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ORIGINAL ARTICLE

Alfin-like transcription factor VqAL4 regulates a stilbene synthase to enhance powdery mildew resistance in grapevine

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Abstract

Resveratrol is a phytoalexin that is synthesized by stilbene synthase (STS). Resveratrol in the human diet is known to have beneficial effects on health. We previously identified six novel STS (*VqNSTS*) transcripts from the transcriptome data of *Vitis quinquangularis* accession Danfeng-2. However, the functions of and defensive mechanisms triggered by these *VqNSTS* transcripts remain unknown. In the present study, we demonstrate that the expression of five of these six novel members, *VqNSTS2*–*VqNSTS6*, can be induced by the powdery mildew-causing fungus *Uncinula necator*. Additionally, overexpression of *VqNSTS4* in the *V. vinifera* susceptible cultivar Thompson Seedless promoted accumulation of stilbenes and enhanced resistance to *U. necator* by activating salicylic acid (SA) signalling. Furthermore, our results indicate that the Alfin-like (AL) transcription factor VqAL4 can directly bind to the G-rich element (CACCTC) in the *VqNSTS4* promoter and activate gene expression. Moreover, overexpression of *VqAL4* in Thompson Seedless enhanced resistance to *U. necator* by promoting stilbene accumulation and activating SA signalling. Conversely, RNA interference-mediated silencing of *VqNSTS4* and *VqAL4* resulted in increased susceptibility to *U. necator*. Collectively, our results reveal that *VqNSTS4*, regulated by VqAL4, enhances grapevine resistance to powdery mildew by activating SA signalling. Our findings may be useful to improve disease resistance in perennial fruit trees.

KEYWORDS
Chinese wild *Vitis quinquangularis*, disease resistance, stilbene, VqAL4 transcription factor, VqNSTS

1 | INTRODUCTION

Grapevine (*Vitis* spp.) is a very important fruit crop of temperate climates that is consumed as table grapes, raisins, grape juice, and wine. The European grapevine, *Vitis vinifera*, is the predominant species in commercial production because of its superior aroma and flavour characteristics. However, most *V. vinifera* cultivars are highly susceptible to powdery mildew caused by the ascomycete fungus *Uncinula necator*, which is an obligate biotrophic fungus (Gadoury et al., 2012; Qiu et al., 2015). Classical disease management methods

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incur both financial and environmental expenses and can directly lead to the development of fungicide resistance in pathogen populations (Jones et al., 2014; Kunova et al., 2021). Therefore, the focus of current research has shifted to development of disease resistance in the grapes themselves.

The stilbene phytoalexin was discovered from *V. vinifera* while exploring the natural mechanisms of disease resistance in grapes (Langcake & Pryce, 1977). Resveratrol is the basic unit of stilbene and is a product of the phenylpropanoid pathway that not only exhibits broad-spectrum resistance to a range of pathogens, including *U. necator*, *Plasmopara viticola*, *Botrytis cinerea*, and *Neofusicoccum parvum* (Hain et al., 1993; Khattab et al., 2021; Pezet et al., 2004b; Xu et al., 2019), but also exhibits important pharmacological properties relevant to human health, exerting anticancer (Jang et al., 1997) and cardioprotective effects (Barger et al., 2008).

Stilbene synthase (STS) is a key enzyme in the resveratrol synthesis pathway and is encoded by a multigenic family. So far, 48 *VvSTS* genes have been identified in *V. vinifera*, of which 32 have potential functions (Parage et al., 2012; Vannozzi et al., 2012). There is considerable interest in the characterization of *STS* genes as part of a strategy to increase stilbene levels and enhance disease resistance in plants. For example, overexpression of two *STS* genes from grapevine in tobacco (*Nicotiana tabacum*) promoted resveratrol accumulation and enhanced resistance to *B. cinerea* (Hain et al., 1993). The function of the *STS* genes has also been analysed in tomato (*Solanum lycopersicum*), rice (*Oryza sativa*), papaya (*Carica papaya*), barley (*Hordeum vulgare*), and wheat (*Triticum* spp.), and it was shown to improve the plant resveratrol content or resistance to pathogens (Leckband & Lörz, 1998; Stark-Lorenzen et al., 1997; Thomzik et al., 1997; Zhu et al., 2004).

A detailed study of the transcriptional regulation of *STS* genes could be important to further enhance disease resistance and the stilbene content of various grape cultivars. Various transcription factors participating in the regulation of *STS* genes in grapevine have been reported, including MYB, WRKY, ERF, and bZIP transcription factors (Höll et al., 2013; Jiang et al., 2021; Mu et al., 2022; Wang et al., 2019, 2020; Wang & Wang, 2019; Yin et al., 2022). However, it remains unclear whether other transcription factors are involved in the regulation of *STS* gene expression.

Plant homeodomain (PHD), a type of epigenetic modification-associated finger protein containing eight conserved metal-binding residues, $Cys_4HisCys_3$, was first reported in *Arabidopsis thaliana* and parsley (*Petroselinum crispum*) to participate in plant disease resistance by binding to the promoter of the *pr2* gene (Kaadige & Ayer, 2006; Korfhage et al., 1994). The Alfin-like (AL) protein belongs to the PHD finger family, and was originally identified as a transcription factor family in alfalfa (*Medicago sativa*) (Bastola et al., 1998). The AL protein family is mainly involved in root development and in responses to abiotic stress, such as salt stress, drought, and low temperatures (Chandrika et al., 2013; Wei et al., 2015; Zhu et al., 2021). There are reports on the functions of AL transcription factors in biotic stress responses. For instance, 10 *BrAL* genes were found to participate in the response of *Brassica rapa* to *Fusarium oxysporum* f. sp. *conglutinans* infection, and two *BoAL* genes showed significant expression in *Brassica oleracea* after inoculation with *Pectobacterium carotovorum* subsp. *carotovorum* (Kayum et al., 2015, 2016). However, studies of the grape AL transcription factor have not been conducted.

China is one of the centres of origin of wild grapevines, serving as an exciting resource providing critical disease resistance genes for resistance breeding of grapevine (Wang et al., 1995, 1998). In total, 61 *VpSTS* genes have been isolated from Chinese wild *Vitis pseudoreticulata* accession Baihe-35-1 (Cao, 2012), and a large number of studies have proved that overexpression of *VpSTS* genes can enhance resistance to powdery mildew (Dai et al., 2015; Jiao et al., 2016; Wang, 2004; Xu, 2010). In particular, *VpSTS29/STS2* overexpression in *V. vinifera* and *A. thaliana* significantly increased resistance to powdery mildew (Xu et al., 2019). Another important Chinese wild resource is *Vitis quinquangularis* accession Danfeng-2, which has not only high resistance to powdery mildew, but also a high content of stilbenes (Duan, 2002; Shi et al., 2014; Wan et al., 2007). Many *VqSTS* genes isolated from Danfeng-2 have been shown to significantly enhance resistance to powdery mildew in the *V. vinifera* susceptible cultivar Thompson Seedless (Ding et al., 2021; Liu et al., 2019a; Wu et al., 2020; Zhao et al., 2020). For example, *VqSTS6*, whose expression is highest in ripe fruit, significantly increased the stilbene content and resistance to powdery mildew when overexpressed in *V. vinifera*, with *trans*-piceid contents 16 times higher than in the wild type (Cheng et al., 2016; Liu et al., 2019a).

So far, 41 *VqSTS* genes have been isolated from Danfeng-2 using the rapid amplification of cDNA ends (RACE) method (Shi et al., 2014), but it is not clear whether there remain any undiscovered *VqSTS* genes in Danfeng-2. To further study the properties of high stilbene content and high resistance to powdery mildew, multiomics analysis of Danfeng-2 and *V. vinifera* 'Cabernet Sauvignon' was carried out. Interestingly, analysis of Danfeng-2 transcriptome data (PRJNA306731) revealed many novel transcripts that could not be mapped on the grapevine reference genome (PN40024), including six novel *STS* transcripts (Li, 2019; Li et al., 2017). However, whether these newly discovered *VqNSTS* genes contribute to the high levels of resveratrol and the high resistance to *U. necator* in Danfeng-2 is still unclear. Furthermore, whether *VqNSTS* genes have novel regulatory mechanisms is a question worthy of further study. The purpose of this study is to investigate how the *VqNSTS* genes contribute to resistance to powdery mildew and to reveal the regulatory mechanisms triggered by these genes that control stilbene biosynthesis.

2 | RESULTS

2.1 | Isolation and characterization of six newly discovered *STS* genes from *V. quinquangularis*

To further explore the relationship between high levels of stilbene and high resistance to powdery mildew in Danfeng-2, we conducted multiomics analysis on Danfeng-2 (Li, 2019).

Transcriptome data for Danfeng-2 that were not mapped to a reference genome were assembled, and 112,266 unigenes were generated. Novel transcripts were grouped into 11 clusters. The numbers of transcripts for clusters 1 to 11 were 311, 322, 76, 53, 205, 380, 324, 460, 385, 328, and 6600, respectively (Figure 1a). Six *STS* transcripts were isolated from cluster 11 with the locus names c50176.graph_c0, c91313.graph_c0, c76190.graph_c0, c56362.graph_c0, c54055.graph_c0, and c11357.graph_c0 (Figure 1b). These newly discovered *STS* transcripts were cloned from Danfeng-2 (designated *VqNSTS1–VqNSTS6*; GenBank: OL589476–OL589481) and they were predicted to be located on chromosome 16 (Table S1).

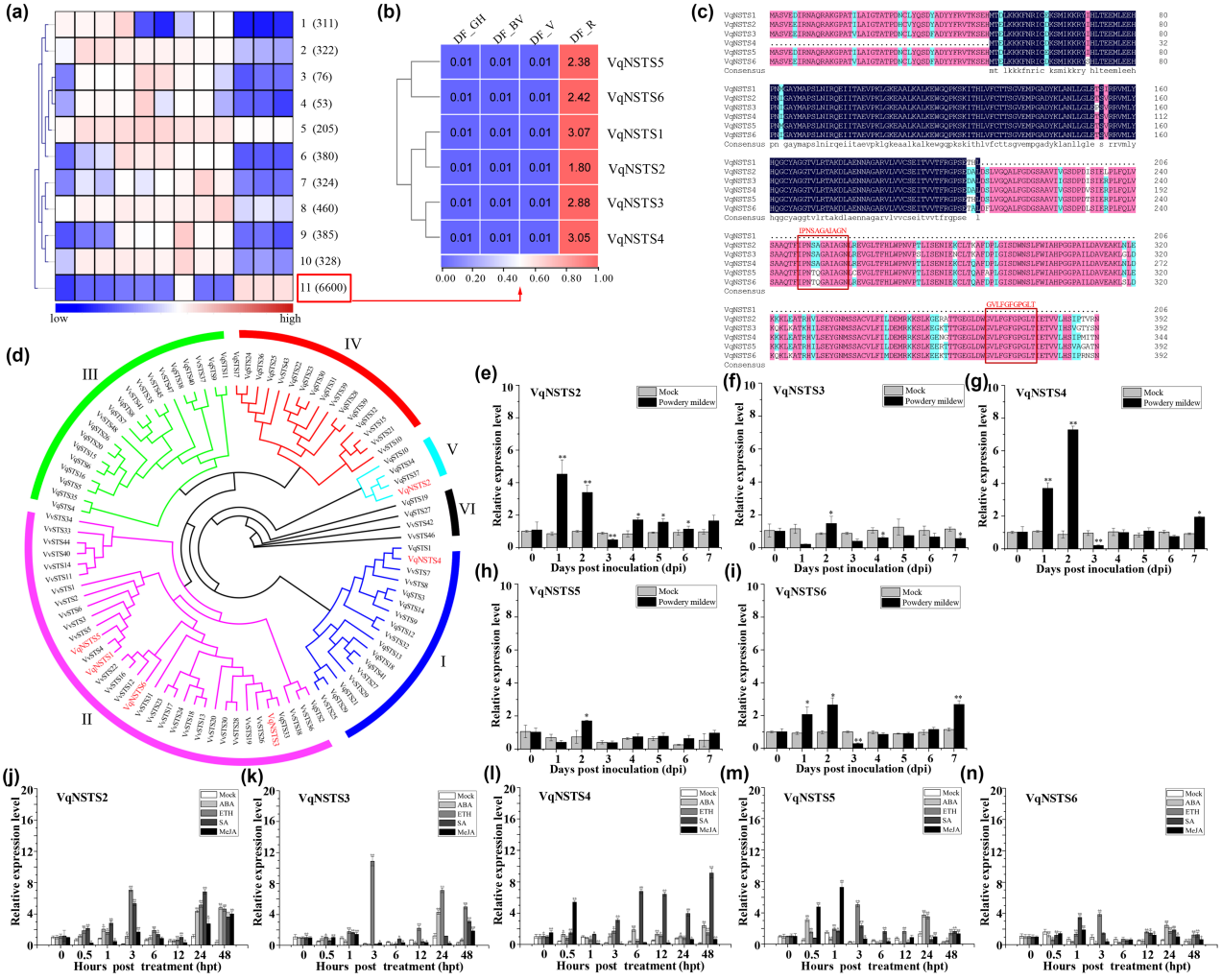

FIGURE 1 Identification and expression analysis of novel stilbene synthase transcripts in *Vitis quinquangularis* accession Danfeng-2. (a) Expression analysis of novel transcripts in *V. quinquangularis* accession Danfeng-2. Novel transcripts are grouped into 11 clusters. The numbers of transcripts from clusters 1 to 11 were 311, 322, 76, 53, 205, 380, 324, 460, 385, 328, and 6600, respectively. Expression of transcripts in cluster 11 was the highest at the ripe stage. (b) Analysis of the expression profiles of novel stilbene synthase transcripts in Danfeng-2. Six stilbene synthase transcripts were isolated from cluster 11, with locus names c50176.graph_c0, c91313.graph_c0, c76190.graph_c0, c56362.graph_c0, c54055.graph_c0, and c11357.graph_c0. The four developmental stages of Danfeng-2 berries were represented by DF_GH (green hard stage, 25 days after blooming), DF_BV (before véraison stage, 40 days after blooming), DF_V (véraison stage, 50 days after blooming), and DF_R (ripe stage, 80 days after blooming). (c) Multiple sequence alignment of VqNSTS1–6 amino acid sequences. The red rectangles represent the two conserved motifs. *VqNSTS1* is a pseudogene, and *VqNSTS5/6* contain mutations in the conserved motif. (d) The phylogenetic tree of VqNSTS1–6 with 41 reported VqSTSs and 48 reported VvSTSs. The 41 reported VqSTSs are from Danfeng-2. All 48 reported VvSTSs are from *V. vinifera* 'Pinot Noir'. They were divided into six subgroups: VqNSTS1, VqNSTS3, VqNSTS5, and VqNSTS6 belong to subgroup II; VqNSTS2 belongs to subgroup V; and VqNSTS4 belongs to subgroup I. (e–i) Reverse transcription-quantitative PCR (RT-qPCR) analysis was conducted to determine the relative transcript levels of *VqNSTS2–6* after inoculation with *Uncinula necator*. *VqNSTS2/4* showed high expression after *U. necator* inoculation, especially *VqNSTS4*. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, Student's *t* test). (j–n) RT-qPCR analysis was conducted to determine the relative transcript levels of *VqNSTS2–6* after treatment with 100 μM abscisic acid (ABA), ethylene (Eth), salicylic acid (SA), or methyl jasmonate (MeJA). *VqNSTS4* responded significantly to SA treatment. The standard deviation (*SD*) was calculated from three independent replicates. One-way analysis of variance (Tukey's test) was carried out, with asterisks indicating significant differences at *$p < 0.05$, **$p < 0.01$.

VqNSTS1 is a 619-bp pseudogene; *VqNSTS4* is a 1035-bp truncated *STS* that encodes 345 amino acids, and *VqNSTS2*, *3*, *5*, and *6* are all 1179 bp and encoded 393 amino acids (Table S1). Interestingly, two conserved motifs of the STS family, IPNSAGAIAGN and GVLFGFGPGLT, were observed in the amino acid sequences of VqNSTS2–6, but not of VqNSTS1 (Figure 1c). Additionally, the phylogenetic tree of VqNSTSs, with 41 reported VqSTSs and 48 reported VvSTSs, showed that VqNSTS1, VqNSTS3, VqNSTS5, and VqNSTS6 belong to subgroup II, VqNSTS2 belongs to subgroup V, and VqNSTS4 belongs to subgroup I (Figure 1d). Moreover, several sequence alignments of VqNSTSs and reported VqSTSs were carried out to confirm that these VqNSTSs were different from the previously reported VqSTSs. The results showed that although VqNSTSs and the reported VqSTSs were highly conserved, there were amino acid deletions and mutations (Figures S1–S5). For example, the sequence similarity of VqNSTS2 and VqSTS37 was 99.49%, with one amino acid mutation, arginine (R)-391 to threonine (T) (Figure S1); the sequence similarity of VqNSTS3 and VqSTS33 was 99.24%, with two amino acid mutations, serine (S)-3 to leucine (L) and serine (S)-276 to phenylalanine (F) (Figure S2); the sequence similarity of VqNSTS4 and VqSTS1 was 87.53%, with 48 amino acid deletions in the N-terminus (Figure S3); the sequence similarity of VqNSTS5 and VqSTS33 was 91.35%, with 33 amino acid mutations (Figure S4); and the sequence similarity of VqNSTS6 and VqSTS33 was 93.89%, with 23 amino acid mutations (Figure S5).

The above results demonstrate that *VqNSTS1* is a pseudogene caused by the deletion of key motifs; although VqNSTS2–6 and the reported VqSTSs have high homology, VqNSTS2–6 are clearly different from the reported VqSTSs based on their sequence alignments, which suggests that *VqNSTS* genes are new members of the *STS* gene family in Danfeng-2.

2.2 | *VqNSTS* genes respond to *U. necator* and phytohormones

To determine if the expression of these *VqNSTS* genes could be induced by *U. necator*, their expression was determined using reverse transcription-quantitative PCR (RT-qPCR) in Danfeng-2 (Figure 1e–i). Here, *VqNSTS1* was not selected for further analysis as it was determined to be a pseudogene, which cannot be translated into a functional protein. The results showed that *VqNSTS2* responded most rapidly to *U. necator*, reaching a maximum at 1 day postinoculation (dpi), 5.3-fold higher than the control (Figure 1e). In particular, *VqNSTS4* showed very high expression levels after *U. necator* inoculation, with the highest expression at 2 dpi, 8.3-fold higher than the control (Figure 1g). These results suggest that *VqNSTS2* and *VqNSTS4* may be involved in the defence response to powdery mildew.

Moreover, many studies have shown that the expression of *STS* genes can be induced by various phytohormones (Jiao et al., 2016; Ma, 2018; Wu et al., 2020). Therefore, RT-qPCR analysis was also conducted to investigate the expression patterns of *VqNSTS* genes after different phytohormone treatments. The results demonstrated that *VqNSTS2* was responsive to four phytohormones, particularly ethylene (Eth) and salicylic acid (SA); its transcript levels peaked at 3 h posttreatment (hpt) and 24 hpt, respectively, and its expression was 71.0-fold and 2.3-fold higher than the control, respectively (Figure 1j). Upon Eth treatment, *VqNSTS3* showed the highest transcript level at 3 hpt, 54.5-fold higher than the control (Figure 1k). *VqNSTS4* responded significantly to SA treatment, with its expression level increasing gradually after 0.5 hpt and reaching a maximum at 48 hpt, 45.5-fold higher than that of the control (Figure 1l). Upon methyl jasmonate (MeJA) treatment, the *VqNSTS5* transcript level increased significantly and peaked at 1 hpt, being 4.9-fold higher than that of the control (Figure 1m). *VqNSTS6* was inducible by Eth and by SA, peaking at 3 hpt and 1 hpt, respectively, being 3.9-fold and 5.0-fold higher than in the control (Figure 1n). These results confirmed that expression of *VqNSTS2–VqNSTS6* could be induced by *U. necator* and by phytohormones, with *VqNSTS2* and *VqNSTS4* being especially inducible.

2.3 | *VqNSTS4* increases the accumulation of stilbenes and enhances grapevine resistance to *U. necator*

Because *VqNSTS4* can be significantly induced by *U. necator* or SA (Figure 1g,l) despite being truncated, we speculated that it might play an important role in plant resistance to powdery mildew. To test this hypothesis, *VqNSTS4* was stably expressed in *V. vinifera* 'Thompson Seedless' through *Agrobacterium*-mediated transformation (Figure S6a,b). A total of 56 kanamycin-resistant grapevine plantlets were obtained and analysed by PCR with specific primers (35S-GFP-F/R), of which seven were identified as transgenic grapevines (12.5%) by western blot (Figure S6c,d). Two transgenic lines—OE*VqNSTS4*-L1 and OE*VqNSTS4*-L2, both with high *VqNSTS4-GFP* expression—were selected for further study (Figure S6e). High-performance liquid chromatography (HPLC) analysis showed that the content of stilbenes was significantly increased in both OE*VqNSTS4*-L1 and OE*VqNSTS4*-L2, particularly of *trans*-piceid, which is the main storage form of stilbene in grapevine (Chong et al., 2009). The contents of *trans*-piceid in OE*VqNSTS4*-L1 and OE*VqNSTS4*-L2 were 2.4-fold and 1.9-fold higher than those in the wild-type Thompson Seedless, respectively (Figure S6f; Table 1). These results suggest that VqNSTS4 can catalyse the synthesis of stilbenes, despite the deletion of 48 amino acids at its N-terminus.

To evaluate disease resistance to powdery mildew, 8-week-old OE*VqNSTS4*-L1 and OE*VqNSTS4*-L2 plants were inoculated with *U. necator* (Figure 2a). As shown in Figure 2b, while many whitish mildew colonies were seen on the infected leaves of the wild-type Thompson Seedless, only scattered and sparse mildew colonies were observed on the leaves of *VqNSTS4* transgenic grapevines at 7 dpi. Trypan blue staining, scanning electron microscopy, and quantification of the number of spores showed that hyphal growth, sporulation, and appressorium formation on the hyphae were significantly restricted in the *VqNSTS4* transgenic lines (Figure 2c,d,f). Also,

TABLE 1 Contents of stilbenes of *VqNSTS4* transgenic *Vitis vinifera* 'Thompson Seedless' lines under natural conditions and 7 days after artificial inoculation with *Uncinula necator* (PM-7d)

Line	*trans*-Piceid (μg/g)	*trans*-Resveratrol (μg/g)	ε-Viniferin (μg/g)	Pterostilbene (μg/g)	*trans*-Piceatannol (μg/g)
Wild type (WT)	125.69 ± 13.23	–	–	84.33 ± 9.07	17.74 ± 2.33
OE*VqNSTS4*-L1	305.01 ± 25.83	85.58 ± 6.46	37.37 ± 7.21	102.01 ± 19.29	67.84 ± 9.52
OE*VqNSTS4*-L2	245.06 ± 19.01	82.97 ± 5.52	38.44 ± 3.94	83.29 ± 10.22	28.93 ± 7.66
WT-PM-7d	131.09 ± 13.95	–	28.66 ± 3.83	86.18 ± 7.67	14.95 ± 2.33
OE*VqNSTS4*-L1-PM-7d	322.08 ± 18.17	135.61 ± 7.72	74.33 ± 10.20	152.54 ± 13.74	63.31 ± 10.44
OE*VqNSTS4*-L2-PM-7d	564.32 ± 27.57	87.93 ± 7.36	57.08 ± 6.04	85.46 ± 9.15	18.34 ± 4.84

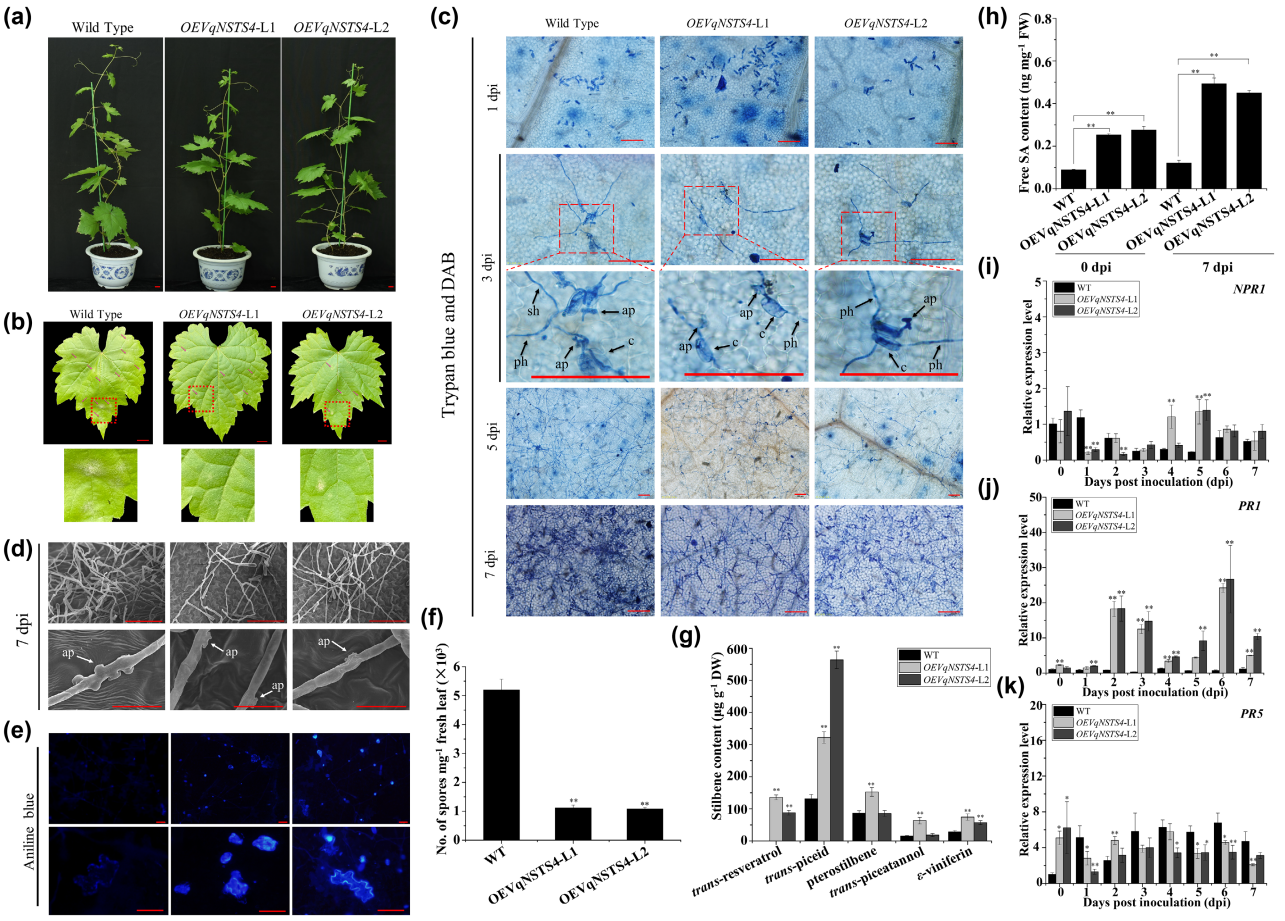

FIGURE 2 *VqNSTS4* transgenic *Vitis vinifera* lines exhibit disease resistance resulting from the accumulation of internal stilbene and the expression of resistance genes. (a) Photograph of *VqNSTS4* transgenic lines and wild-type Thompson Seedless before *Uncinula necator* inoculation. Bars = 1 cm. (b) Photograph of leaves of *VqNSTS4* transgenic lines and wild-type Thompson Seedless at 7 days postinoculation (dpi). Bars = 1 cm. (c) Trypan blue staining and 3,3′-diaminobenzidine (DAB) staining show the hyphal growth of *U. necator* and H_2O_2 accumulation at 1, 3, 5, and 7 dpi. c, conidium; ap, appressorium; ph, primary hypha; sh, secondary hypha. Bars = 100 μm. (d) Scanning electron micrographs of the hyphae and appressoria (ap) of *U. necator* in *VqNSTS4* transgenic lines and wild-type Thompson Seedless. Upper figures, bars = 100 μm; lower figures, bars = 20 μm. (e) Aniline blue staining showing callose depositions in *U. necator*-infected epidermal cells at 7 dpi. Bars = 50 μm. (f) Quantification of spores per mg fresh leaves from *VqNSTS4* transgenic lines and wild-type Thompson Seedless at 7 dpi. (g) Determination of five stilbene contents in the leaves of *VqNSTS4* transgenic mutants and wild-type Thompson Seedless at 7 dpi. (h) Free salicylic acid (SA) content in the leaves of *VqNSTS4* transgenic lines and wild-type Thompson Seedless at 0 dpi and 7 dpi. (i–k) Reverse transcription-quantitative PCR analysis was conducted to determine the relative transcript levels of SA-related genes in *VqNSTS4* transgenic lines following *U. necator* inoculation. WT, wild-type Thompson Seedless. The standard deviation (*SD*) was calculated from three independent replicates. One-way analysis of variance (Tukey's test) was carried out. Asterisks indicate significant differences at $^{*}p < 0.05$, $^{**}p < 0.01$.

plentiful callose accumulated in the epidermal cells of the *VqNSTS4* transgenic line leaves (Figure 2e). Interestingly, *U. necator* inoculation promoted the accumulation of *trans*-resveratrol, *trans*-piceid, and ε-viniferin in the two *VqNSTS4* transgenic lines, while pterostilbene and *trans*-piceatannol levels were markedly elevated only in OE*VqNSTS4*-L1, at 1.8-fold and 4.2-fold higher levels than in wild-type Thompson Seedless (Figure 2g and Table 1). Taken together, these results demonstrate that overexpression of *VqNSTS4* significantly promotes the accumulation of stilbenes and enhances resistance to *U. necator* in grapevine. To further understand the resistance mechanisms activated by *VqNSTS4* overexpression, the content of free SA was measured. As shown in Figure 2h, the content of free SA was 2.8-fold and 3.1-fold higher in *VqNSTS4* transgenic lines than in wild-type Thompson Seedless at 0 dpi and 4.1-fold and 3.8-fold higher than in the wild type at 7 dpi (Figure 2h). Therefore, we also examined the transcript levels of the SA-related genes *nonexpressor of pathogenesis-related gene 1* (*NPR1*), *pathogenesis-related gene 1* (*PR1*), and *PR5* in *VqNSTS4* transgenic lines and wild-type Thompson Seedless after *U. necator* inoculation. Although the transcript levels of *NPR1* and *PR5* were not continuously up-regulated after *U. necator* inoculation in *VqNSTS4* transgenic lines, the transcript level of *PR1* was markedly higher than in wild-type Thompson Seedless (Figure 2i–k). To further study the function of *VqNSTS4* in disease resistance, we used RNA interference (RNAi) and investigated the resistance of transiently transformed Danfeng-2 leaves to *U. necator* (Figure 3a). Because of the high sequence similarity of *VqNSTS4* and *VqSTS1*, we knocked down *VqNSTS4* and *VqSTS1* at the same time. However, knockdown with RNAi-*VqNSTS4*/*STS1* in Danfeng-2 resulted in the opposite results compared with transgenic overexpression plants (Figure 3b–j, Table 2). Taken together, these results indicate that VqNSTS4 can positively regulate the defence response to *U. necator* in grapevine by activating the SA signalling pathway.

2.4 | *VqNSTS4* is regulated by the novel transcription factor VqAL4

To identify the transcription factors that regulate the expression of *VqNSTS* genes, the promoters of *VqNSTS2*–*VqNSTS6* were cloned from Danfeng-2 and analysed (Figures S7–S11). Several well-known WRKY and MYB binding sites were identified in the promoters, such as MBS and W-box elements (Figure 4a, Table S2). The WRKY and MYB transcription factors that have been reported to regulate *VqSTS* genes directly or indirectly include three WRKY proteins (VqWRKY2, VqWRKY3, and VqWRKY53) (Wang et al., 2020) and three MYB proteins (VqMYB14, VqMYB15, and VqMYB154) (Höll et al., 2013; Jiang et al., 2021). Yeast one-hybrid (Y1H) analysis was conducted to identify the regulatory relationships between these six transcription factors and *VqNSTS* promoters (ProVqNSTS). As shown in Figure 4b, VqMYB154 can bind to the promoter of *VqNSTS2*, VqWRKY3 and VqWRKY53 can bind to the promoter of *VqNSTS3*, and VqMYB14 can bind to the promoter of *VqNSTS6*. None of these six VqWRKY/MYB transcription factors was found to bind to the promoters of *VqNSTS4* or *VqNSTS5* (Figure 4b).

However, a G-rich element (CACCTC/GAGGTG), which could be recognized by an AL transcription factor, was found in the promoter of *VqNSTS4* (Figure 4a) (Bastola et al., 1998; Wei et al., 2017). Meanwhile, coexpression analysis using the transcriptome data of Danfeng-2 revealed that the Pearson correlation coefficient value between *VqNSTS4* and one AL transcription factor (VIT_11s0016g01500) was 0.89. Based on the grapevine gene nomenclature system, this AL transcription factor was designated VqAL4 (Grimplet et al., 2014). To examine whether the VqAL4 transcription factor could directly bind to the *VqNSTS4* promoter, Y1H assays were carried out. As shown in Figure 4b, the pGADT7-VqAL4 plasmid was transferred into the bait strains containing *VqNSTS* promoters. Only the yeast cells containing *ProVqNSTS4* were able to grow normally on SD/−Leu medium containing 200 ng/ml Aureobasidin A (AbA) (Figure 4b). In addition, *ProVqNSTS4* could not be bound by the VqAL4 transcription factor after deletion or mutation of the G-rich element (Figures 4c and S12). These results demonstrate that VqAL4 binds only to the G-rich element in *ProVqNSTS4*. Subsequently, dual-luciferase and chromatin immunoprecipitation (ChIP)-qPCR assays were conducted to further demonstrate that VqAL4 binds specifically to the G-rich element in *ProVqNSTS4* (Figure 4d–f). The above results indicate that *VqNSTS4* is not regulated by reported VqMYB or VqWRKY transcription factors but by a novel transcription factor, VqAL4, through binding to the G-rich element.

2.5 | VqAL4 up-regulates the expression of *VqNSTS4* in grapevine

To analyse the effects of VqAL4 protein accumulation on the transcription of *VqNSTS4*, a transient expression assay was carried out in Danfeng-2 leaves using *Agrobacterium*-mediated infiltration (Figure 5a–c). As shown in Figure 5d,e, western blot assays demonstrated that 35S-VqAL4-GFP and RNAi-VqAL4-GFP were expressed in the Danfeng-2 leaves. The transcript levels of *VqAL4* were more elevated in 35S-VqAL4-GFP leaves than in those containing empty vector (EV), with 11.4-fold and 8.7-fold higher expression, respectively (Figure 5f). Meanwhile, the expression levels of *VqNSTS4* in 35S-VqAL4-GFP leaves were increased 5.1-fold and 5.6-fold compared with those in EV leaves (Figure 5f). However, RNAi-VqAL4-GFP leaves of Danfeng-2 showed the opposite results compared with 35S-VqAL4-GFP (Figure 5i). Consistent with the expression of *VqNSTS4*, the content of stilbenes was increased in 35S-VqAL4-GFP leaves and decreased in RNAi-VqAL4-GFP leaves, especially for the stilbene associated with strong antifungal activity (Pezet et al., 2004a) ε-viniferin (Figure 5g,j, Table 3). In addition, the content of free SA was also increased in 35S-VqAL4-GFP leaves but decreased in RNAi-VqAL4-GFP leaves (Figure 5h,k). These results suggest that VqAL4 can positively regulate the expression of *VqNSTS4*, thereby promoting the accumulation of stilbene and the synthesis of free SA.

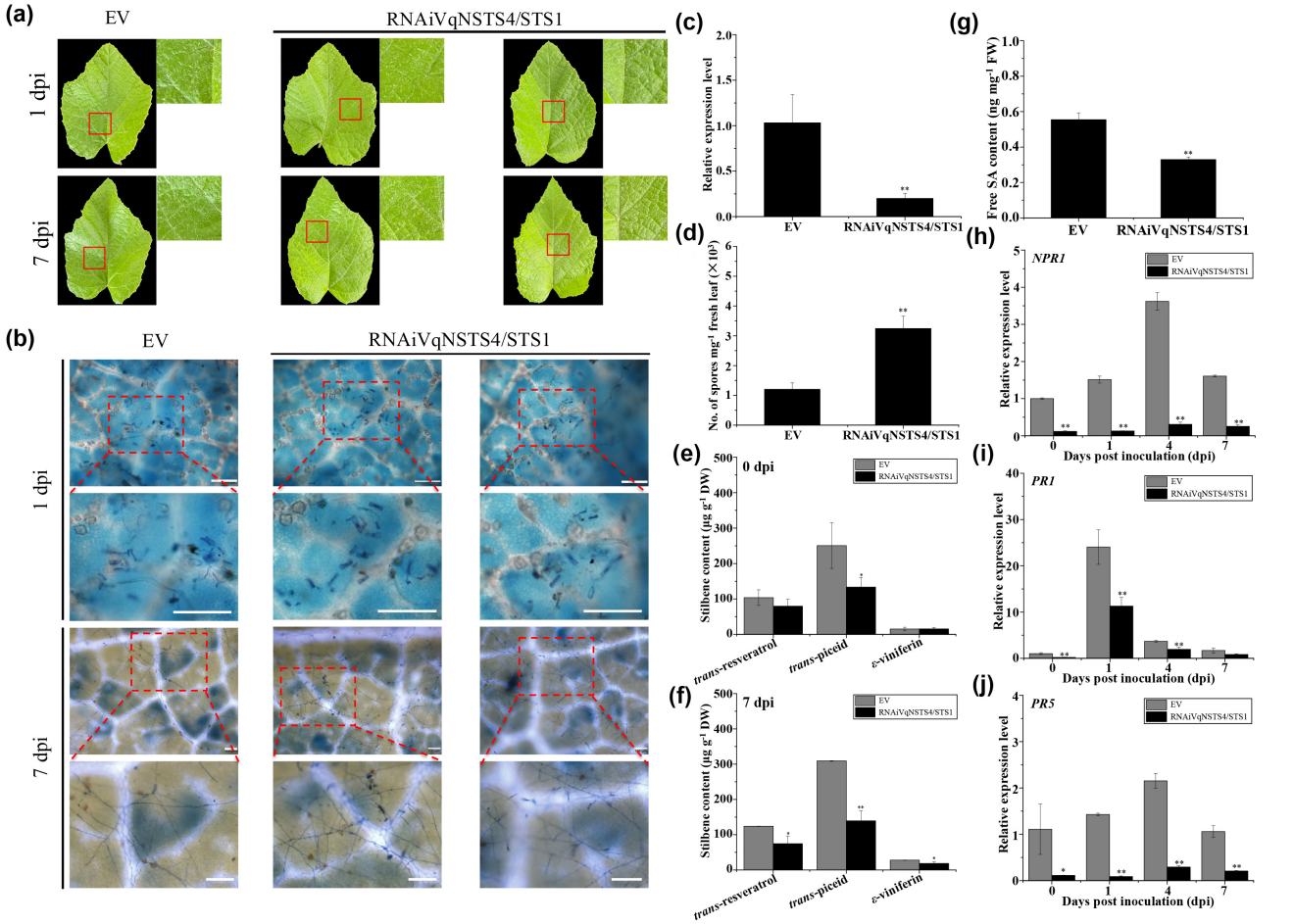

FIGURE 3 Transient silencing of *VqNSTS4/STS1* in leaves of *Vitis quinquangularis* accession Danfeng-2 reduces resistance to *Uncinula necator*. (a) Phenotypes of control EV and transiently silenced RNAi-VqNSTS4/STS1-GFP Danfeng-2 leaves at 1 day postinoculation (dpi) and 7 dpi. EV, the empty vector pK7GWIWG2(II)-35S-GFP. (b) Trypan blue staining of leaves from EV and transiently silenced RNAi-VqNSTS4/STS1-GFP leaves at 1 dpi and 7 dpi. Bars = 100 μm. (c) Reverse transcription-quantitative PCR (RT-qPCR) analysis was conducted to determine the relative expression levels of *VqNSTS4/STS1* in transiently transformed Danfeng-2 leaves. (d) Quantification of spores per mg fresh leaves from EV and transiently silenced RNAi-VqNSTS4/STS1-GFP leaves at 7 dpi. (e, f) Stilbene contents in EV and transiently silenced RNAi-VqNSTS4/STS1-GFP leaves. (g) Free salicylic acid (SA) content in the leaves of EV and transiently silenced RNAi-VqNSTS4/STS1-GFP leaves at 7 dpi. (h–j) RT-qPCR analysis was conducted to determine the relative transcript levels of SA-related genes in EV and transiently silenced RNAi-VqNSTS4/STS1-GFP leaves following *U. necator* inoculation. The standard deviation (*SD*) was calculated from three independent replicates. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, Student's *t* test).

TABLE 2 Contents of stilbenes in *Vitis quinquangularis* accession Danfeng-2 leaves in which *VqNSTS4* is transiently silenced before (PM-0d) and after artificial inoculation with *Uncinula necator* (PM-7d)

Line	*trans*-Piceid (μg/g)	*trans*-Resveratrol (μg/g)	ε-Viniferin (μg/g)
EV-PM-0d	250.64 ± 64.63	103.88 ± 22.01	15.25 ± 4.83
RNAiVqNSTS4-PM-0d	133.57 ± 27.28	79.73 ± 19.59	15.49 ± 3.89
EV-PM-7d	309.27 ± 1.04	124.02 ± 0.29	27.87 ± 0.43
RNAiVqNSTS4-PM-7d	139.38 ± 27.94	74.72 ± 21.57	18.73 ± 4.88

2.6 | VqAL4 is localized in the nucleus and responds to *U. necator* and phytohormones

To investigate the function of the transcription factor VqAL4, the structural characteristics and the expression of *VqAL4* were analysed. *VqAL4* was predicted to be located on chromosome 11, and VqAL4 consists of two functional domains: an Alfin-like domain (DUF3594) at the N-terminus and a PHD finger domain at the C-terminus (Figures 6a and S13). Phylogenetic analysis showed that VqAL4 was clustered into subgroup IV with three VvALs (VvAL1, VvAL3, and VvAL4), one AtAL (AtAL4), and three BrALs (BrAL3, BrAL7, and BrAL10) (Figure 6b). Amino acid sequence comparison between VqAL4 and VvAL4 revealed that the 57th amino acid was changed from leucine in VvAL4 to methionine in VqAL4 (Figure 6c).

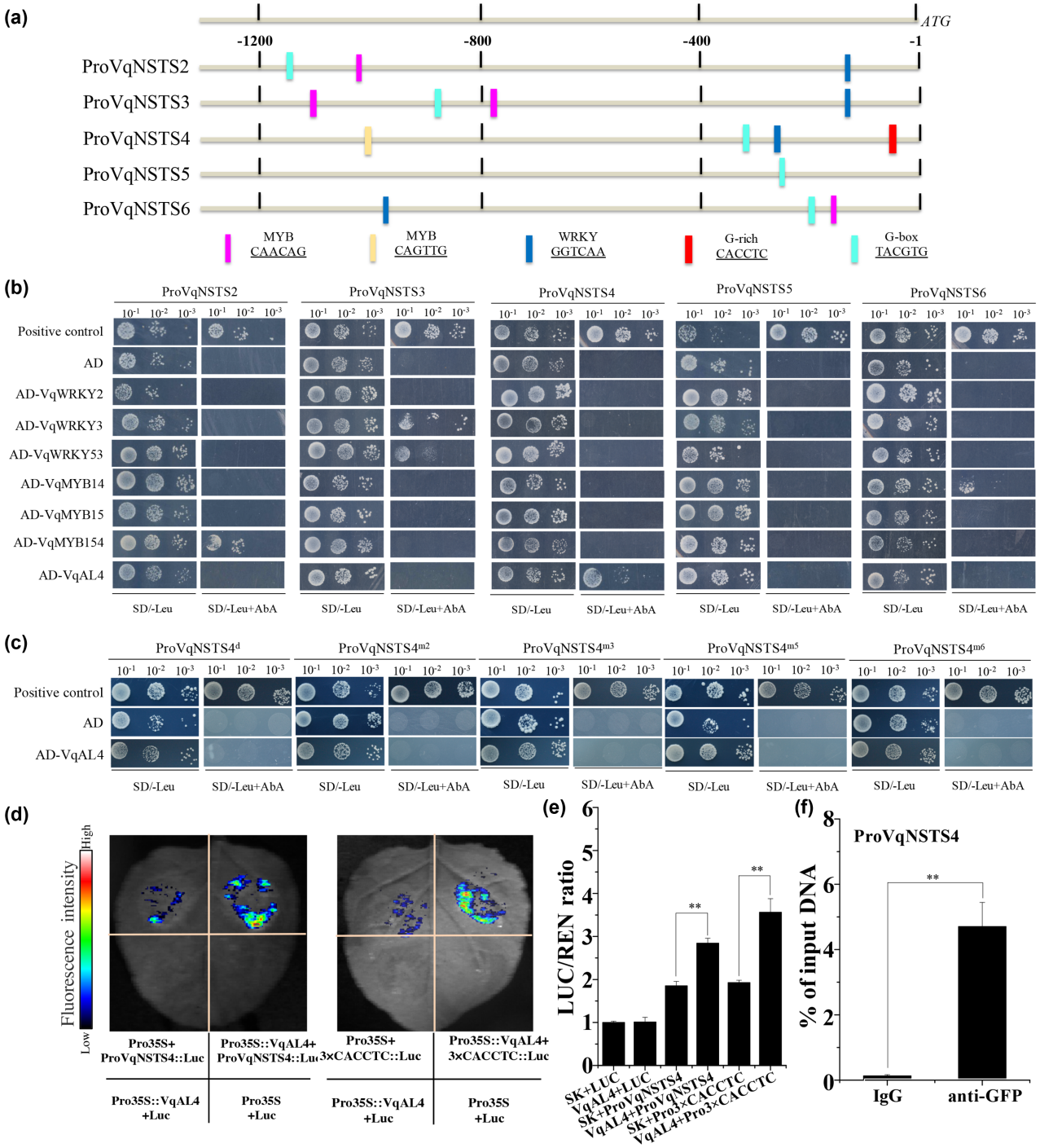

FIGURE 4 *VqNSTS4* has unconventional transcriptional regulation activity and carries specific VqAL4 binding sites. (a) *cis*-Regulatory element analysis in the promoters of *VqNSTS* genes. MYB and WRKY binding elements were found in the *ProVqNSTS2*, *ProVqNSTS3*, and *ProVqNSTS6* promoters. The *ProVqNSTS4* promoter included a G-rich element in addition to the MYB and WRKY binding elements. (b) Yeast one-hybrid assays were carried out to determine whether VqWRKYs, VqMYBs, and VqAL4 could bind directly to the promoters of *VqNSTS* genes. VqMYB154 can bind to *ProVqNSTS2*, VqWRKY3 and VqWRKY53 can bind to *ProVqNSTS3*, VqMYB14 can bind to *ProVqNSTS6*, and VqAL4 can bind to *ProVqNSTS4*. (c) Yeast one-hybrid assays were conducted to demonstrate that VqAL4 cannot bind to *ProVqNSTS4*^{m2}, *ProVqNSTS4*^{m3}, *ProVqNSTS4*^{d}, *ProVqNSTS4*^{m5}, or *ProVqNSTS4*^{m6}. (d) Luminescence intensity. (e) Ratio of firefly luciferase (LUC) to Renilla luciferase (REN) activity. The standard deviation (*SD*) was calculated from three independent replicates. One-way analysis of variance (Tukey's test) was carried out. Asterisks indicate significant differences at $^{*}p < 0.05$, $^{**}p < 0.01$. (f) Chromatin immunoprecipitation–quantitative PCR assays were carried out to demonstrate that VqAL4 binds to the promoter of *VqNSTS4* via the CACCTC/GAGGTG element. The *SD* was calculated from three independent replicates. Asterisks indicate significant differences ($^{*}p < 0.05$, $^{**}p < 0.01$, Student's *t* test).

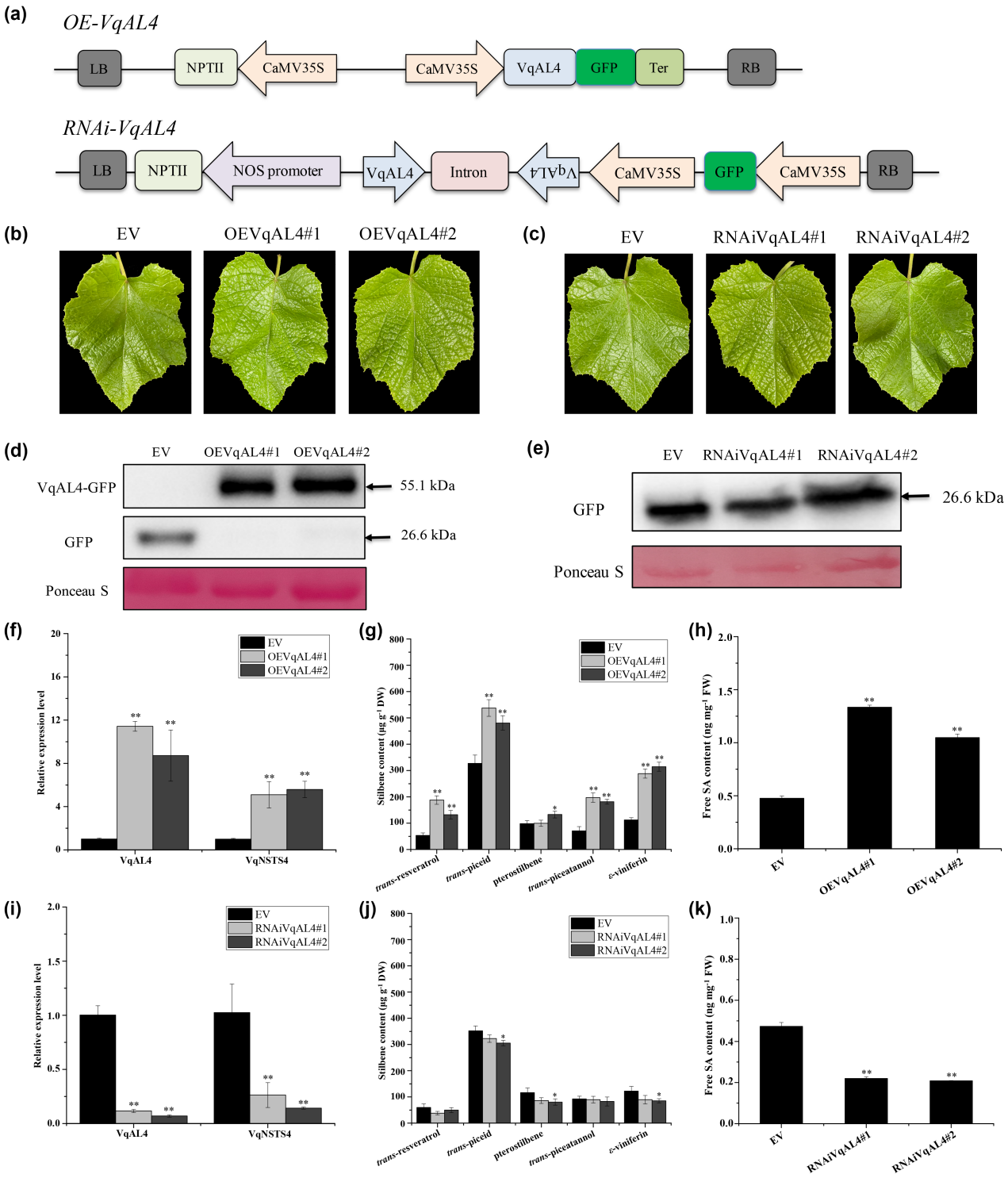

FIGURE 5 Transient expression of transcription factor VqAL4 in *Vitis quinquangularis* accession Danfeng-2 promoted transcription of *VqNSTS4* and antifungal stilbene accumulation. (a) Schematic diagram of overexpression and RNAi vectors. (b, c) Transient transformation of 35S-VqAL4-GFP and RNAi-VqAL4-GFP in Danfeng-2 leaves using the *Agrobacterium* vacuum infiltration method. (d) Western blot assays were carried out to determine the expression of 35S-VqAL4-GFP in transiently transformed Danfeng-2 leaves. EV, the empty vector pCAMBIA2300-35S-GFP was used as the control. (e) Western blot assays were carried out to determine the expression of RNAi-VqAL4-GFP in transiently transformed Danfeng-2 leaves. EV, the empty vector pK7GWIWG2(II)-35S-GFP was used as the control. (f, i) Reverse transcription-quantitative PCR analysis was conducted to determine the relative expression levels of *VqAL4* and *VqNSTS4* in transiently transformed Danfeng-2 leaves. (g, j) Determination of five stilbene contents in transiently transformed Danfeng-2 leaves. (h, k) Free salicylic acid (SA) content in transiently transformed Danfeng-2 leaves. The standard deviation (*SD*) was calculated from three independent replicates. One-way analysis of variance (Tukey's test) was carried out. Asterisks indicate significant differences at $^{*}p < 0.05$, $^{**}p < 0.01$.

TABLE 3 Contents of stilbenes in *Vitis quinquangularis* accession Danfeng-2 leaves transiently expressing *VqAL4* overexpression (OE) and RNA interference (RNAi) constructs *VqAL4*

Line	*trans*-Piceid (μg/g)	*trans*-Resveratrol (μg/g)	ε-Viniferin (μg/g)	Pterostilbene (μg/g)	*trans*-Piceatannol (μg/g)
EV	327.33 ± 31.37	53.51 ± 9.53	112.24 ± 8.57	98.33 ± 11.37	70.76 ± 15.90
OEVqAL4#1	537.56 ± 31.66	187.69 ± 16.06	288.05 ± 17.22	99.45 ± 11.87	197.09 ± 17.98
OEVqAL4#2	480.74 ± 27.30	131.91 ± 16.43	314.60 ± 17.13	132.87 ± 12.90	181.57 ± 9.11
EV	352.02 ± 18.50	59.94 ± 13.41	122.14 ± 17.87	116.12 ± 17.80	92.12 ± 10.60
RNAiVqAL4#1	322.45 ± 14.18	38.18 ± 6.83	89.38 ± 16.25	85.98 ± 11.01	89.48 ± 12.70
RNAiVqAL4#2	305.26 ± 10.32	49.77 ± 9.43	85.27 ± 7.84	80.31 ± 12.17	82.59 ± 16.86

Furthermore, we performed a subcellular localization assay. The location of the green fluorescence signal from the VqAL4-GFP fusion protein was identical to the red fluorescence signal from mCherry-tagged AtHY5, which is a nuclear-localized protein from *Arabidopsis*. This indicated that VqAL4 was localized in the nucleus (Figure 6d). A yeast two-hybrid (Y2H) assay showed that VqAL4 has transcription activation functions in yeast (Figure 6e), suggesting that VqAL4 may function as a transcriptional activator. Additionally, expression analysis showed that the transcript level of *VqAL4* was highest in mature leaves, followed by young leaves, and lowest in roots (Figure S14b). Moreover, the expression of *VqAL4* could be induced by *U. necator* and various phytohormones, such as SA and abscisic acid (ABA) (Figure S14c–g). These results indicate that *VqAL4* may be involved in powdery mildew resistance in mature leaves as a transcriptional activator.

2.7 | *VqAL4* enhances resistance to *U. necator* in grapevine

Based on the finding that *VqAL4* expression can be induced by *U. necator* (Figure S14c), we speculated that *VqAL4* is involved in resistance to powdery mildew. Therefore, transgenic *VqAL4* overexpression lines were created to evaluate disease resistance (Figure S15a). Two transgenic lines (OE*VqAL4*-L1 and OE*VqAL4*-L3) were identified from 21 PCR-positive grapevine plantlets (9.5%) by western blot (Figure S15b). RT-qPCR analysis showed that *VqAL4* was up-regulated 6.6-fold in OE*VqAL4*-L1 and 8.8-fold in OE*VqAL4*-L3 (Figure S15c). Intriguingly, *VvSTS* genes were also up-regulated, and stilbene levels were significantly increased in the *VqAL4* transgenic lines (Figure S15c,d, Table 4).

Eight-week-old OE*VqAL4*-L1 and OE*VqAL4*-L3 were inoculated with *U. necator* to assess their disease resistance (Figure 7a). The results showed that the infected leaves of the *VqAL4* transgenic grapevines were more tolerant to *U. necator* than the wild-type Thompson Seedless, as indicated by fewer spores and appressoria, more hypersensitive response (HR)-like cell death and callose deposition, and higher H_2O_2 and free SA contents (Figure 7b–h). In addition, HPLC analysis showed that stilbene levels were significantly increased in the two *VqAL4* transgenic lines after *U. necator* inoculation (Figure 7i, Table 4). These results indicate that *VqAL4* can enhance resistance to *U. necator* and promote the accumulation of stilbenes. Given that plentiful H_2O_2 was detected in the process of resistance to *U. necator* in the two *VqAL4* transgenic grapevine lines (Figure 7g), the expression of *respiratory burst oxidase homologue D* (*RBOHD*) and *glutathione S-transferase 1* (*GST1*) was also examined in addition to *NPR1*, *PR1*, and *PR5*. As shown in Figure 7j–n, the transcript levels of *RBOHD*, *GST1*, *NPR1*, *PR1*, and *PR5* were higher in OE*VqAL4*-L1 and OE*VqAL4*-L3 than in wild-type Thompson Seedless. These results demonstrate that overexpression of *VqAL4* in grapevine activates the SA signalling pathway. In contrast, transient silencing of *VqAL4* in Danfeng-2 leaves decreased resistance to powdery mildew by preventing SA production (Figure 8).

The up-regulated expression of *VvSTS* and the increased stilbene content in the *VqAL4* transgenic lines compared with wild-type Thompson Seedless indicate that VqAL4 may also be involved in the regulation of *VvSTS* genes. We cloned and analysed the promoters of seven *VvSTS* genes classified in subgroup I along with *VqNSTS4* and found a G-rich element in the promoter of *VvSTS32* (Figures 1d, S16a, and S17–S23). Y1H, dual-luciferase, and ChIP-qPCR assays demonstrated that VqAL4 could bind directly to the *VvSTS32* promoter via the CACCTC element (Figure S16b–e). The above results demonstrate that VqAL4 can also up-regulate *VvSTS32* expression by binding to the G-rich (CACCTC) element.

3 | DISCUSSION

3.1 | Disease resistance of stilbene synthase genes in grapevines

STS, which exhibits broad-spectrum resistance and plays defensive roles against a variety of pathogens, is a key enzyme in the synthesis of stilbene (Hain et al., 1993; Khattab et al., 2021; Pezet et al., 2004b; Xu et al., 2019). The Chinese wild *V. quinquangularis* accession Danfeng-2 is an important natural resource for investigating the potential relationship between STS and powdery mildew resistance that not only has a high content of stilbene but also exhibits strong disease resistance (Duan, 2002; Shi et al., 2014; Wan et al., 2007). Although 41 *VqSTS* genes have been cloned from Danfeng-2 using the RACE method, six novel *STS* transcripts were identified from the RNA-seq data of Danfeng-2 (Li, 2019; Shi et al., 2014). We isolated

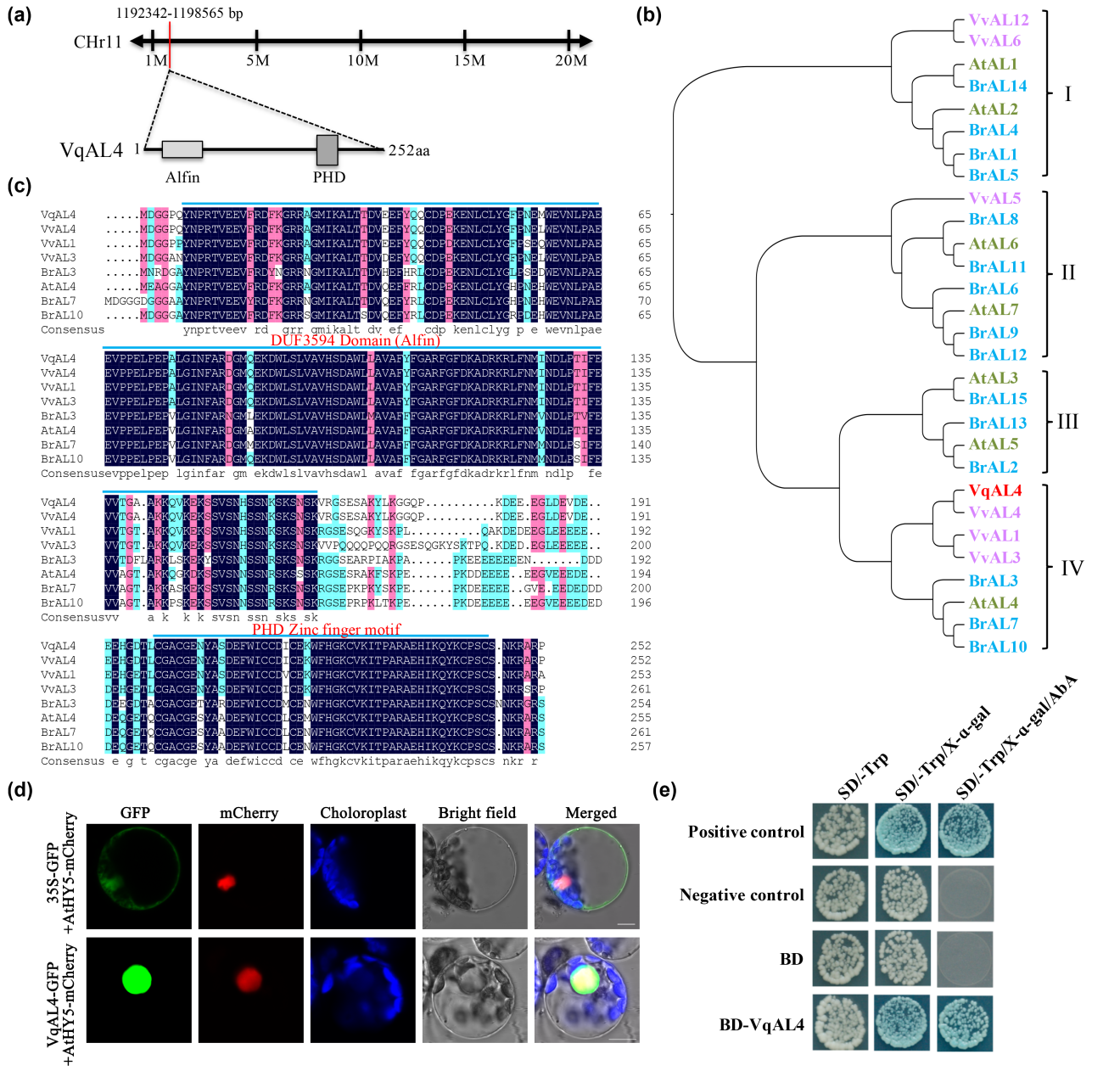

FIGURE 6 Location and structural analysis of VqAL4 isolated from *Vitis quinquangularis* accession Danfeng-2. (a) Chromosomal localization of *VqAL4*. (b) Phylogenetic analysis of VqAL4 and the Alfin-like proteins in *Vitis vinifera*, *Brassica rapa*, and *Arabidopsis thaliana*. (c) Multiple sequence alignment of amino acid sequences of VqAL4 and other group IV members, including VvAL4, VvAL1, VvAL3, AtAL4, BrAL3, BrAL7, and BrAL10. (d) Subcellular localization of VqAL4 in *A. thaliana* protoplasts. (e) Transcriptional activation of VqAL4 in yeast. pGADT7-T/pGBKT7-53 was used as a positive control, and pGADT7-T/pGBKT7-Lam was used as a negative control.

these six *VqNSTS* genes from Danfeng-2 and found that *VqNSTS1* was a pseudogene (Figure 1, Table S1). Similarly, several *VvSTS* genes and *VpSTS* genes are incomplete or are pseudogenes (Cao, 2012; Parage et al., 2012). Furthermore, we found that the IPNSAGAIAGN conserved motif in VqNSTS5 and VqNSTS6 has been replaced by IPNTQGAIAGN (Figure 1c); the same phenomenon was also observed in VvSTSs (He, 2012). We cannot rule out the possibility that these mutations affect the functions of STS.

Many complete *STS* genes have been confirmed to have enzymatic activities for resveratrol biosynthesis and to enhance resistance to powdery mildew (Cheng et al., 2016; Liu et al., 2019; Xu et al., 2019). However, in the present study, we identified an N-terminally truncated *STS* gene (Figure 1c), *VqNSTS4*, and showed that its expression was significantly induced by *U. necator* and SA (Figure 1g,l). In a previous report, it was speculated that two truncated *STS* genes, *VvSTS1* and *VvSTS4*, may also contribute to stilbene biosynthesis (Vannozzi et al., 2012). Therefore, we created *VqNSTS4* transgenic grapevines and demonstrated that *VqNSTS4* overexpression significantly promoted the accumulation of stilbenes and enhanced resistance to powdery mildew (Figure 2). Conversely,

TABLE 4 Contents of stilbenes in *VqAL4* transgenic *Vitis vinifera* 'Thompson Seedless' lines under natural conditions and 7 days after artificial inoculation with *Uncinula necator* (PM-7d)

Line	*trans*-Piceid (μg/g)	*trans*-Resveratrol (μg/g)	ε-Viniferin (μg/g)	Pterostilbene (μg/g)	*trans*-Piceatannol (μg/g)
Wild type (WT)	126.22 ± 10.03	–	23.24 ± 2.86	42.91 ± 7.64	17.89 ± 2.48
OE*VqAL4*-L1	156.19 ± 12.01	37.64 ± 4.22	23.09 ± 2.42	37.45 ± 5.90	19.08 ± 2.12
OE*VqAL4*-L3	133.95 ± 9.63	64.84 ± 4.36	26.46 ± 3.35	61.96 ± 8.00	37.20 ± 9.19
WT-PM-7d	170.62 ± 11.24	–	27.26 ± 5.78	47.12 ± 5.69	16.71 ± 1.93
OE*VqAL4*-L1-PM-7d	242.61 ± 14.34	56.72 ± 5.65	42.64 ± 5.89	43.67 ± 7.58	60.69 ± 5.73
OE*VqAL4*-L3-PM-7d	215.72 ± 10.76	128.69 ± 9.95	75.04 ± 7.41	74.01 ± 8.46	111.78 ± 8.99

transient silencing of *VqNSTS4* in Danfeng-2 leaves decreased resistance to powdery mildew (Figure 3). The above results indicate that the deletion of 48 amino acids at the N-terminus of VqNSTS4 did not affect the disease resistance function of VqNSTS4.

3.2 | Regulatory mechanisms of stilbene synthase genes in grapevine

Many studies have focused on the transcriptional regulation of stilbene biosynthesis in grapevine (Orduña et al., 2022; Wong & Matus, 2017). However, most of these focused on two transcription factor families, MYB and WRKY. For example, MYB14 and MYB15 can not only promote the expression of *STS* genes independently, but also interact with other WRKY transcription factors to regulate the expression of *STS* genes (Höll et al., 2013; Jiang et al., 2019; Mu et al., 2022; Vannozzi et al., 2018; Wang et al., 2020). In the present study, although several MYB and WRKY binding sites were identified in the promoter of *VqNSTS4*, VqWRKY2/3/53 and VqMYB14/15/154 transcription factors could not directly regulate *VqNSTS4* expression (Figure 4a,b), suggesting that MYB and WRKY transcription factors are specific to the regulation of *STS* genes.

In addition, other transcription factors have been reported to be involved in the regulation of *STS* expression. Thus, VqERF114 indirectly regulates the expression of *VqSTS* genes by interacting with VqMYB35 (Wang & Wang, 2019), while VqbZIP1 interacts with VqSnRK2.4 and VqSnRK2.6 to enhance the regulation of the *STS* promoters (Wang et al., 2019). In the present study, we also identified a novel transcription factor, VqAL4, that can bind directly to the G-rich element on the promoter of *VqNSTS4* and positively regulate its expression (Figures 4b–f and 5). Similar results were reported in other plants, such as *Atriplex hortensis*, *Glycine max*, and *A. thaliana*, where AL transcription factors could bind to G-rich elements on target gene promoters to promote their expression (Tao et al., 2018; Wei et al., 2009, 2015). However, it is unclear whether the mechanism of *VqNSTS4* regulation by VqAL4 is related to the regulation of *STS* genes by MYBs and WRKYs. Furthermore, *VqAL4* expression was significantly induced by *U. necator* and *VqAL4* enhanced resistance to *U. necator* in grapevine (Figures 7, 8, and S14c). This is consistent with previous reports that the expression of AL transcription factors is induced by pathogens (Kayum et al., 2015; Kayum et al., 2016). Together, the above findings show that the AL transcription factor in grapevines is involved in disease resistance and reveal a novel regulatory mechanism of STS genes in grapevine.

3.3 | Disease resistance pathways activated by stilbene synthase genes in grapevine

Plant defence responses to pathogens are usually viewed as a two-tiered immune system, constituted by pattern-triggered immunity (PTI) and effector-triggered immunity (ETI), which function together as a unified interdependent system that exerts a mutually potentiating effect (Jones & Dangl, 2006; Ngou et al., 2022; Pruitt et al., 2021; Wan et al., 2021). When grapevine is affected by powdery mildew, the two-tiered immune system is stimulated and a series of typical disease resistance reactions is initiated, including the formation of a haustorium complex and callose-rich papillae, the accumulation of reactive oxygen species (ROS) and stilbene phytochemicals, and HR-like cell death (Hu et al., 2021; Qiu et al., 2015; Ramming et al., 2010; Yin et al., 2022). Consistent with previous studies (Liu et al., 2019a; Xu et al., 2019), we observed a large amount of powdery mildew-induced callose deposition in infected epidermal cells of the leaves of *VqNSTS4* transgenic lines after artificial inoculation with *U. necator* (Figure 2e). Unlike typical disease resistance reactions, in the current and previous studies (Cheng et al., 2016; Liu et al., 2019a), no obvious H_2O_2 accumulation and HR-like cell death were observed (Figure 2c). However, overexpression of *VqAL4* enhanced grapevine resistance to *U. necator* and showed typical signs of an antifungal immune response, including a decrease in spore count, an H_2O_2 burst, HR-like cell death, and callose accumulation (Figure 7b–g). This difference in the observed strength of the immune reaction may have been due to the difference in the disease resistance pathways activated by different genes as well as the deletion of 48 amino acids at the N-terminus of VqNSTS4, and should be investigated in greater detail in future studies.

Plant immunity is also often tightly associated with SA signalling (Huang et al., 2020; Zhang & Li, 2019). In the present study, the transcript levels of *VqNSTS4* and *VqAL4* were significantly upregulated after SA treatment (Figures 1l and S14d). Meanwhile, we also observed that the content of free SA and the expression levels of SA-related genes were significantly increased in transgenic

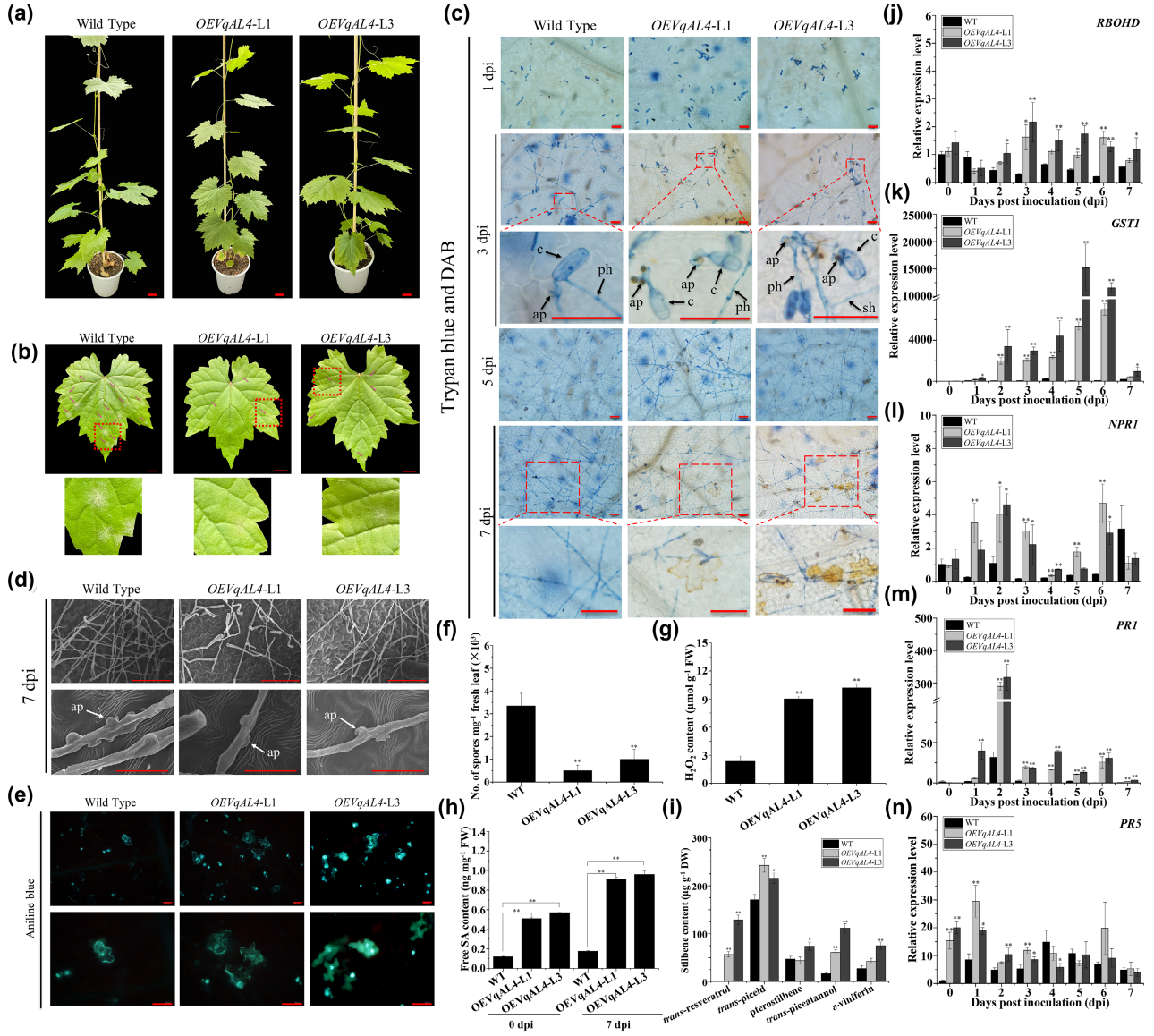

FIGURE 7 Overexpression of *VqAL4* in grapevine enhances resistance to *Uncinula necator* by promoting stilbene accumulation and activating expression of resistance genes. (a) Photograph of *VqAL4* transgenic lines and wild-type Thompson Seedless before *U. necator* inoculation. Bars = 1 cm. (b) Photograph of leaves of *VqAL4* transgenic lines and wild-type Thompson Seedless at 7 days postinoculation (dpi). Bars = 1 cm. (c) Trypan blue staining and 3,3′-diaminobenzidine (DAB) staining show the hyphal growth of *U. necator* and H_2O_2 accumulation at 1, 3, 5, and 7 dpi. c, conidium; ap, appressorium; ph, primary hypha; sh, secondary hypha. Bars = 100 μm. (d) Scanning electron micrographs of the hyphae and appressoria (ap) of *U. necator* in *VqAL4* transgenic lines and wild-type Thompson Seedless. Upper figures, bars = 100 μm; lower figures, bars = 20 μm. (e) Aniline blue staining showing callose depositions in *U. necator*-infected epidermal cells at 7 dpi. Bars = 50 μm. (f) Quantification of spores per mg fresh leaves from *VqAL4* transgenic lines and wild-type Thompson Seedless at 7 dpi. (g) H_2O_2 content of *VqAL4* transgenic lines and wild-type Thompson Seedless leaves at 7 dpi. (h) Free salicylic acid (SA) content in the leaves of *VqAL4* transgenic lines and wild-type Thompson Seedless at 0 dpi and 7 dpi. (i) Determination of five stilbene contents in the leaves of *VqAL4* transgenic lines and wild-type Thompson Seedless at 7 dpi. (j, k) Reverse transcription-quantitative PCR (RT-qPCR) analysis was conducted to determine the relative transcript levels of H_2O_2-related genes in *VqAL4* transgenic lines after *U. necator* inoculation. WT, wild-type Thompson Seedless. (l–n) RT-qPCR analysis was conducted to determine the relative transcript levels of SA-related genes in *VqAL4* transgenic mutants after *U. necator* inoculation. The standard deviation (*SD*) was calculated from three independent replicates. One-way analysis of variance (Tukey's test) was carried out. Asterisks indicate significant differences at $^{*}p < 0.05$, $^{**}p < 0.01$.

VqNSTS4 and *VqAL4* overexpression grapevines (Figures 2h–k and 7h,l–n). In RNAi-*VqNSTS4* and RNAi-*VqAL4* leaves, the content of free SA and the expression levels of SA-related genes were significantly decreased (Figures 3g–j and 8d–g). These results indicate that *VqNSTS4*-mediated or *VqAL4*-mediated disease resistance not only promotes the biosynthesis of stilbenes but also activates the SA signalling pathway. Therefore, we speculate that the relationship between the biosynthesis of stilbenes and SA is mutually reinforcing. This is consistent with previous reports that the SA signalling pathway participates in stilbene-mediated resistance to powdery mildew

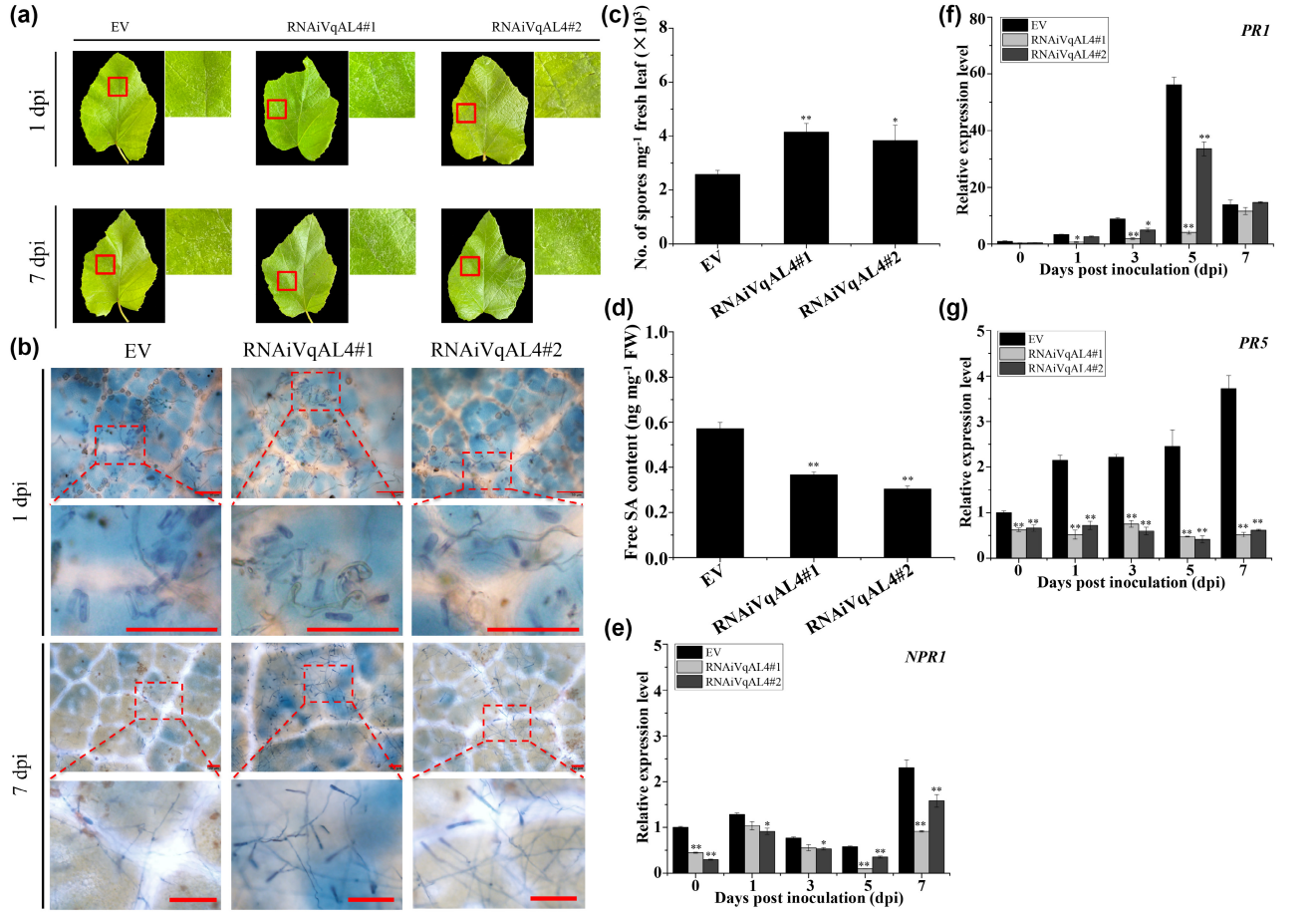

FIGURE 8 Transient silencing of *VqAL4* in leaves of Danfeng-2 reduces resistance to *Uncinula necator*. (a) Phenotypes of control EV and transiently silenced RNAi-VqAL4-GFP Danfeng-2 leaves at 1 day postinoculation (dpi) and 7 dpi. EV, the empty vector pK7GWIWG2(II)-35S-GFP. (b) Trypan blue staining of leaves from EV and transiently silenced RNAi-VqAL4-GFP leaves at 1 dpi and 7 dpi. Bars = 100 μm. (c) Quantification of spores per mg fresh leaves from EV and transiently silenced RNAi-VqAL4-GFP leaves at 7 dpi. (d) Free salicylic acid (SA) content in the leaves of EV and transiently silenced RNAi-VqAL4-GFP leaves at 7 dpi. (e–g) Reverse transcription-quantitative PCR analysis was conducted to determine the relative transcript levels of SA-related genes in EV and transiently silenced RNAi-VqAL4-GFP leaves following *U. necator* inoculation. The standard deviation (*SD*) was calculated from three independent replicates. One-way analysis of variance (Tukey's test) was carried out. Asterisks indicate significant differences at $^{*}p < 0.05$, $^{**}p < 0.01$.

(Jiao et al., 2016; Xu et al., 2019; Yin et al., 2022). However, the specific cause–consequence relationship between the biosynthesis of stilbenes and SA needs further study.

In conclusion, we have identified novel *VqNSTS* genes from Danfeng-2 according to six novel *STS* transcripts. Among them, *VqNSTS4* expression is significantly induced by *U. necator*, enhancing resistance to *U. necator* in transgenic overexpression grapevine by activating SA signalling. Additionally, VqAL4 promotes *VqNSTS4* expression by binding to the G-rich element (CACCTC) on the *VqNSTS4* promoter, resulting in higher antifungal stilbene levels. Moreover, overexpression of *VqAL4* promotes the accumulation of stilbenes and enhances resistance to *U. necator* by activating SA signalling (Figure 9). These results reveal not only novel disease resistance *VqNSTS* genes but also a novel transcription factor that promotes their expression. All of these findings prove that *VqNSTS4* regulated by VqAL4 improves grapevine resistance to powdery mildew by activating SA signalling. The present study has enriched our knowledge of disease resistance in perennial fruit trees.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials

The Chinese wild *V. quinquangularis* accession Danfeng-2 was grown in the vineyard of Northwest A&F University, located in Yangling, Shaanxi, China (34°20′ N, 108°24′ E). Proembryonic masses (PEMs) of *V. vinifera* 'Thompson Seedless', to be used as transgenic receptor materials, were cultured on Murashige and Skoog medium supplemented with 1.5 g/L activated carbon at 25°C in the dark. *A. thaliana* (Col-0), to be used for subcellular

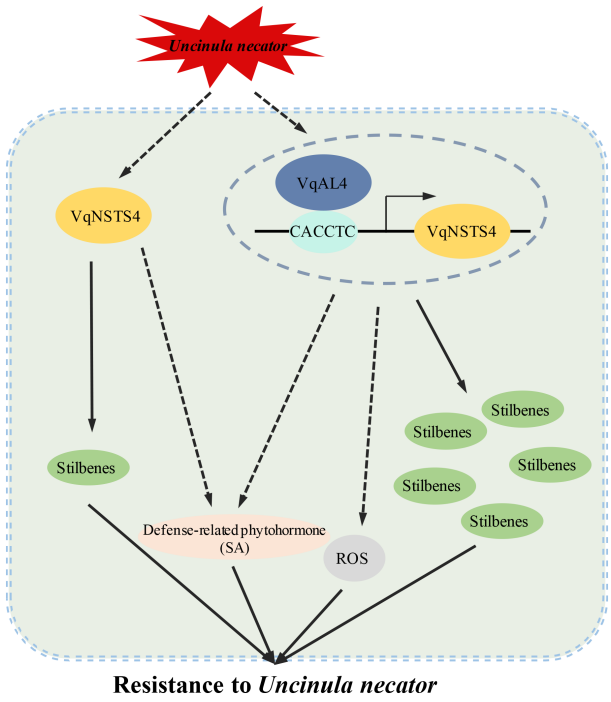

FIGURE 9 Proposed model of *VqNSTS4*-mediated disease resistance to *Uncinula necator* and its regulatory mechanism. *U. necator* infection induces expression of *VqNSTS4* and *VqAL4*. VqAL4 positively regulates the transcription of *VqNSTS4*. *VqNSTS4* or *VqAL4* transgenic grapevines show enhanced resistance to *U. necator* by producing more phytoalexin and activating SA signalling. ROS, reactive oxygen species. SA, salicylic acid.

localization, was planted in a chamber at 23/19°C under a light/dark cycle of 16/8 h. *Nicotiana benthamiana* used for dual-luciferase assays was grown in a growth chamber at 25°C with a photoperiod of 16 h.

4.2 | Identification of novel transcripts in Danfeng-2

Danfeng-2 berries were collected at four stages: the green hard stage (DF_GH, 25 days after blooming), the before véraison stage (DF_BV, 40 days after blooming), the véraison stage (DF_V, 50 days after blooming), and the ripe stage (DF_R, 80 days after blooming). Total RNA of grape berries was extracted with a Plant RNA extraction Kit (Omega). Library preparation and sequencing were performed using Illumina HiSeq 2500. After filtering out reads containing adapters, the remaining high-quality sequencing reads were mapped to the available genome of *V. vinifera* (Jaillon et al., 2007) using Bowtie2 (Langmead & Salzberg, 2012) with default parameters. Unmapped data were assembled by Trinity (Grabherr et al., 2011). Gene expression levels were calculated and normalized using fragments per kilobase per million mapped reads value (Mortazavi et al., 2008). Cluster analysis was carried out according to gene expression levels in four different developmental stages using MultiExperiment Viewer software (MeV, DFCI Boston).

4.3 | Gene cloning and sequence analysis

The coding sequences (CDSs) of *VqNSTS* genes and *VqAL4* were cloned from the cDNA of Danfeng-2, while the promoters of *VqNSTS* genes and *VvSTS* genes were isolated from the genomic DNA of Danfeng-2 and Thompson Seedless. The *cis*-elements in *STS* promoters were predicted using the Plant-CARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The chromosomal location of *VqAL4* was predicted according to the physical location information from the Grape Genome Browser (12X) Database (https://www.genoscope.cns.fr/vitis/). Amino acid sequence alignments were carried out using DNAMAN software v. 7.0.2 (LynnonBiosoft), while phylogenetic trees were constructed with MEGA 5.0 software using the neighbour-joining approach with 1000 bootstrap replications. The primers used in this study are listed in Table S3.

4.4 | Generation of transgenic grapevine using an *Agrobacterium*-mediated method

The CDSs of *VqNSTS4* and *VqAL4* were inserted into the pCAMBIA2300-GFP expression vector. The recombinant constructs were introduced into *Agrobacterium tumefaciens* GV3101 and then transformed into PEM of Thompson Seedless according to previously described methods (Zhou et al., 2014). *VqNSTS4-GFP* and *VqAL4-GFP* transgenic lines were identified by western blot and RT-qPCR. To confirm the expression of VqNSTS4-GFP and VqAL4-GFP fusion proteins, western blot analysis was conducted. Total protein extraction and detection were carried out as described previously (Xu et al., 2019), with the primary antibody anti-GFP. GFP fluorescence (488 nm) of the fusion protein was examined with a confocal laser scanning microscope (TCS SP8; Leica). For RT-qPCR, total RNA was extracted using the HiPure Plant RNA Mini Kit (Magen), and 1 μg of RNA was used for cDNA first-stand synthesis using SumOnetube RT Mixture III (gDNA removal) (SUM 7806). PCR 2×FastSYBR Green Mixture (Cofitt LBQ7505-05) and an IQ5 RT-PCR system (Bio-Rad) were used for qPCR. The PCR mixture was 20 μl, comprised of 0.8 μl of forward primer, 0.8 μl of reverse primer, 1 μl of sample cDNA, 10 μl of SYBR, and 7.4 μl of RNase-free water. Grape *GAPDH* (CB973647) was used as a reference gene (Xu et al., 2019). The $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression levels (Livak & Schmittgen, 2001). The primers used for RT-qPCR are listed in Table S3.

4.5 | Artificial inoculation of *U. necator* and phytohormone treatments

Fully expanded and healthy growing leaves at the third to sixth position of the grape shoot tip were inoculated with *U. necator* using a previously described method (Wang et al., 1995). Briefly, the surfaces of mature leaves of Danfeng-2 or transgenic Thompson Seedless grapevines were sprayed with 0.78% glucose solution

containing 2×10^5 conidia/ml to the point where tiny liquid droplets appeared but no run-off occurred. Samples were collected at 0, 1, 2, 3, 4, 5, 6, and 7 dpi. Exogenous ABA (100 μM), Eth (100 μM), SA (100 μM), or MeJA (100 μM) (Wang et al., 2020) was sprayed on the leaves of Danfeng-2, which were collected at 0, 0.5, 1, 3, 6, 12, 24, and 48 hpt. Mock samples from leaves sprayed with sterile water were collected at the same sampling times as the exogenous treatment samples.

4.6 | Y1H assay

Y1H assays were carried out with the Matchmaker Gold Yeast One-Hybrid System (Clontech). The promoters of *VqNSTS* genes and *VvSTS* genes were inserted into pAbAi. The mutations of *ProVqNSTS4* (*ProVqNSTS4*^{m2}, *ProVqNSTS4*^{m3}, *ProVqNSTS4*^{d}, *ProVqNSTS4*^{m5}, and *ProVqNSTS4*^{m6}) were inserted into the pAbAi vector. These pAbAi-baits were linearized using *Bbs*I or *Bst*BI (NEB) and integrated into the genome of the Y1HGold yeast strain. The transformed yeast cells were cultured on SD/−Ura medium to select for positive clones (cultured at 30°C for 3 days). The CDSs of *VqAL4*, *VqWRKY2*, *VqWRKY3*, *VqWRKY53*, *VqMYB14*, *VqMYB15*, and *VqMYB154* were inserted into pGADT7 to create the AD-prey vectors. These AD-prey vectors were transferred into the bait strains and subsequently growth of varying yeast transformants on SD/−Leu medium supplemented with AbA (200 ng/ml) was examined. pGADT7-p53/p53-pAbAi was used as a positive control, and an empty AD vector was used as a negative control. The primers used in this study are listed in Table S3.

4.7 | Dual-luciferase assays

To further demonstrate that transcription factors can activate *STS* promoters, dual-luciferase assays were carried out using a previously described method (Wang & Wang, 2019). Briefly, the promoters of *VqNSTS4* or *VvSTS32* were inserted into the pGreenII 0800-LUC vector and the CDS of *VqAL4* was cloned into the pGreenII 62-SK vector. These recombinant vectors were then transferred into *A. tumefaciens* GV3101 and transiently expressed in *N. benthamiana* leaves by *Agrobacterium*-mediated infiltration. The leaves were collected for protein extraction after culture for 3 days. The enzyme activities of firefly luciferase (LUC) and Renilla luciferase (REN) were examined using the Dual Luciferase Reporter Gene Assay Kit (Beyotime).

4.8 | ChIP-qPCR analysis

ChIP-qPCR analysis was conducted to validate VqAL4 binding to the G-rich element. For ChIP analysis, 3 g of Danfeng-2 leaves transiently overexpressing 35S-VqAL4-GFP or young leaves from 35S-VqAL4-GFP transgenic grapevine were crosslinked in 0.5% formaldehyde for 10 min using vacuum infiltration. Chromatin was isolated using the EpiQuik Plant ChIP Kit (Epigentek) according to the manufacturer's manual and immunoprecipitated by anti-GFP. The immunoprecipitated and input DNA samples were used for qPCR analysis, and the ChIP-qPCR results are presented as percentages of the input DNA. The specific primers are listed in Table S3.

4.9 | *Agrobacterium*-mediated transient expression in grape leaves

Transient transformation in Danfeng-2 leaves was performed to confirm that VqAL4 regulates *VqNSTS4* expression and enhances resistance to *U. necator*. The *A. tumefaciens* GV3101 strains containing the fusion vectors *35S-VqAL4-GFP*, *RNAi-VqAL4-GFP*, and *RNAi-VqNSTS4-GFP* and the empty vectors pCAMBIA2300-GFP and pK7GWIWG2(II)-35S-GFP were cultured in Luria–Bertani (LB) liquid medium with the OD_{600} value adjusted to 0.6–0.8. Leaves of Danfeng-2 were immersed in the bacterial solution and held under vacuum for 30 min. These leaves were then cultured in a growth chamber with the petioles wrapped in moist cotton for 72 h and harvested for further analysis. For disease resistance analysis, the transiently transformed leaves of Danfeng-2 were inoculated with *U. necator* after 24 h of culture.

4.10 | Subcellular localization of the VqAL4 transcription factor

The isolation and transformation of *Arabidopsis* protoplasts were carried out using a previously described method (Wang et al., 2019). Briefly, 5 μl (1 μg/μl) of 35S-VqAL4-GFP and 35S-AtHY5-mCherry plasmid were mixed in a 2-ml centrifuge tube and subsequently transformed into protoplasts using polyethylene glycol–Ca^{2+}-mediated transformation. The transformed protoplasts were cultured in the dark for 20 h at 25°C, and the expression of fusion protein was examined with a confocal laser scanning microscope (TCS SP8; Leica).

4.11 | Y2H assay

For transcriptional activation assays, the full-length CDS of *VqAL4* was inserted into the vector pGBKT7 (BD) to construct the pGBKT7-VqAL4 (BD-VqAL4) fusion vector. The plasmid DNA of BD-VqAL4 was transferred into the Y2HGold yeast strain (Clontech) according to the instructions of the Yeastmaker Yeast Transformation System 2 User Manual (Clontech). pGADT7-T co-transformed with pGBKT7-53 was used as a positive control, and pGADT7-T co-transformed with pGBKT7-Lam was used as a negative control. The transformants were cultured on SD/−Trp, SD/−Trp + X-α-gal (40 μg/ml), and SD/−Trp + X-α-gal (40 μg/ml) + AbA (200 ng/ml) media at 30°C for 3 days (Wang et al., 2019). The primers used in this study are listed in Table S3.

4.12 | Observation of spore growth and development on the leaves of grapevines after artificial inoculation with *U. necator*

The fungal spores were quantified as previously reported (Weßling & Panstruga, 2012). Briefly, infected leaves were collected at 7 dpi, cut into pieces, and immersed in 5 ml of sterile water containing 0.01% Tween 20. This was shaken for 30 min at 500 rpm and then a haemocytometer was used to count the spores under a microscope. Scanning electron microscopy and aniline blue, trypan blue, and 3,3′-diaminobenzidine staining were used to visualize hyphae, callose deposition, H_2O_2 accumulation, and HR-like cell death (Hu et al., 2021). Samples were taken from three different plants per line for three biological replicates. The extraction and measurement of free SA were carried out using a previously described method (Wang et al., 2020). Briefly, 100 mg of leaves was ground and extracted with 1 ml of extraction solvent (methanol:isopropanol, 20:80 [vol/vol] with 1% of glacial acetic acid) using ultrasonication (4°C). After centrifugation (10,000 rpm for 15 min at 4°C), the supernatant was collected, dried completely under a nitrogen stream, redissolved in 300 µl of methanol, centrifuged (10,000 rpm for 5 min), and filtered through a 0.22-µm membrane film. Samples were then analysed by HPLC–tandem mass spectrometry with a Shim-pack XR-ODS (2.0 mm internal diameter, 75 mm × 1.6 µm) column coupled to a triple quadrupole mass spectrometer (LC-MS8040; Shimazu) with an electrospray ionization source. A standard sample of free SA (cat. #B21197; Yuanye) was used to confirm the retention time. The H_2O_2 content was measured by fluorescence spectrophotometry using the Hydrogen Peroxide Assay Kit (Solarbio). All samples were analysed in triplicate.

4.13 | Detection and analysis of stilbenes using HPLC

Leaves were collected from different transgenic grapevines and wild-type Thompson Seedless for stilbene detection using HPLC. Extraction and determination of stilbenes were carried out using a previously described method (Liu et al., 2019a). The leaves were freeze-dried for 24 h before being extracted in methanol at 4°C for 12 h in the dark. The extracted liquor was filtered through a 0.22-µm membrane film for HPLC analysis. Standard samples of stilbenes (Sigma) were dissolved in methanol and used to confirm the retention times. HPLC analyses were conducted using a Waters HPLC System (Waters ACQUITY Arc). The gradient elution programme was as follows: 0–1 min, 20% acetonitrile and 80% water; 1–30 min, 20%–75% acetonitrile and 80%–25% water; 30–33 min, 75%–100% acetonitrile and 25%–0% water; 33–36 min, 100% acetonitrile and 0% water; 36–38 min, 100%–20% acetonitrile and 0%–80% water; 38–49 min, 20% acetonitrile and 80% water. Standard samples of *trans*-resveratrol (cat. #R5010; Sigma-Aldrich), *trans*-piceid (cat. #15721; Sigma-Aldrich), pterostilbene (cat. #P1499; Sigma-Aldrich), *trans*-piceatannol (cat. #CFN99024; ChemFaces), and ε-viniferin (cat. #CFN97067; ChemFaces) were used to confirm the retention times.

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CONFLICT OF INTEREST

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the results are included in this article and its supplementary materials.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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