

Genetic transformation of a fruit-specific, highly expressed stilbene synthase gene from Chinese wild *Vitis quinquangularis*

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Received: 28 October 2015 / Accepted: 22 December 2015 / Published online: 18 January 2016
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Abstract

Main conclusion The stilbene synthase gene *VqSTS6*, from Chinese wild type *Vitis quinquangularis* accession Danfeng-2, increases the resveratrol content and pathogen resistance of transgenic plants of *V. vinifera* Thompson Seedless.

This study successfully created transgenic plants of *V. vinifera* Thompson Seedless which overexpressed *VqSTS6*, cloned from Chinese wild type *V. quinquangularis* accession Danfeng-2. Western blot and qRT-PCR showed a variable range in transcript levels among transgenic lines. The resistance to powdery mildew (*Uncinula necator*) was particularly enhanced in lines most highly expressing *VqSTS6*. Compared with the non-transformed controls, *trans*-resveratrol and other stilbene compounds were significantly increased in the transgenic lines. The correlation between high resveratrol content and high pathogen resistance in transgenic grapes is discussed. We hypothesize that the fruit-specific, highly expressed gene *VqSTS6* from Chinese wild

V. quinquangularis accession Danfeng-2, is directly involved in the resveratrol synthesis pathway in grapes, and plays an important role in the plant's defense against pathogens. Genetic transformation of *VqSTS6* explored the potential of the wild Chinese grape species for use in breeding, the results of which would raise both the disease resistance and the fruit quality of *V. vinifera* grapevines.

Keywords Grapevine · *VqSTS6* · Resveratrol · *Uncinula necator*

Abbreviations

PEM	Pro-embryogenic masses
SE	Somatic embryo
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
AC	Activated charcoal
BAP	6-Benzylaminopurine
PM	Powdery mildew
HPLC	High-performance liquid chromatography
DW	Dry weight

Introduction

The grape (*Vitis* sp.) is an ancient fruit crop that is becoming increasingly important throughout the developed world. All along, the prominence of this crop has been based on its high economic value and also its cultural significance. More recently, however, the health benefits of its products are also being recognized (Bouquet et al. 2008). Significant research is now being focused on grape's high resveratrol content—resveratrol having anti-inflammatory, antiplatelet, anticarcinogenic, antifungal, and antibacterial activities (Adrian and Jeandet 2006; Anekonda 2006; Athar et al. 2007; Barger

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et al. 2008; Jang et al. 1997; King et al. 2006; Saiko et al. 2008; Xu et al. 2014). While the European grapevine (*Vitis vinifera* L.) is the predominant grape species cultivated, most *V. vinifera* cultivars are very susceptible to fungal pathogens (Gomès and Coutos-Thévenot 2009). In particular, powdery mildew (*Uncinula necator* (Schw.) Burr.) severely impacts both the yield and the quality of grapes (He et al. 1991). However, most of the Chinese wild species of *Vitis* possess excellent resistance to powdery mildew (Wang and He 1997; Wang et al. 1995). Moreover, the high level of resveratrol contained in their ripe berries has been reported previously by our research group (Shi et al. 2014; Zhou et al. 2015). Among these Chinese *Vitis* species, *Vitis quinquangularis* accession Danfeng-2 (with white berries) is naturally highly resistant to powdery mildew and it is also rich in resveratrol (Shi et al. 2014; Wan et al. 2015). Therefore, it is very interesting to see if it is possible to use this Chinese wild germplasm Danfeng-2, to improve the disease resistance and resveratrol contents in some of the European *V. vinifera* grapevine cultivars. Due to the long juvenile stage, polyploidy and high level of heterozygosity (Gray et al. 1992; Nakano et al. 1994), conventional crossing between Chinese wild and European grapevines was severely limited. Precision breeding through genetic transformation enable to transfer fungi-resistant and high level resveratrol traits of Danfeng-2 into European grape cultivars without changing their horticultural traits (Bornhoff et al. 2005; Franks et al. 2006; Vidal et al. 2003; Yamamoto et al. 2000).

Stilbene synthase (STS), belonging to the polyketide synthase family, is a key enzyme in the biosynthesis of resveratrol (Rupprich and Kindl 1978). Since STS was first purified from cell suspension cultures in 1984 (Schöppner and Kindl 1984), a considerable amount of research has been carried out on it. In Hain et al. (1990), successfully introduced a complete *STS* gene from groundnut into tobacco. Later, they transformed more *STS* genes from *V. vinifera* into tobacco, which increased its resistance to *Botrytis cinerea* (Hain et al. 1993). Since then, grapevine *STS* genes have been transferred into a number of plants, including *Arabidopsis* (Christine et al. 2006), pea (Richter et al. 2006), lettuce (Liu et al. 2006), kiwifruit (Kobayashi et al. 2000), apple (Rühmann et al. 2006) and banana (Vishnevetsky et al. 2009), in each case resulting in significant improvements in pathogen resistance.

However, disease symptoms in transgenic lines are often only partially relieved (Delaunoy et al. 2009; Hipskind and Paiva 2000; Serazetdinova et al. 2005). In some cases, fungal infection is merely delayed, not prevented, such as in transgenic papaya plants (Zhu et al. 2004). (Serazetdinova et al. 2005) suggest that different transgenic lines express varying resistance to the same pathogen. Moreover, in other cases, STS-overexpressing plants have shown no signs of increased resistance to pathogens (Kobayashi et al.

2000), even though the transformed plants produced higher amounts of stilbene (Giorcelli et al. 2004). Therefore, the relationship between pathogen resistance and resveratrol content in transgenic plants requires further investigation.

Previously, our research group has isolated and characterized a number of stilbene synthase genes from the wild Chinese grapevine species, *V. pseudoreticulata* and *V. quinquangularis*. Transcript abundances of six *VpSTS* genes cloned from *V. pseudoreticulata* ‘Baihe-35-1’ were significantly up-regulated by inoculation with the powdery mildew fungus, *U. necator* (Wang et al. 2007). One *VpSTS* gene was successfully transformed into *V. vinifera* L. cv. Thompson Seedless and this resulted in a marked increase in resveratrol concentration, compared to non-transformed plants (Fan et al. 2008). More recently, one stilbene synthase gene *VpSTSgDNA2* was transferred to *V. vinifera* cv. Chardonnay and the transgenic plants exhibited increased levels of stilbene and H₂O₂ in comparison with wild type plants and, therefore, enhanced the transgenic plants’ resistance to *U. necator* (Dai et al. 2015). Our research group has also verified that all genotypes from *V. quinquangularis* have higher resveratrol contents than most *V. vinifera* grapevine cvs. This is especially true for cv. Danfeng-2, which contains much higher levels of resveratrol than other *V. quinquangularis* genotypes (Shi et al. 2014). Moreover, the expression of gene *VqSTS6* from cv. Danfeng-2 was markedly higher than the other *VqSTS* genes in the six genotypes examined. Therefore, we suggest that *VqSTS6* may be a key to achieving the best biological properties for the European grapevine, *V. vinifera* with respect to resistance to biotic stress.

In this paper, we generate transgenic *V. vinifera* Thompson Seedless plants overexpressing *VqSTS6* isolated from Chinese wild *V. quinquangularis* accession Danfeng-2, through embryogenesis. Our interest focusses on the role of over-expressed *VqSTS6* in influencing the resveratrol content and also tolerance to powdery mildew of the transgenic Thompson Seedless plants. Genes related to the resveratrol synthesis pathway were also analyzed. It is expected the findings of this study will stimulate interest in exploring the high intrinsic potential of the wild Chinese grape germplasm both for conventional breeding (hybridization) and also as a source of genes for transgenic work to enhance the disease resistance and resveratrol content of a wide range of table and wine grape cultivars.

Materials and methods

Plant materials

Chinese wild *Vitis quinquangularis* accession Danfeng-2 (leaves) and *Vitis vinifera* cv. Thompson Seedless (anthers)

were harvested from vines grown under natural conditions in the grape germplasm resources orchard of Northwest A&F University, in Yangling, Shaanxi, People’s Republic of China.

Binary vector construction and genetic transformation of grape

Genetic transformation assays of Chinese wild *V. quinquangularis* accession Danfeng-2 *STS* cDNA (*VqSTS6*) (GenBank Accession No. JQ868692) were carried out using *A. tumefaciens* strain GV3101 harboring pART-CAM-S binary vector (Fig. 1). This vector embodies the *VqSTS6* cDNA and *nptII* coding region, which confers kanamycin resistance as selectable marker.

Proembryonic masses (PEM) of *V. vinifera* Thompson Seedless which were initiated from immature anthers were used for genetic transformation of *VqSTS6* (Fig. 2). Transformation was carried out via the *Agrobacterium*-mediated transformation system as previously described by our group (Dai et al. 2015; Fan et al. 2008; Zhou et al. 2014). Germinated embryos were transferred to 50 ml woody plant medium (Aguero et al. 2006), supplemented with 0.20 mg l⁻¹ IBA, 0.15 mg l⁻¹ BAP and 1.0 g l⁻¹ activated charcoal (AC). Well-developed plants were transplanted into pots and kept in greenhouse conditions.

DNA extraction and semi-quantitative RT-PCR

Grapevine DNA was isolated from 0.5 to 1.0 g of in vitro grown transformed grape plants (Thomas et al. 1993). Specific oligonucleotide primers for gene sequences were designed to identify the presence of *VqSTS6* in genomic DNA. Primers for *VqSTS6* gene (sense primer 5'-ATGGCTTCAGTTGAGGAATTTA-3' and antisense primer 5'-TTAATTTGTAAGTGTAGG AATG-3') were used for the PCR reactions.

Protein extraction and Western blot

The protein extraction procedure was carried out according to the method of Méchin et al. (2007). About 20–25 µg of protein was used for SDS-PAGE and blotted onto a

polyvinylidene fluoride membrane (Roche, Product No. 03010040001). Immunodetection was carried out using Anti-Flag Mouse Monoclonal Antibody (TransGen Biotech, Beijing, China) combined with a secondary Goat Anti-Mouse IgG (H + L) horseradish peroxidase-conjugate antibody (TransGen Biotech, Beijing, China). Detection was carried out using Pierce ECL Western Blotting Substrate (Thermo Scientific) and imaged on a Chemi-Doc™ XRS + System with Image Lab™ Software (BIO-RAD).

Inoculation with powdery mildew fungus

Transgenic and non-transformed lines were assayed for powdery mildew *U. necator* resistance as described by Vidal et al. (2006). Three plants of each line were evaluated. Fully expanded leaves in the third or fourth positions from the shoot tip were chosen for inoculation. Symptoms of powdery mildew infection were recorded 24, 48, 72 and 168 h after inoculation. Leaves were fixed and prepared for microscopy by an established procedure (Vanacker et al. 2000).

Measurement of resveratrol contents

Fully expanded leaves collected from transgenic and non-transgenic *V. vinifera* cv. Thompson Seedless were used for resveratrol analysis by HPLC. Leaf samples (1.0 g) were ground to a fine powder in liquid nitrogen then transferred to a vacuum freeze drier (CS110-4, LaboGene, Denmark). After freeze drying for 48 h in the dark, samples were extracted in 1:20 (W/V) methanol overnight at 4 °C and centrifuged (J-LITE®JLA-16.250, Beckman Coulter Inc., CA, USA) for 15 min at 5500 rpm to remove insoluble debris. The supernatant was collected in separate glass tubes and filtered through a 0.22 µm membrane film before HPLC analysis. All these steps were performed under low-light conditions.

The extracts obtained were subjected to chromatographic analysis on an Agilent 1200 HPLC system with an Inertsil ODS-3 column (5.0 µm particle size, 4.6 × 250 mm; GL Sciences Inc., Tokyo, Japan). Elution was carried out using mobile phase A (acetonitrile) and

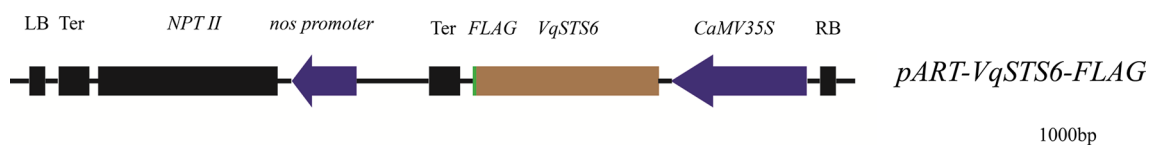


Fig. 1 Molecular analysis of transgenic lines of *Vitis vinifera* cv. Thompson Seedless. Schematic representation of the T-DNA region of the binary vector *pART-CAM-S*; *LB* left T-DNA border, *RB* right T-DNA border, *Ter* terminator, *CaMV35S* cauliflower mosaic virus

35S promoter *nos* promoter nopaline synthase promoter, *NPTII* neomycin phosphotransferase gene, *FLAG* a short, hydrophilic protein tag used for Western blot

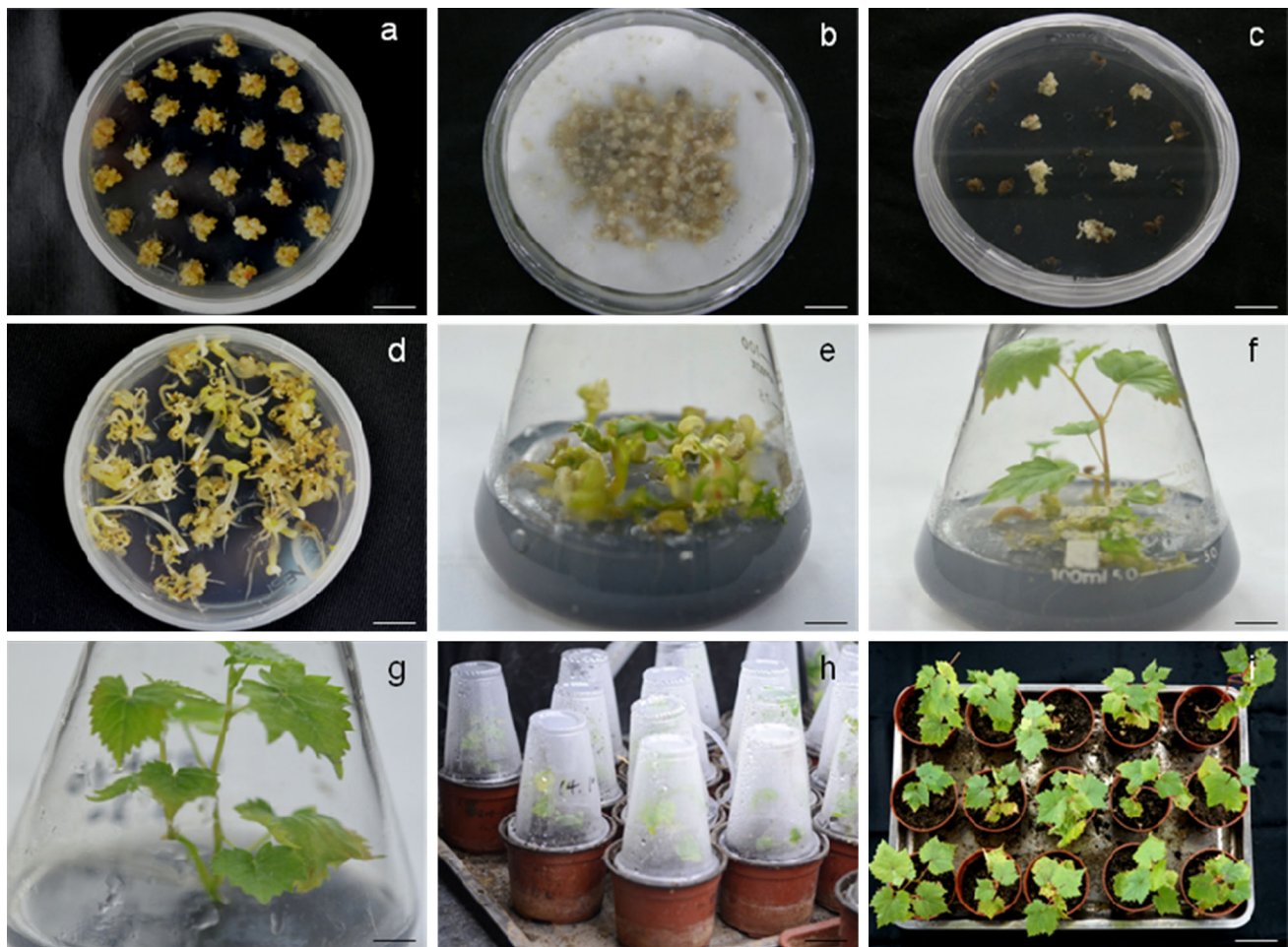


Fig. 2 Genetic transformation of gene *VqSTS6* via *Agrobacterium tumefaciens* and regeneration of *Vitis vinifera* cv. Thompson Seedless transgenic lines. **a** Pro-embryogenic masses (PEM) (*bar*: 1.5 cm); **b** co-culture of embryogenic callus and *Agrobacterium tumefaciens*

(*bar*: 1.5 cm); **c**, **d** resistant embryos formed (*bar*: 1.5 cm); **e**, **f** resistant plantlet germination (*bar*: 1 cm); **g** transgenic plantlet propagation (*bar*: 8 mm); **h** transgenic lines grown in growth room (*bar*: 5 cm); **i** transgenic lines grown in greenhouse (*bar*: 10 cm)

mobile phase B (water). The following linear gradient was used: 0 min: 20 % A, 80 % B; 0–30 min: 75 % A, 25 % B; 30–32 min: 100 % A, 0 % B; 32–35 min: 100 % A, 0 % B; 35–36 min: 20 % A, 80 % B; 36–45 min: 20 % A, 80 % B. The flow rate was 1.0 ml/min. Injection volume was 5 μ l with a diode array detector (Agilent Technology, Palo Alto, CA, USA) set to the absorbance wavelengths of 288 and 306 nm. The retention times for each of the components in the pool analyzed were compared to the following standards: ϵ -viniferin, pterostilbene, *trans*-resveratrol, *cis*-resveratrol, *trans*-piceid, and *cis*-piceid.

Quantitative real-time PCR

Total RNA was isolated from grape tissues using a small-scale method based on the RNeasy plant mini kit (Qiagen). DNase-treated total RNA (3 μ g) was reversely transcribed using the SuperScript III First-Strand Synthesis System for

RT-PCR (Invitrogen) using the Oligo (dT)₂₀ primer according to the manufacturer's instructions.

Specific primer pairs of six selected genes were designed (Table 1) using Primer Express 3.0 and tested by Real-time RT-PCR. Primers (10 μ M) 5'-CCCTTGCC TCCCAACTCT-3' and 5'-CCTTCTCAGCACTGTCCC T-3' were used for amplifying grapevine *GAPDH* (GenBank Accession No. GR883080) as an internal control. Quantitative real-time PCR was carried out using a SYBR Green method in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative expression level of each gene analyzed was calculated by comparison with the internal control.

Accession numbers

VqSTS6 (GenBank Accession No. JQ868692).
GAPDH (GenBank Accession No. GR883080).

Table 1 Genes selected for real-time RT-PCR

Gene	Description	Forward primer	Reverse primer	Target size
MYB14	NM_001281203	TCTGAGGCCGGATATCAAAC	GGGACGCATCAAGAGAGTGT	221
MYB15	KC514110	CAAGAATGAACAGATGGAGGAG	TCTGCGACTGCTGGGAAA	124
CHS	JF808008	CATCACAAATAGCGAACACAAG	CCTAGCATCCAGGGAAGC	158
STS	JX673940	CAAGCCCTTTTTGGTGATG	CCACAAGTGAAAGGTGAGTCC	171
PAL	XM_002268737	CTGAAGTTTTATCGGCTGTTTT	GATGGATTTAGTTGATGCTCG	275
RSGT	DQ832169	CTACCATGGCTTAGTCCCTTTC	AGCTGGCGATTTTCATCATACT	99

MYB14 (GenBank Accession No. NM_001281203).
MYB15 (GenBank Accession No. KC514110).
CHS (GenBank Accession No. JF808008).
STS (GenBank Accession No. JX673940).
PAL (GenBank Accession No. XM_002268737).
RSGT (GenBank Accession No. DQ832169).

Statistical analysis

Mean values of all experimental results were compared by one-way ANOVA and differences among results were tested by post hoc comparison test (Student–Newman–Keuls) at $p < 0.05$ with SPSS 21.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Molecular analysis of transformants

To further study the function of *VqSTS6* in grapevine, transgenic *V. vinifera* Thompson Seedless plants with the overexpression construct *VqSTS6* (Fig. 1) were obtained through embryogenesis via *Agrobacterium*-media transformation (Fig. 2). Gene introgression was confirmed by semi-quantitative RT-PCR analysis using specific primers for introduced *STS* and original genomic DNA in transgenic plants (Fig. 3). As *VqSTS6* is homologous to *VvSTS48* in *V. vinifera* and they both have an intron in the genes, PCR assay amplified two bands, including the genomic DNA model (1538 base pairs) and the complementary DNA model (1179 base pairs) from transgenic

lines, while only the genomic DNA model appeared in wild type and only the complementary DNA model presents in samples with transformed plasmid being amplified template. Moreover, no PCR signals for foreign genes were detected in the water control. Putative transgenic plants were transferred to a greenhouse for further analysis.

VqSTS6 is fused with a short peptide DYKDDDDK, known as FLAG, to avoid non-specific binding with other STS proteins, when conducting a Western blot experiment to confirm the protein expression level of *VqSTS6* in overexpression lines.

As shown in Fig. 4, Western blot analysis of leaf extracts with polyclonal antibody confirmed the presence of the expected proteins in these positive transgenic plants. Western blot results demonstrated various expression levels of STS in individual transgenic lines of *V. vinifera* cv. Thompson Seedless. Transgenic plants expressing STS could be recognized by the polyclonal antibody. No bands were detected from the non-transformed controls. A comparison of STS levels in transgenic plants with that in the positive control suggested that transgenic plants expressed more or less proteins than the positive control, and the transgenic plants (lines 2, 5 and 6) with higher expressions were selected for gene transcript analysis.

Assessment of transgenic plants for resistance to *Uncinula necator*

To investigate whether *VqSTS6* overexpression enhances resistance to pathogens, the lines 2, 5, 6 and the control lines were inoculated with *U. necator* (Fig. 5). We conducted a study of conidial germination and hyphal

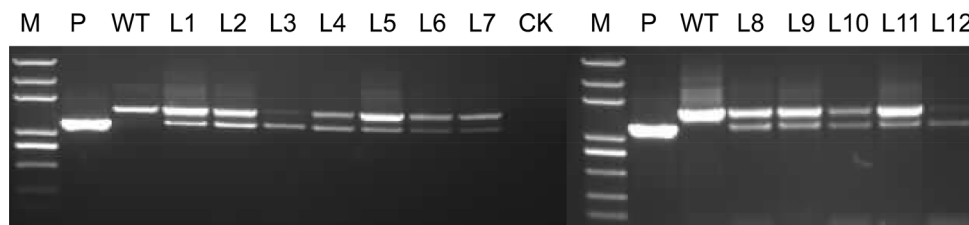
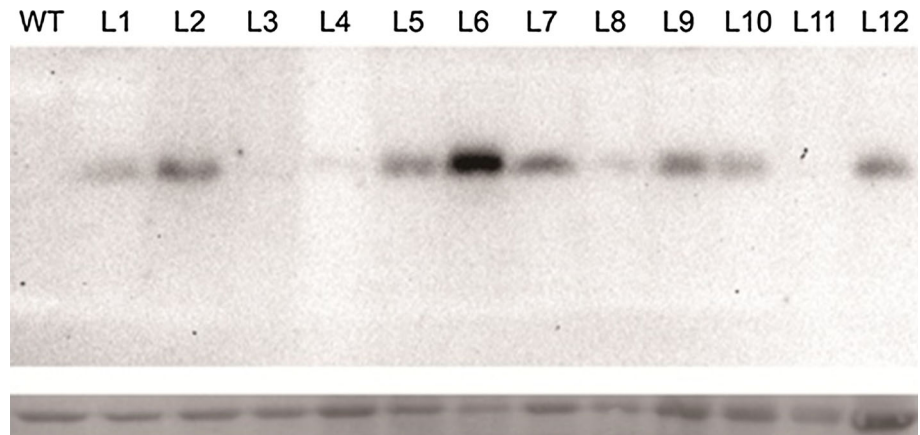


Fig. 3 PCR application of a 1538 bp DNA fragment of genomic DNA and complementary DNA fragments from the transgenic and wild type (WT) lines. *M* marker, *P* plasmid, *L1* to *L12* different transgenic lines containing construct of *VqSTS6*

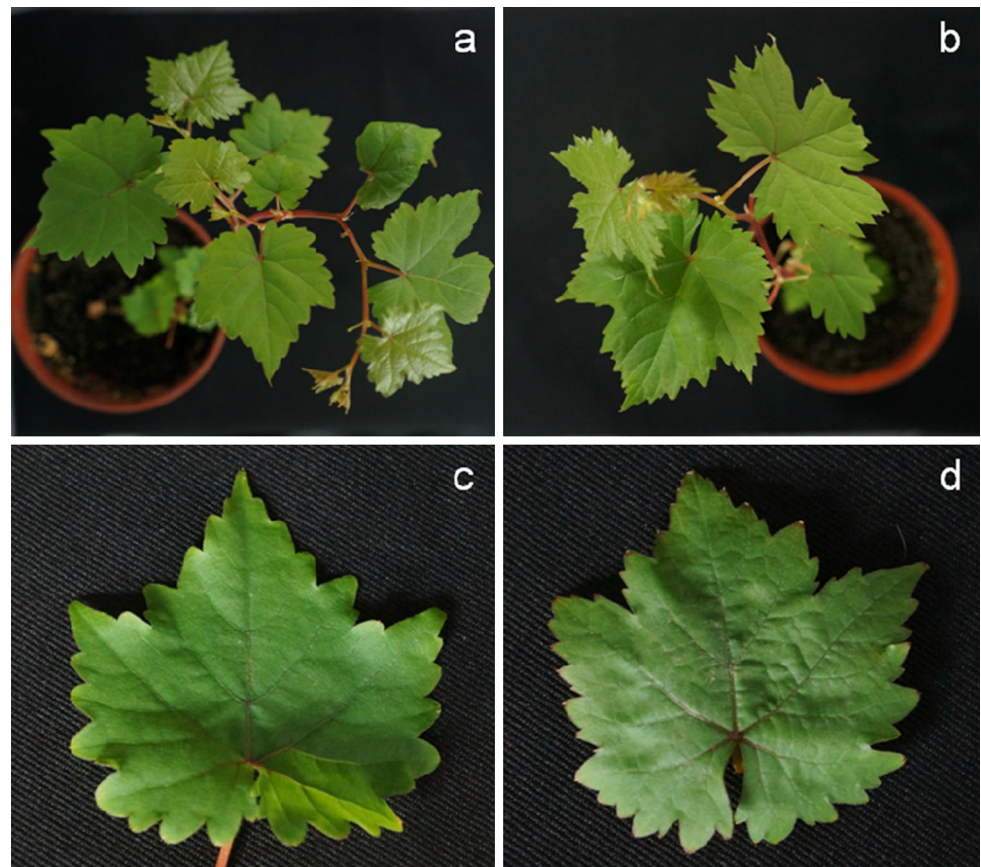
Fig. 4 Western blot detection for protein expression level of *VqSTS6* in different transgenic lines. *WT* wild type line of *VqSTS6*, *L1–L12* twelve transformed lines containing construct of *VqSTS6*



development over a 7-day period to compare the characteristics of powdery mildew-induced symptoms in the transgenic and control plants. Images at 24, 48, 72 and 168 hpi (hours post inoculation) are presented in Fig. 6, when the degrees of infection were evaluated (Table 2). The percentage of powdery mildew occurrence on the leaf surface after inoculation was used to measure the extent of resistance in transgenic plants. Severity was recorded at six positions on each (inoculated) leaf. Conidia produced

appressoria on both *V. vinifera* cv. Thompson Seedless transgenic and control leaves at 24 hpi (Fig. 6a, b). Hyphae were generated in both transgenic and control leaves at 48 hpi (Fig. 6c, d). The numbers of *U. necator* conidia produced at 24, 48 and 72 hpi were not significantly different between the transgenic lines and the wild type (Table 2). Moreover, the severity of infection was no higher in the control leaves than in the transgenic ones at 24 and 48 hpi. Brown-colored cells were observed beneath appressoria

Fig. 5 Comparison of powdery mildew-induced symptoms in transgenic plants (a, c) and non-transformed plants (b, d) at 168 h post inoculation, under natural growth conditions



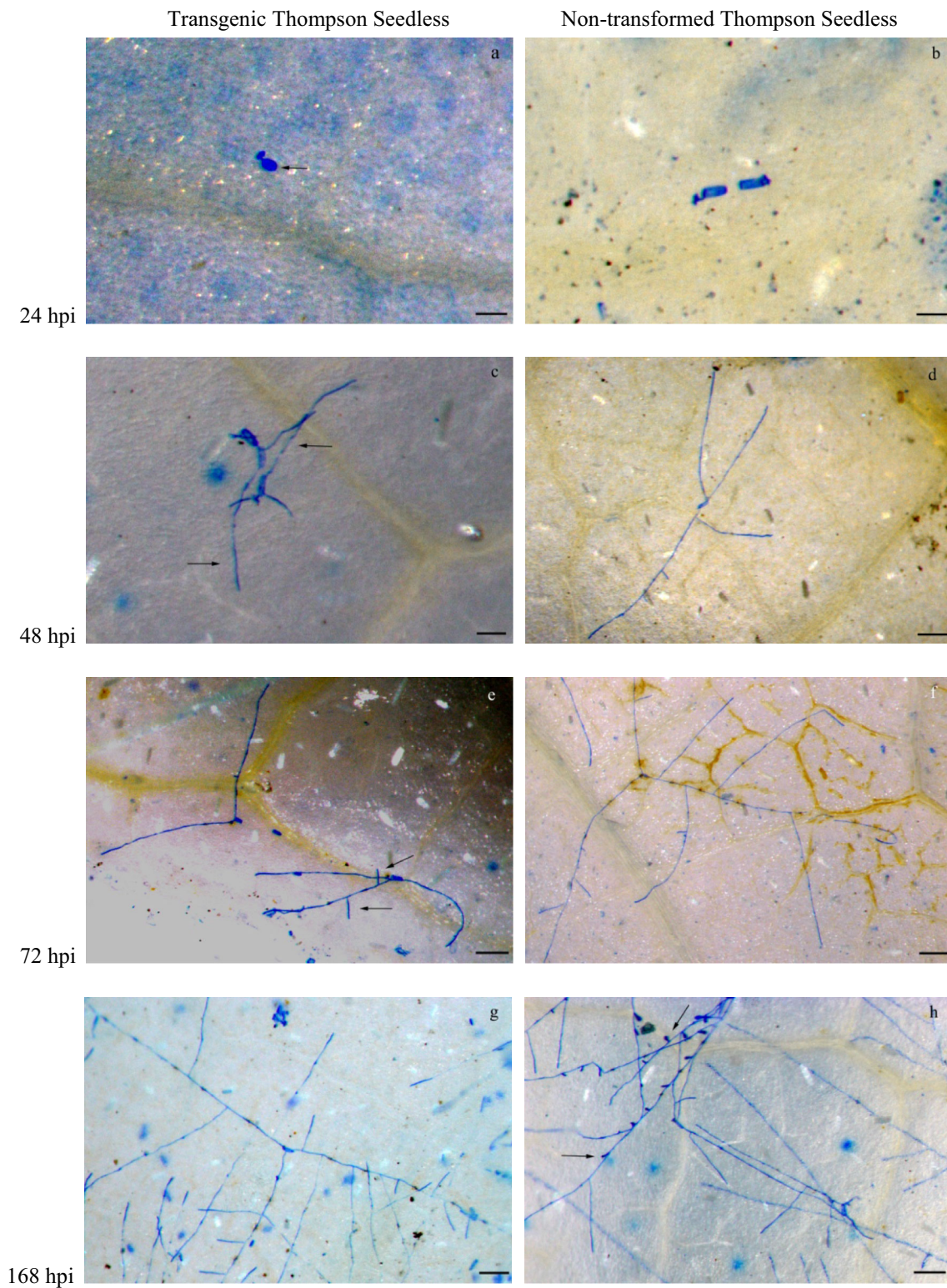


Fig. 6 Progression of powdery mildew on leaves of *Vitis vinifera* cv. Thompson Seedless. Images were taken at 24 (a, b), 48 (c, d), 72 (e, f), and 168 (g, h) hours post inoculation (hpi) of transgenic plants (a, c, e and g) and non-transformed plants (b, d, f and h). Scale bar 50 μ m

that had developed from secondary hyphae at 72 hpi (Fig. 6e, f). The numbers of secondary hyphae growing on the leaves of transgenic plants at 72 hpi, were significantly

lower than in the controls, revealing a lower susceptibility of the *VqSTS6* overexpressors, compared with the non-transformed controls. The infection led to the formation of

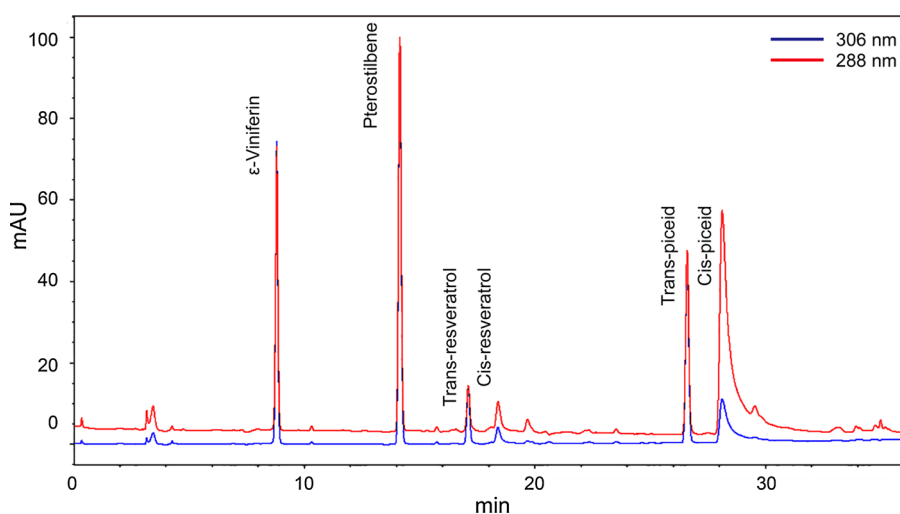
Table 2 Differences in response to powdery mildew *Uncinula necator* inoculations between transgenic and non-transformed plants of *Vitis vinifera* cv. Thompson Seedless

Inoculation Type	24 hpi			48 hpi			72 hpi			168 hpi
	No. of conidia	No. of hyphae	No. of secondary hyphae	No. of conidia	No. of hyphae	No. of secondary hyphae	No. of conidia	No. of hyphae	No. of secondary hyphae	No. of conidiophores
C	13 ± 2.1 ^a	0	0	34 ± 6.7 ^a	13 ± 1.9 ^a	0	49 ± 1.3 ^a	15 ± 0.9 ^a	11 ± 0.9 ^a	35 ± 5.2 ^a
L-2	14 ± 0.6 ^a	0	0	36 ± 5.1 ^a	15 ± 3.4 ^a	0	51 ± 1.9 ^a	17 ± 3.1 ^a	5 ± 1.0 ^b	17 ± 4.2 ^b
L-5	10 ± 1.6 ^a	0	0	40 ± 12.1 ^a	12 ± 4.3 ^a	0	46 ± 1.8 ^a	19 ± 4.7 ^a	3 ± 1.7 ^b	12 ± 1.0 ^b
L-6	13 ± 1.1 ^a	0	0	42 ± 2.4 ^a	10 ± 1.0 ^a	0	43 ± 7.4 ^a	13 ± 1.5 ^a	4 ± 0.6 ^b	15 ± 2.3 ^b

Each value represents the mean ± SE of three different experiments. Means with different letters are significantly different ($p < 0.05$). No significant differences were found between 24 and 48 hpi

No. number, C non-transformed wild type control line, L transgenic lines, hpi hours post inoculation

Fig. 7 HPLC chromatogram of standards at 306 nm (blue line) and 288 nm (red line). The six main peaks indicate (left to right): ϵ -viniferin, pterostilbene, *trans*-resveratrol, *cis*-resveratrol, *trans*-piceid, and *cis*-piceid



colonies with dense conidiophores on non-transformed leaves but only small colonies with conidiophores on transgenic leaves by 168 hpi (Fig. 6g, h). Furthermore, the number of conidiophores counted at 168 hpi showed that the difference between the *VqSTS6* overexpressors and the non-transformed control was statistically significant. Conidiophore formation on transgenic lines was lower than on non-transformed plants. To sum up, these data clearly demonstrate that overexpression of *VqSTS6* contributed to a significant reduction in the susceptibility of the transformed *V. vinifera* plants to *U. necator*.

Measurement of resveratrol contents of transgenic grapes

We used the optimized extraction conditions above coupled with HPLC analytical methods to compare the accumulation of resveratrol in leaves between transgenic lines (L2, L5, L6, L7 and L9) with non-transformed controls. We

specifically quantified the resultant ϵ -viniferin, pterostilbene, *trans*-resveratrol, *cis*-resveratrol, *trans*-piceid and *cis*-piceid (Fig. 7). As analyzed from the chromatograms, neither *cis*-resveratrol nor *cis*-piceid was detected in extracts from either transgenic or wild type plants, whereas ϵ -viniferin, pterostilbene, *trans*-resveratrol and *trans*-piceid accumulations at various levels were observed in transgenic lines (Table 3). In transgenic lines 2, 5 and 6, over-expressed *VqSTS6* greatly increased all the components of the stilbenes tested for, with ϵ -Viniferin being the predominant one. Line 5 had the highest *trans*-resveratrol concentration (108.94 $\mu\text{g/g}$ DW), followed by lines 2 and 6 (80.04 and 77.90 $\mu\text{g/g}$ DW, respectively). These were significantly higher than in lines 7, 9 and the controls. Pterostilbene and *trans*-piceid concentrations from lines 7 and 9 were very low, although the ϵ -viniferin concentrations of these two lines were significantly higher than in the controls. Moreover, lines 2, 5 and 6 had the highest resveratrol accumulations, which is in agreement with the Western blot result.

Table 3 Effects of genetic transformation on stilbenoid content (µg/g DW)

Line ¹	ε-Viniferin	Pterostilbene	Trans-resveratrol	Cis-resveratrol	Trans-piceid	Cis-piceid
WT#1 ²	23.38 ^b	2.00 ^b	–	–	5.87 ^b	–
WT#2	34.47 ^b	2.02 ^b	2.73 ^b	–	0.79 ^b	–
WT#3	55.72 ^b	2.57 ^b	–	–	–	–
L2 ³	155.86 ^a	78.53 ^a	80.04 ^a	–	93.88 ^a	–
L5	187.74 ^a	78.65 ^a	108.94 ^a	–	76.23 ^a	–
L6	144.96 ^a	77.10 ^a	77.90 ^a	–	75.65 ^a	–
L7	108.77 ^a	2.78 ^b	5.95 ^b	–	16.22 ^b	–
L9	109.90 ^a	2.25 ^b	10.71 ^b	–	16.11 ^b	–

Means followed by different letters within each column indicate significant differences ($p < 0.5$)

–, not determined

¹ Transgenic and control lines used in the comparisons

² Non-transformed wild type control

³ Transgenic lines

Quantitative real-time PCR analysis

To validate *STS* expression profiles, the steady-state transcript levels of six related genes were analyzed in transgenic lines 2, 5 and 6 using qRT-PCR. Among these, two genes (*STS* and *RSGT*) were up-regulated and two genes (*PAL* and *MYB14*) were down-regulated (Fig. 8). However, *MYB15* and *CHS* transcripts were not significantly changed compared with the controls. *GAPDH*, shown to be stable in previous work (Guan et al. 2011), was chosen as a reference gene for data normalization.

Discussion

Grapevine (*Vitis* spp.) is one of the world’s most important fruit crops. Powdery mildew (*Uncinula necator* (Schw.) Burr.) is one of the most damaging fungal diseases of the European grapevines (*Vitis vinifera* L.). Resistance of grapevines to this serious fungal disease has been reported in Chinese wild *Vitis* germplasm (He et al. 1991; Wan et al. 2015; Wang et al. 1995, 1998). In addition to their potential as sources of disease resistance genes, resveratrol contents were also significantly higher in most Chinese wild grapevines than in European *Vitis vinifera* grapevine cvs (Shi et al. 2014). Fruit of the Chinese wild *V. quinquangularis* accession Danfeng-2 were shown to contain much higher levels of resveratrol than other Chinese wild samples tested. Moreover, ‘Danfeng-2’, which originates from north-west China, is highly resistant to powdery mildew (Wan et al. 2015).

Here, we report the successful transformation of the Chinese wild *V. quinquangularis* accession Danfeng-2 *STS* cDNA gene (*VqSTS6*) into the European *V. vinifera* grapevine cultivar Thompson seedless, to show that *VqSTS6* plays a crucial role in enhancing the cultivar’s

biological properties and its resistance to biotic stress. The expression of resistance genes in transgenic plants, resulting in enhanced resistance, is one more approach of value to breeding research which is both precise and methodical (Mitra 2001). Recently, a number of natural and synthetic resistance genes from foreign varieties were successfully transformed into transgenic Indica rice (Chen et al. 2005), broccoli (Zhao et al. 2003), tomato (Ruf et al. 2001) and tobacco (Lindbo et al. 1993), causing enhanced resistance to pathogen infection. Transgenic grapevines with improved pathogen resistance have been reported in previous research. Yamamoto et al. (2000) introduced the rice chitinase gene (*RCC2*) into the somatic embryos of grapevine (*Vitis vinifera* L. cv. Neo Muscat) by *Agrobacterium* infection, and an increased defense against powdery mildew caused by *U. necator* was found. ‘Freedom’ grape expressing a *rpjF* gene, encoding the synthase for diffusible signal factor (DSF), from *Xylella fastidiosa*, reduced the severity of Pierce’s disease and also pathogen mobility in the plant (Lindow et al. 2014). A biotech-based solution for controlling Root-knot nematodes (RKNs) by introducing RNA interference (RNAi) to silence RKN effector gene (*16D10*), was used to enhance nematode resistance in transgenic grape hairy roots (Yang et al. 2013). Grapevine rootstock of *V. berlandieri* × *V. rupestris* cv. ‘Richter 110’ was transformed with an *Agrobacterium* oncogene-silencing transgene to establish crown gall-resistant lines (Galambos et al. 2013). To overcome production losses caused by chilling and freezing, an identified grapevine C-repeat binding factor (CBF) gene, *VvCBF4*, was over-expressed in grape vine cv. ‘Freedom’, which exhibited improved freezing survival (Tillett et al. 2012). Whereas, transgenic grapevines over-expressing *VvPIP2;4N*, which is an aquaporin gene, improved growth performance by modifying water metabolism in the absence of water stress, but drought stress resistance was

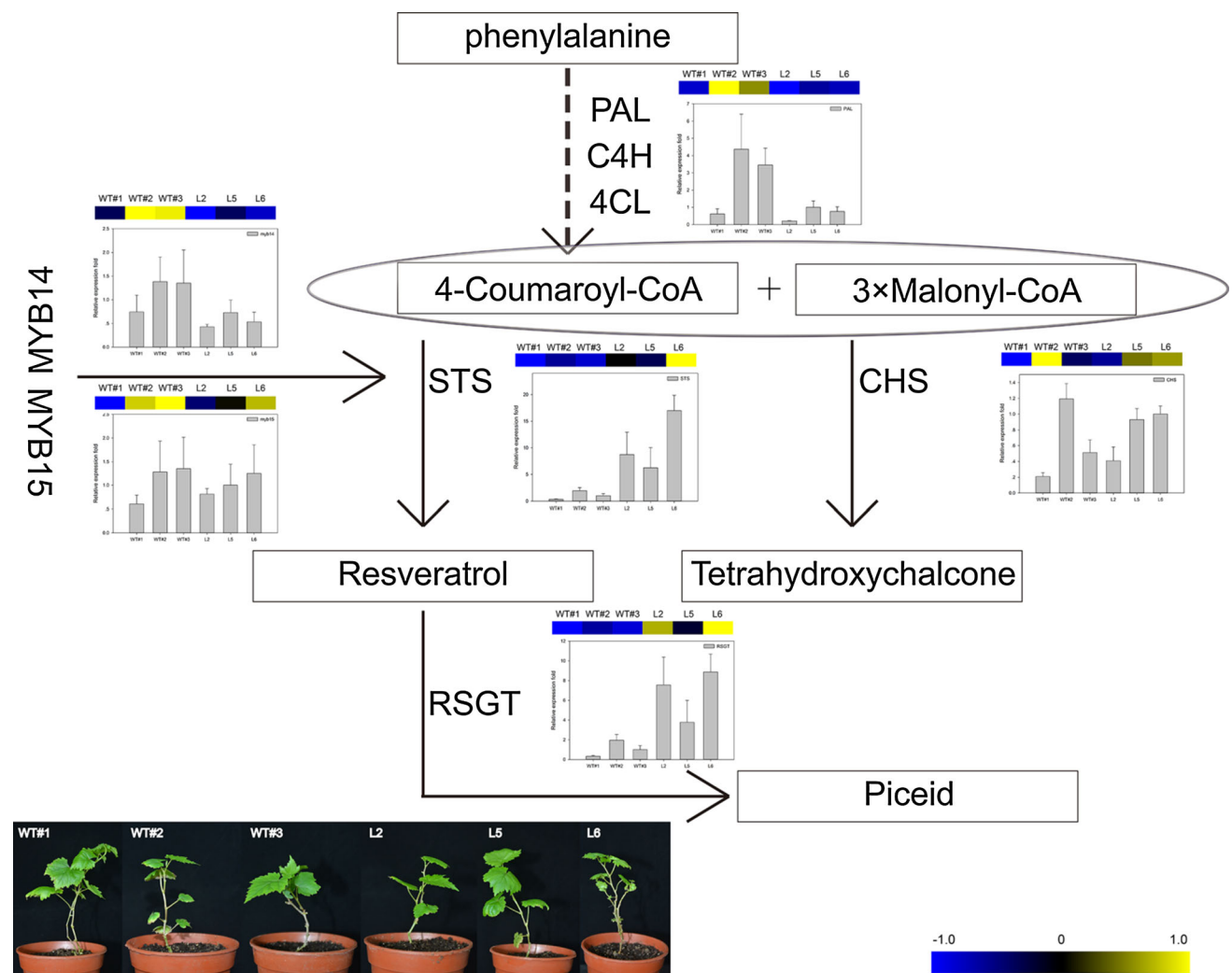


Fig. 8 A hypothetical model of the phenylalanine pathway for gene regulation in transgenic lines. RT-PCR expression data were standardized to create the color heat maps, where differences in gene expression are color coded (see key, *bottom, right*)

not induced in the research of Perrone et al. (2012). In the present study, we report the transformation of Thompson Seedless containing *VqSTS6* gene, which was selected from Chinese wild *V. quinquangularis* accession Danfeng-2. Transgenic plants regenerated from transformed PEM conferred lower susceptibility to *U. necator* and higher resveratrol accumulation, compared with non-transformed plants. Transgenic plants also displayed enhanced disease resistance to phytopathogenic fungi, although sometimes they did not contain high levels of free resveratrol (Liu et al. 2006). In our work, field trials are currently underway to evaluate their powdery mildew resistance under natural conditions but this study will take a number of years to complete.

The HPLC analyses showed that *trans*-resveratrol was present in some transgenic Thompson Seedless lines at higher concentrations than in the non-transformed control lines. To date, *trans*-resveratrol has also been detected in

transgenic apples (Szankowski et al. 2003), white poplars (Giorcelli et al. 2004) and grapes (Fan et al. 2008). Resveratrol also exists in the chemical form of *trans*-piceid. Kiwifruit (Kobayashi et al. 2000) and hop (Schwekendiek et al. 2007) transformed with a grape stilbene synthase gene produced piceid at concentrations reaching 182 $\mu\text{g/g}$ fresh weight (kiwifruit) and 560 $\mu\text{g/g}$ fresh weight (hop). In this study, however, the maximum amount of *trans*-piceid in transgenic leaves was only 93.9 $\mu\text{g/g}$ fresh weight.

Induced expression of key genes and increased resveratrol levels strongly suggest the resveratrol synthesis pathway is activated in the transgenic *V. vinifera* cv. Thompson Seedless leaf tissue. We found different expressions of a set of genes, including transcription factor genes and enzyme genes. Transcription factors, *MYB14* and *MYB15*, considered to interact with *STS* genes, were demonstrated to specifically activate the promoters of *STS*

genes, regulating stilbene biosynthesis in grapevine at transcriptional levels (Höll et al. 2013). However, results obtained in this study show that, compared with non-transformed controls, transcript levels of *MYB14* and *MYB15* were not increased along with *STS* overexpression. This may find its explanation in that obvious feedback did not take place through this pathway from transcription factors, MYB14 and MYB15, to *STS*. A possible reason for this may be due to competition between the introduced *STS* and the endogenous *CHS* for the substrates 4-coumaroyl CoA and malonyl CoA. *STS* and *CHS* are key enzymes of the flavonoid biosynthesis pathway (Jez and Noel 2000; Suh et al. 2000). It is reasonable to assume that the expression of an exogenous *STS* may lead to substrate competition with *CHS*, which possibly decreases *CHS* activity or that of other related pathways (Delaunoy et al. 2009). Such competition has also been observed between *STS* activity and resveratrol accumulation in transgenic white poplars (Giorcelli et al. 2004), and between anthocyanin activity and piceid accumulation in red-pigmented apple fruit (Rühmann et al. 2006). Similar results were found in this work. The transcript levels of chalcone synthase (*CHS*) in transgenic plants were not changed very much, although *STS* transcript levels were 22-times higher than in the controls. These results indicate that over-expression of *STS* affected secondary biosynthetic pathways in grapevine. The expression of *RSGT* in transgenic plants was significantly (30 %) higher than in non-transformed plants. Hall and De Luca (2007) found that *Vitis labrusca* grape berries accumulated both stilbene glucosides and hydroxycinnamic acid glucose esters, consistent with the bi-functional role of *RSGT* in stilbene and hydroxycinnamic acid modification. It may be that our over-expressed *STS* led to a rise in levels of hydroxycinnamic acid glucosyltransferase, with the result that accumulation of piceid was increased (Table 3). Stilbenes, including resveratrol, are synthesized via the phenylpropanoid pathway (Langcake and Pryce 1977). Phenylalanine ammonia-lyase (*PAL*) is the first enzyme in this pathway which catalyzes monooxidative deamination of phenylalanine (Phe) leading to cinnamate production (Jeandet et al. 2002). Our results indicate that the expression of *PAL* was down-regulated in the transgenic lines. A similar conclusion was reached in previous research. Resveratrol contents were increased in *V. amurensis* calli via an enhancement of expression of individual *STS* genes by coumaric acid (CA) treatment (Dubrovina et al. 2010). Meanwhile, the expression of *PAL* genes remained unchanged or was decreased by various concentrations of CA treatment, compared with non-transformed control plants (Shumakova et al. 2011). In summary, qRT-PCR analyses indicate that *VqSTS6* was involved in the resveratrol synthesis pathway in transgenic plants (Fig. 8).

In conclusion, we demonstrate here the genetic transformation of *VqSTS6* from Chinese wild *V. quinquangularis* accession Danfeng-2 into *V. vinifera* Thompson seedless plants. Following this successful integration of *VqSTS6* into the grapevine genome, we also detected the expression of this gene, protein accumulation and metabolite product accumulations, as well as an interaction with pathogen resistance of transgenic Thompson seedless plants. Genetic transformation of *VqSTS6* represents a significant step towards the goal of increasing the resveratrol content and pathogen resistance of *V. vinifera* grape cultivars—especially of the well-established winegrape cvs—for the improvement of their key horticultural traits.

Author contribution statement YW designed research. XX, SC, YX, CZ, XW, JZ and YW performed experiments. XX, YW analyzed the results. XX wrote the manuscript. All authors read and approved the manuscript.

Acknowledgments This work was supported by the Key Laboratory of Horticultural Plant Biology and Germplasm Innovation in Northwest China. The research was done with Grants from the National Science Foundation of China (Grant No. 31372039) and from the Program for Innovative Research Team of Grape Germplasm Resource and Breeding (2013KCT-25). The authors thank Dr. Alexander (Sandy) Lang from RESCRIPT Co. (New Zealand) for useful comments and language editing which have greatly improved the manuscript.

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