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ORIGINAL ARTICLE

Expression patterns and promoter characteristics of the *Vitis quinquangularis VqSTS36* gene involved in abiotic and biotic stress response

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Abstract Resveratrol is a stilbene compound that is synthesized by plants in response to biotic stress and has been linked to health benefits associated with the consumption of certain foods and food products, such as grapes and wine. The final step in the biosynthesis of resveratrol is catalyzed by the enzyme stilbene synthase (STS). Here, we assessed the expression of two *STS* genes (*VqSTS36* and *VpSTS36*) from the wild grape species *Vitis quinquangularis* (accession 'Shang-24'; powdery mildew (PM) resistant) and *Vitis pseudoreticulata* (accession 'Hunan-1'; PM susceptible) following infection by *Uncinula necator* (Schw.) Burr, the causal agent of PM disease. Some correlation was observed between the relative levels of *STS36* transcript and disease resistance. We also cloned the 5′ upstream sequence of both *VpSTS36* and *VqSTS36* and generated a series of 5′ *VqSTS36* promoter deletions fused to the *GUS* reporter gene in order to analyze expression in response to wounding, the application of exogenous stress-associated hormones, and biotic stress in tobacco leaves. The promoter was shown to be induced by the hormone salicylic acid (SA), inoculation with the fungal pathogen *Erysiphe cichoracearum*, and by wounding. These results suggest that *VqSTS36* is regulated by biotic stresses and that it plays an important role in mediating disease resistance in grape.

Keywords *Vitis* · Powdery mildew · Stilbene synthase · Gene expression · Promoter activity analysis · GUS

Introduction

Grapevine (*Vitis vinifera*) is an agriculturally and economically important fruit crop that is grown worldwide, which also provides a significant source of functional compounds, such as resveratrol and other derivatives. Grape production can be severely affected by powdery mildew (PM) disease caused by

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the fungus *Uncinula necator*, which can result in substantial production losses as well as significant decline of fruit quality. It has been shown that most European varieties of grapevine are highly susceptible to PM, while other *Vitis* species, such as *Vitis aestivalis*, *Vitis labrusca*, and *Vitis rupestris*, exhibit various levels of resistance (Mullins et al. 1992). Due to the high cost and negative effects associated with fungicide applications, the development of resistant cultivars using genetic material derived from wild grape species, which have better disease resistance than cultivated varieties, represents an attractive strategy to reduce the threat of PM disease.

Previous research focusing on PM resistance in grape has centered mainly on the cloning and functional analysis of disease resistance genes (Guo et al. 2016; Li et al. 2010), as well as the elucidation of defense mechanisms (Ficke et al. 2004). These defense mechanisms can include various modes of action, such as the generation of structural barriers or the production of antimicrobial compounds (Gurr and Rushton 2005). Stilbenes are one example of a defense-related compound belonging to a small family of phenylpropanoids that are synthesized by a broad taxonomic range of plant species, including monocotyledons such as sorghum (*Sorghum bicolor*) and dicotyledonous angiosperms, such as peanut (*Arachis hypogaea*) and grapevine (*V. vinifera*). In addition to their participation in defense processes, some stilbenes have also been attributed with valuable pharmacological properties, the most notable example being resveratrol (3,5,4′-trihydroxy-transstilbene), which can be found in red wine (Renaud and Delorgeril 1992).

Resveratrol is a phytoalexin with antifungal activity (Fung et al. 2008; Serazetdinova et al. 2005), which is synthesized via the phenylalanine pathway through the condensation of one molecule of p-coumaroyl-CoA and three molecules of malonyl-CoA. This reaction is catalyzed by the enzyme stilbene synthase (STS) (Rupprich and Kindl 1978), which is a member of the type III polyketide synthase superfamily. The heterologous expression of *Vitis STS* genes in various plant species, such as tobacco (Hain et al. 1993), alfalfa (Hipskind and Paiva 2000), tomato (Thomzik et al. 1997), papaya (Zhu et al. 2004), wheat (Fettig and Hess 1999), barley, and rice (Leckband and Lorz 1998), to name a few, has been found previously to confer increased pathogen resistance (Jeandet et al. 2010). While the strong constitutive CaMV35S promoter has been widely used for overexpressing *STS* genes (Christine et al. 2006; Delaunois et al. 2009; Fan et al. 2008; Fischer et al. 1997; Giorcelli et al. 2004; Giovinazzo et al. 2005; Kobayashi et al. 2000; Liu et al. 2006; Morelli et al. 2006; Nicoletti et al. 2007; Schwekendiek et al. 2007; Yu et al. 2005), it has often been observed that this high level of expression can cause negative effects (Osusky et al. 2004) by interfering with the growth of transgenic plants (Gatz and Lenk 1998) or by enhancing their susceptibility to other pathogens (Kim et al. 2006). The use of pathogen-inducible promoters can mitigate this problem, as they reduce unwanted transgenic expression under disease-free conditions (Gurr and Rushton 2005). For example, a grapevine promoter derived from the *VST1* gene of *V. vinifera*, which is a disease-susceptible cultivar, has been used for the purpose of engineering plant disease resistance previously (Hain et al. 1993; StarkLorenzen et al. 1997; Thomzik et al. 1997; Zhu et al. 2004). In addition, pathogen-responsive promoter elements of an *STS* gene from a PM-resistant accession of Chinese wild *Vitis pseudoreticulata* have been found to be an important motif (Xu et al. 2010). However, there remains a paucity of information concerning stress-responsive promoter elements controlling the expression of *STS* genes.

Interestingly, it has been shown that the production of stilbenes in pathogen-inoculated grape leaves is not as effective in reducing PM infection in resistant cultivars as it is in susceptible species (Schnee et al. 2008). This raises the question of whether the function of *STS* genes is distinct in different grapevine genotypes with disparate pathogen susceptibilities or whether their regulatory networks exhibit significant differences during a defense response. In order to explore this aspect, we investigated the expression of *STS36* genes associated with *U. necator* infection from both a susceptible cultivated grapevine, *V. pseudoreticulata* 'Hunan-1', and a resistant genotype, Chinese wild *Vitis quinquangularis* 'Shang-24'. Chinese native wild *Vitis* species hold potential as important resources for grapevine disease resistance (Wang et al. 1995), and Shang-24 is known to be a highly PM-resistant accession. Specifically, we cloned the *STS36* gene promoter sequences from both Shang-24 and Hunan-1 and carried out alignments and phylogenetic analyses. We also characterized the activities of a series of 5′ promoter deletions from the *VqSTS36* gene promoter following *Erysiphe cichoracearum* infection, salicylic acid (SA) application, and wounding in transgenic tobacco (*Nicotiana benthamiana)* leaves, to determine its potential use in increasing tolerance to pathogens and as a possible means of enhancing the resveratrol content of crops.

Materials and methods

Plant material and treatments

Chinese wild *V. quinquangularis* Shang-24 and *V. pseudoreticulata* Hunan-1 were collected from the Grape Repository of the Northwest A&F University, Yangling, Shaanxi, China. Shang-24 is highly resistant to *U. necator*, while Hunan-1 is susceptible to this pathogen (Wang et al. 1995). Both Shang-24 and Hunan-1 were grown in a greenhouse with appropriate environmental conditions. When vine shoots were 25–35 cm in length, young grapevine leaves were selected for inoculation with *U. necator*, which was collected from leaves of the field-grown *Vitis adstricta*, as previously described (Fung et al. 2008; Gao et al. 2012; Wang et al. 1995). The

inoculated leaves were enclosed within plastic bags for 12 h to maintain appropriate humidity. Treated leaves were collected at different time points (0, 6, 12, 24, 48, 72, 96, and 120 h) after pathogen inoculation.

Hormone treatment was conducted by spraying grape leaves with 100 μM salicylic acid solution, and treated leaves were sampled at 0, 1, 3, 6, 12, and 24 h post-treatment. All samples were frozen in liquid nitrogen and stored at −80 °C. Three biological replicate samples were collected for each time point.

Expression of *STS36* by semi-quantitative PCR and real-time PCR analysis

Total RNA was extracted as previously described (Zhang et al. 2003). First-strand complementary DNA (cDNA) was synthesized from 1 μg DNase-treated total RNA using a mixture of PolydT and random hexamer PrimeScript™ RTase (TaKaRa Biotechnology, Dalian, China). In the following experiments, the reverse transcription products were diluted 6-fold. The grape *ACTIN1* (GenBank accession no. AY680701) gene was used as a reference in order to adjust the concentration of the cDNA. Gene-specific primers designed using Primer Premier 5.0 for the *VqSTS36* genes are listed in Table 1. For semi-quantitative RT-PCR, a 20 μl reaction volume containing 2.0 μl of gene-specific primers (1.0 μM), 10.0 μl PCR Master Mix (Tiangen, Beijing, China), 1.0 μl of cDNA template, and 7.0 μl sterile distilled water was used. The semi-quantitative PCR reaction parameters were 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 25 s, and a final step at 72 °C for 2 min. Each PCR reaction was replicated three times, and three independent analyses displayed similar trends. Quantitative real-time PCR analysis was conducted using SYBR Green (TaKaRa Biotechnology) with an IQ5 real-time PCR machine (Bio-Rad, Hercules, CA, USA). Each reaction was carried out in triplicate, each with a final volume of 20 μl containing 1.0 μl cDNA template, 0.8 μl each primer (1.0 μM), 10.0 μl SYBR® *Premix Ex Taq*™ II (TaKaRa Biotechnology), and 7.4 μl sterile distilled H_2O. Cycling parameters were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Melting curve analysis was carried out at 95 °C for 15 s, followed by a constant increase from 60 to 95 °C after the PCR cycles. Grape *ACTIN1* was amplified as an internal control. Relative expression levels were analyzed using IQ5 software and the normalized expression method (Hou et al. 2013). A one-sided paired *t* test was performed using SigmaPlot 10.0 (Ashburn, VA, USA) to assess significant differences.

Table 1 Primer sequences used in the study

Primer name	Primer sequence (5′-3′)
STS36-F (qPCR)	CGGGTATAAATTAAGTGAAGGGGAA
STS36-R (qPCR)	GGGGGGATAATGAAACAGTGAGATA
STS36-F (clone)	ATGGCTTCAGTTGAGGAAATCAG
STS36-R (clone)	GATAATGAAACAGTGAGATA
P*VqSTS36*-R	TCTGCAGCAGCATAATCAGACTGGTAGA
P*VqSTS36*-Del-F1	GGGATCCGACCCTTTATCGTAGTTCA
P*VqSTS36*-Del-F2	CGGATCCTTGCCTCAATCCTTATCC
P*VqSTS*36-Del-F3	CGCGGATCCAATATGTTTGATGCTTAT
P*VqSTS*36-Del-F4	CGCGGATCCCTTTGAACTTGAAATG
P*VqSTS*36-Del-F5	CGCGGATCCATGAGATATTTGTTGAA
P*VqSTS*36-Del-F6	CGGATCCTGGATTAGGGTTGGTGA
ACTIN1-F	GATTCTGGTGATGGTGTGAGT
ACTIN1-R	GACAATTTCCCGTTCAGCAGT

Cloning of *STS* genes and promoters from two *Vitis* genotypes

Genomic DNA was extracted with phenol/chloroform and precipitated with ethanol. Genomic DNA and cDNA was dissolved in sterile water for subsequent cloning. PCR with gene-specific primers (Table 1) was performed to clone *STS* genes and isolate promoter fragments, each of which were subsequently inserted into the pMD 19-T cloning vector (TaKaRa Biotechnology) using the *Pst* I and *Bam*H I restriction sites. The constructs were transformed into the *Escherichia coli* strain DH5a (TaKaRa Biotechnology). Plasmid extraction kits, DNA gel extraction kits, and DNA and protein markers were obtained from Tiangen Biotechnology. A positive candidate clone was sequenced at Invitrogen Biotechnology, and the promoter sequences were analyzed using the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) database (Lescot et al. 2002).

Construction of S-24 STS::GUS (β-glucuronidase) fusion vectors

For this study, we used the pC0380GUS and pC35SGUS vectors (Xu et al. 2010) for transient expression assays. The 1.9 kb 5′ upstream region of *PvqSTS36*, as well as five nested 5′ deletion fragments (1.7, 1.4, 0.8, 0.6, and 0.2 kb), was cloned using PCR amplification from extracted DNA of *V. quinquangularis* Shang-24. Five forward primers and one reverse primer (Table 1) were designed to separate the −1991, −1750, −1444, −872, −680, and −243 sequences of the *VqSTS36* promoter. A *Pst* I site was introduced at the 3′ end of the reverse primer, while a *Bam*H I site was added to the 5′ end of each of the five forward primers (Table 1). These five promoter fragments were all double digested with the appropriate restriction enzymes and subsequently inserted into the pC0380GUS vector. Deletion constructs that were verified by sequencing and digestion

were transformed into *Agrobacterium tumefaciens* GV3101 by electroporation.

Agrobacterium-mediated transient expression assay in tobacco plants

Agrobacterium-mediated transient expression assays were performed as previously described (Sparkes et al. 2006). Fully expanded tobacco (*N. benthamiana*) leaves that had been infiltrated with *Agrobacterium* harboring pC35SGUS as a positive control were collected and used to determine GUS activity, as previously described (Xu et al. 2010).

Biotic and abiotic stress treatments

Agrobacterium-infiltrated (as above) tobacco (*N. benthamiana*) leaves were inoculated with *E. cichoracearum* DC, wounded, or treated with SA 48 h after infiltration. The *E. cichoracearum* DC isolate was collected from leaves of greenhouse-grown accession 'qinyan96'. *N. benthamiana* leaves were inoculated by touching with sporulating colonies from the surface of pathogen-infected leaves. Each inoculation was repeated three times. Tobacco leaves were sampled at 24 h for the GUS assay or the specific time points (0, 4, 8, 12, 24, 36, 48, and 60 h) for quantitative real-time PCR analysis after *E. cichoracearum* inoculation. To induce mechanical wounding, a sterile inoculation needle was used to generate three wounds along the veins of the leaves. The treated tobacco leaves and control leaves were collected for GUS assays 24 h after wounding treatment. For the hormonal treatment, tobacco leaves were sprayed with 100 μM SA or 0.1% ethanol solution (mock treatment). GUS assays using the SA-sprayed and mock-treated leaves were carried out 24 h after treatment.

Analysis of *GUS* transcript levels

To quantify *GUS* transcript levels in inoculated and untreated *N. benthamiana* leaves, quantitative real-time PCR analysis was conducted using SYBR Green (TaKaRa Biotechnology) with an IQ5 real-time PCR machine (Bio-Rad, Hercules, CA, USA). Total RNA was extracted from tobacco leaf samples using the E.Z.N.A.® Plant RNA Kit (Omega Bio-Tek, USA, R6827-01), and the concentration was adjusted to 1 μg prior to reverse transcription. These reactions were performed in 96-well plates (20 μl/well) with mixtures including 1 μl of template cDNA, 0.8 μl of each primer (each as a 10 μM stock), 10.0 μl of SYBR Premix Ex™ TaqII, and 7.4 μl of ddH_2O. Reactions were conducted in triplicate. Cycling parameters were described previously. *GUS* transcript levels were calculated using the normalized-expression method and normalized using the tobacco ubiquitin gene (*UBI*: GenBank accession no. U66264). Real-time PCR amplifications were conducted using the following primers: *GUS*, F 5′-ATT ATG CGG GCA ACG TCT GGT ATC AG-3′ and R 5′-CAT CGG CTT C AA ATG GCG TAG C-3′ and *UBI*, F 5′-ATG AAC GCT GGC GGC ATG CTT A-3′ and R 5-AGA TCT GCA TTC CTC CCC TCA GCT A-3′.

GUS activity assay

Histochemical and quantitative GUS assays were conducted as described previously and expressed as nanomolar of 4-methylumbelliferon (4-MU; Sigma-Aldrich) produced per minute per milligram of soluble protein (Jefferson 1987). To calculate the fluorescence of the 4-MU output, a Hitachi 850 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) was used. The total concentration of proteins was determine using the protein dye binding assay (Bradford 1976) using a Nicolet Evolution 300 UV-Vis spectrophotometer (Thermo Electron Corp., Madison, WI, USA) with bovine serum albumin as the standard. For data analysis, one-way ANOVA tests were used to calculate means comparison. *t* Tests were conducted to show significant differences between treatment and control samples using the software package of SigmaPlot 10.0 (Ashburn, VA, USA). Differences at $P < 0.05$ and $P < 0.01$ were considered significant.

Results

Expression patterns of the grape *VqSTS36* and *VpSTS36* genes following PM and SA treatment

Expression of the *VqSTS36* gene from a PM-resistant accession of *V. quinquangularis* in response to PM was analyzed using semi-quantitative RT-PCR in leaves at different time points (0, 6, 12, 24, 48, 72, 96, and 120 h) after inoculation. The grapevine *ACTIN1* gene was used as an internal control to normalize expression levels. *VqSTS36* transcript levels increased substantially following PM infection from 0 to 24 hpi, decreased from 24 to 48 hpi, and then increased again from 48 to 120 hpi (Fig. 1). In contrast, the expression levels of *VpSTS36* in infected leaves from a highly susceptible genotype of *V. pseudoreticulata* were lower than in the PM-resistant leaves, and between 0 and 120 hpi, the expression levels in the former were much lower than those in the latter. As for hormone treatment, results showed that both *VqSTS36* and *VpSTS36* exhibited increased expression levels following SA treatment (Fig. S1).

Analysis of the *VqSTS36* promoter and corresponding sequences from other Chinese wild species

To determine whether the differential expression patterns of the *VqSTS36* and *VpSTS36* genes correlate with the regulation of their promoters, the non-coding upstream genomic regions

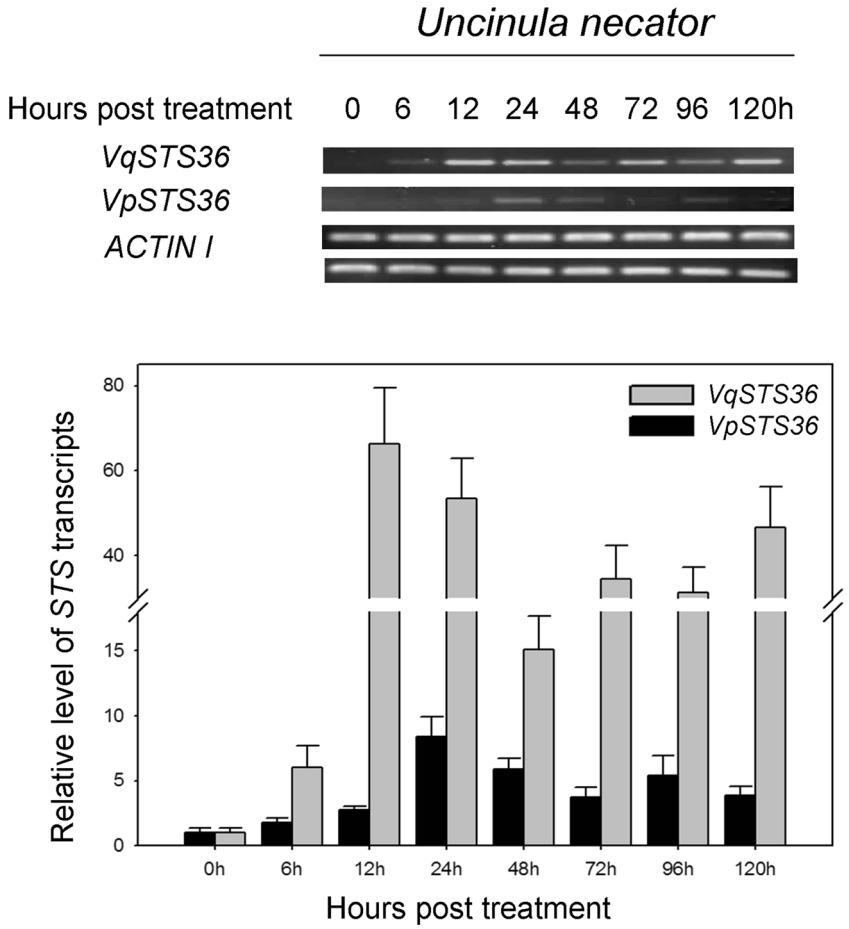

Fig. 1 Expression of *VqSTS36* and *VpSTS36* in response to *Uncinula necator* inoculation. For preparation of total RNA, grape leaves were collected at the indicated times after *U. necator* inoculation. *ACTIN1* was used as a constitutive control

from *VqSTS36* and *VpSTS36* were compared with those from *VvcSTS* (GenBank accession no. GU269272), *VvtSTS* (GenBank accession no. GU269273), *VpSTS* (GenBank accession no. FJ605484), and *VvSTS36*. The upstream regions of *VqSTS36* and *VpSTS36* were 1991 and 1976 bp in length, respectively. A sequence alignment showed that the *VqSTS36* promoter demonstrated 58% homology with the promoters of *VvcSTS* and *VvtSTS*, but the latter two were 94% identical to each other (Fig. 2). Finally, there was 98% identity between the 1991 bp *VqSTS36* promoter sequence and the 1976 bp *VpSTS36* promoter sequence (Fig. 3). In addition, we also aligned the coding regions and 5′ UTR regions of the *STS36* genes from the Hunan-1 and 'Baihe 35-1' accessions. The results are shown in Figs. S2 and S3.

Based on a detailed analysis of responsive functions of *cis*-regulatory elements within their promoters, three functional groups were classified: defense and stress-responsive elements (DSREs), hormone-responsive elements (HREs), and light-responsive elements (LREs). As shown in Fig. 4 and Table 2, the DSREs consisted of MYB binding site (MBS), defense and stress-responsive element (TC-rich repeats), hypoxia-responsive element (GC motif), high transcription level-related element (5′ UTR Py-rich stretch), low-temperature-responsive element (LTR), and heat stress-

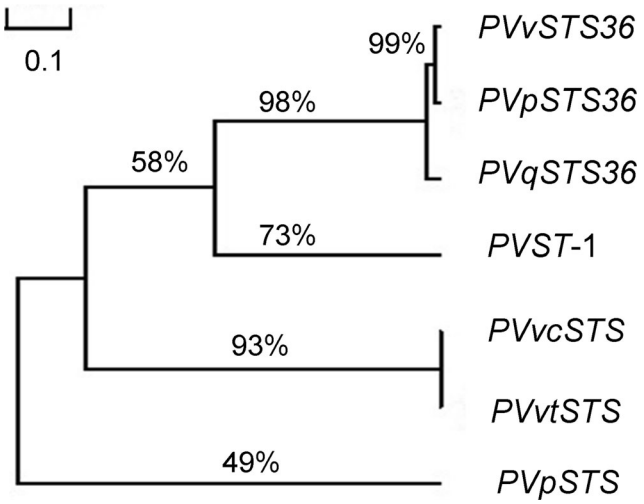

Fig. 2 Phylogenetic tree based on ClustalX multiple sequence alignment of *STS* promoters from a range of grape species (Thompson et al. 1997). The *scale bar* represents 0.1 substitutions per site, and the *numbers* next to the nodes are bootstrap values from 1000 replicates. Phylogenetic tree of stilbene synthase promoter sequences constructed from *V. pseudoreticulata* (Genebank No. FJ605484), *V. thompson* (GU269273), *V. carignane* (GU269272), and *V. optima Vst1* (Y18532)

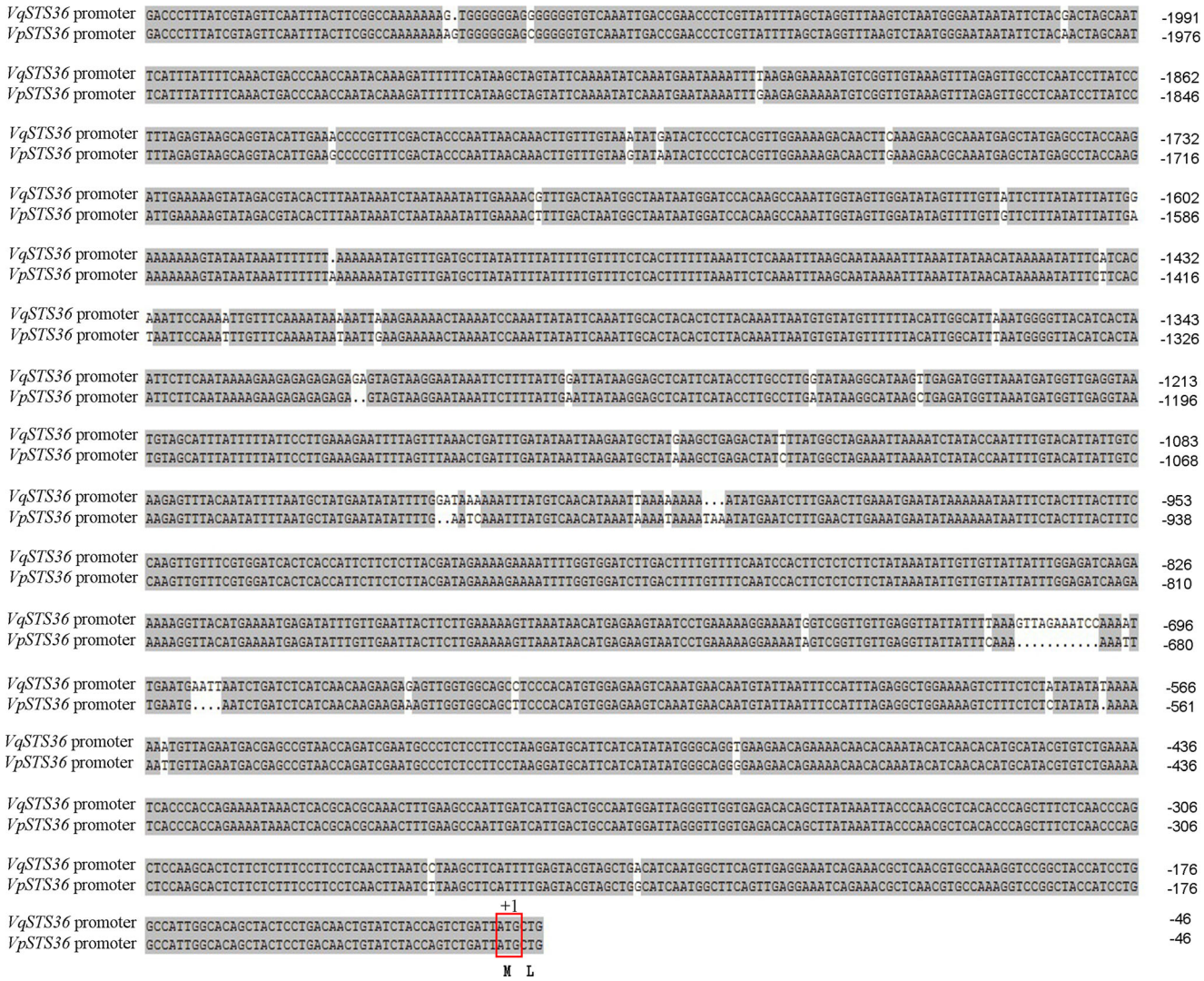

Fig. 3 A comparison of the *PVpSTS36* and *PVqSTS36* promoter sequences. Conserved sequences are shown with *shaded nucleotides*. The translational start sites (+1) are shown in *red*

responsive element (HSE) (Daraselia et al. 1996; Diaz-De-Leon et al. 1993; Dolferus et al. 1994; Nash et al. 1990; Pastuglia et al. 1997; White et al. 1994). The HREs included abscisic acid (ABA)-responsive elements (ABRE), MeJA-responsive elements (CGTCA motif and TGACG motif, ethylene-responsive element (ERE), and salicylic acid-responsive element (TCA element) (Baker et al. 1994; Fink et al. 1988; Itzhaki and Woodson 1993). The LREs included relevant elements such as Box I, G-box, and L-box (ArguelloAstorga and HerreraEstrella 1996; Foster et al. 1994; Logemann et al. 1995). Furthermore, in the promoter region of *VqSTS36*, additional predicted *cis*-regulatory elements such as an AC-I element, GCN4 motif, TCCC motif, GT1 motif, and circadian element were also confirmed. As shown in Fig. 4 and Table 2, the majority of the predicted *cis*-regulatory elements are associated with response to different abiotic or biotic stress conditions, indicating that the *VqSTS36* promoter may have a significant function in response to defense and stress regulation.

Responsiveness of the *VqSTS36* promoter to abiotic stress

To investigate the potential regulation of the *VqSTS36* promoter by the defense-related hormone SA, the 1991 bp promoter fragment was inserted immediately upstream of the promoterless *GUS* reporter gene, as were a variety of promoter deletions (−1750, −1444, −872, −680, and −243) (Fig. 5). All constructs were transformed into tobacco leaves via infiltration with *Agrobacterium* and then sprayed with 100 μM SA or 0.1% ethanol solutions and harvested 24 h after treatment. A promoterless construct (pC0380GUS) served as a negative control, and a *CaMV35S*::GUS (pC35SGUS) construct was regarded as a positive control. The highest SA-inducible promoter activity, corresponding to a 5.3-fold increase in GUS activity compared to mock-treated leaves, was detected in leaves expressing the P1750 construct (Fig. 5). After 24 h of SA treatment, GUS activities of the 1991 bp promoter fragment and deletion constructs of −1444, −872, and −680 bp

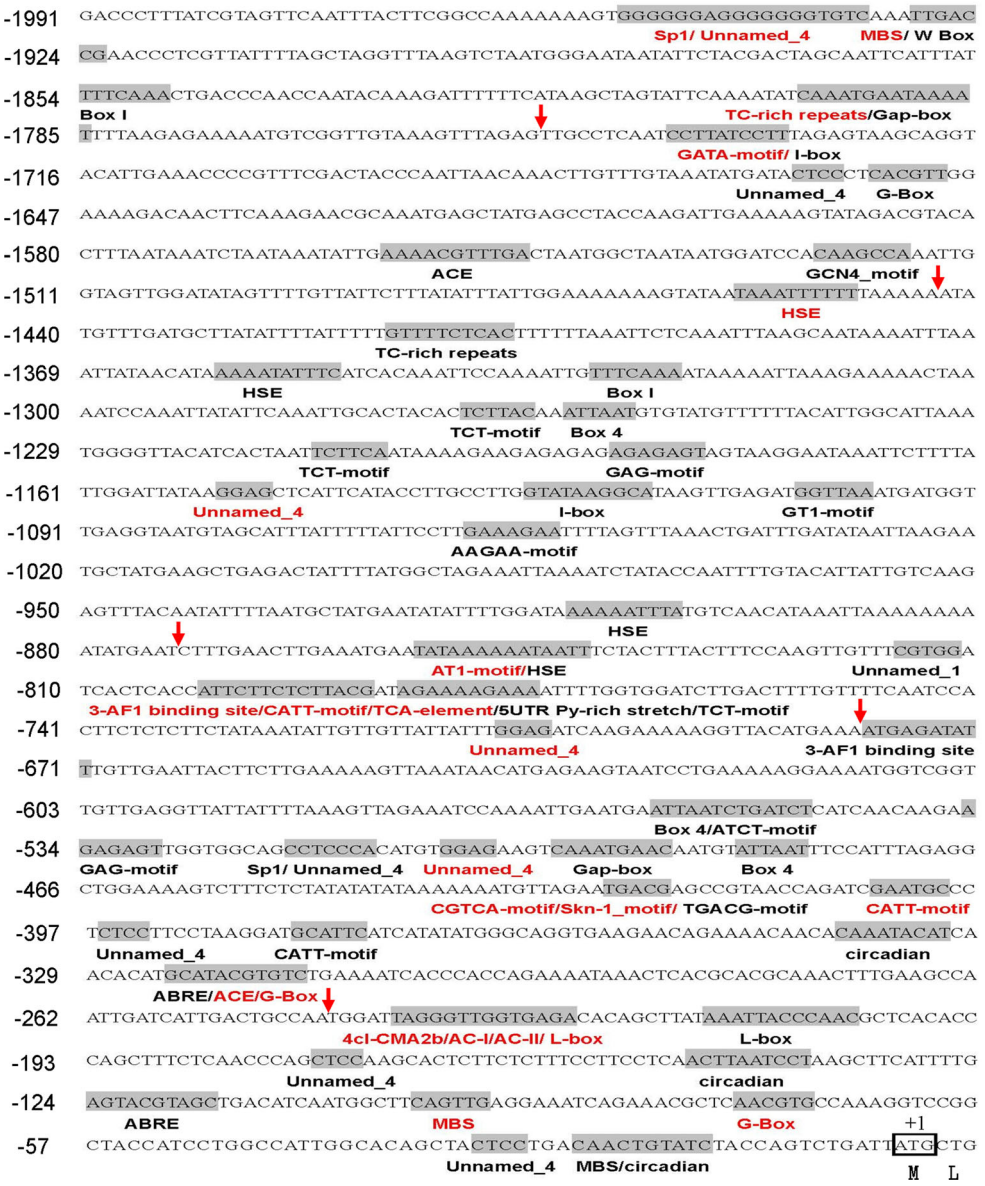

Fig. 4 Nucleotide sequence of the promoter of *VqSTS36* from Chinese wild *Vitis quinquangularis* 'Shang-24'. Identified *cis*-acting elements are *shaded*, and the names are shown under the elements. *Arrowheads* represent starting points of 5′ deleted derivatives. The translational start sites (+1) are shown in *black*. *Red typeface* represents the reverse direction of *cis*-elements compared to promoter orientation (5′-3′)

promoter regions were also increased significantly when compared with mock-treated leaves.

We also evaluated the activity of the *VqSTS36* promoter in response to wounding. The highest inducible GUS activity was detected in the −1991-bp-long *VqSTS36* promoter region, which was approximately 2.4-fold higher after wounding than that in the control leaves (Fig. 6). Although the induction of GUS activity with the P1750, P1444, and P872 deletion constructs was lower than when the full promoter was utilized, we still observed a 1.9-, 1.6-, and 1.7-fold increase compared to mock-treated leaves. However, when −680 and −243 bp *VqSTS36* promoter regions were used, only comparatively low levels of GUS activity were detected, compared with mock-treated leaves. These results indicate that regulatory elements for activation are present in the *VqSTS36* upstream promoter sequence and that these activation elements may be located in the region between −1991 and −1458.

Responsiveness of the *VqSTS36* promoter to defense pathogen attack

With the aim of testing pathogen inducibility and locating pathogen-responsive *cis*-regulatory regions within the *VqSTS36* promoter, tobacco leaves were inoculated with *E. cichoracearum* DC or sterile water, and each deletion construct was tested for GUS activity (Fig. 7a). A *CaMV35S*::GUS (pC35SGUS) construct and a promoterless construct (pC0380GUS) served as a positive control and a negative control, respectively.

Table 2 Number of *cis*-acting elements involved in stress-responsive and pathogen-responsive expression among five *Vitis* genotypes

Name	Sequences	Number of *cis* elements					Function
		V. vinifera Carignane	*V. vinifera* Thompson seedless	*V. pseudoreticulata* Baihe35-1	*V. quinquangularis* Shang-24	*V. pseudoreticulata* Hunan-1	
ABRE	CACGTG	2	2	2	2	2	*Cis*-acting element involved in the abscisic acid responsiveness
AuxRR-core	GGTCCAT	0	0	1	0	0	*Cis*-acting regulatory element involved in auxin responsiveness
Box S	AGCCACC	0	0	1	0	0	Elicitation, wounding and pathogen Responsiveness
W-box	TTGACC	2	2	1	1	1	Fungal elicitor responsive element
CCAAT-box	CAACGG	1	0	0	0	0	MYBHv1 binding site
CGTCA motif	CGTCA	1	1	0	1	0	*Cis*-acting regulatory element involved in the MeJA responsiveness
ERE	ATTTCAAA	1	1	0	0	1	Ethylene-responsive element
GARE motif	AAACAGA	1	1	0	0	0	Gibberellin-responsive element
GC motif	CCCCCG	0	0	0	0	1	Enhancer-like element involved in anoxic-specific inducibility
HSE	AGAAAATTCG	1	1	0	4	4	*Cis*-acting element involved in heat stress responsiveness
LTR	CCGAAA	0	0	2	0	0	*Cis*-acting element involved in low-temperature responsiveness
MBS	CAACTG	2	2	2	3	3	MYB binding site
P-box	CCTTTTG	0	0	1	0	0	Gibberellin-responsive element
TCA element	GAGA AGAATA	0	0	0	1	1	*Cis*-acting element involved in salicylic acid responsiveness
TC-rich repeats	ATTCTCTAAC	0	0	1	2	3	*Cis*-acting element involved in defense and stress responsiveness
TGACG motif	TGACG	1	1	0	1	1	*Cis*-acting regulatory element involved in the MeJA responsiveness
WUN motif	TCATTACGAA	1	1	0	0	0	Wound-responsive element
5′ UTR Py-rich stretch	TTTCTTCTCT	2	2	1	2	3	*Cis*-acting element conferring high transcription levels

Table 2 (continued)

Name	Sequences	Number of *cis* elements					Function
		V. vinifera Carignane	*V. vinifera* Thompson seedless	*V. pseudoreticulata* Baihe35-1	*V. quinquangularis* Shang-24	*V. pseudoreticulata* Hunan-1	
AC-I	CCCACCTACC	3	3	2	1	1	Enhanced xylem expression and repressed phloem expression
AC-II	TCAA CCAACTCC	0	0	0	1	1	Enhanced xylem expression and repressed phloem
ATGC AAAT motif	ATACAAAT	1	1	0	0	0	*Cis*-acting regulatory element associated to the TGAGTCA motif
CAAT-box	CCAAT	62	66	45	55	59	Common *cis*-acting element in promoter and enhancer regions
CAT-box	GCCACT	0	0	1	0	0	*Cis*-acting regulatory element related to meristem expression
Circadian	CAANNNNATC	1	1	3	3	3	*Cis*-acting regulatory element involved in circadian control
GCN4_ motif	CAAGCCA	1	1	3	1	1	*Cis*-regulatory element involved in endosperm expression
Skn-1_motif	GTCAT	8	9	5	1	1	*Cis*-acting regulatory element required for endosperm expression
TATA-box	TATA	70	88	47	78	76	Core promoter element around −30 of transcription start
as-2-box	GATAATGATG	0	0	1	0	0	Involved in shoot-specific expression and light responsiveness
ATCT motif	AATCTAATCC	0	0	1	1	1	Part of a conserved DNA module involved in light responsiveness
Box 4	ATTAAT	3	3	1	3	2	Part of a conserved DNA module involved in light responsiveness

The *CaMV35S* promoter displayed no significant induction after leaf inoculation with *E. cichoracearum*, and leaves expressing either the negative control or wild-type leaves had much lower levels of GUS activity than leaves transformed with the *VqSTS36* promoter. The highest levels of GUS activity were observed when the full −1991 *VqSTS36* promoter sequence was present, with GUS activity being induced up to 7.97-fold 24 h following *E. cichoracearum* inoculation (*$P < 0.05$ or at **$P < 0.01$). Compared with the highest GUS activity that was detected with the *PvqSTS36* (−1991) and *PvqSTS36* (−680) promoter regions, the −1444 region of the *VqSTS36* promoter led to a considerably lower level of GUS activity. Finally, transformation with the −243 bp region also yielded a significant increase in *E. cichoracearu*-inducible GUS expression, showing a 4-fold enhancement compared to mock-treated leaves. These results suggest that regulatory activation elements are present in the *VqSTS36* promoter and that they may be located in the regions between −1991 and −1750 and −680 and −243. As expected, little or no GUS activity was detected in wild-type leaves, but in the tobacco leaves containing

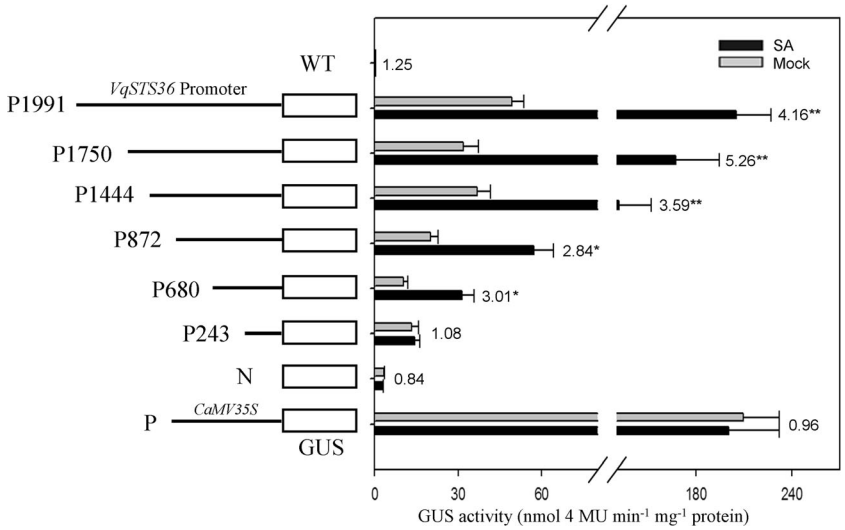

Fig. 5 Deletion analysis of *VqSTS36* promoter GUS activity in response to SA treatment in transiently transformed tobacco leaves. Schematic diagram of vector constructs used for transient expression assays is indicated on the *left*. Mean GUS activity (±SD) is averaged from triplicate experiments, and SD is shown on each *bar*. *WT* wild type, *N* negative control (no promoter), *P* positive control (CaMV 35S promoter). *Numbers* above the bars show the fold induction in GUS activity with SA over mock-treated sample. Significant differences were evaluated using a one-sided paired *t* test (**$P < 0.01$ or at *$P < 0.05$)

the promoterless GUS construct, a small amount of background GUS activity was detected. Compared with untreated wild-type tobacco leaves, GUS activity was induced after *E. cichoracearu* inoculation (Fig. 7a). Moreover, histochemical staining patterns showed in Fig. 7b also support these findings.

Regulation of the *VqSTS36* promoter in response to pathogen attack

To further elucidate how the *VqSTS36* promoter confers the differential transcriptional regulation of *VqSTS36* expression following *E. cichoracearum* inoculation, quantitative real-time RT-PCR assays were used to detect *GUS* expression levels in tobacco leaves that had been transiently transformed with each of the promoter–*GUS* constructs. Infiltrated tobacco leaves were harvested after being treated with *E. cichoracearum* for 48 h. Compared with untreated tobacco leaves, *GUS* expression was higher after *E. cichoracearum* inoculation (Fig. 7c). A low and comparable basal expression level was detected in transformed leaves at 0 h, and then, a transient increase occurred in the inoculated leaves at 0–12 h. A peak in *GUS* messenger RNA (mRNA) levels occurred at 12 h in transformed tobacco leaves.

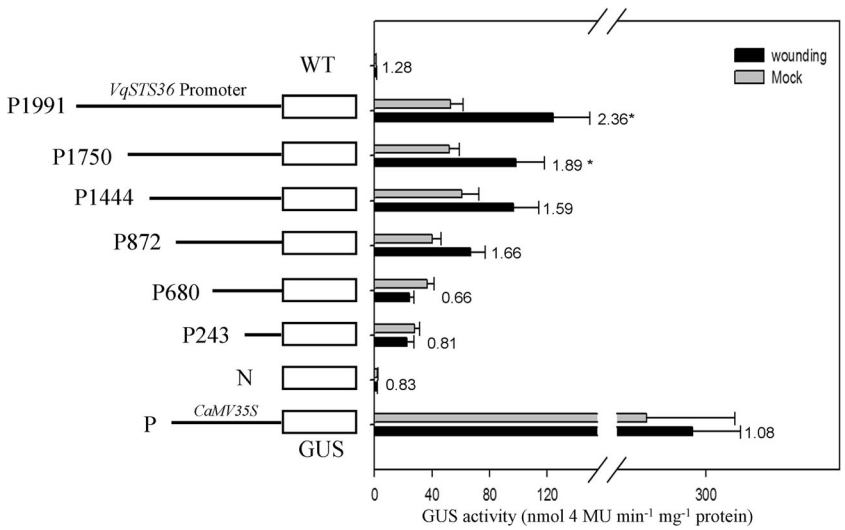

Fig. 6 Deletion analysis of the *VqSTS36* promoter GUS activity induced by wounding in transiently transformed tobacco leaves. Schematic diagram of vector constructs used for transient expression assays is indicated on the *left*. Mean GUS activity (±SD) is averaged from triplicate experiments, and SD is shown on each *bar*. *WT* wild type, *N* negative control (no promoter), *P* positive control (CaMV 35S promoter). *Numbers* above the bars show the fold induction in GUS activity with wound-treated over mock-treated sample. Significant differences were evaluated using a one-sided paired *t* test (**$P < 0.01$ or at *$P < 0.05$)

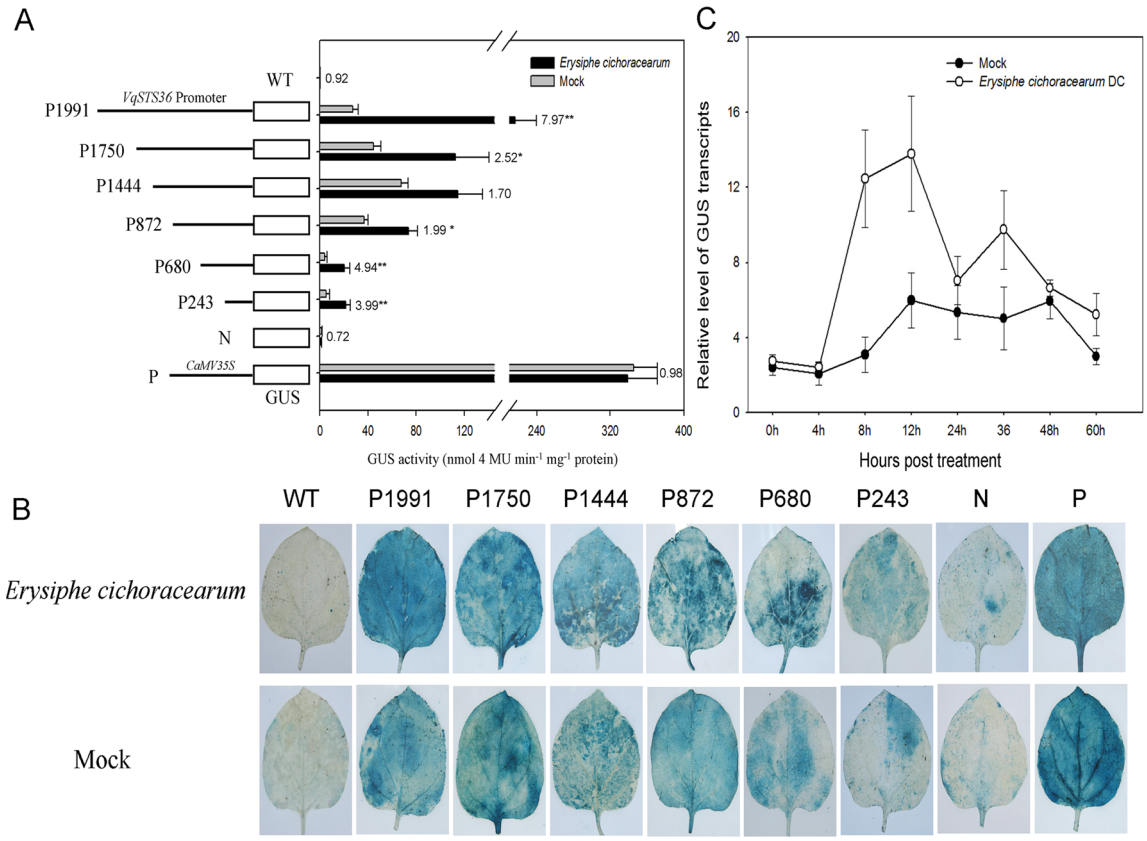

Fig. 7 **a** Deletion analysis of *VqSTS36* promoter activity induced by *Erysiphe cichoracearum* in transiently transformed tobacco leaves. *WT* wild type, *N* negative control (no promoter), *P* positive control (CaMV 35S promoter). Mean GUS activity (±SD) represents an average of triplicate experiments, and SD is indicated on each *bar*. *Numbers* above the bars indicate the fold difference in *E. cichoracearum* infection-induced GUS activity compared to that of the mock-inoculated sample. Significant differences were evaluated using a one-sided paired *t* test (**$P < 0.01$ or at *$P < 0.05$). **b** Histochemical assays of GUS activity in transiently transformed *N. benthamiana* leaves inoculated with *Erysiphe cichoracearum*. Tobacco leaves were infiltrated with each of the deletion constructs. GUS staining was conducted 24 h after inoculation with *E. cichoracearum* (*first line*) or mock-treated leaves (*second line*). **c** Activity of the *VqSTS36* promoter in tobacco at different time points after *Erysiphe cichoracearum* inoculation, performed by real-time RT-PCR. Results are averaged from triplicate experiments, and SD is shown on each *bar*

Subsequently, *GUS* mRNA abundance decreased to considerably lower levels in the treated tobacco leaves, while there was no significant change in mock-treated plants. A second peak was observed in inoculated leaves expressing the *VqSTS36* promoter constructs at 36 h.

Discussion

It has previously been shown that in peanut, grapevine, and pine tree, stilbene biosynthesis can be induced by a variety of biotic and abiotic stresses as a result of upregulation of *STS* genes (Fliegmann et al. 1992; Lanz et al. 1990; Sparvoli et al. 1994; Suzuki et al. 2015; Yin et al. 2016). Responses to biotic stresses, such as pathogen infection and insect herbivory, can be mediated by all kinds of signaling molecules including the hormones jasmonic acid (JA), SA, and ethylene (Bari and Jones 2009; Giraud et al. 2012). Stress-mediated regulation of stilbene biosynthesis is especially well understood in grapevine, where expression of *STS* genes and synthesis of stilbenes can occur in response to infection with various fungal pathogens, such as the causal agent of PM, *U. necator* (Fung et al. 2008; Schnee et al. 2008), and *Plasmopara viticola*, which causes downy mildew (Adrian et al. 1997; Langcake and Pryce 1976). In this study, we focused on PM, a crucial fungal disease of cultivated grapevine, although wild grapevine species, such as *V. quinquangularis*, can show considerable PM resistance (Wang et al. 1995).

Our results indicate that *VqSTS36* transcript from the PM-resistant *V. quinquangularis* Shang-24 accumulated transiently after *U. necator* inoculation. In agreement with previous studies (Langcake and Pryce 1976), expression of *VqSTS36* was relatively low prior to PM inoculation. However, after pathogen infection, expression of *VqSTS36* was induced between 12 and 24 hpi. In contrast, the *VpSTS36* expression pattern in PM-susceptible *V. pseudoreticulata* Baihe 35-1

(Xu et al. 2010) was quite different, suggesting a distinct transcriptional regulation pattern.

We assumed that this difference might reflect the use of different cultivars and experimental conditions, suggesting complex modes of regulation of *STS* genes in response to various stress conditions. In order to analyze the mode of resistance between two accessions of the same species, we aligned the coding regions and 5′ UTR regions of Hunan-1 and Baihe 35-1 accessions, which both belong to Chinese wild *V. pseudoreticulata* (Figs. S2 and S3). In Fig. S2, the sequence alignment indicated that the *STS36* coding region from the Hunan-1 accession demonstrated 90.59% homology with the *STS36* coding region from Baihe 35-1. This high homology suggests that *STS* genes are highly conserved. In this case, we surmised that the promoter regions of these two accessions (Hunan-1 and Baihe 35-1) might correlate with the regulation of the differential expression patterns of *STS36* genes. Indeed, there was only 47.17% identity between the *STS36* non-coding upstream promoter regions from these two accessions (Fig. S3). Based on our detailed sequence alignment analysis, we inferred that the differences between the *STS36* promoter regions may play a key role in their response to *U. necator*. Only low levels of *STS36* transcript were detected in the leaves of the susceptible accession Hunan-1, and expression of *VpSTS36* increased gradually with time after *U. necator* infection. These results demonstrate that the expression levels of *VqSTS36* and *VpSTS36* may be involved in a novel defense profile. In agreement with our findings, *STS* was found to be upregulated in non-pathogen-challenged *V. aestivalis* compared to *V. vinifera* (Fung et al. 2007). As such, we further hypothesized that the differential regulation pattern of *STS36* between PM-resistant *V. quinquangularis* Shang-24 and PM-susceptible *V. pseudoreticulata* Hunan-1 is a consequence of diverse regulatory mechanisms achieved by *cis*-regulatory elements in the promoter regions.

The genomic organization and promoter activation of the *VqSTS36* gene were subsequently examined. Our results demonstrated that the *V. quinquangularis* promoter shared 73% identity with the *V. vinifera VST*-1 gene promoter, but only 49% identity with the *VpSTS36* promoter from *V. pseudoreticulata* Baihe 35-1. These significant differences may provide information concerning the regulatory mechanisms behind the phenylpropanoid pathway in Shang-24 and also suggest differences in the upstream regulatory sequences of the *STS* promoters of *V. quinquangularis* Shang-24 and *V. pseudoreticulata* Baihe 35-1. Based on the above findings, we decided to identify the functional regions of the *VqSTS36* promoter and to anatomize these elements with respect to their responses to a pathogen, SA, and wounding. To this end, we generated a series of 5′ deletion *VqSTS36* promoter constructs and evaluated their expression in tobacco leaves.

The *VqSTS36* promoter was found to contain a TCA element in the −820 bp region (Fig. 4). Since this *cis*-acting element is known to be involved in the response to SA, we investigated whether the application of 100 μM SA for 24 h to infiltrated tobacco leaves had an effect on promoter activity. After SA exposure, *VqSTS36* promoter activity was substantially higher compared with the control, and it is likely that the TCA element was involved in this activation. It is now well-established that phytohormones play significant roles in plant responses to stimuli. SA plays an important role in host stress responses as a key signaling molecule, and increases in endogenous SA levels are associated with the expression of pathogenesis-related genes (Jayakannan et al. 2015; Shah 2003). Furthermore, the W-box sequences located in the promoter region of the *Arabidopsis thaliana NPR1* gene, which is regulated specifically by SA-induced WRKY DNA-binding proteins, have been well-studied (Yu et al. 2001). It is worth noting that a W-box element exists in the −1800 *VqSTS36* promoter region, and the increased *GUS* expression demonstrated by this particular promoter deletion construct provides further evidence for the activity of the sequence located in this region.

In the wounding experiments, *VqSTS36* promoter activity was highly induced, in agreement with a previous study (Jeandet et al. 1997). We also observed that wounding was linked to high induction mediated by the −1991, −1744, −1450, or −872 promoter regions, whereas a reduction in GUS activity was conferred by the −680 region, suggesting that regulatory sequences suppressing promoter activity may be present in the region between −680 and −243 bp. These findings indicate that mechanical wounding may function in much the same way as exogenous stimuli, which can activate the promoter (Xu et al. 2010).

Next, a tobacco-based pathogen-inducible system was used to perform deletion analyses of the *VqSTS36* promoter. Slight differences were found in the −1450 and −872 regions. However, GUS activity was significantly induced when the entire −1991 region was present, suggesting that regulatory sequences suppressing promoter activity may be present in the region between −1444 and −872. Analyses using the PlantCARE program indicated the *VqSTS36* promoter contains several pathogen-responsive elements. One example is the W-box, which has previously been identified as the binding site of WRKY transcription factors, which regulate the transcriptional plant defense response (Eulgem and Somssich 2007; Guo et al. 2014; Liu et al. 2015; Pandey et al. 2010). A 6 bp W-box (5′-TTGACC-3′) was discovered in the promoter region located between −1929 and −1924, and this motif might contribute to the significantly higher levels of activity of the full *VqSTS36* promoter when leaves were inoculated by *E. cichoracearum*. TC-rich repeats have also been identified as defense and stress-responsive elements in *Nicotiana tabacum* (Diaz-De-Leon et al. 1993), and two TC-rich-like repeat motifs were detected located between −1794 and −1786 and −1415 and −1407 of the *VqSTS36* promoter

region, although the first was in the reverse direction relative to the *VqSTS36* promoter.

When taken together with data from our inoculation experiments, it appears that pathogen-inducible regulatory elements may be located in the *VqSTS36* promoter and that they may interact with each other. *GUS* gene expression in infiltrated tobacco leaves (Fig. 7c) suggests that the *VqSTS36* promoter exhibits significantly higher levels of activity when leaves were inoculated with *E. cichoracearum*. In agreement with our results, it has been reported that the *STS* promoter from *V. pseudoreticulata* Baihe 35-1 is activated in response to pathogen inoculation (Xu et al. 2010, 2011). These observations indicated that the *VqSTS36* promoter regulates gene expression under different inoculation conditions. Our analysis also identified other pathogen-responsive elements that have been reported in the promoter regions of genes from other plant species, including *A. thaliana* (Eulgem and Somssich 2007). Although pathogen-responsive elements can differ among species, all have the same role in controlling gene expression under inoculated or uninoculated conditions (Li et al. 2013). In this case, the *VqSTS36* promoter, in controlling gene expression following pathogen inoculation, might make a significant contribution towards enhanced disease resistance.

In conclusion, the differential expression patterns of *STS36* genes were explored in two grapevine accessions following PM inoculation. Comparative analysis of the *STS36* promoter regions in these two accessions demonstrated that their *cis*-elements from non-coding upstream sequences are divergent from each other, implying new methods for studying gene regulation mechanisms. Promoter regions include highly divergent sequences, and its activities are under the control of combination of multiple proteins. However, knowledge concerning the complicated interactions that command gene expression is very limited (Agius et al. 2005). Additional studies are therefore needed in the future to enable the expression of native genes or transgenes for use in genetic engineering or plant biotechnology.

CaMV35S, cauliflower mosaic virus 35S promoter; GUS, β-glucuronidase; MES, 2-(*N*-morpholino)ethanesulphonic acid; MUG, 4-methyl umbelliferyl glucuronide; PM, powdery mildew; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; SA, salicylic acid; *VpSTS36*, *Vitis pseudoreticulata* stilbene synthase36; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid; 4-MU, 4-methylumbelliferone.

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Author contributions XW and XY: conceived and designed the experiments. XY, LH, and HW: performed the experiments. XW, XZ, CG, and ZL: contributed reagents/materials/analysis tools. XW: provided guidance for the entire study. XY and XW: wrote the manuscript. All authors approved the final manuscript.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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