvSTS* copies on the grapevine genome stress the significance of STS-mediated plant metabolism in the adaptation of grapevine to the changing context (Dai et al. 2012).

Likewise, it is hard to think that the large size of the *VvSTS* family is not related to a diversification of expression among different groups of genes within the family. Despite the high similarity of *VvSTSs*, Vannozzi et al. (2012) showed through a microarray and RNA-seq study, that genes could be rearranged in subfamilies exhibiting different expression profiles. Transcriptional expression of *VvSTS* is regulated by pathogen infection, abiotic stresses, mechanical wounding or hormones in a developmentally regulated way (Lijavetzky et al. 2012; Vannozzi et al. 2012; Almagro et al. 2014). Thus, many researchers have focused their investigation in a set of *VvSTSs* and had analyzed their transcript levels to a particular condition. For example, transcriptional studies have been carried on the powdery mildew fungus infection (Dai et al. 2012; Shi et al. 2014), the UV light exposure (Parage et al. 2012; Vannozzi et al. 2012), and on wounding and the downy mildew infection (Vannozzi et al. 2012). At the same time, it is well known that the response elements of the promoter sequences are essential for the temporal, spatial, and cell type-specific control of gene expression, as well as to respond to different types of stress (Higo et al. 1999; Lescot et al. 2002). A number of studies over the p*VvSTS* have been reported dealing with induction under the influence of pathogen infection (e.g., *Uncinula necator* and *Alternaria alternata*), low temperature, wounding, and hormone and signaling molecule treatment (e.g., salicylic acid, ozone, methyl jasmonate, and ethylene) (Schubert et al. 1997; Grimmig et al. 2002; Xu et al. 2010, 2011). Despite this, much remains to be known about CREs and transcription factors controlling expression of *VvSTS* in response to various environmental

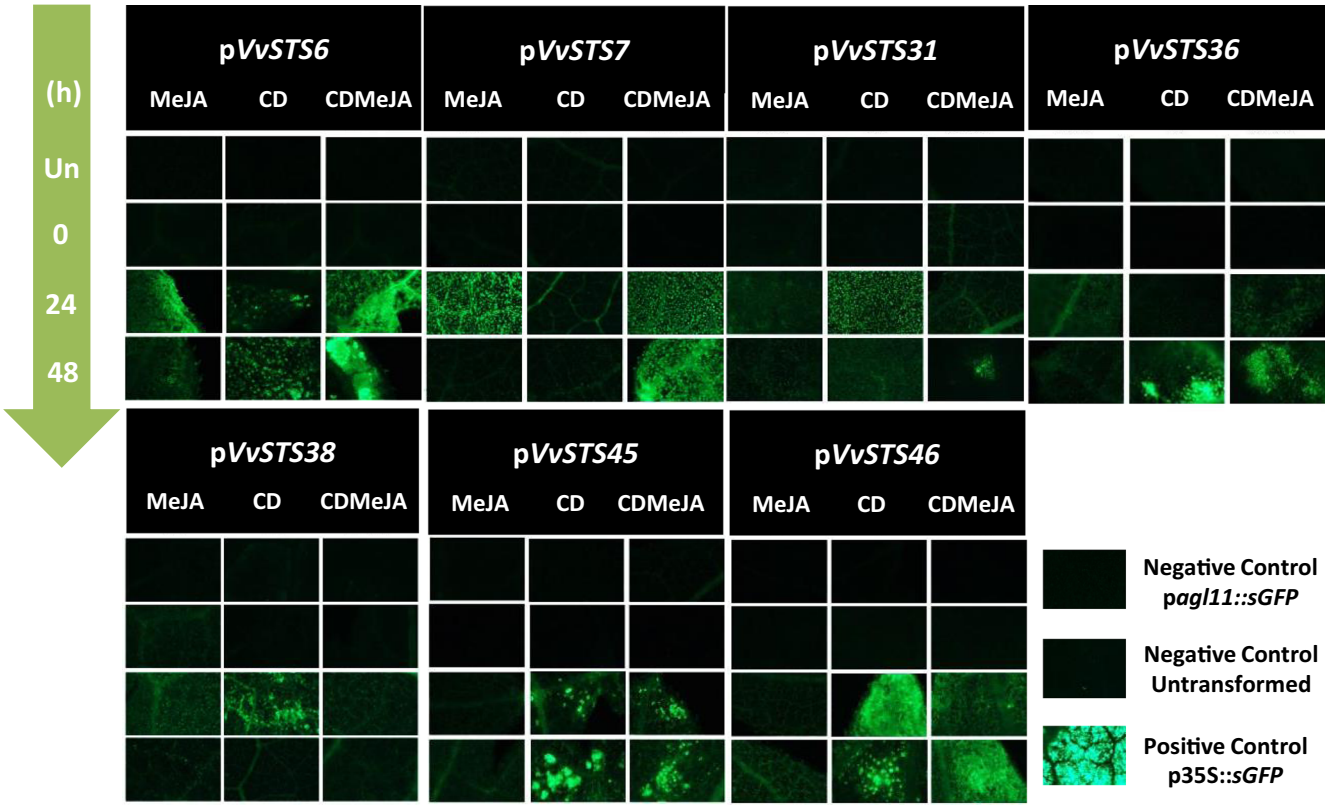

Fig. 3 Functional and temporal qualitative analysis of the activity of *VvSTS* promoters (p*VvSTSs*) on the expression of the *sGFP* reporter gene. The emission of fluorescence was observed as an indicator of the inductive effect of the promoters when the plantlets were treated with different elicitors and at different time points. Images for positive, negative, and untransformed controls were also included

stimuli. Hypothesizing, it is predictable that the differential responsiveness of *VvSTS* conducted by elicitor treatment is associated with differences in the upstream regulatory regions of the genes.

For this reason, we considered necessary to carry out a dissection of the CREs in the regulatory region of *VvSTSs* to better understand the specific responses to an array of external and internal signals. This idea was supported since some evidence indicated that the nucleotide sequence analysis of *pVvSTS* differ significantly not only between individual *VvSTS* but also between the same *VvSTS* of different *Vitis* species (Wiese et al. 1994; Hou et al. 2002; Xu et al. 2010, 2011). To asses this possibility and elucidate the molecular basis of their expression, we first isolated a subset of p*VvSTS* (Table 3) and then evaluated their phylogenetic relationships. From the phylogenetic tree, we inferred a higher variability at the regulatory sequences level compared to that of the VvSTS protein sequences (Fig. 1a, b). This could be deduced from a model of tree having longer branch topology. This result demonstrated that regulatory sequences could be eligible over the gene sequences to easily identify each member of the *VvSTS* family. Moreover, we could recognize two putative pairs of paralogues supported by the high bootstrap values that were obtained from the tree: p*VvSTS42*, *46* and p*VvSTS9*, *15* (Fig. 1b). This observation was quite consistent with the phylogenetic tree built from the protein sequences (Fig. 1a). Secondly, we made an *in silico* analysis of the promoter sequences based on the regulatory elements. We focused our attention in the CREs related to NAA, MeJA, and ethylene. These were the elicitors that we further use in the gene expression analysis. In general, we observed that each promoter has a specific constitution and distribution of CREs that differs from each of the other genes under study (Table 4). Although the higher number of matches was found between members that also had a high percentage of identity, the divergences could explain the basis of the differential transcriptional profiles that could turn them into functionally specialized genes.

We also investigated the transcriptional responses of eight *VvSTSs* to three abiotic stress treatments (NAA, MeJA, and ethylene) for which the genes contain in their regulatory sequences unless one response element (Table 4). In an attempt to validate the differential regulation of their expressions within the *VvSTS* family, we undertook an experiment using cellular suspension cultures of *V. vinifera* cv. Cabernet Sauvignon treated with the different elicitors and quantified the transcripts using RT-qPCR at six time points (0, 5, 10, 24, 48, and 72 h). The transcriptional responses of the *VvSTSs* to a phytohormone, such as the auxin NAA, have not been studied before. Auxins play a very important control in almost every

aspect of plant growth, development and differentiation (Davies 1995). In grape, auxin levels decrease before the onset of véraison. Therefore, auxin attenuation may be important in stopping pre-véraison berry-related processes, allowing those related to activation of maturation to be induced. On the other hand, transcripts related to phytoalexin biosynthesis are positively regulated during berry maturation, mainly due to their antifungal properties, which would act as defense against pathogen attack (Dai et al. 2012; Lijavetzky et al. 2012). Accordingly, NAA would be expected to exert an inhibition of the response of the *VvSTSs*. Although the cell culture-based study model did not allow the definition of a maturation stage for the cells, according to the results obtained and represented in Fig. 2 and Fig. A3, NAA exerted an inducing effect on the expression of *VvSTS* genes at different time points with one group of genes having mainly an early and oscillating response (*VvSTS45*, *VvSTS46*, *VvSTS38*, *VvSTS31*, *VvSTS7*, and *VvSTS42*) and another group of genes having mainly a late response (*VvSTS36* and *VvSTS6*). Although this phytohormone would participate in the regulation of the development of grapevine plants and would also affect the expression of the *VvSTS* genes, it is still unknown if the signaling pathway involved would play any role in *VvSTS* transcription.

In addition, grapevine is affected by many diseases caused by bacteria, fungi, and viruses (Jeandet et al. 2002). To contain the development of these pathogens, plants possess physical barriers and inducible defense mechanisms, such as the production of phytoalexins (Hammond-Kosack and Jones 1997). For instance, necrotrophic-type infection of *Vitis vinifera* activates the signaling pathway controlled by the molecule jasmonate (JA) and the phytohormone ethylene (JA/ET pathway) along with the induction of genes related to phytoalexin biosynthesis (Zhao et al. 2005; Armijo et al. 2016). In grapevine cellular cultures, the effect of MeJA is very effective in stimulating the accumulation of endogenous resveratrol as well as in its release to the culture medium (Tassoni et al. 2005; Vezzulli et al. 2007; Lijavetzky et al. 2008; Belchí-Navarro et al. 2012). On the other hand, the ethylene treatment of grapevine leaf explants increases the resistance to *Erysiphe necator* by inducing the expression of several genes for PR proteins and the production of stilbenes (Belhadj et al. 2008). In this way, Faurie et al. (2009) showed that ethylene and jasmonate cooperate synergistically to stimulate the production of phytoalexins in grapevine (Faurie et al. 2009). The results derived from this experiment showed, according to Fig. 2 and Figs. A1 and A2, that the expression of the *VvSTS* was induced by MeJA and ethylene. What is more interesting is that their response patterns were similar, presenting an early induction of expression, especially for ethylene, which was maintained stable until 10 h post-treatment (Fig. 2 and Fig. A2). However, on both the ethylene and MeJA treatments, it was possible to recognize the differential behavior of two genes, *VvSTS45* and *VvSTS46*, which expressions were induced again from 48 and 72 h for ethylene and MeJA, respectively. The *VvSTS42* also showed this late response at 72 h for the MeJA treatment, and the *VvSTS6* was only induced until 5 h post-treatment. As can be seen from the results of this experiment, the elicitors under study appeared to be related in the way they exert their induction. The signaling pathways of ethylene and JA interact through the production of phytoalexins (Zhao et al. 2005; Faurie et al. 2009) and have previously been shown to have a role in *VvSTS* transcription (Tassoni et al. 2005; Vezzulli et al. 2007; Belhadj et al. 2008).

In this way, RT-qPCR analysis confirmed significant differences in the transcript quantification of these genes having different responses to different treatments. This analysis also demonstrated evident differences in the expression pattern of the *VvSTS* genes that may allow arranging them in subfamilies according to their responses. As can be seen in Fig. 2 and Figs. A1–A3, the profile expression for each gene varied independently according to the nature of the stimulus but was significantly similar between genes that had a high sequence identity (*VvSTS*45 and 46; *VvSTS*7 and 31). Although the different patterns of transcriptional response between the *VvSTS* groups suggest that these genes may be responding to different signaling pathways, the information contained in their regulatory sequences widens the divergence and therefore the specificity of the response for each gene.

To this end, in this work we proposed to evaluate the differential activation of several p*VvSTS* through a transient expression experiment and using elicitors that were reviewed to induce a transcriptional response (Tassoni et al. 2005; Vezzulli et al. 2007; Lijavetzky et al. 2008; Belchí-Navarro et al. 2012; Almagro et al. 2014). Studies using transient transformation in grapevine are scarce and only focus on embryogenic tissues and cells, with the main objective of regenerating stable transformed plants (Vidal et al. 2010; Chialva et al. 2016). Thus, we designed a novel transient expression assay based on the use of complete grapevine plantlets. By the use of this experimental procedure, we infiltrated seven different grapevine cultivars (Fig. A4) with a bacterial suspension carrying a 35S:GUS construct. The described procedure relies on the use of axillary bud micro-cuttings technique for plant propagation and maintenance, which is simple to reproduce and widespread in many laboratories. Also, the infiltrated plantlets were subjected to physiological recovery for 2 days prior elicitation assays, reducing importantly the mechanical damage caused by the agro-infiltration procedure. In addition, we observed that the survival of the infiltrated plantlets was maintained for at least 2 weeks with no *Rhizobium* occurrence, enabling the use of the plantlets over this time.

Leaf infiltration with a suspension of *Rhizobium radiobacter* proves to be an easy and non-invasive technique that has been employed in different plant species in order to study, among other things, the in vivo analysis of promoters and transcription factors (Yang et al. 2000).

Taking advantage of the higher variability found through the analysis of the p*VvSTS* we designed specific primers for the promoters in order to perform the in vivo analysis of their function. In this way, the promoters were cloned into vectors carrying a *sGFP* reporter gene at the 3′ end of the insertion site. Subsequently, these constructs were used to transform *Rhizobium radiobacter* and to carry out the transient transformation assay of in vitro grapevine cv. Thompson Seedless plantlets. As elicitors, we chose MeJA and CDs as they constitute two abiotic elicitors that in cellular suspension cultures were able to induce the expression of the *VvSTSs* and the production of phytoalexins (Vezzulli et al. 2007; Lijavetzky et al. 2008; Faurie et al. 2009; Belchí-Navarro et al. 2012; Almagro et al. 2014). Microscopic observation of induced leaves showed that the promoter sequences were capable of respond to the elicitors in a specific and differential way and representative to the gene sequence they regulate. What is more interesting was that differences in induction could be observed both temporally and probably dependent on the presence of CREs present in the promoters. For instance, the induction of the promoters with the abiotic elicitors at 0 h was imperceptible and equivalent to that observed for untreated (Un) transformed plantlets (Fig. 3). At 24 and 48 h post-treatment, the elicitors individually and combined, induced in general a higher fluorescent intensity of *sGFP*, which for some constructs was more evident than for others. Although this technique is basically a qualitative functional analysis of promoters (Hernandez-garcia and Finer 2014), it was useful to confirm that the elicitor nature and the presence of CREs for its regulation, direct the transcription of the nearby gene. This regulation results specific to each gene and explains the expression profile of this type of stress-inducible genes. Further experiments, including the stable transformation of plants, are a requirement to evaluate with certainty the role of the promoters in the differential regulation of the expression of *VvSTSs*.

Conclusion

This research describes the isolation and characterization of the regulatory regions of *VvSTSs* in order to test the differential expression of these stress-inducible genes. Therefore we made an *in silico* analysis of these *VvSTS* promoter sequences allowing the identification of the elicitors used for the RT-qPCR comparative analysis of *VvSTS* transcripts obtained from treated cellular suspension cultures. Moreover, as the main achievement of our work, we analyzed the activity of the *VvSTS* promoters using a novel transient expression approach. Consequently, by means of the latter experiments, we confirmed that the *STSs* respond differentially to the elicitors and that these responses are specific to their promoter sequences. The information reported in these experiments allows a better understanding of the role that the regulatory sequences of a gene or gene family could play in leading their expression. In particular, when most of the members of large gene families are functional and exhibit differential response patterns to signals caused by external stimuli. This work is expected to provide evidence of the specific expression functions of *VvSTS* promoters and may benefit future research in understanding the regulation of gene expression.

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Authors' Contribution C.C., H.P., and D.L. conceived and designed the experiments. C.C. performed the in silico, in vitro, and transcriptomic experiments. C.M. provided the technical support on the transcritomic analysis. L.C. helped with the general data analysis and graphical presentations. H.P. and M.M. designed and tested the transient transformation assay. M.M and E.E. provided the technical support on the in vitro experiments. C.C. and D.L. wrote the paper. All the authors critically revised the manuscript and approved its final version.

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Compliance with Ethical Standards

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Conflict of Interest The authors declare that they have no conflict of interest.

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