VqWRKY53 enhances stilbene synthesis and disease resistance by interacting with VqMYB14 and VqMYB15 from Chinese wild grape

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Highlight

VqWRKY53 acts as a positive regulator of stilbene synthesis by forming a transcriptional regulatory complex with VqMYB14 and VqMYB15. Overexpression of Vqwrky53 in *Arabidopsis* accelerates leaf senescence and promotes disease resistance.

Abstract

Resveratrol is notable not only for its functions in disease resistance in plants but also for its health benefits when it forms part of the human diet. Identification of new transcription factors helps to reveal the regulatory mechanisms of stilbene synthesis. Here, the WRKY transcription factor Vqwrky53 was isolated from the Chinese wild grape, *Vitis quinquangularis*. Vqwrky53 was expressed in a variety of tissues and responded to powdery mildew infection and to exogenous hormone application. VqWRKY53 was located in the nucleus and had transcriptional activation activity in yeast. The yeast two-hybrid (Y2H) assay and the bimolecular fluorescence complementation (BiFC) assay confirmed that VqWRKY53 interacted physically with VqMYB14 and VqMYB15 which have previously been reported to regulate stilbene synthesis. When Vqwrky53 was overexpressed in grape leaves, the expressions of *VqSTS32* and *VqSTS41* and the content of stilbenes were increased. The yeast one-hybrid assay demonstrated that Vqwrky53 could bind directly to the promoters of *STS* genes. Overexpression of Vqwrky53 activated GUS expression, driven by *STS* promoters and co-expressing Vqwrky53 with VqMYB14 and VqMYB15 showed stronger regulatory functions. Heterologous overexpression of Vqwrky53 in *Arabidopsis* accelerated leaf senescence and disease resistance to *Pst*DC3000.

Keywords: Chinese wild *Vitis quinquangularis*, stilbene, WRKY transcription factor, transcriptional regulation, leaf senescence, disease resistance

Introduction

Stilbenes are important phytoalexins, produced in response to a range of biotic and abiotic stresses including to pathogen infection (Fung *et al.*, 2007; Langcake and Pryce, 1976; Schnee *et al.*, 2008), UV-C light (Adrian *et al.*, 2000; Fritzemeier and Kindl, 1981; Selma *et al.*, 2008; Wang *et al.*, 2010), ozone (Rosemann *et al.*, 1991; Schubert *et al.*, 1997) and hormone treatment (Belhadj *et al*., 2008). In 1939, resveratrol which is the basic unit of stilbenes was isolated from *Veratrum grandiflorum* (Takaoka, 1939). Later, it was detected in the roots of *Polygonum cuspidatum* (Nonomura *et al.*, 1963). Now, 72 plant species belonging to 31 genera and 12 families have been shown to produce resveratrol. Some of these plants form components of a normal human diet, including blueberries (*Vaccinium myrtillus*), cranberries (*Vaccinium oxycoccos*), peanuts (*Arachis hypogaea*) and grapes (*Vitis vinifera*) (Jang *et al.*, 1997). Particularly high levels of stilbenes occur in grapes (Jeandet *et al*., 1991; Jang *et al.*, 1997). Resveratrol is notable not only for its function in disease resistance in plants but also for its beneficial effects in human health, where it provides a degree of protection against cancer, inflammation, cardiovascular disease and diabetes (Baur and Sinclair, 2006; Jang *et al.*, 1997; Kalantari and Das, 2010). With the discovery of stilbenes in fruits, there is growing interest in the study of stilbenes, as fruits are likely to become an increasing component of the human diet (Chong *et al*., 2009). Among the stilbene-producing plants, grapevine is one of the most important, with increased consumption of its fruit offering significant health benefits. The stilbenes are derived from the phenylpropanoid pathway. Stilbene synthase (STS) competes with chalcone synthase (CHS) in the last step for the same substrates to catalyse the synthesis of resveratrol (Halls and Yu, 2008; Rolfs and Kindl, 1984). STS is the key enzyme in stilbene synthesis and belongs to the type III polyketide synthases family, first extracted from peanut (Schoppner and Kindl, 1984). With the completion of genome sequencing of Pinot Noir PN40024, 48 *STS* genes have been identified. These are located on two chromosomes 10 and 16 (Jaillon *et al.*, 2007; Vannozzi *et al.*, 2012). After the discovery of resveratrol, various derivatives, such as piceid (Waterhouse and Lamuela-Raventós, 1994), viniferins and pterostilbene (Langcake, 1981; Jeandet *et al*., 2019) have been detected in grapevine. Stilbenes play important

roles in plant disease resistance. Stilbene synthesis occurs in cells infected by pathogens, such as powdery mildew (Schnee *et al*., 2008). The content of resveratrol and its derives δ- and ε- viniferins can increase rapidly in the leaves following pathogen infection (Alonso-Villaverde *et al*., 2011).

In recent years, transcription factors (TFs) participating in regulating the *STS* genes in grapevine have been reported. Thus, *MYB14* and *MYB15* were reported to regulate stilbene synthesis which increases the promoter activity of *VvSTS29* and *VvSTS41* (Höll *et al.*, 2013). Then, *Myb14* was shown to bind directly to the promoter of the *STS* gene and to promote its expression (Fang *et al.*, 2014). *VviMYB13*, which shares the same co-expressed *STS* genes as *VviMYB14* and *VviMYB15*, is likely a pivotal TF involved in stilbene accumulation (Wong *et al.*, 2016). Another study showed that promoters of *STS* genes contain enriched MYB and WRKY binding sites. So, in addition to MYB, the WRKY TFs are also important TF families involved in regulating *STS* genes (Wong and Matus, 2017). Later, WRKY family was identified as the main transcription factor family regulating *STS* genes. *VviWRKY24* acts as an effector of *STS29* promoter and *VviWRKY03* up-regulates the promoter of *STS2*9 through co-expression with *VviMYB14* (Vannozzi *et al.*, 2018). Recently, VvWRKY8 which is a negative regulator of *STS* genes, has been shown to suppress the expression of *VvSTS15/21* by interacting with VvMYB14 (Jiang *et al.*, 2019). The discovery of these TFs provides new opportunities for discovering the regulation of stilbenes biosynthesis (Jeandet *et al*., 2019). In addition, other TF family members, such as bZIP TF and ERF TF, have been reported to participate in regulating stilbene synthesis. *VvABF2* has been demonstrated to promote the accumulation of stilbenes (Nicolas *et al.*, 2014). VqbZIP1 positively regulates the expression of *VqSTS6*, *VqSTS16* and *VqSTS20* by interacting with the key components of ABA signal transduction, VqSnRK2.4 and VqSnRK2.6 (Wang *et al.*, 2019). VqERF114 promotes the expression of *STS* genes by interacting with VqMYB35 (Wang and Wang, 2019).

The WRKY TF family is one of the most important TF families in plants. According to data from the Plant Transcription Factor Database, compared

with other transcription factor families in 165 plant species, the total number of WRKY family ranks 8th (Jin *et al.*, 2017). As plant genome sequencing has progressed, WRKY families have been identified in numerous species, including 75 members in *Arabidopsis*, 83 in rice and 59 in grape (Goff *et al.*, 2002; Guo *et al.*, 2014; Riechmann and Ratcliffe, 2000). WRKY TFs contain at least one highly-conserved WRKY domain containing 60 amino acids. The WRKY domain contains a conserved WRKYGQK sequence and a zinc-finger motif (Rushton *et al.*, 1995). WRKY TFs have been divided into three subgroups - I, II and III (Rushton *et al.*, 2010). Members of subgroup I contain two WRKY domains while those belonging to subgroups II and III contain only one. WRKY TFs can recognise the conserved DNA binding site W-box (TTGACC/T) (Eulgem *et al.*, 2000; Rushton *et al.*, 1996). A number of WRKY TFs involved in senescence and in disease resistance have been reported in *Arabidopsis*. For example, AtWRKY6 positively regulates senescence by targeting a receptor-like protein kinase (SIRK) and increasing the promoter activity of the *PR1* (PATHOGENESIS-RELATED GENE 1) (Robatzek and Somssich, 2002). *AtWRKY75* accelerates leaf senescence through a tripartite amplification loop and promotes plant defence responses (Guo *et al.*, 2017). In crops, the WRKY TF *BnaWRG1* from oilseed rape regulates reactive oxygen species (ROS) accumulation and leaf senescence (Yang *et al.*, 2018). Studies of WRKY TFs involved in senescence in grapevine have seldom been reported. However, a number of WRKY TFs from grapevine have been shown to be involved in plant disease resistance. For example, overexpression of *VvWRKY1* up-regulates the expression of disease-resistant genes in the jasmonic acid (JA) pathway, thereby increasing the resistance of transgenic lines to grey mould and downy mildew (Marchive *et al.*, 2013; Marchive *et al.*, 2007). Heterologous expression of *VpWRKY1* and *VpWRKY2* in *Arabidopsis* can increase the resistance of transgenic lines to powdery mildew (Li *et al.*, 2010). Heterologous expression of *VqWRKY52* in *Arabidopsis* enhances the resistance of transgenic lines to powdery mildew and *Pseudomonas syringae*, but increases their susceptibility to grey mould (Wang *et al.*, 2017a). Knockout of *VvWRKY52* using the CRISPR/Cas9 system can enhance the resistance of transgenic grape to grey mould (Wang *et al.*, 2017b).

China is one of grapevine's main centres of origin with abundant wild grape germplasm resources. In previous studies, we showed that the Chinese wild grape *V. quinquangularis* accession Danfeng-2 contains much higher levels of resveratrol than the *V. vinifera* cultivars examined (Shi *et al.*, 2014; Zhou *et al.*, 2015). Later, we used the berries of Danfeng-2 at four developmental stages to carry out transcriptome sequencing (the results are not yet released). Later, we detected the expressions of 59 grape WRKY members in Danfeng-2 under induction by powdery mildew and found that 16 WRKY TFs in Danfeng-2 were up-regulated (Supplementary Figure S1). According to the transcriptomic data, co-expression analysis was carried out to identify TFs participating in regulating the *STS* genes. Here, a WRKY type transcription factor, Vqwrky53, which can be induced by pathogen infection, was isolated from Danfeng-2. This study demonstrates that VqWRKY53 positively regulates the accumulation of stilbenes by directly regulating *STS* genes or forming transcriptional regulation complex with VqMYB14 and VqMYB15. In addition, Vqwrky53 accelerates leaf senescence and promotes disease resistance in transgenic *Arabidopsis*.

Materials and methods

Plant materials

Various organs of Danfeng-2 including leaves, stems, inflorescences, tendrils and berries were collected from the Grape Germplasm Resources orchard of Northwest A&F university, Yangling, Shaanxi, China (34°20´N, 108°24´E). The tobacco (*Nicotiana benthamiana*) used for the GUS activity assay was grown in a phytotron (25°C; photoperiod 16/8 h). *Arabidopsis thaliana* used for subcellular localisation, the BiFC assay and the transgenic experiment, were grown in a growth chamber at 22°C under a 16/8 photoperiod.

Gene cloning and sequence analysis

The RNA of Danfeng-2 berries was extracted with an Omega Plant RNA Kit (Omega, Norcross, Georgia). The RNA (1 μg) was used to synthesise the cDNA following the instructions of the FastKing RT Kit (TIANGEN, Beijing, China). Then, the cDNA was used as a template for gene cloning. Full-length

coding sequence (CDS) of Vqwrky53 (VIT_17s0000g01280) were amplified using the primers VqWRKY53-F/ VqWRKY53-R (Supplementary Table S2) which were designed according to the homologous sequence in Pinot Noir. DNAMAN (Lynnon Biosoft, San Ramon, USA) was used for sequence alignment. The online website Clustal W2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/) and the FigTree v1.4.4 software (Andrew Rambaut Institute of Evolutionary Biology, University of Edinburgh, UK) was used for cluster analysis. The Grape Genome Browser website (http://www. geno scope.cns.fr/externe/GenomeBrowser/Vitis/) was used for chromosomal localisation analysis and The SMART online analysis website (http://smart.embl-heidelberg.de/) was used to analyse protein structure.

Subcellular localisation of VqWRKY53

The CDS of Vqwrky53 was inserted into the pCAMBIA2300 vector (Ma *et al*., 2018) to generate 35S-VqWRKY53-GFP fusion vector using the primers VqWRKY53-GFP-F-Kpn I/ VqWRKY53-GFP-R-Sal I (Supplementary Table S2). The fusion vector 35S-AtHY5-mCherry, which was constructed previously by our team, was used as a nuclear localisation marker gene (Yao *et al.*, 2017). Empty pCAMBIA2300 was used as control. Different combinations of plasmids (35S-VqWRKY53-GFP+35S-AtHY5-mCherry and 35S-GFP+35S-AtHY5-mCherry) were transformed into *Arabidopsis* protoplasts according to the PEG-mediated method as described previously (Zhao *et al*., 2016). The protoplasts were then cultured at 22°C in the incubator. The green fluorescent protein (GFP) signal and mCherry signal were observed with a laser scanning confocal microscope (Carl Zeiss LAM510, Germany). Here, the chloroplast signal is shown in blue which distinguishes it from the red fluorescence signal of mCherry.

Inoculation of powdery mildew

Grape powdery mildew was collected from the Grape Germplasm Resources orchard of Northwest A&F University. Inoculation of Danfeng-2 leaves with *Uncinula necator* was done as described previously (Wang et al., 1995). Leaves treated with distilled water were used as controls. After inoculation, petioles of Danfeng-2 leaves were inserted in wet, degreased cotton wool and

cultured for 120 h in a phytotron at constant temperature (25°C; photoperiod 16/8 h). Samples were collected 0, 12, 24, 48, 72, 96 and 120 h after inoculation. The leaves were immediately frozen in liquid nitrogen and stored at -80°C for later use.

Hormone treatment

Leaves of Danfeng-2 were sprayed with different hormone solutions including with salicylic acid (SA, 100 μM), methyl jasmonate (MeJA, 100 μM), ethylene (Eth, 100 μM) and abscisic acid (ABA, 100 μM). All four hormones solutions were first dissolved in absolute ethanol and then diluted with water to final concentration (100 μM). Leaves treated with distilled water were used as controls. Samples were collected at 0, 0.5, 1, 2, 6 and 10 h post treatment. The leaves were immediately frozen in liquid nitrogen and stored at -80°C for later use.

Yeast two-hybrid (Y2H) assay

Vqwrky53 was amplified using primers VqWRKY53-BD-F-EcoR I/ VqWRKY53 -BD-R-Sal I (Supplementary Table S2) and inserted into the vector pGBKT7 (Clontech, Mountain View, CA, USA) to generate BD-VqWRKY53. The CDSs of *VqMYB14* and *VqMYB15* were separately inserted into the vector pGADT7 (Clontech, Mountain View, CA, USA) to generate AD-VqMYB14 and AD-VqMYB15 using primers VqMYB14-AD-F-EcoR I/VqMYB14-AD-R-BamH I and VqMYB15-AD-F-

EcoR I/VqMYB15-AD-R-BamH I (Supplementary Table S2). The fusion vector BD-VqWRKY53 was separately co-transformed with AD-VqMYB14 and AD-VqMYB15 into the Y2HGold yeast strain (Clontech, Mountain View, CA, USA). The pGADT7 empty vector was used as negative control. The yeast transformation experiment was conducted using the method provided in the 'Yeastmaker Yeast Transformation System 2 User Manual' (Clontech, Mountain View, CA, USA). The transformed Y2HGold strain was cultured on the medium SD/-Leu/-Trp and SD/-Trp /-Leu/-Ade/-His with aureobasidin A (AbA) and X-α-gal and cultured at 28°C for three days.

Bimolecular fluorescence complementation (BiFC) assay

The CDS of Vqwrky53 was subcloned into the pSPYNE vector to generate pSPYNE/VqWRKY53. The CDSs of *VqMYB14* and *VqMYB15* without termination codon were subcloned into pSPYCE vector to generate pSPYCE/VqMYB14 and pSPYCE/VqMYB15 (Waadt *et al*., 2008). The vector pSPYNE/VqWRKY53 was separately co-transformed with pSPYCE/VqMYB14 and pSPYCE/VqMYB15 into *Arabidopsis* protoplasts. Transformation used the PEG method previously reported (Zhao *et al.*, 2016). Different combinations including pSPYNE/ VqWRKY53 with pSPYCE, pSPYCE/VqMYB14 with pSPYNE and pSPYCE/ VqMYB15 with pSPYNE were transformed into protoplasts as negative controls. After transformation for 20 h, the yellow fluorescent protein (YFP) signal was observed using a confocal laser microscope (LSM 510, Zeiss, Oberkochen, Germany). Here, the chloroplast signal is shown in red.

Yeast one-hybrid (Y1H) assay

The Matchmaker™ Gold Yeast One-Hybrid System (Clontech, Mountain View, CA, USA) was used in this experiment. The promoters of *VqSTS32* and *VqSTS41* were inserted into the pAbAi vector to generate pAbAi-ProVqSTS32 and pAbAi-Pro- VqSTS41. The three tandem repeats of TTGACC (W-box 1) and TTGACT(W-box 2) were also inserted into pAbAi. The pAbAi fusion vectors were then linearised using the restriction enzyme Bbs I (NEB, USA) and transformed into the Y1H gold yeast strain as described in the 'Yeastmaker Yeast Transformation System 2 User Manual' (Clontech, Mountain View, CA, USA). Y1H gold strains harbouring the *STS* promoters and W-boxes were used as the bait strain. The full length CDS of Vqwrky53 and the N-terminal of VqWRKY53(58-151 aa) were inserted into the pGADT7 to generate AD-VqWRKY53 and AD-VqWRKY53$^{58\text{-}151}$. AD-VqWRKY53 and AD-VqWRKY53$^{58\text{-}151}$ were then transformed into different bait strains. The empty pGADT7 vector was transformed to the bait strain as the negative control. The single colony was cultured on to the SD/-Leu medium with AbA.

Agrobacterium-mediated transient overexpression assay in grape leaves

The vector 35S-VqWRKY53-GFP and empty pCAMBIA2300 vector were separately transformed into agrobacterium strain GV3101 and cultured in Luria-Bertani (LB) liquid medium. This was centrifuged to pelletize the cells and then resuspended to an OD_{600} value of 0.6. Leaves of Danfeng-2 were put upside-down into glasses containing 100 ml of the bacterial suspension to conduct the transient overexpression assay using the vacuum-infiltration method described previously (Xu *et al.*, 2010). After vacuuming for 30 min, the leaves were then placed with the petioles inserted between humidified absorbent cotton wool in preservative film-sealed trays for 48 h and collected for further analysis.

Stilbene content determination

The detection of stilbene content in grapevine leaves was carried out as reported previously (Cheng et al., 2016). Leaves were ground to a powder in liquid nitrogen and freeze-dried for 24 h. Samples were then transferred to methanol for extraction at 4°C for 12 h in the dark and centrifuged at 5500 rpm for 15 min and the insoluble debris was discarded. The supernatant was filtered through a 0.22 μm membrane film separately and collected for HPLC analysis. HPLC analyses were conducted on a Waters 600E-2487 HPLC system (Waters, USA). Well-prepared samples (5 μl) of the extracts were subjected to this system and eluted with mobile phase A (acetonitrile) and mobile phase B (water) with a flow rate of 1.0 ml/min under the absorbance wavelength of 306 nm. The linear gradient settings (0 min: 20% A and 80% B; 0-30 min: 75% A and 25% B; 30-32 min: 100% A and 0% B; 32-35 min: 100% A and 0% B; 35-36 min: 20% A and 80% B; 36-45 min: 20% A and 80% B) were consistent with previous studies (Cheng *et al.*, 2016). Standard samples of trans-piceid, trans-resveratrol and *ε*-viniferin (Sigma-Aldrich, St Louis, MO, USA) were used to confirm the retention times.

GUS activity assay

The promoters of *VqSTS32* and *VqSTS41* were inserted into pCAMBIA1391 to generate $P_{VqSTS32}$-GUS and $P_{VqSTS41}$-GUS and transformed into GV3101 strain (Xu *et al.*, 2010). The CDSs of VqMYB14 and VqMYB15 were

inserted into pCAMBIA2300 to generate 35S-VqMYB14-GFP and 35S-VqMYB15-GFP fusion vectors. Then, the vectors 35S-VqWRKY53-GFP, 35S-VqMYB14-GFP and 35S- VqMYB15-GFP were separately transformed into GV3101 strain. The GV3101 strain harbouring different vectors was infiltrated into tobacco leaves following the method described previously (Liu *et al*., 2010). After infiltration, the tobacco plants were grown in a phytotron (25°C; photoperiod 16/8 h) for 72 h and the leaves then collected for GUS activity detection. GUS activity was detected as described previously (Jefferson, 1987; Xu *et al*., 2010) using a Nicolet Evolution 300 UV-VIS spectrophotometer (Thermo, USA).

Arabidopsis transformation

The GV3101 harbouring 35S-VqWRKY53-GFP was cultured in 50 ml LB liquid medium supplemented with the corresponding antibiotic at 28°C until they reached an OD_{600} of 0.8. The bacterial solution was collected in a 50 ml centrifuge tube, centrifuged at 5000 rpm for 10 min and the supernatant discarded. The pellet was resuspended in the solution (½ MS supplemented with 5% sucrose and 0.02-0.03% Silwet L-77) and adjusted to an OD_{600} of 0.6. The inflorescence dip method was used for *Arabidopsis thaliana* transformation as reported previously (Clough and Bent, 2010). The harvested T0 transgenic seeds were surface sterilised in 10% NaClO for 10 min and immediately washed four times with sterile water. The seeds were then selected in ½ MS medium supplemented with 50 mg/ml kanamycin for 12 d and the resistant seedlings transplanted to soil and cultured in an incubator. The harvested T1 transgenic seeds were selected to T3 using the same methods.

Chlorophyll content measurement

When measuring the chlorophyll content, 0.05 g of fresh leaves were placed in 5 ml of 96% ethanol and held overnight at 4°C. Chlorophyll absorbance was measured at 665 and 649 nm using an ultraviolet spectrophotometer. Chlorophyll content (mg/g FW) = (18.08 × A649 + 6.63 × A665) / leaf fresh weight (Zhang *et al.*, 2012).

SA content measurement

The 7th leaves of *Arabidopsis* from wild type (Col-0) and three transgenic lines (OE# 2, OE#5 and OE#6) were collected and 0.1 g weighed out for free SA measurement. The extraction and measurement of free SA were carried out using the method described previously (Han et al., 2019).

***Pst*DC3000 inoculation, trypan blue and DAB staining**

The *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000) (Staskawicz *et al*., 1987) were grown in the medium (King *et al*., 1954) supplemented with rifampicin at 28°C for pathogen inoculation. Leaves from five-week-old *Arabidopsis* were inoculated with *Pst*DC3000 using the infiltration method described previously (Kiedrowski *et al*., 1992; Varet et al., 2003). Leaves after inoculation for 3 dpi were used for detection of bacterial growth following the method described previously (Varet et al., 2003). Samples were collected after inoculation for 0, 24, 48 and 72 h and stored at -80°C for further qRT-PCR analysis. Leaves were collected 72 h after inoculation for trypan blue and diaminobenzidine (DAB) staining. Trypan blue staining was used to detect cell death. Selected leaves were immersed in 8 ml trypan blue staining solution, boiled for 5 min, let stand for 8 h at room temperature. Then the staining solution was discarded and chloral hydrate added to decolourise. Then the leaves were placed in 10% glycerol and photographed. DAB staining was carried out to detect the accumulation of ROS in leaves of *Arabidopsis*. The selected leaves were immersed in 8 ml of DAB staining solution for 8 h at room temperature. Then, the DAB staining solution was discarded, 95% ethanol was added and boiled for 5 min. After decolourisation, the leaves were placed in 10% glycerol and photographed.

Quantitative real-time PCR (qRT-PCR)

RNA extraction of samples from grape and *Arabidopsis* was carried out using an Omega Plant RNA Kit (Omega, Norcross, Georgia). Then, 1 μg RNA was used to synthesise the first strand of cDNA with the FastKing RT Kit (TIANGEN, Beijing, China). The qRT-PCR analysis was carried out in volumes of 20 μl reaction buffer, containing 0.8 μl of each primer, 1 μl of cDNA previously synthesised, 10 μl of 2×SYBR Premix Ex Taq (Takara,

Japan), and 7.4 μl deionised water. Reactions were run under the amplification program of 95°C for 3 min, then 95°C for 10 s, 60°C for 15 s, 72°C for 20 s, for 45 cycles. qRT-PCR was run on an iCycler iQ5 thermal cycler (Bio-Rad, Hercules, CA, USA). The $2^{-\Delta\Delta c(t)}$ method was used to calculate gene relative expression levels. Grape *GAPGH* (GR883080) and *Arabidopsis Actin* (AT3G18780) were used as internal controls. Data are means (±SD) of three biological replicates. Significant differences were analysed with SPSS using One-Way ANOVA with the *Tukey* test (**P*<0.05; ***P*<0.01). Primers used are listed in Supplementary Table S2 and Supplementary Table S3.

Results

Isolation and sequence analysis of Vqwrky53

To find potential TFs involved in stilbene synthesis, co-expression analysis was carried out using the transcriptomic data for Danfeng-2 and the Pearson's correlation coefficient (PCC) value was used to measure the degree of co-expression relationship (Sedgwick *et al*., 2012). The WRKY TF gene, Vqwrky53 (VIT_17s0000g01280) was identified as co-expressed with the *STS32* (VIT_16s0100g01040) and *STS41* (VIT_16s0100g01130) genes, with high PCC values of 0.90 and 0.98 respectively (Supplementary Table S1). By searching in the Grape Genome Browser, Vqwrky53 was predicted to be located on chromosome 17. The full length of Vqwrky53 was 2100 bp. One intron was inserted in position 276-1919 bp in this gene. Vqwrky53 was flanked by UDP-arabinopyranose mutase 1 (XP_002283867.1) and SUMO-activating enzyme subunit 1B-1 (XP_002283880.1). The CDS of Vqwrky53 (GenBank accession No. MN240482) was isolated from Danfeng-2. The full length CDS of Vqwrky53 was 456 bp and encoded 151 amino acids. The C-terminus of VqWRKY53 contained a conserved WRKY domain (residues 71-130 aa) (Fig. 1A, B). Cluster analysis of VqWRKY53 with WRKY family members from grape, *Arabidopsis* and rice showed that VqWRKY53 shows high homology with VvWRKY53, AtWRKY75 and OsWRKY72 and belongs to the subgroup IIc subfamily (Fig. 1C).

Vqwrky53 expresses in various organs, responds to powdery mildew infection and to different hormone treatments

qRT-PCR analysis was used to analyse the expression of Vqwrky53 in various organs including stems, tendrils, flowers, leaves and berries of Danfeng-2 (Fig. 2A). Results show that Vqwrky53 was expressed in all the organs tested. The expression level was higher in mature fruits than in young berries and higher in mature leaves than in young ones (Fig. 2B). Vqwrky53 responded to induction by powdery mildew by 12 h after the inoculation and reached its highest level after 72 h (Fig. 2C). The expression level of Vqwrky53 also increased after exogenous SA treatment for 1 h. It had increased 1.9-fold after 10 h (Fig. 2D). The transcript level of Vqwrky53 had increased 3.35-fold by 1 h following treatment with MeJA (Fig. 2E). Vqwrky53 also responded to induction by ABA and by Eth, reaching its highest level (an 8.02-fold increase) 2 h after ABA treatment and (a 3.04-fold increase) 10 h after Eth treatment (Fig. 2F, G).

VqWRKY53 has a transcriptional activation function in yeast

To detect whether VqWRKY53 had a transcription activation function in yeast, the full-length CDS of Vqwrky53 was inserted into pGBKT7 vector to generate BD-VqWRKY53 fusion vector. The vector BD-VqWRKY53 was transformed into the Y2HGold strain. After transformation for three days, the yeast harbouring BD-VqWRKY53 was streaked on to SD/-Trp medium with AbA and X-α-gal. The empty pGBKT7 vector (BD) was used as negative control. Results show that only the Y2H strain harbouring BD-VqWRKY53 grew and developed to blue on the SD/-Trp medium supplemented with AbA and X-α-gal (Fig. 3A). This indicates VqWRKY53 functions as a transcription activator in yeast.

VqWRKY53 localises in the nucleus

The CDS of Vqwrky53 removed termination codon was inserted into pCAMBIA2300 to generate the fusion vector 35S-VqWRKY53-GFP for further determining its subcellular location. In our previous studies, AtHY5 (AT5G11260) a nuclear location protein from *Arabidopsis*, was combined with mCherry (35S-AtHY5-mCherry) and used as nuclear localisation marker

gene (Fig. 3B). Here, the vector 35S-VqWRKY53-GFP was co-transformed into *Arabidopsis* protoplasts with 35S-AtHY5-mCherry. At the same time, empty pCAMBIA2300 was used as control. Results show the VqWRKY53 signal overlaps with the AtHY5 signal (Fig. 3B). VqWRKY53 is located in the nucleus.

VqWRKY53 interacts with VqMYB14 and VqMYB15 in yeast and *Arabidopsis* protoplasts

Previous studies have shown that MYB14 and MYB15 are two important transcription factors involved in the synthesis of stilbenes and that they are in the same branch of the MYB family and with high sequence similarity (Höll *et al.*, 2013). VvWRKY8 (the same gene as VvWRKY3, named after the article of Guo (2014)) was shown to interact with MYB14 (Jiang *et al.*, 2019). In our study, cluster analysis shows that Vqwrky53 and *VvWRKY3* belong to the group IIc subfamily and show high sequence similarity (Fig. 1C). Therefore, we hypothesise there is a protein interaction between VqWRKY53 and VqMYB14 or VqMYB15. To confirm this hypothesis, a Y2H assay was carried out. VqMYB14 and VqMYB15 were isolated from the cDNA of Danfeng-2. Sequence alignment results show the similarity between VqMYB14 and VvMYB14 is 97.03%. The amino acid sequence of VqMYB14 lacks just two threonine residues (Supplementary Figure S2A). The similarity between VqMYB15 and VvMYB15 is 99.21%. There are two mutations in the 24th amino acid (asparagine in VqMYB15 and L-isoleucine in VvMYB15) and the 117th amino acid (threonine in VqMYB15 and lysine in VvMYB15) (Supplementary Figure S2B). Full-length CDSs of VqMYB14 and VqMYB15 were inserted in the pGADT7 vector to obtain AD-VqMYB14 and AD-VqMYB15. BD-VqWRKY53 was co-transformed into the Y2HGold strain with AD-VqMYB14 and AD-VqMYB15, respectively. Results show that Y2HGold strain harbouring BD-VqWRKY53/AD-Vq

MYB14 and BD-VqWRKY53/AD-VqMYB15 grew and turned blue on SD/-Leu/-

Trp/-His/-Ade medium supplemented with AbA and X-α-gal (Fig. 4A). The result indicates VqWRKY53 can interact with VqMYB14 and VqMYB15 in yeast. To further prove the interaction between VqWRKY53 and VqMYB14,

VqMYB15, the full-length CDS of Vqwrky53, was inserted into the vector pSPYNE to generate pSPYNE/VqWRKY53 and CDSs of *VqMYB14*, *VqMYB15* without termination codons were inserted into the pSPYCE vector to generate pSPYCE/VqMYB14 and pSPYCE/VqMYB15 (Fig. 4B). Then, a BiFC assay was carried out in *Arabidopsis* protoplasts. Different combinations including, pSPYNE/VqWRKY53+pSPYCE/Vq MYB14, pSPYNE/VqWRKY53+pSPYCE/VqMYB15, pSPYNE/VqWRKY53+ pSPYCE, pSPYNE+ pSPYCE/VqMYB14 and pSPYNE+pSPYCE/VqMYB15 were co-transformed into *Arabidopsis* protoplasts. The YFP signal could be detected only when pSPYNE/VqWRKY53 was co-transformed with pSPYCE/VqMYB14 or pSPYCE/VqMYB15. This result demonstrates that VqWRKY53 interacts physically with VqMYB14 and VqMYB15 in the nucleus (Fig. 4C).

Vqwrky53 up-regulates the expression of *VqSTS32*, *VqSTS41* and promotes the accumulation of stilbenes

To further investigate the function of Vqwrky53 on *STS* gene expression and stilbene synthesis, a series of experiments were carried out. First, the promoters of *VqSTS32* and *VqSTS41* were isolated from gDNA of Danfeng-2. Sequence analysis shows that the promoter of *VqSTS32* contains the WRKY binding site TTGACC, TTGACT and MYB binding site CCAACC, TTGTTG. The promoter of *VqSTS41* contains the WRKY binding site TTGACT and MYB binding site TTGTTG (Fig. 5A). This suggests VqWRKY53 may recognise W-boxes and bind directly to the promoters of *VqSTS32* and *VqSTS41*. To demonstrate this, a yeast one hybrid assay was carried out. The promoters of *VqSTS32* , *VqSTS41* and the three tandem repeats of the WRKY binding site TTGACT, TTGACC were separately inserted into the pAbAi vector and then separately transformed into the Y1H yeast strain as bait. The full-length CDS of Vqwrky53 and the C-terminal of Vqwrky53 including the WRKY domain were inserted into the pGADT7 vector to generate AD-VqWRKY53 and AD-VqWRKY53$^{58\text{-}151}$. The two vectors were then transformed into Y1H strain harbouring pAbAi bait. Empty pGADT7 vector was used as negative control. After transformation for three days, the single

colony was dropped on SD/-Leu medium with AbA and cultured at 28°C for three days. Results show that VqWRKY53 can bind directly to the promoter of *VqSTS41* and the C-terminal VqWRKY53$^{58\text{-}151}$ can bind to the promoters of *VqSTS32* and *VqSTS41* (Fig. 5B). At the same time, our results show that VqWRKY53 and VqWRKY53$^{58\text{-}151}$ can bind to both types of WRKY binding site TTGACC and TTGACT (Fig. 5B).

A GUS activity experiment was carried out with tobacco leaves to further investigate whether the Vqwrky53 could activate *STS* promoters. Promoters of *VqSTS32* and *VqSTS41* were inserted into the vector pCAMBIA139 1 to generate $P_{VqSTS32}$-GUS and $P_{VqSTS41}$-GUS fusion vectors. Results show that transient overexpression of Vqwrky53 activates the promoter of *VqSTS32* and *VqSTS41* (Fig. 5C). The combination effect of *VqWRKY53* with *VqMYB14* and *VqMYB15* induced higher promoter activity of *VqSTS32* and *VqSTS41* compared with the expression of Vqwrky53 only (Fig. 5C). To detect the influence of Vqwrky53 on stilbene accumulation, Vqwrky53 was transiently overexpressed in young leaves of Danfeng-2 using the agrobacterium-mediated method. Transient expression of the empty vector (EV) was used as control. We found that the overexpression of Vqwrky53 up-regulated the expression levels of *VqSTS32* and *VqSTS41* (Fig. 5D). HPLC analysis showed that the content of *trans*-resveratrol and *trans*-piceid was increased but there was no difference in the level of *ε*-viniferin (Fig. 5E).

Vqwrky53 accelerates leaf senescence in transgenic *Arabidopsis*

To discover more about the functions of Vqwrky53, transgenic *Arabidopsis* of Vqwrky53 were generated and three independent transgenic lines (OE#2, OE#5 and OE#6) were selected for the following experiments (Fig. 6A). When the wild type (Col-0) and the transgenic lines were cultured, the leaves of transgenic lines were prematurely senescent compared with the wild type. In the transgenic *Arabidopsis*, the first to the 7th rosette leaves from bottom to top showed the senescence phenotype but the leaves of the Col-0 remained green (Fig. 6B). Then, the chlorophyll content of the leaves from the transgenic lines and wild type were measured. Results show that the chlorophyll content of the 3rd, 5th and 7th leaves of the transgenic lines was

significantly lower than in the wild type (Fig. 6C). Further, qRT-PCR was used to detect the expression level of *AtSAG12* which has been reported to be an important senescence associated gene in *Arabidopsis* (Pontier et al., 1999). We found in the 5th and 7th leaves, the transcript level of *AtSAG12* in the three transgenic lines was significantly higher than in the wild type (Fig. 6D).

Previous studies have shown that ROS and SA can induce leaf senescence (Vanacker *et al*., 2006; Morris *et al*., 2000; Lim *et al*., 2007). DAB staining shows that leaves from the transgenic lines accumulate more ROS than those from the wild type. Also, the accumulation of ROS was more obvious at the leaf margins in the transgenic lines where aging occurred (Fig. 7A). When measuring the SA content, we found that the SA levels in the 7th leaves of the transgenic lines were higher than in the wild type (Fig. 7B). Further qRT-PCR was carried out to detect the expression of genes involved in ROS and SA accumulation. The accumulation of ROS in plants depends on the production of ROS on the one hand and on the inhibition of ROS scavenging on the other. The respiratory burst oxidase homologues (RBOHs), also known as NADPH oxidases are important ROS producers in plants (Marino *et al*., 2012). *Arabidopsis* contains 10 RBOH genes, *AtRBOHA-AtRBOHJ* and AtRBOHA and AtRBOHB are two important RBOH in producing ROS (Marino *et al*., 2012; Kaya *et al*., 2019). Three catalase genes including *CAT1*, *CAT2* and *CAT3* have been identified in *Arabidopsis* and CAT2 is an important catalase which shows 90% of catalase activity (Frugoli *et al*., 1996; Queval *et al*., 2007; Mhamdi *et al*., 2010). Results show that the expression levels of *AtRBOHA* and *AtRBOHB* in the transgenic lines were higher than in the wild type, while the expression level of *AtCAT2* was significantly down-regulated, compared with the wild type (Fig. 7C). Previous studies report that two pathways, the isochorismate pathway and the phenylalanine ammonia-lyase pathway, are involved in SA biosynthesis (Dempsey *et al*., 2011). The SALICYLIC ACID INDUCTION DEFICIENT 2 (*SID2*) (Nawrath and Métraux, 1999) and the PHE AMMONIA LYASE1 (*PAL1*) (Cochrane *et al*., 2004) are the key enzymes of the two pathways, respectively. Our results showed that the expression levels of *AtPAL1* and *AtSID2* were significantly increased in the transgenic lines (Fig. 7C). At the same time the transcript

level of *AtWRKY28* (van Verk *et al*., 2011) which is the regulator SID2 was also increased in transgenic lines. This may explain the higher content of ROS and SA in the transgenic lines.

Vqwrky53 enhances disease resistance to *Pst*DC3000 in transgenic *Arabidopsis*

The results demonstrate that Vqwrky53 can respond to pathogen induction and to treatment with the exogenous hormones SA and MeJA (Fig. 2D, E). Overexpression of Vqwrky53 promotes the accumulation of ROS and SA. Therefore, we hypothesise that Vqwrky53 may increase disease resistance. To demonstrate this, the transgenic lines and wild-type *Arabidopsis* were inoculated with *Pst*DC3000 (Staskawicz *et al*., 1987). After inoculation for 72 h, the transgenic lines showed stronger disease resistance than the wild-type plants which was reflected in less yellowing of the leaves of the transgenic plants (Fig. 8A). At the same time, the numbers of bacteria in the leaves were measured. The results show bacterial growth in the transgenic lines was slower than in the wild type (Fig. 8B). Trypan blue staining was carried out to compare cell death in transgenic lines and in the wild type after *Pst*DC3000 inoculation for 72 h. The transgenic lines exhibited greater cell death (Fig. 8C). At the same time, after 72 h inoculation the leaves were stained with DAB to detect the accumulation of ROS. The staining results indicate the transgenic lines accumulate more ROS (Fig. 8D). Further, qRT-PCR was carried out to analyse the expressions of defence-related genes. Leaves of the transgenic lines and wild type were collected after inoculation for 0, 24, 48 and 72 h for qRT-PCR. We detected the transcript levels of three marker genes involved in the SA signalling pathway. *ICS1* (ISOCHORISMATE SYNTHASE 1) is involved in SA synthesis (Nawrath and Métraux, 1999). *AtPR1* (PATHOGENESIS-RELATED GENE 1) and *PR5* (PATHOGENESIS-RELATED GENE 5) are important SA-responsive defence-related genes (Blanco *et al*., 2009). We found the expression levels of *AtICS1* and *AtPR5* in the transgenic lines were significantly higher than in the wild type at all four times (Fig. 8E). The transcript level of *AtPR1* gene in the transgenic lines was higher than in the wild type 72 h after inoculation with *Pst*DC3000 (Fig. 8E). However, the expression level of LIPOXYGENASE 3 (*LOX3*) (Halitschke

and Baldwin, 2003), which is involved in the JA signalling pathway, was significantly lower than in the wild type 0, 48 and 72 h after inoculation (Fig. 8E). Based on these results, we hypothesise that Vqwrky53 increases disease resistance to *Pst*DC3000 via the SA pathway.

Discussion

Stilbenes are important phytoalexins in grapes. Not only do they protect plants against pathogen infection but when consumed they can also benefit human health (Jang *et al.*, 1997). In recent years, studies of the transcriptional regulation mechanisms of stilbenes have reported that various transcription factor families participate in regulation of stilbene synthesis, including MYB (Höll *et al.*, 2013; Fang *et al.*, 2014), WRKY (Jiang *et al.*, 2019; Vannozzi *et al.*, 2018), bZIP (Wang *et al.*, 2019) and ERF (Wang and Wang, 2019). However, full elucidation of the regulatory network for the synthesis of stilbenes requires further work. In this study, we characterise the functions of Vqwrky53 in the regulation of stilbene synthesis and further show that Vqwrky53 is involved in leaf senescence and disease resistance.

The WRKY family is an important family of TFs which has been widely studied in plants. Previous studies have divided the WRKY family members into three subfamilies, group I, group II and group III, based on the conserved WRKY domain and the structure of the zinc finger motif (Rushton *et al.*, 2010). In previous studies, WRKY genes co-expressed with *STS* genes were mainly identified as belonging to the WRKY group II. So far only one WRKY gene, *VviWRKY24*, has been identified as belonging to group I (Vannozzi *et al.*, 2018). The *VvWRKY8* belonging to group IIc has been reported as a negative regulator of resveratrol synthesis (Jiang *et al.*, 2019). Vqwrky53 isolated here contained one WRKY domain at the C-terminal and clustered with Vqwrky53, *VvWRKY3*, *AtWRKY75* and *OsWRKY72* which belong to group IIc (Fig. 1). Sequence analysis shows that a nuclear location signal from 30-57 aa was predicted in the N-terminal of VqWRKY53 and further study also demonstrated VqWRKY53 is located on the nucleus. This indicates that VqWRKY53 could enter the nucleus to exercise a transcriptional regulation function.

WRKY TFs are recognised as bound to the *cis*-element W-box (TTGACT/C) (Eulgem *et al.*, 2000; Rushton *et al.*, 1996). In *Arabidopsis*, AtWRKY57 was found to bind to the W-box elements on promoters of *RD29A* and *NCED3* (Jiang *et al.*, 2012). OsWRKY6 can bind directly to the three tandem repeats of W-box from the promoter of *OsPR1* (Seon-Hee *et al.*, 2011). BnaWGR1 from oilseed rape can bind to the W-box (TTGACC) (Yang *et al.*, 2018). In this study, the promoter of *VqSTS32* contained both W-box type I TTGACT and type II TTGACC. The promoter of *VqSTS41* contained W-box type I TTGACT (Fig. 5A). The presence of W-boxes in *STS* promoters suggests that VqWRKY53 may directly regulate the expression of *VqSTS32* and *VqSTS41*. From the results of the yeast one-hybrid assay, VqWRKY53 was confirmed as binding directly to the two types of W-box.

Until now, 48 *STS* members have been identified in grapevine but only a few TFs have been shown to regulate the expressions of these genes (Vannozzi et al., 2012). The reported TFs of *STS* genes include positive regulators, for example, MYB14 and MYB15 which up-regulate the transcript levels of *STS29* and *STS41* (Höll *et al.*, 2013). Recently, a negative regulator of *STS* genes, *VvWRKY8*, was reported to reduce the expression level of *STS15/21* and the accumulation of trans-piceid and trans-resveratrol (Jiang *et al.*, 2019). In our study, when Vqwrky53 is transiently overexpressed, the expression levels of *VqSTS32*, *VqSTS41* and the accumulations of trans-resveratrol and trans-piceid were also promoted. This result demonstrates that Vqwrky53 is a positive regulator of *STS* genes. Previous studies showed that WRKY TFs can interact with different proteins to participate in signal transduction, transcriptional regulation and chromatin remodelling (Chi *et al.*, 2013). Recently, WRKY proteins have been were reported as interacting with MYB proteins. In grapevine, VvWRKY8 binds directly to VvMYB14 to negatively regulate stilbene synthesis (Jiang *et al.*, 2019). VviWRKY03 has been reported to act in a combined manner with VviMYB14 to up-regulate *STS* genes. *VviWRKY03* cannot increase the activity of VviSTS29 alone but when transfected with VviMYB14, the luciferase activity can increase 8-fold compared with the control (Vannozzi *et al.*, 2018). In our study, VqWRKY53

was shown to interact with VqMYB14 and VqMYB15 in the nucleus (Fig. 4). Co-expression of Vqwrky53 with *VqMYB14* or *VqMYB15* can enhance the promoter activities of *VqSTS32* and *VqSTS41* compared with the expression of Vqwrky53 alone (Fig. 5C).

In plants, WRKY TFs participate widely in growth and development processes including in leaf senescence (Fei *et al.*, 2018). For example, *AtWRKY57* represses leaf senescence induced by JA and binds directly to the W-box region in the promoters of *SEN4* and *SAG12* and down-regulates their expression (Jiang *et al.*, 2014). In *Arabidopsis*, *AtWRKY75* functions as a positive regulator of leaf senescence and can be induced by age, SA and H_2O_2 (Guo *et al.*, 2017; Li *et al.*, 2012). In our study, the expression level of Vqwrky53 in mature berries and leaves of Danfeng-2 was higher than in young berries and leaves (Fig. 2A). As the homologous gene of *AtWRKY75*, Vqwrky53 may be involved in regulating leaf senescence. Further study demonstrates that heterologous expression of Vqwrky53 in *Arabidopsis* can accelerate leaf senescence. Overexpression of Vqwrky53 significantly increases the expression of *AtSAG12* and causes the accumulation of active oxygen and SA. This result is similar to that with *AtWRKY75* in *Arabidopsis* (Guo *et al.*, 2017). Previous studies have suggested that the synthesis of SA in plants goes through two different pathways, the isochorismate pathway and the phenylalanine ammonia-lyase pathway (Dempsey *et al.*, 2011). PAL1 is the key enzyme in the latter. In transgenic lines of Vqwrky53, the expression level of *PAL1* was up-regulated (Fig. 7C). PAL1 is also the key catalytic enzyme upstream of the phenylalanine metabolic pathway. The synthesis of stilbenes is a branch of the phenylpropanoid pathway, so we hypothesise that Vqwrky53 may play important roles in phenylpropanoid metabolic pathways and participate in the regulation of the accumulation of stilbenes with aging.

WRKY TFs are widely reported to be involved in disease resistance (Fei *et al.*, 2018). In grapevine, *VlWRKY3* responds to the exogenous hormones MeJA and Eth and offers enhanced resistance to *Golovinomyces cichoracearum* in transgenic *Arabidopsis* which overexpresses *VlWRKY3* (Guo *et al.*, 2018). *VqWRKY52*, which responds to SA treatment, was shown to promote disease

resistance to *Pst*DC3000 in *Arabidopsis*. Overexpression of *VqWRKY52* in *Arabidopsis* can repress the transcript level of PDF1.2 which is a defence-related gene involved in the MeJA signalling pathway (Wang *et al.*, 2017a). Here, we found that Vqwrky53 responds to powdery mildew infection, SA and MeJA treatment in Danfeng-2 (Fig. 2C, D, E). Vqwrky53 may participate in resistance to pathogens. Further study shows that overexpression of Vqwrky53 in *Arabidopsis* can induce the accumulation of SA (Fig. 7B). Previous studies have demonstrated that accumulation of SA in plants increases disease resistance to pathogens (Chen *et al.*, 2009; Nawrath and Métraux, 1999). Here, we found that overexpression of Vqwrky53 in *Arabidopsis* can promote plant resistance to *Pst*DC3000 and up-regulate the defence related genes *AtICS1*, *AtPR1* and *AtPR5* involved in SA signalling pathway (Fig. 8).

In summary, our study reports the functioning of Vqwrky53 isolated from Chinese wild *V. quinquangularis*. Vqwrky53 can be induced by different signals including by pathogen infection. The expression of VqWRKY53 then acts as a positive regulator of stilbene synthesis by directly binding to the promoter of *STS* genes or interacting with VqMYB14 and VqMYB15 to form a transcriptional complex to regulate *STS* genes. The accumulation of stilbenes can further promote plant disease resistance (Fig. 9). On the other hand, Vqwrky53 positively promotes the accumulation of ROS and SA which leads to leaf senescence and disease resistance. At the same time, the accumulation of SA can, in turn, induce the expression of Vqwrky53. This may form a positive feedback amplification function to further enhance the function of Vqwrky53 in regulating stilbene synthesis and disease resistance.

Author contributions

Y.J.W. conceived this project. D.W. performed experiments in this study and prepared the manuscript. C.Y.J. and W.D.L. participated in conducting the experiments. Y.J.W. revised the manuscript.

Conflict of interest

All authors have no conflict of interest to declare.

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Figure legends

Figure 1. Sequence analysis of Vqwrky53 isolated from *Vitis quinquangularis* accession Danfeng-2. (A) Chromosomal location analysis of Vqwrky53. Vqwrky53 was predicted located on chromosome 17 from the position of 922916 to 925015. The WRKY domain located at the C-terminal of VqWRKY53 from 71 to 130 aa. (B) Multiple sequence alignment between VqWRKY53 and its homologous genes from other species. The WRKY domain (WRKYGQK-X13-C-X4-C-X23-HXH) is shown with a black line. Proteins used for sequence alignment: VvWRKY1 (NP_001268218.1), AtWRKY75 (NP_196812.1), NtWRKY75 (XP_016446514.1), SlWRKY1 (NP_001310244.1), OsWRKY56 (XP_015615223.1), ZmWRKY75 (XP_008657250.1), MdWRKY75(XP_008389898.1). (C) Cluster analysis of VqWRKY53 with WRKY transcription factors from grape, *Arabidopsis* and rice. VqWRKY53 belongs to the subgroup IIc subfamily and shows high homology with VvWRKY53, AtWRKY75 and OsWRKY72.

Figure 2. Expression analysis of Vqwrky53 **in different organs and under different treatments.** (A) Stems, tendrils, inflorescences, leaves and berries from Danfeng-2 for qRT-PCR analysis. (B) Expression analysis of Vqwrky53 in different organs. (C) Leaves from Danfeng-2 were infected with powdery mildew and collected 0, 12, 24, 48, 72, 96 and 120 h later for qRT-PCR analysis. (D-G) Leaves from Danfeng-2 were treated with 100 μM SA, MeJA, ABA and Eth. Samples were collected 0, 0.5, 1, 2, 6 and 10 h after treatment. Data are means (±SD) of three biological replicates. Significance was analysed with SPSS using One-Way ANOVA with the *Tukey* test (**P*<0.05; ***P*<0.01).

Figure 3. VqWRKY53 has transcriptional activation activity and is localised at the nucleus. (A) Transcriptional activation experiment of VqWRKY53 in yeast. The yeast harbouring BD-VqWRKY53 and BD empty vector were placed on SD/-Trp, SD/-Trp+AbA and SD/-Trp+AbA+X-α-Gal plates and cultured at 28°C for three days. Only Y2HGold strain habouring

BD-VqWRKY53 can grow on SD/-Trp+AbA plates and turned blue on SD/-Trp+AbA+X-*α*-Gal plates. (B) VqWRKY53 localises in the nucleus. VqWRKY53 was combined with GFP (VqWRKY53-GFP) and the reported nuclear protein AtHY5 from *Arabidopsis* was combined with mCherry (AtHY5-mCherry). VqWRKY53-GFP and AtHY5-mCherry were together transformed into *Arabidopsis* protoplasts. At the same time, transformation of 35S-GFP was used as control. The transformed protoplasts were cultured at 22°C for 20 h. Then, GFP and mCherry signal was observed by confocal laser microscopy. Here, the chlorophyll signal is marked in blue. Bars = 5 μm.

Figure 4. VqWRKY53 interacts with VqMYB14, VqMYB15 in yeast and *Arabidopsis* protoplasts. (A) The Y2H assay confirms the interaction between VqWRKY53 and VqMYB14, VqMYB15 in yeast. Yeast Y2HGold strain carrying BD-VqWRKY53 and AD-VqMYB14 or AD-VqMYB15 were cultured on medium SD/–Leu/–Trp and SD/–Trp/–Leu/–Ade/–His+AbA+X-*α*-Gal at 28°C for three days. The empty pGADT7 was used as control. (B) Schematic diagrams of the pSPYNE and pSPYCE vectors used in the BiFC assay. The N-terminal of VqWRKY53 was fused with $eYFP_{N173}$ and the C-terminals of VqMYB14 and VqMYB15 were fused with $eYFP_{C155}$. (C) BiFC assay was conducted to further demonstrate the interaction between VqWRKY53 and VqMYB14, VqMYB15 in *Arabidopsis* protoplasts. Different combinations of (pSPYNE/VqWRKY53+pSPYCE/VqMYB14, pSPYNE/VqWRKY53+pSPYCE/VqMYB15, pSPYNE/VqWRKY53+pSPYCE, pSPYNE+pSPYCE/VqMYB14 and pSPYNE+pSPYCE/VqMYB15) were co-transformed into *Arabidopsis* protoplasts and cultured at 22°C for 20 h. Then, the YFP signal was observed by confocal laser microscopy. Bars = 5 μm .

Figure 5. Vqwrky53 up-regulates the expressions of *VqSTS32, VqSTS41* and promotes the accumulation of stilbenes. (A) Promoter analysis of *VqSTS32* and *VqSTS41*. WRKY type I (TTGACT) and WRKY type II (TTGACC) represents the WRKY transcription factor binding site. MYB type

I (CCAACC) and MYB type II (TTGTTG) represent the MYB transcription factor binding sites. (B) The yeast one-hybrid assay demonstrates that VqWRKY53 binds directly to *STS* promoters. The full-length CDS of VqWRKY53 and the C-terminal of VqWRKY53$^{58\text{-}151}$ were inserted into pGADT7 vector to generate AD-VqWRKY53 and AD-VqWRKY53$^{58\text{-}151}$. Empty AD vector was used as control. The Y1H strain harbouring the promoter of *VqSTS32* and *VqSTS41*, three tandem repeats of TTGACT and TTGACC were used as bait. The transformants were cultured on SD/-Leu medium with AbA. (C) VqWRKY53 activates promoters of *VqSTS32* and *VqSTS41*. The vectors $P_{VqSTS32}$-GUS and $P_{VqSTS41}$-GUS were separately transformed with various transcription factors (VqWRKY53, VqMYB14, VqMYB15, VqWRKY53+VqMYB14, VqWRKY53+VqMYB15) into tobacco leaves. Leaves were collected at three days after transformation for GUS activity assay. (D) Transient overexpression of Vqwrky53 promoted the expression levels of *VqSTS32* and *VqSTS41*. VqWRKY53 was overexpressed in leaves of Danfeng-2 using the Agrobacterium-mediated method. At the same time, empty vector (EV) was expressed as control. (E) Overexpression of Vqwrky53 promoted the accumulation of stilbenes. Here, we detected the content of trans-piceid, trans-resveratrol and *ε*-viniferin using HPLC. Data are means (±SD) of three biological replicates. Significance was analysed with SPSS using One-Way ANOVA with the *Tukey* test (**P*<0.05; ***P*<0.01).

Figure 6. Overexpression of Vqwrky53 accelerates leaf senescence in transgenic *Arabidopsis*. (A) Vqwrky53 was overexpressed in OE#2, OE#5 and OE#6 *Arabisopsis* transgenic lines. (B) Phenotype observation of wild-type *Arabidopsis* (Col-0) and three Vqwrky53 transgenic lines (OE#2, OE#5 and OE#6). Rosette leaves of Col-0 and transgenic lines (OE#2, OE#5 and OE#6) are presented from bottom to top. Compared with Col-0, leaves of transgenic lines show senescence at 7th rosette leaves which were still green in Col-0. (C) Detection of the chlorophyll content of leaves from Col-0 and transgenic lines. The 3rd, 5th, 7th and 9th leaves from the bottom were collected for this experiment. (D) Expression analysis of *AtSAG12* which has been reported to be an important senescence associated gene in *Arabidopsis*. Data

are means (±SD) of three biological replicates. Significance was analysed with SPSS using One-Way ANOVA with the *Tukey* test (**P*<0.05; ***P*<0.01).

Figure 7. Overexpression of *VqWRKY53* promotes the accumulation of ROS and SA in transgenic *Arabidopsis.* (A) DAB staining of leaves from wild type and transgenic lines. The 5th and 7th leaves were collected for DAB staining. The brown colour represents ROS accumulation. (B) Measurement of the free SA content of leaves from wild type and transgenic lines. The 7th leaves were collected for the measurement of SA. (C) qRT-PCR analysis of the ROS- and SA-related gene expressions. The 5th and 7th leaves from Col-0 and transgenic lines were used for the qRT-PCR analysis. Data are means (±SD) of three biological replicates. Significance was analysed with SPSS using One-Way ANOVA with the *Tukey* test (**P*<0.05; ***P*<0.01).

Figure 8. Overexpression of Vqwrky53 enhances disease resistance to *Pst*DC3000 in transgenic *Arabidopsis*. (A) Morphological observation of the leaves from wild-type (Col-0) and transgenic lines (OE#2, OE#5 and OE#6) after inoculation of *Pst*DC3000 for 72 h. (B) The number of bacteria in the leaves was measured 72 h after inoculation with *Pst*DC3000. (C) Trypan blue staining was used to detect cell death 72 h after inoculation with *Pst*DC3000. (D) DAB staining was used to detect the accumulation of reactive oxygen species 72 h after inoculation. (E) qRT-PCR analysis of the expression patterns of defence-related genes in transgenic lines and Col-0 at 0, 24, 48 and 72 h after inoculation. Data are means (±SD) of three biological replicates. Significance was analysed with SPSS using One-Way ANOVA with the *Tukey* test (**P*<0.05; ***P*<0.01).

Figure 9. A hypothetical working model of Vqwrky53 regulating stilbene synthesis and enhancing disease resistance. Pathogen infection induces the expression of Vqwrky53. Vqwrky53 positively regulates the expression of *STS*

genes in two ways. On the one hand, VqWRKY53 can bind directly to the W-box cis-elements in the promoter of *STS* genes to up-regulate their expressions. On the other hand, VqWRKY53 can form a transcriptional regulatory complex with VqMYB14 and VqMYB15 to more effectively regulate the expressions of *STS* genes. Then, the stilbene synthases catalyse the synthesis of more stilbenes. Further, the accumulation of stilbenes can increase disease resistance.

Figure 1

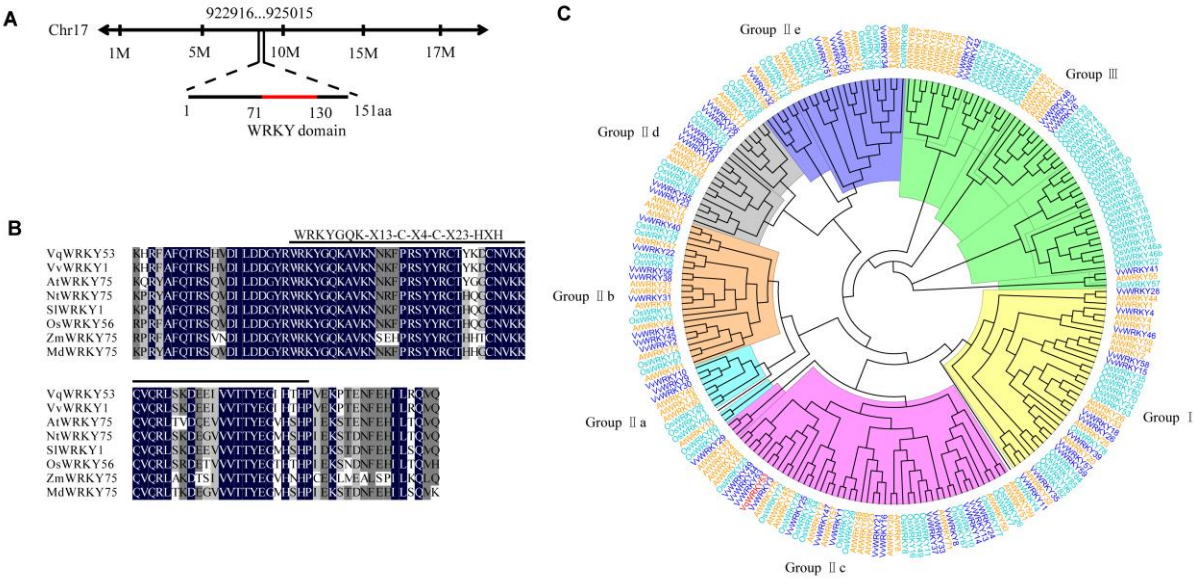


Figure 2

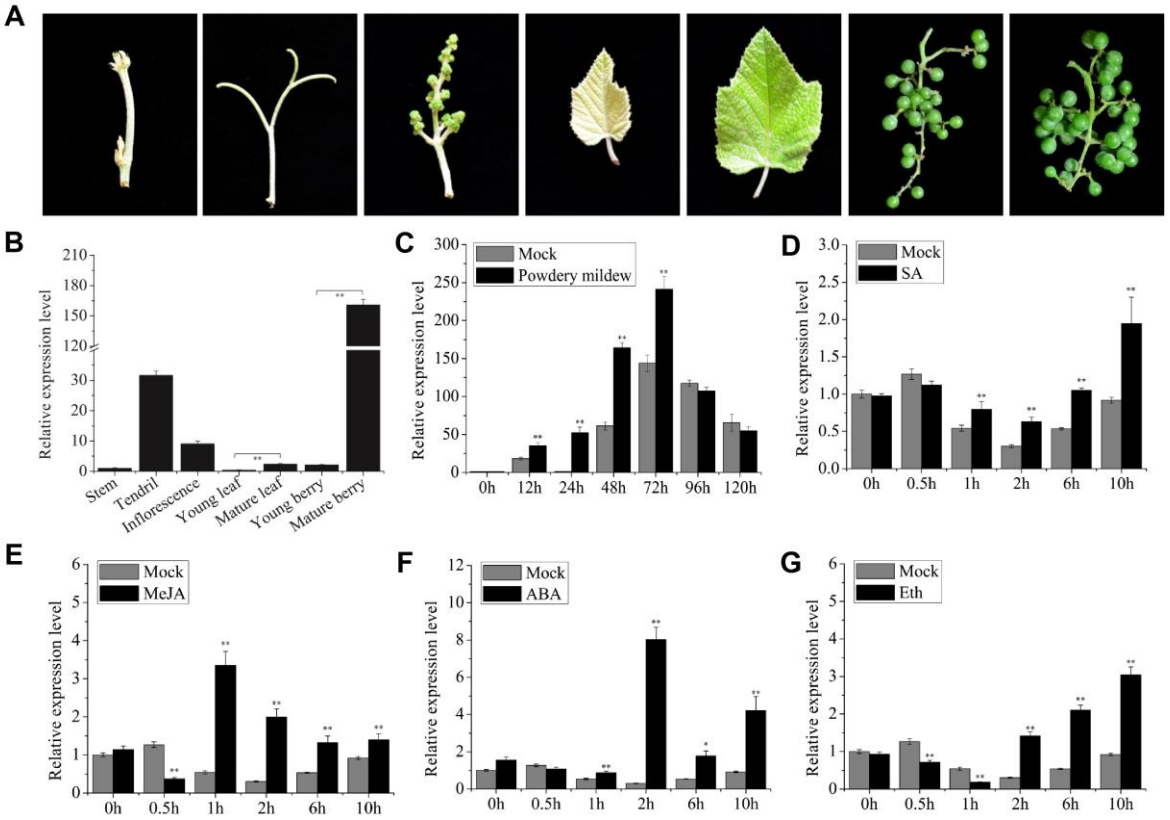


Figure 3

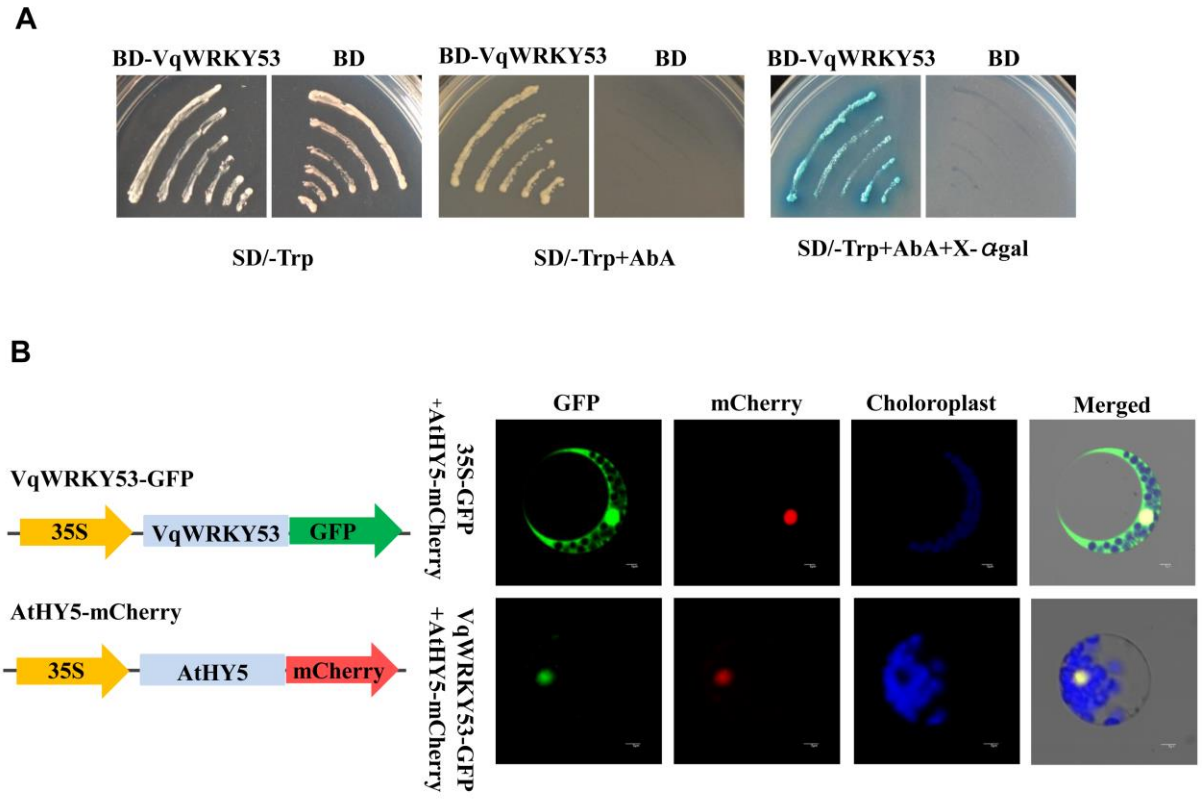


Figure 4

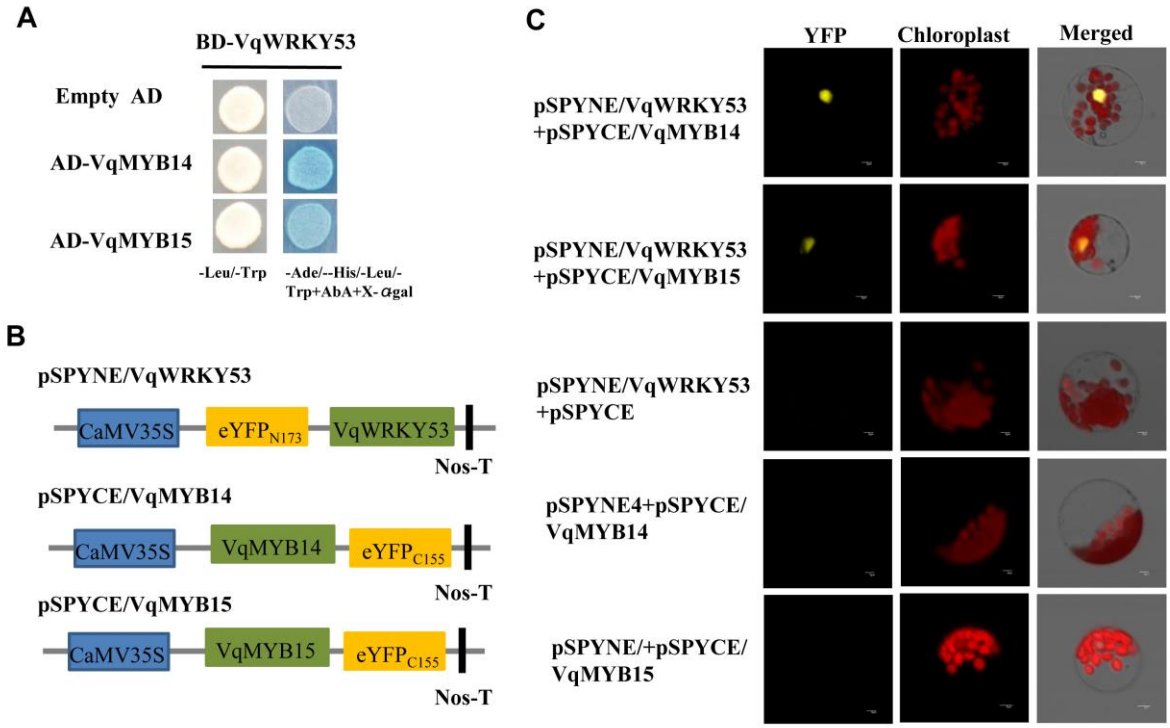
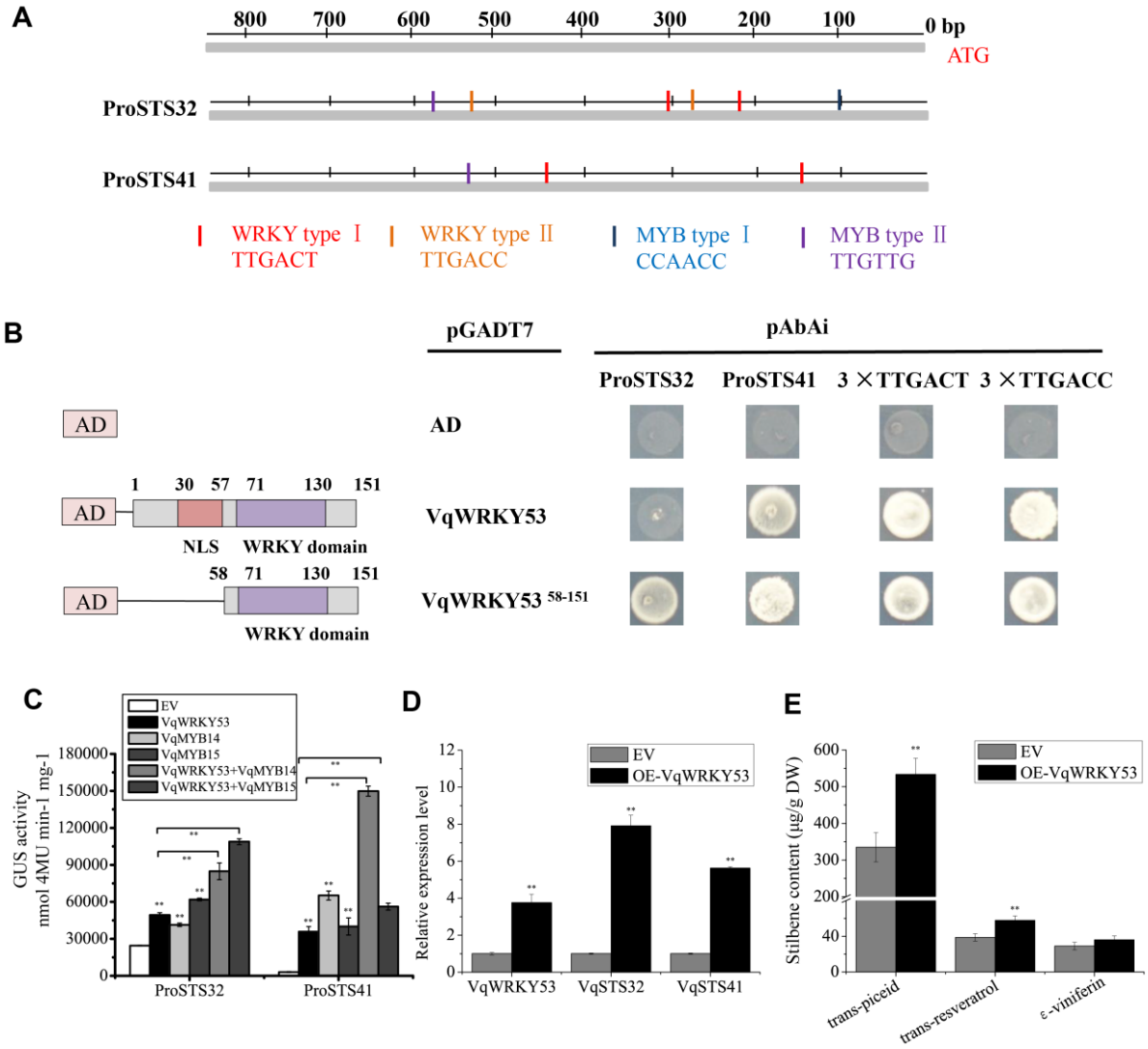


Figure 5



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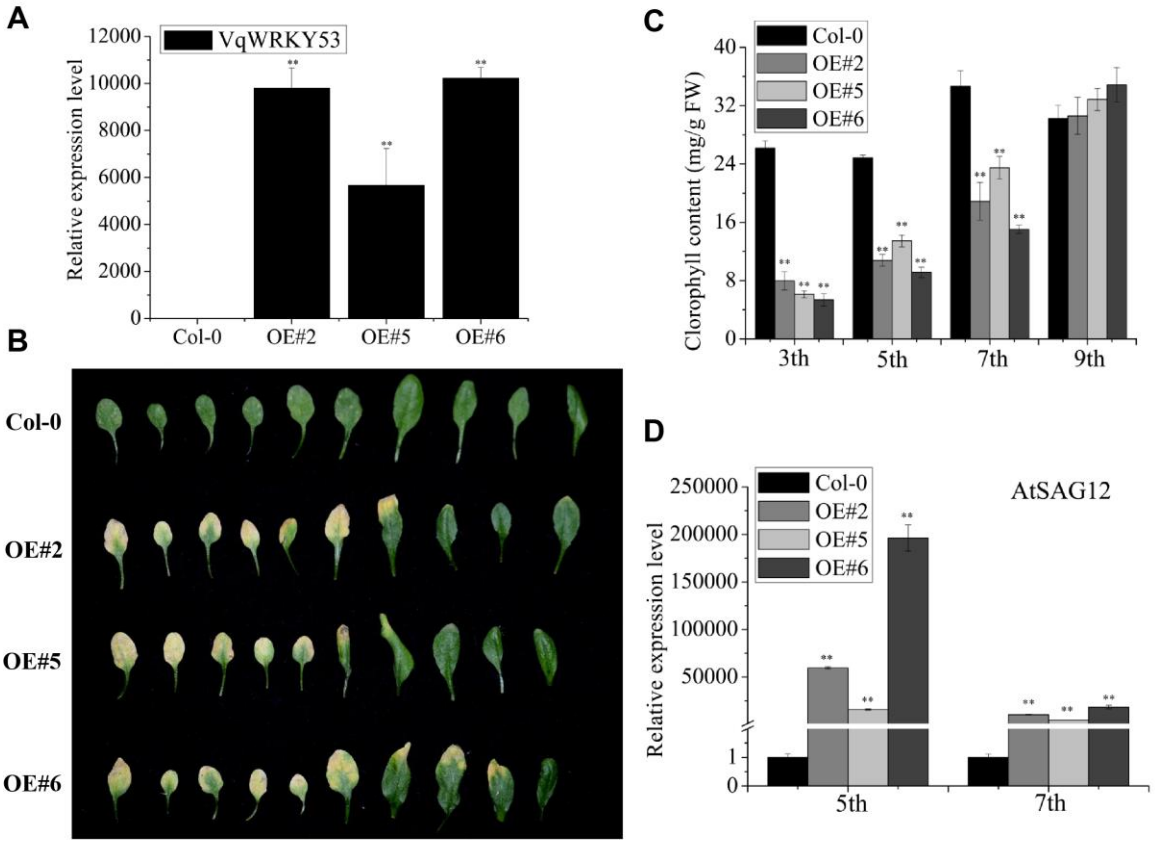

Figure 7

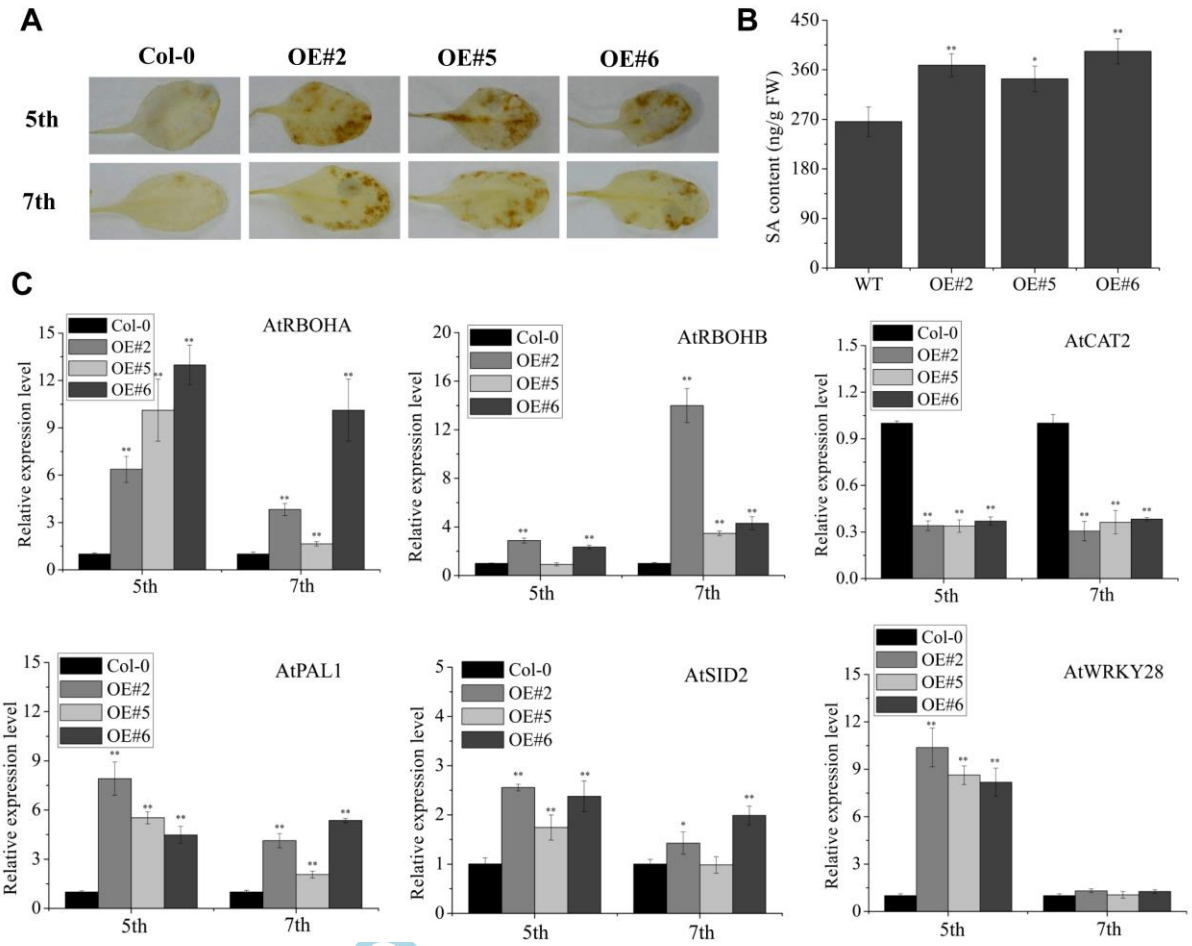

Figure 8

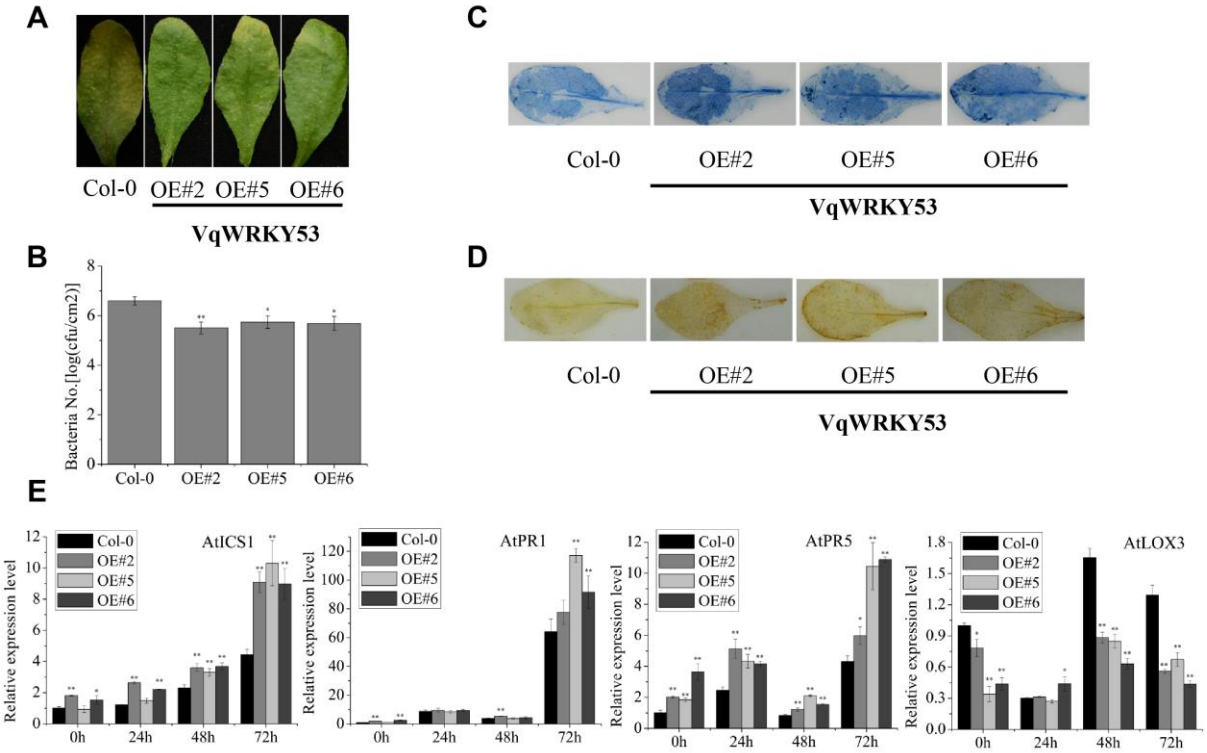

Figure 9