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ORIGINAL ARTICLE

Expression and functional analysis of two genes encoding transcription factors, *VpWRKY1* and *VpWRKY2*, isolated from Chinese wild *Vitis pseudoreticulata*

Huie Li · Yan Xu · Yu Xiao · Ziguo Zhu · Xiaoqing Xie · Heqing Zhao · Yuejin Wang

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Abstract In this study, two *WRKY* genes were isolated from *Erysiphe necator*-resistant Chinese wild *Vitis pseudoreticulata* W. T. Wang 'Baihe-35-1', and designated as *VpWRKY1* (GenBank accession no. GQ884198) and *VpWRKY2* (GenBank accession no. GU565706). Nuclear localization of the two proteins was demonstrated in onion epidermal cells, while *trans*-activation function was confirmed in the leaves of 'Baihe-35-1'. Expression of *VpWRKY1* and *VpWRKY2* was induced rapidly by salicylic acid treatment in 'Baihe-35-1'. Expression of *VpWRKY1* and *VpWRKY2* was also induced rapidly by *E. necator* infection in 11 grapevine genotypes; the maximum induction of *VpWRKY1* was greater in *E. necator*-resistant grapevine genotypes than in susceptible ones post *E. necator* inoculation. Furthermore, ectopic expression of *VpWRKY1* or *VpWRKY2* in *Arabidopsis* enhanced resistance to powdery mildew *Erysiphe cichoracearum*, and enhanced salt tolerance of transgenic plants. *VpWRKY2* also enhanced cold tolerance of transgenic plants. In addition, the two proteins were shown to regulate the expression of some defense marker genes in *Arabidopsis* and grapevine. The data suggest that *VpWRKY1* and *VpWRKY2* may underlie the resistance in transgenic grapevine to *E. necator* and tolerance to salt and cold stresses.

Keywords Chinese wild *Vitis* · Disease resistance · Grapevine · Powdery mildew · Transcription factor · WRKY

Abbreviations

qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
VpWRKY1	*Vitis pseudoreticulata WRKY1*
VpWRKY2	*Vitis pseudoreticulata WRKY2*
SA	Salicylic acid
MeJA	Methyl jasmonate
Eth	Ethephon
Hpi	Hours post inoculation
dpi	Days post inoculation
hpt	Hours post treatment
EST	Expressed sequence tag

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H. Li · Y. Xu · Y. Xiao · Z. Zhu · X. Xie · H. Zhao · Y. Wang (✉)
College of Horticulture, Northwest A&F University, Yangling 712100, Shaanxi, People's Republic of China
e-mail: wangyj@nwsuaf.edu.cn; wangyuejin@263.net

H. Li · Y. Xu · Y. Xiao · Z. Zhu · X. Xie · H. Zhao · Y. Wang
Key Laboratory of Horticultural Plant Germplasm Resource Utilization in Northwest China, Ministry of Agriculture of the People's Republic of China, Northwest A&F University, Yangling 712100, Shaanxi, People's Republic of China

H. Li · Y. Xu · Y. Xiao · Z. Zhu · X. Xie · H. Zhao · Y. Wang
Shaanxi Key Laboratory of Molecular Biology of Agriculture, Northwest A&F University, Yangling 712100, Shaanxi, People's Republic of China

Introduction

Abiotic and biotic stresses cause major losses in crop productivity worldwide. The responses of plants to these stresses are regulated by multiple signaling pathways (Singh et al. 2002). A considerable number of genetic and molecular approaches have demonstrated that stress-responsive signaling pathways in plants involve a complex

network of multiple components including receptors, kinases, phosphatases, and transcription factors (Gutterson and Reuber 2004). These studies have shown that plants are capable of extensive, highly dynamic, and temporal reprogramming of their transcriptome to generate a defense response. Regulation for adaptive plasticity is mainly achieved by the enforcement of a network of various transcription factors (Pandey and Somssich 2009).

WRKY proteins comprise a large family of transcription factors (Ulker and Somssich 2004) potentially involved in the regulation of transcriptional reprogramming responsible for plant immune responses (Eulgem and Somssich 2007). This family is defined by the conserved amino acid sequence WRKY together with a novel zinc-finger-like motif. WRKY proteins are classified into three groups based on the number of WRKY domains and the type of zinc-finger-like motif (Eulgem et al. 2000). Those with two WRKY domains belong to group I, whereas most proteins with one WRKY domain belong to group II or III. Group I and group II members have the finger motif C_2–H_2 ($\underline{C}$–$X_{4–5}$–$\underline{C}$–$X_{22–23}$–$\underline{H}$–X–$\underline{H}$). Group II members are further divided into five distinct subgroups (IIa–e) based on ten additional conserved motifs. Instead of a C_2–H_2 pattern, group III WRKY proteins contain a C_2–HC finger motif ($\underline{C}$–X_7–$\underline{C}$–X_{23}–$\underline{H}$–X_1–$\underline{C}$) (Eulgem et al. 2000).

Grape is an important fruit crop, but cultivated grapevine (*Vitis vinifera*) is susceptible to many pathogens. For instance, powdery mildew caused by *Erysiphe necator* (Schw.) Burr. is an economically important disease that infects green tissues of vines, and causes significant losses in yield and reduces berry quality (Fung et al. 2008). Wild species are often a valuable source of resistance to crop pathogens (Pavlousek 2007). In recent years, there has been a new trend of generating novel varieties of *V. vinifera* by introducing disease resistance gene(s) from resistant species into susceptible species (Bisson et al. 2002). Resistance genes *Run1* and *Ren1* against powdery mildew and *Rpv1*, *Rpv2* and *Rpv3* against downy mildew have been isolated from resistant grapevine species (Bellin et al. 2009; Coleman et al. 2009). Although two *WRKY* transcription factors genes (*VvWRKY1* and *VvWRKY2*) isolated from susceptible *V. vinifera* L. cv. Cabernet Sauvignon have been shown to enhance resistance to fungal pathogens in transgenic tobacco plants (Marchive et al. 2007; Mzid et al. 2007; Guillaumie et al. 2010), transcription factors from powdery mildew-resistant grapevine species have not yet been studied.

In a previous study, Chinese wild grapevine genotype *Vitis pseudoreticulata* W. T. Wang ‘Baihe-35-1’ was identified as resistant to *E. necator* (Wang et al. 1995). The goal of the present research was to characterize two genes encoding transcription factors, *VpWRKY1* and *VpWRKY2*, isolated from Chinese wild *V. pseudoreticulata* W. T. Wang ‘Baihe-35-1’. We focused on the roles of *VpWRKY1* and *VpWRKY2* in resistance to biotic and abiotic stresses. Expression of *VpWRKY1* and *VpWRKY2* post *E. necator* inoculation and signaling molecule treatments were determined by qRT-PCR. In addition, expression of some SA- and JA/ET-dependent defense marker genes was tested in transgenic *Arabidopsis* and grapevine. We also analyzed resistance of transgenic *Arabidopsis* plants to *Erysiphe cichoracearum*, salt and cold stresses.

Materials and methods

Plant materials

Grapevines were grown in grape germplasm resources orchard of Northwest A&F University, Yangling, China (34°20′N, 108°24′E). Eleven grapevine genotypes were tested in this study including five *E. necator*-resistant and six *E. necator*-susceptible genotypes. The *E. necator*-resistant genotypes were Chinese wild *V. pseudoreticulata* W. T. Wang ‘Baihe-35-1’, ‘Baihe-13’, ‘Baihe-13-1’, ‘Guangxi-1’, and ‘6-12-6’ (a cross between Chinese wild *V. pseudoreticulata* W. T. Wang ‘Baihe-35-1’ and *V. vinifera* L. cv. Carignane). The six *E. necator*-susceptible grapevine genotypes were *V. vinifera* L. cv. Carignane, Chinese wild *V. pseudoreticulata* W. T. Wang ‘Guangxi-2’, ‘Hunan-1’, ‘Shangnan-2’, ‘Baihe-35-2’, and ‘6-12-2’ (another cross between Chinese wild *V. pseudoreticulata* W. T. Wang ‘Baihe-35-1’ and *V. vinifera* L. cv. Carignane). The susceptibility index and resistance ratings to *E. necator* of the 11 grapevine genotypes are listed in Supplementary Table S1. In vitro cultivation of grapevines used for transient experiments was performed as described by Guan et al. (2010).

Arabidopsis thaliana L. ecotype Col-0 was used for over-expression experiments and was grown in a chamber at 22°C in long-day conditions (16 h of light and 8 h of dark). Onion (*Allium cepa* L.) was purchased from a local market.

Biotic and abiotic treatments

Grapevine *E. necator* was maintained as described by Guan et al. (2010). When shoots of vines were 25–35 cm in length, the third to fifth fully expanded young grapevine leaves beneath the apex were selected for biotic and abiotic treatments. Inoculation by *E. necator* was performed on the selected leaves under field conditions as described by Wang et al. (1995), and was repeated three times on three independent plants for each species. Leaves sprayed with sterile water were used as negative control. Inoculated leaves were then covered with plastic bags for 12 h to

maintain humidity. Field leaves were collected 0, 6, 12, 24, 48, 72, 96 and 120 h post inoculation (hpi), and immediately frozen in liquid nitrogen for further study.

Arabidopsis powdery mildew *E. cichoracearum* was identified in Col-0 plants. Fungal isolate was purified via single colony inoculation of clean Col-0 plants for five consecutive generations. The isolate was then maintained on live Col-0 plants at 22°C (16 h light, 8 h dark) in a separate growth chamber for generation of fresh inocula. *E. cichoracearum* inoculation was conducted on leaves of selected 6-week-old T_2 transgenic and wild type plants as described by Xiao et al. (1997). Visual scoring of disease reaction phenotypes was done 12 days post inoculation (dpi) as described previously (Xiao et al. 2005). Spore count of the most susceptible leaves was determined as following: leaf samples were collected and weighed, and placed in 50-ml tubes containing 40 ml of sterilized dH_2O and 0.02% of Tween 20, after which the tubes were stirred vigorously for 60 s using a vortex mixer. The resulting spore suspension was diluted 1:10 with 0.02% Tween 20 solution. Spores were then counted under a dissecting microscope in a large area using a hemocytometer to get a more reliable spore density estimate.

To test if *VpWRKY1* and *VpWRKY2* expression is induced by plant defense signaling molecules, 100 μM salicylic acid (SA) (Wang and Li 2006), 50 μM methyl jasmonate (MeJA) (Repka et al. 2004) and 0.5 g/l ethephon (Eth) (Belhadj et al. 2008) were sprayed on selected leaves of 'Baihe-35-1' under field conditions. Three leaves were selected from three independent plants for each treatment at 0, 3, 6, 12, 24, 48, 72 and 96 h post treatment (hpt). Samples were immediately frozen in liquid nitrogen.

To determine the salt tolerance of the transgenic plants, T_2 transgenic *Arabidopsis* seeds were sowed in MS medium with and without 150 mM NaCl. Germination rate was calculated based on the presence of green cotyledons 10 days after sowing. Cold treatment was accomplished by transferring 2-week-old seedlings grown in MS media to a 4°C refrigerator for 6 h, after which they were returned to the growth chamber. Fresh weight was determined 1 week after the cold treatment.

RNA isolation and cDNA library construction

Total RNA of *Arabidopsis* was extracted as described by Ulker et al. (2007), whereas total RNA of grapevines was extracted using improved SDS/phenol method (Zhang et al. 2003) at 0, 6, 12, 24, 48, 72, 96 and 120 h post *E. necator* infection of 'Baihe-35-1' leaves. cDNA library was constructed as described by Xu et al. (2009) with an equal amount of mRNA pooled at each time point. All sequences in this study were determined by Generay Biotech Co. Ltd (Shanghai, China).

Full length cloning and sequence analysis

Rapid amplification of cDNA ends (RACE) was conducted using BD SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA), and cDNA synthesized from *E. necator*-infected leaves of 'Baihe-35-1'. Specific primers were designed based on the *WRKY* EST sequences (GenBank accession no. GR883935 and GR883939). Primers GSP1: 5′ TTCACGATGACGGTTAT GCCTGGCG for 3′ RACE and GSP2: 5′ TGCACTCCAT TGTGCTCATGGTGGC for 5′ RACE were used to obtain the full length of *VpWRKY1*, while primers GSP3: 5′ CA TTTCCAAAGGCTAACAGTGAA for 3′ RACE and GSP4: 5′ ATACATTCACTGTTAGCCTTTGG for 5′ RACE were used to obtain the full length of *VpWRKY2*. RACE results were compiled using Seqman. Nuclear-localization signals were predicted by PSORT WWW Server (http://psort.ims.u-tokyo.ac.jp/) and the phylogenetic tree was constructed using the CLUSTALW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Sequence alignment was performed using DNAMAN.

Subcellular localization

Coding sequences of *VpWRKY1* and *VpWRKY2* without the termination codon were introduced into pCAMBIA1302 vector to generate 35S::VpWRKY1–GFP and 35S::VpWRKY2–GFP. Sequenced plasmids were delivered into onion epidermal cells using PDS-1000/He gene gun at 1,100 psi as described by Mare et al. (2004), and then cultured in MS media in darkness at 22°C for 18 h. After cultivation, GFP visualization at excitation wavelength 480 ± 20 nm and emission wavelength 510 ± 20 nm was conducted using a Zeiss confocal microscope (LSM510; Carl Zeiss Thornwood, NY, USA).

Trans-activation assay

Primers 5′ CTATTAGGAGGAGTTGGTTG and 5′ CTCATGGTGGCGTCTGTG were designed based on previously released whole genome sequences of *V. vinifera* (Jaillon et al. 2007; Velasco et al. 2007) and the cDNA sequence of *VpWRKY1* to clone the *VpWRKY1* promoter. The promoter fragment was amplified from the genomic DNA of 'Baihe-35-1'. Sequence analysis showed that *VpWRKY1* promoter (GenBank accession no. GU565705) was enriched in W-boxes (TGAC). Minimal-100 CaMV35S promoter (m35S) was inserted upstream of the *GUS* gene of pC0390GUS (Xu et al. 2010) to generate an m35S-GUS construct (Fig. 3a). The 140-bp fragment

containing three W-boxes (A/TGAC/A, −215 to −354 bp) from *VpWRKY1* promoter was amplified by PCR, and then inserted upstream of m35S to generate W-box-m35S-GUS reporter construct (Fig. 3a). The *gusA* of pCAMBIA1301 was replaced with the coding region of *VpWRKY1* or *VpWRKY2* to generate over-expression constructs p1301-VpWRKY1 and p1301-VpWRKY2, respectively (Fig. 3a). *Agrobacterium* strain GV3101 harboring recombinant plasmids was transformed in vitro into 6-week-old plantlet leaves of *V. pseudoreticulata* W. T. Wang ‘Baihe-35-1’ via *Agrobacterium*-mediated transient assay. GUS staining was performed at 3 dpi as described by Xu et al. (2010).

Gene expression analysis by semi-quantitative RT-PCR and qRT-PCR

First-strand cDNA was synthesized from 1 μg of DNase-treated total RNA using PrimeScript^{TM} RTase (TaKaRa Biotechnology, Dalian, Liaoning, China). Semi-quantitative RT-PCR was performed at 94°C for 3 min, 25 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by final elongation at 72°C for 5 min. qRT–PCR was conducted using SYBR green (Takara Biotechnology) on an IQ5 real time PCR machine (Bio-Rad, Hercules, CA, USA). Each reaction was done in triplicates with a reaction volume of 25 μl. Cycling parameters were 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 30 s. To analyze the quality of the dissociation curves, the following program was added after 40 PCR cycles: 95°C for 15 s, followed by a constant increase from 60 to 95°C. Grapevine *VpGAPDH* or *Arabidopsis tubulin* was amplified as internal control. Primers used for qRT-PCR are listed in Supplementary Table S2. Each relative expression level was analyzed with IQ5 software using the normalized-expression method. A one-side paired *t* test using SigmaPlot 11.0 (Ashburn, VA, USA) was performed to assess significant differences between the negative control and the treatment.

Generation of transgenic *Arabidopsis*, and transient over-expression and silencing of *VpWRKY1* and *VpWRKY2* in grapevine

Agrobacterium GV3101 harboring over-expression construct p1301-VpWRKY1 or p1301-VpWRKY2 was used for *Arabidopsis* transformation via the floral dip method (Clough and Bent 1998). *Arabidopsis* transformants were selected based on hygromycin B resistance on MS plates. Semi-quantitative RT-PCR was used to select T_2 lines with the highest expression level of *VpWRKY1* or *VpWRKY2*. The fourth to seventh rosette leaves of 6-week-old *Arabidopsis* plants were selected for qRT-PCR analysis. Fragment of *VpWRKY1* from 131 to 488 bp was amplified as sense and antisense fragments, and inserted into pKANNIBAL (Wesley et al. 2001) to generate the *VpWRKY1* silencing construct. The same procedure was done on the fragment of *VpWRKY2* from 24 to 323 bp to generate the *VpWRKY2* silencing construct. Transient over-expression and silencing of *VpWRKY1* and *VpWRKY2* in ‘Baihe-35-1’ leaves was performed as described by Xu et al. (2010). qRT-PCR was used to analyze expression of defense marker genes at 3 days post infiltration.

Results

Cloning and sequence analysis of *VpWRKY1* and *VpWRKY2*

To identify candidate genes from Chinese wild *Vitis* and facilitate the molecular breeding of resistant varieties, a cDNA library was constructed from *E. necator*-inoculated ‘Baihe-35-1’ leaves and sequenced. Among more than 4,300 sequences in the library, two ESTs (GenBank accession no. GR883935 and GR883939) with single copy for each were found to contain conserved WRKY domain(s). By using RACE technique, full lengths of the two *WRKY* genes were obtained and designated as *VpWRKY1* (GenBank accession no. GQ884198) and *VpWRKY2* (GenBank accession no. GU565706). Full length of *VpWRKY1* cDNA was 1,157 bp, encoding a polypeptide of 322 amino acids (Supplementary Fig. S1), while *VpWRKY2* was 1,607 bp, encoding a polypeptide of 499 amino acids (Supplementary Fig. S2). Sequence analysis showed that VpWRKY1 contains one WRKY domain, one C_2–HC zinc-finger motif ($\underline{C}$–X_7–$\underline{C}$–X_{23}–$\underline{H}$–X_1–$\underline{C}$) and one predicted nuclear-localization signal (KRRK) (Supplementary Fig. S1), whereas VpWRKY2 contains two WRKY domains, one C_2H_2 zinc-finger motif ($\underline{C}$–X_4–$\underline{C}$–X_{23}–$\underline{H}$–X–$\underline{H}$), and four putative nuclear-localization signals (Supplementary Fig. S2). The phylogenetic tree based on the classification method of Eulgem et al. (2000) showed that VpWRKY1 belongs to group III and VpWRKY2 belongs to group I of the WRKY superfamily (Supplementary Fig. S3). *AtWRKY70* from *Arabidopsis* is the most closely related gene to *VpWRKY1* (Supplementary Fig. S3). Multiple alignments of the amino acid sequences of this two proteins and reported VvWRKY1 and VvWRKY2 indicated that they share an overall 22–45% sequence similarity. However, the deduced amino acid sequence of VpWRKY1 has 100% similarity with the predicted protein acc no. XP 002272504 from *V. vinifera* with the corresponding gene located in chromosome 8, while VvWRKY2 shares 99% similarity with the predicted protein acc no. XP 002276194 from *V. vinifera* with the corresponding gene located in chromosome 11. The corresponding gene of VvWRKY1 is located in chromosome 17,

whereas the location of the corresponding gene of VvWRKY2 is unknown.

Expression of *VpWRKY1* and *VpWRKY2* are induced rapidly by *E. necator* and some plant defense signaling molecules

Results of qRT-PCR showed that both *VpWRKY1* and *VpWRKY2* were induced rapidly by *E. necator* in Chinese wild *V. pseudoreticulata* W. T. Wang 'Baihe-35-1', from which they were originally isolated (Fig. 1a, b). To determine whether *VpWRKY1* and *VpWRKY2* respond to *E. necator* in different grapevine genotypes, qRT-PCR was conducted on the ten other grapevine genotypes. Findings indicated that expression of *VpWRKY1* and *VpWRKY2* was induced by *E. necator* infection in all 11 grapevine genotypes tested (Fig. 1a, b). Expression levels of *VpWRKY1* and *VpWRKY2* peaked at 6–12 hpi, and then decreased to original levels at 96–120 hpi in all genotypes. Maximum induction of *VpWRKY1* was observed in *E. necator*-resistant genotype 'Baihe-35-1' at 12 hpi (Fig. 1a).

Though there were differential levels of basal expression of *VpWRKY1* or *VpWRKY2* between the genotypes (Supplementary Fig. S4, S5), they are not consistent with maximum induction levels (Fig. 1a, b) and degree of disease resistance of these genotypes (Supplementary Table S1).

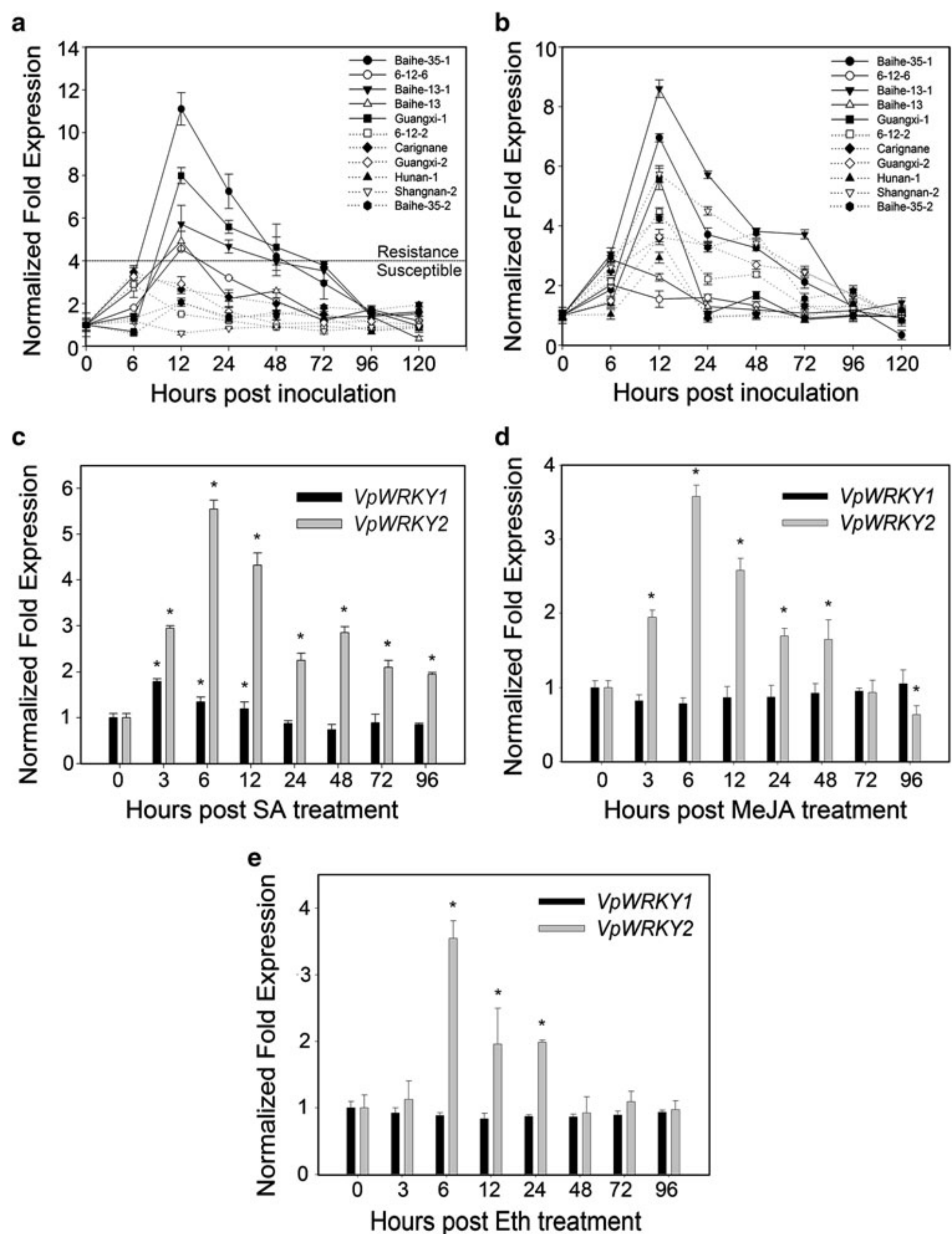

Fig. 1 Expression profiles of *VpWRKY1* and *VpWRKY2*. **a** *VpWRKY1* was induced by *E. necator* in eleven grapevine genotypes, the five grapevines with *VpWRKY1* induction levels of more than fourfold are all resistant to *E. necator* infection (*solid lines*), while the six genotypes with *VpWRKY1* induction levels lower than fourfold are all susceptible to *E. necator* infection (*dotted lines*). **b** *VpWRKY2* was induced by *E. necator* in five resistant (*solid lines*) and six susceptible grapevine genotypes (*dotted lines*). Expression profiles of *VpWRKY1* and *VpWRKY2* in response to SA (**c**), MeJA (**d**), and Eth (**e**) treatments in leaves of Chinese wild *V. pseudoreticulata* W. T. Wang 'Baihe-35-1'. *VpGAPDH* was used as internal control for qRT-PCR and fold expressions indicate expression level in treated leaves of each genotype compared with the negative control, which was set to 1. *Asterisks* indicate a significant difference ($P < 0.05$) in *VpWRKY1* expression. Mean values and SDs were obtained from three technical and three biological replicates

However, all *E. necator*-resistant grapevine genotypes had a maximum *VpWRKY1* induction of more than fourfold, while susceptible genotypes had a maximum *VpWRKY1* induction less than fourfold (Fig. 1a). Therefore, maximum *VpWRKY1* induction levels correlate well with the degree of disease resistance of the 11 grapevine genotypes. Although *VpWRKY2* was induced by *E. necator* in all genotypes tested, the maximum induction of *VpWRKY2* does not correlate with disease resistance levels of the grapevine genotypes (Fig. 1b).

To determine whether *VpWRKY1* and *VpWRKY2* were induced by defense signaling molecules, the selected leaves of Chinese wild *V. pseudoreticulata* W. T. Wang 'Baihe-35-1' were treated with SA, MeJA or Eth. Basal *VpWRKY1* transcript level in 'Baihe-35-1' was not significantly induced by Eth and MeJA, but was slightly induced by SA at 3 hpt (Fig. 1c, d, e). In contrast, *VpWRKY2* was induced rapidly by all three signaling molecules with SA as the strongest inducer (Fig. 1c, d, e).

VpWRKY1 and VpWRKY2 proteins are localized in the nucleus

Sequence analysis of the two genes revealed that their proteins contain putative nuclear-localization signal(s) (Supplementary Fig. S1, S2). To investigate the subcellular localization of VpWRKY1–GFP and VpWRKY2–GFP proteins, plasmids 35::VpWRKY1–GFP, 35::VpWRKY2–GFP, and negative control 35::GFP were transiently transformed into onion epidermal cells by particle bombardment. Results showed that the fusion proteins VpWRKY1–GFP and VpWRKY2–GFP targeted the nucleus of onion epidermal cells (Fig. 2). In contrast, control GFP was observed throughout the whole cell (Fig. 2). These indicate that VpWRKY1 and VpWRKY2 are nuclear proteins, which are consistent with their roles as transcription factors.

VpWRKY1 and VpWRKY2 function as potential transcriptional activators

Since reported WRKY factors have shown high binding affinity to W-box (TGAC), which is regarded as the pivotal sequence in numerous defense genes promoters (Ulker and Somssich 2004; Eulgem and Somssich 2007), transient co-expression was performed in a homologous system to test the effects of VpWRKY1 and VpWRKY2 on reporter gene expression. Leaves co-transformed with reporter and over-expression constructs were stained dark blue, leaves transformed with only W-box–m35S–GUS showed a slight blue background, and the leaves transformed with only m35S–GUS or co-transformed with m35S–GUS and over-expression constructs did not stain blue (Fig. 3b). Results imply that VpWRKY1 and VpWRKY2 can activate *GUS* expression by binding to the 140-bp promoter fragment that contains three W-boxes.

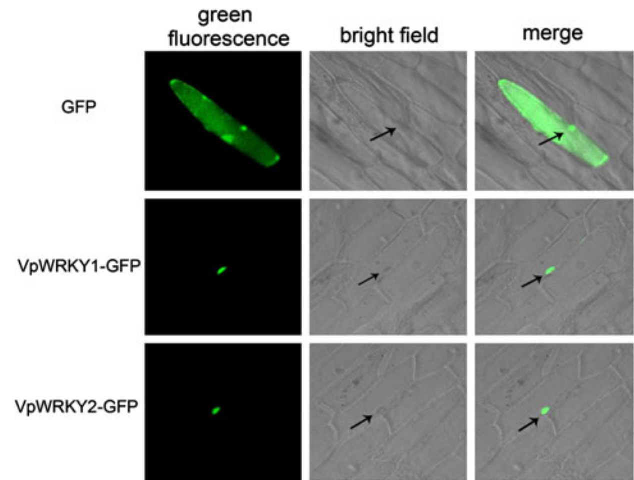

Fig. 2 Subcellular localization of VpWRKY1 and VpWRKY2. VpWRKY1–GFP (*middle row*) and VpWRKY2–GFP (*bottom row*) localized in the nucleus of onion epidermal cells, GFP alone (*top row*) localized throughout the whole cell. Cells were analyzed for GFP fluorescence by fluorescent microscopy

Ectopic over-expression of *VpWRKY1* and *VpWRKY2* in *Arabidopsis* results in enhanced resistance to *E. cichoracearum*

To analyze the biological function of *VpWRKY1* and *VpWRKY2*, the coding sequences of the two genes were transformed into *Arabidopsis* under the control of 35S promoter. T_2 transgenic *Arabidopsis* were generated with the lines *VpWRKY1*-T_2-4 (W1-4) and *VpWRKY1*-T_2-13 (W1-13) exhibiting the greatest expression of *VpWRKY1*, and *VpWRKY2*-T_2-2 (W2-2) and *VpWRKY2*-T_2-7 (W2-7) exhibiting the greatest expression of *VpWRKY2*, as determined by semi-quantitative RT-PCR (Fig. 4a). Therefore, W1-4 and W1-13, W2-2 and W2-7 were selected for further studies. These transgenic *Arabidopsis* lines exhibited enhanced resistance to *E. cichoracearum* at 12 dpi compared to wild type (Fig. 4b). To quantify the *E. cichoracearum* resistance, the number of spores on the most susceptible leaf of each plant was determined. The number of *E. cichoracearum* spores was significantly decreased in the four transgenic lines at 12 dpi compared with the wild type (Fig. 4c).

Since *VpWRKY1* and *VpWRKY2* are induced by defense signaling molecules, the defense response of the selected lines was analyzed by comparing the expression of SA- and JA/ET-dependent *Arabidopsis* defense marker genes *AtPR1*, *AtPR10*, *AtNPR1*, *AtCOR1*, and *AtPDF1.2* with 35S::VpWRKY1, 35S::VpWRKY2, and wild type controls

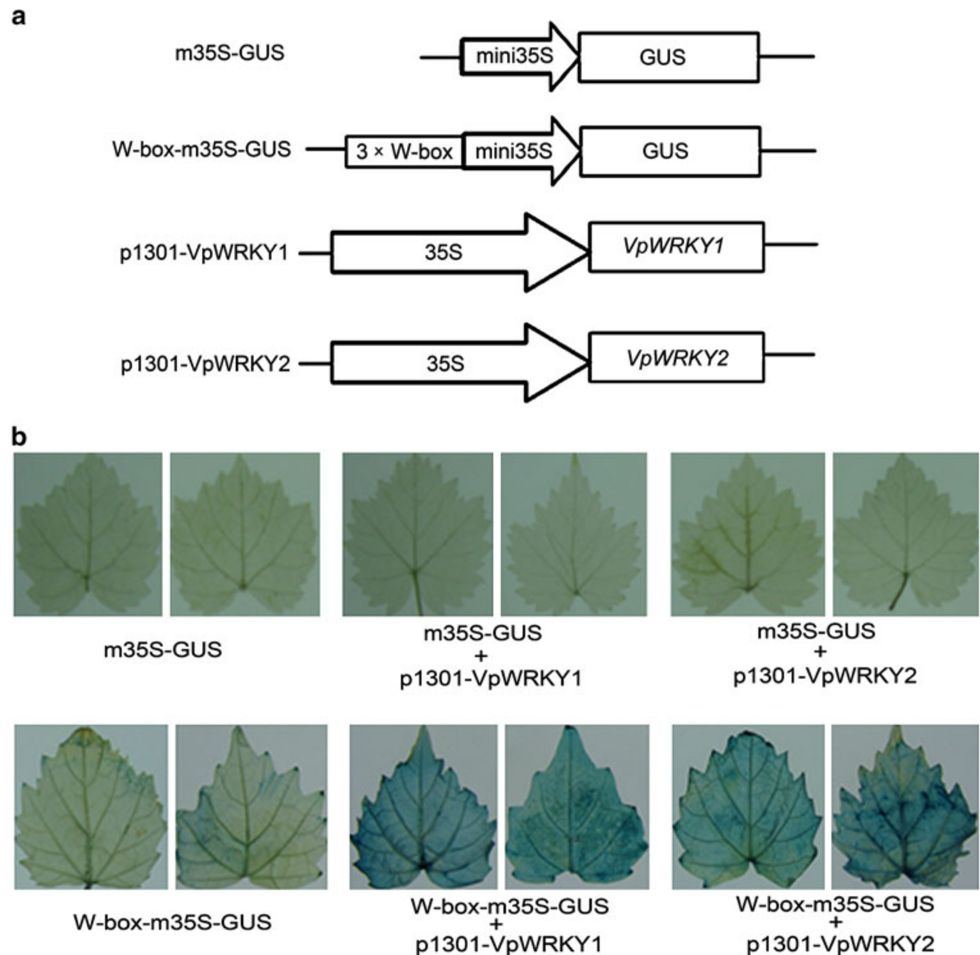

Fig. 3 *Trans*-activation of VpWRKY1 and VpWRKY2. **a** Schematic diagram of reporter and over-expression constructs (1301-VpWRKY1 and 1301-VpWRKY2) used for transient transformation of grapevine leaves. **b** GUS staining of two representative leaves transformed with constructs ($n = 20$). GUS staining was performed 3 days after transformation with m35S-GUS alone or co-transformed with m35S-GUS and over-expression constructs (*top row*). GUS staining was performed 3 days after transformation with W-box-m35S-GUS alone or co-transformed with W-box-m35S-GUS and over-expression constructs (*bottom row*). Fully expanded leaves from 6-week-old in vitro plantlets of Chinese wild grape clone *V. pseudoreticulata* 'Baihe-35-1' were used. Similar staining results were obtained in three biological experiments

under normal conditions. qRT-PCR showed that the amount of *AtPR10* and *AtNPR1* transcripts were much higher in the two *VpWRKY1* transgenic lines compared with the wild type (Fig. 4e, f). Transcription of *AtPR10* was up-regulated only in the *VpWRKY1* transgenic *Arabidopsis* lines under normal conditions (Fig. 4f). In contrast, *AtPR1* transcription was dramatically down-regulated in all the four transgenic *Arabidopsis* lines compared with the wild type (Fig. 4d). Transcripts of *AtCOR1* and *AtPDF1.2* were down-regulated only in *VpWRKY2* transgenic *Arabidopsis* lines (Fig. 4g, h). These results suggest that SA-dependent *Arabidopsis* defense marker genes are likely regulated by transcription factors VpWRKY1 and VpWRKY2, while JA/ET-dependent *Arabidopsis* defense marker genes are likely regulated only by VpWRKY2.

Ectopic over-expression of *VpWRKY1* and *VpWRKY2* in *Arabidopsis* results in enhanced tolerance to abiotic stress(es)

About 7% wild type *Arabidopsis* seeds were able to germinate in MS media with 150 mM NaCl, whereas germination rates of *VpWRKY1* and *VpWRKY2* transgenic seeds were significantly higher than that of the wild type, and transgenic seedlings grew very well under the same condition (Fig. 5a, b). In addition, cold tolerance test revealed that the two *VpWRKY2* transgenic *Arabidopsis* seedlings grew to almost twice the size of wild type seedlings 1 week after cold treatment (Fig. 5c), biomass of the two *VpWRKY2* transgenic lines was significantly higher than the wild type 1 week after cold treatment (Fig. 5d), whereas the two *VpWRKY1* transgenic lines did not show any difference in growth compared with the wild type (data not shown). These indicate that over-expression of *VpWRKY2* enhanced tolerance of transgenic seedlings to both salinity and cold stresses, while over-expression of *VpWRKY1* only enhanced tolerance of transgenic seedlings to salinity stress.

VpWRKY1 and VpWRKY2 regulate expression of grapevine defense marker genes in a transient transformation assay

The roles of VpWRKY1 and VpWRKY2 in the regulation of grapevine defense marker genes in a homologous system were studied using transient over-expression and silencing

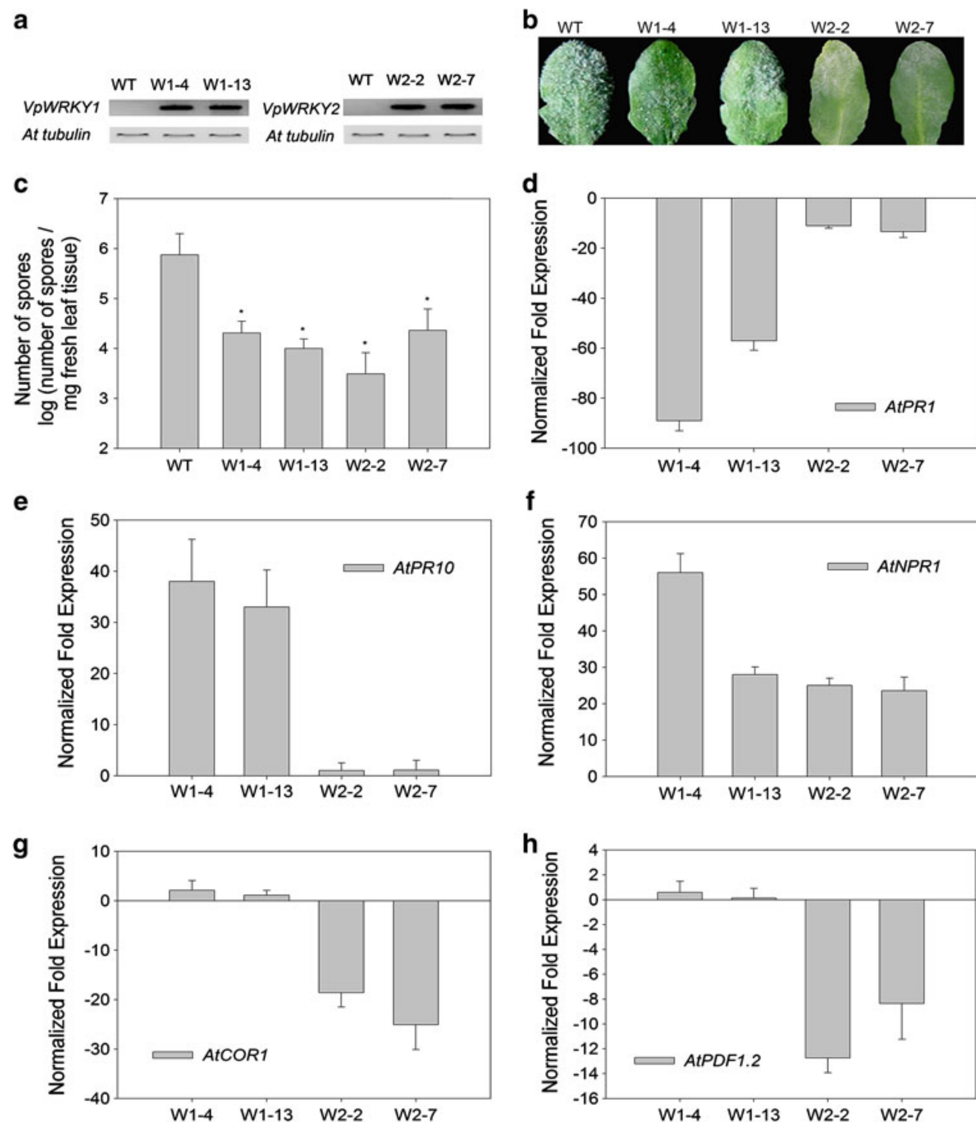

Fig. 4 VpWRKY1 (*W1*) and VpWRKY2 (*W2*) over-expression enhanced *E. cichoracearum* resistance of *Arabidopsis*. Six-week-old T_2 lines were used for resistance and expression tests. **a** Semi-quantitative RT-PCR analysis of *VpWRKY1* and *VpWRKY2* expression in wild type and transgenic lines. The *tubulin* gene was amplified as control. **b** Disease reaction phenotypes of representative wild type and transgenic *Arabidopsis* at 12 dpi. **c** The ten most susceptible leaves from ten seedlings from each genotype at 12 dpi were pooled and the number of spores per milligram of fresh tissue was determined. Similar results were obtained in two biological experiments. *Asterisks* indicate a significant difference ($P < 0.05$). Expression of defense marker genes *AtPR1* (**d**), *AtPR10* (**e**), *AtNPR1* (**f**), *AtCOR1* (**g**), and *AtPDF1.2* (**h**) in non-inoculated transgenic *Arabidopsis* under normal conditions. Fold changes indicate relative expression levels in transgenic lines compared with the wild type, which was set as 1. Mean values ± SD were obtained from three technical and three biological replicates

assay. qRT-PCR results showed that over-expression of *VpWRKY2* resulted in increased *VpPR1* transcripts, and silencing of *VpWRKY1* reduced *VpPR1* transcripts (Fig. 6a, b). In contrast, over-expression of *VpWRKY1* enhanced expression level of *VpPR10*, and silencing of *VpWRKY2* resulted in decreased expression of *VpPR10* (Fig. 6a, b). Moreover, over-expression of *VpWRKY1* or *VpWRKY2* enhanced accumulation of *VpNPR1*, while silencing of *VpWRKY1* or *VpWRKY2* reduced *VpNPR1* transcripts (Fig. 6a, b). Results indicate that VpWRKY1 and VpWRKY2 play a role in regulating the expression of the defense marker genes in homologous system.

Discussion

Plant WRKY transcription factors are a superfamily of regulatory proteins forming a network of genes that regulate plant responses to variable environmental conditions (Pandey and Somssich 2009). In grape, 43 assembled *WRKY* genes have been predicted from susceptible *V. vinifera* using PlantGDB independent from the released whole genome sequences of *V. vinifera* (Jaillon et al. 2007; Velasco et al. 2007; Guo et al. 2008), and two WRKY transcription factor genes (*VvWRKY1* and *VvWRKY2*) isolated from susceptible *V. vinifera* have been studied (Marchive et al. 2007; Mzid et al. 2007; Guillaumie et al. 2010). In the present study, both *VpWRKY1* and *VpWRKY2* were isolated from *E. necator*-resistant Chinese wild *V. pseudoreticulata* W. T. Wang ‘Baihe-35-1’. Sequence analysis showed that VpWRKY1 and VpWRKY2 belong to group III and group I of the WRKY superfamily, respectively (Supplementary Fig. S3). Both VpWRKY1 and VpWRKY2 proteins were shown to localize in the nucleus of onion epidermal cells (Fig. 2). *Trans*-activation assays showed that both proteins obviously activate *GUS*

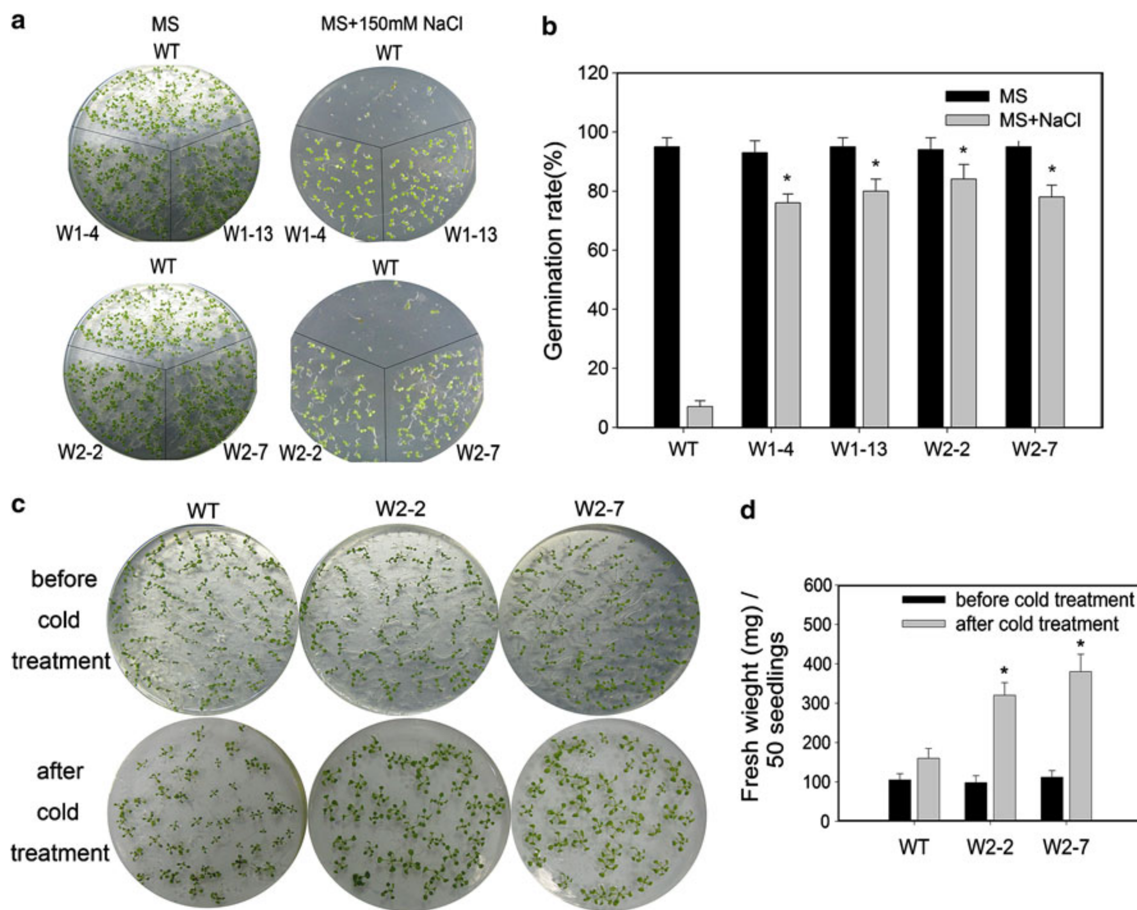

Fig. 5 VpWRKY1 and VpWRKY2 enhanced tolerance to abiotic stresses in transgenic *Arabidopsis* plants. **a** VpWRKY1 and VpWRKY2 enhanced salt tolerance in *Arabidopsis*. Seedlings were grown in MS medium with and without 150 mM NaCl. Phenotypes were scored 10 days after sowing. **b** Quantitative analysis of germination rate. Average germination rates and standard errors were calculated using results of three replicated experiments ($n = 50$). **c** VpWRKY2 enhanced cold tolerance. Seedlings were grown on MS medium before and after cold treatment (4°C for 6 h). Seedlings grown on MS medium for 2 weeks before cold treatment are shown in top row. Seedlings grown on MS medium 1 week after cold treatment are shown in *bottom row*. **d** Quantitative analysis of fresh weights 1 week after cold treatment. Average fresh weights and standard errors were calculated using results of three replicates ($n = 50$)

expression in a homologous system by binding to a 140-bp promoter fragment that contains three W-boxes. However, leaves transformed with only W-box-m35S-GUS also present with a slight blue background (Fig. 3b), suggesting that the endogenous WRKY proteins may be responsible for the background *GUS* expression by binding to the 140-bp fragment.

The response to pathogenic attack requires large-scale transcriptional reprogramming of *WRKY* genes (Pandey and Somssich 2009), and most of these processes have been proven to be induced by pathogens (Eulgem and Somssich 2007). In a previous report, powdery mildew-induced transcriptional change of *VaWRKY30* was observed in the powdery mildew-susceptible *V. vinifera* only, and not in the powdery mildew-resistant *V. aestivalis* (Fung et al. 2008). However, in the current study, expression of *VpWRKY1* and *VpWRKY2* can rapidly be induced by *E. necator* in their genotype of origin (‘Baihe-35-1’) and ten other grapevine genotypes (Fig. 1b, c). In particular, expression levels of *VpWRKY1* correlate well with the degree of resistance to *E. necator* of all 11 grapevine genotypes (Fig. 1a), whereas *VpWRKY2* does not exhibit such correlation (Fig. 1b). *E. necator*-induced expression patterns provide preliminary evidence for the role of *VpWRKY1* and *VpWRKY2* in regulating powdery mildew resistance in grapevine. The deduced amino acid sequences for *VpWRKY1* and *VpWRKY2* show very high similarities with the predicted proteins from susceptible *V. vinifera*, whereas the differences in the post-inoculation expression levels between powdery mildew-resistant *V. pseudoreticulata* and powdery mildew-susceptible *V. vinifera* might be attributed to the different regulatory mechanisms controlled by *cis*-regulatory elements in the promoter region (Xu et al. 2010).

To investigate the role of *VpWRKY1* and *VpWRKY2* in powdery mildew resistance, the two genes were

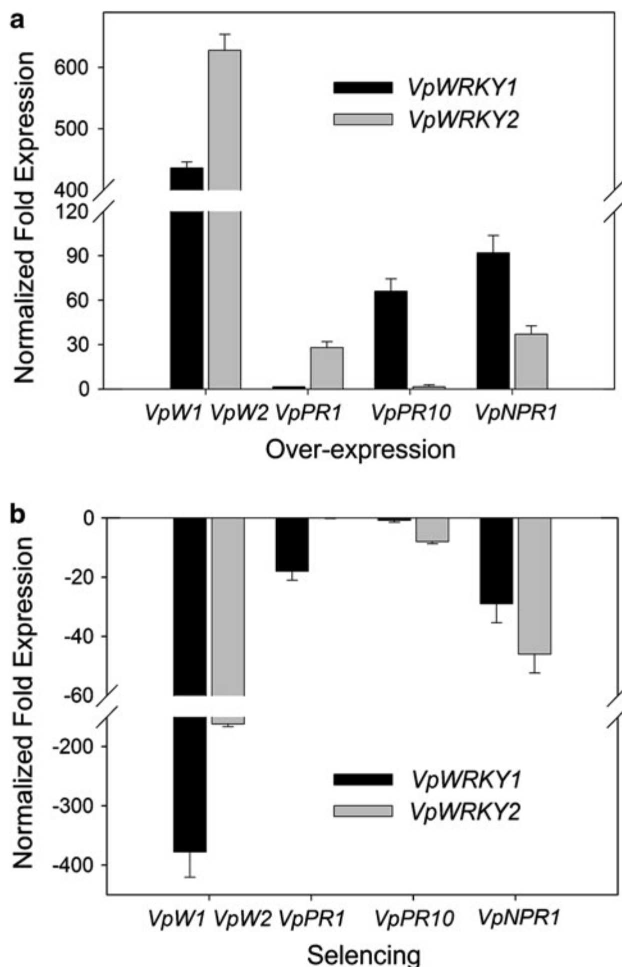

Fig. 6 Expression of grapevine defense marker genes *VpPR1*, *VpPR10*, and *VpNPR1* when *VpWRKY1* and *VpWRKY2* are transiently over-expressed (**a**) and silenced (**b**) in grapevine leaves. *VvGAPDH* was used as internal control, and fold expression indicates expression levels in transiently over-expressed or silenced leaves compared with expression in GV3101 infiltrated leaves, which was set as 1. Mean values and SDs were obtained from three technical and three biological replicates

over-expressed in *Arabidopsis* Col-0 and the resistance of the transgenic plants was tested against *E. cichoracearum*, a fungal biotroph that is virulent to *Arabidopsis* Col-0. Results showed that *VpWRKY1* and *VpWRKY2* enhanced resistance to *E. cichoracearum* (Fig. 4b, c). Similarly, *AtWRKY3*, *AtWRKY4*, *AtWRKY18*, *AtWRKY33*, *AtWRKY53* and *AtWRKY70* in *Arabidopsis* also have been shown to enhance resistance to pathogens (Xu et al. 2006; Knoth et al. 2007; Lai et al. 2008; Pandey and Somssich 2009). In rice, *OsWRKY13*, *OsWRKY31*, *OsWRKY45*, *OsWRKY53* and *OsWRKY71* positively contribute to resistance to pathogens (Chujo et al. 2007; Liu et al. 2007; Qiu et al. 2007; Zhang et al. 2008; Qiu and Yu 2009). Expression of a *Medicago truncatula WRKY* (W109669) can enhance the response to the tobacco mosaic virus in transgenic tobacco plants (Naoumkina et al. 2008). In addition, over-expression of *WRKY* transcription factor genes isolated from susceptible *V. vinifera* leads to enhanced resistance to tobacco mildew (Marchive et al. 2007). However, the closest homolog of *VpWRKY1* (Supplementary Fig. S3), *AtWRKY70*, acts as a negative regulator of disease resistance in *Arabidopsis* (Ulker et al. 2007). This may be attributed to the different roles that *WRKY* genes play in decreasing or enhancing susceptibility toward pathogens (Eulgem and Somssich 2007). Results of this study suggest that *VpWRKY1* and *VpWRKY2* play a positive role in *Arabidopsis* powdery mildew resistance.

Plant defense responses to microbial attack is regulated through a complex network of signaling pathways that involve SA, JA, and Eth (Glazebrook 2005), and *WRKY* genes are often induced by these signaling molecules (Eulgem et al. 2000). In this study, both *VpWRKY1* and *VpWRKY2* were induced rapidly by SA treatment and *E. necator* infection in *E. necator*-resistant Chinese wild *V. pseudoreticulata* W. T. Wang 'Baihe-35-1' (Fig. 1a, b, c). Similarly, 43 out of 72 *WRKY* genes tested in *Arabidopsis* were induced by SA treatment or bacterial infection (Dong et al. 2003). Studies on *WRKY* genes predominantly indicate an involvement in the SA signaling pathway (Wang et al. 2009). However, SA, JA, and Eth defense signaling pathways do not function independently, but exhibit complicated cross-talk and interaction including synergism and antagonism, during defense response (Glazebrook 2005). In the present study, *VpWRKY2* was also shown to be induced rapidly by signaling molecules MeJA and Eth in 'Baihe-35-1', whereas *VpWRKY1* was unaffected by MeJA or Eth (Fig. 1d, e). Though these are not conclusive evidence for synergistic and/or antagonistic relationships among the three signaling molecules during *VpWRKY2* expression, results suggest that *VpWRKY2* is involved in a more complex defense signaling network than *VpWRKY1* in grapevine. Transcription factors are essential components of the defense signaling pathways, since they regulate the expression of defense-related marker genes (Eulgem 2005). In this study, over-expression of *VpWRKY1* and *VpWRKY2* were shown to be capable of regulating the expression of SA-dependent maker genes *AtPR1* and *AtNPR1* in transgenic *Arabidopsis* plants (Fig. 4d, f). However, another SA-dependent maker gene, *AtPR10*, was up-regulated only in *VpWRKY1* transgenic *Arabidopsis* plants and not in *VpWRKY2* transgenic *Arabidopsis* (Fig. 4e). This may be because *AtPR10* cannot be regulated by VpWRKY2 in transgenic *Arabidopsis* plants. Among the three SA-dependent defense marker genes, only *AtPR1* was down-regulated in all four transgenic lines (Fig. 4d), which concurs with previous reports that *AtPR1* appears to function negatively in disease resistance in *Arabidopsis* (Ulker et al. 2007; Savitch et al. 2007). Moreover, expression of *AtCOR1* and *AtPDF1.2* was

down-regulated in *VpWRKY2* transgenic lines, suggesting that VpWRKY2 acts as a repressor of some JA/Eth-dependent genes in *Arabidopsis*. This is in agreement with previous studies stating that AtWRKY70 acts as a repressor of JA/Eth response genes *AtCOR1* and *AtPDF1.2* (Li et al. 2004; Ulker et al. 2007). Therefore, VpWRKY1 and VpWRKY2 may enhance resistance to *E. cichoracearum* through repression of some defense genes that function negatively and increasing the expression of other genes that regulate positively in disease resistance.

Defense marker genes are up- or down-regulated in transgenic *Arabidopsis* plants, possibly as a result of the transcriptional output of individual downstream target genes that are either positively or negatively affected by WRKY proteins (Journot-Catalino et al. 2006). Heterologous expression in *Arabidopsis* has shown that *VpWRKY1* and *VpWRKY2* regulate the expression of defense marker genes. To evaluate the effect of *VpWRKY1* and *VpWRKY2* in a homologous system, the transient expression system was utilized. The results confirmed that *VpWRKY1* and *VpWRKY2* regulate some defense marker genes in a homologous system thus suggesting that these defense marker genes in *Arabidopsis* and grapevine are regulated by VpWRKY1 or VpWRKY2. In this study, *AtPR1* expression was decreased in *VpWRKY2* transgenic *Arabidopsis* plants, while *VpPR1* was increased in *VpWRKY2* transgenic grapevine leaves. What may be the reason which causes the different expression models of *AtPR1* and *VpPR1* in both *VpWRKY2* transgenic plants? First, though they are both PR1 proteins, deduced amino acid sequences of the two PR1 proteins only share 57% similarity. Second, there is a difference in promoter sequences of *PR1* genes between *Arabidopsis* and grapevine, which may result in VpWRKY2 protein specific binding to different *cis*-elements of the *PR1* promoters. Third, other factors that interact with VpWRKY2 protein may be involved in enhancing or inhibiting the expression of different *PR1* genes in grapevine or *Arabidopsis*.

WRKY proteins specifically interact with the W-box, a major class of *cis*-element in the context of promoters of pathogen- or elicitor-responsive genes, such as pathogenesis-related proteins, receptor protein kinases, or WRKY transcription factors (Journot-Catalino et al. 2006; Xu et al. 2006; Pandey and Somssich 2009; Wang et al. 2009). Thus, this study analyzed the 1-kb sequence upstream of the translation start sites of all eight defense marker genes that were selected based on the released whole genome sequences of *V. vinifera* in NCBI and *Arabidopsis* in TAIR. Results revealed that the promoters of these eight defense marker genes are enriched with W-boxes, containing three to six W-boxes within the 1-kb sequences upstream of *VpPR1*, *VpPR10*, *VpNPR1*, *AtPR1*, *AtPR10*, *AtNPR1*, *AtCOR1*, and *AtPDF1.2* were found. These indicate that VpWRKY1 and VpWRKY2 may enhance resistance to *E. cichoracearum* by regulating the expression of defense marker genes. Their ability to regulate the expression of grapevine defense marker genes provides important insights into the molecular basis of VpWRKY1 and VpWRKY2 in grapevine powdery mildew resistance.

Another important role of *WRKY* genes is the enhancement of tolerance to abiotic stresses. Numerous studies have reported that expression of *WRKY* genes in plants is induced by abiotic stresses (Rizhsky et al. 2002; Ulker and Somssich 2004; Marchive et al. 2007; Jing et al. 2009). In the present study, *VpWRKY1* and *VpWRKY2* transgenic *Arabidopsis* plants are more capable of adapting to the salt stress during germination (Fig. 5a, b). Similarly, recent reports have revealed that *Arabidopsis WRKY2* mediates seed germination, and *OsWRKY08* improves osmotic stress tolerance of transgenic *Arabidopsis* (Jiang and Yu 2009; Yu et al. 2009). Moreover, *VpWRKY2* transgenic *Arabidopsis* plants have enhanced cold tolerance in growing seedlings (Fig. 5c, d). These findings concur with an earlier report that over-expression of soybean *GmWRKY21* in *Arabidopsis* can enhance tolerance to cold stress (Zhou et al. 2008). Therefore, results of the present study confirm that grapevine *WRKY* also functions in regulating tolerance to abiotic stresses.

Despite the similar expression patterns of the two genes in post *E. necator* infection of grapevine and the similar roles in enhancing resistance of transgenic *Arabidopsis* plants to *E. cichoracearum* infection, the two *WRKY* genes differ in some other characteristics. First, VpWRKY1 can activate reporter genes in yeast, while VpWRKY2 is unable to do (Supplementary Fig. S6); second, MeJA and Eth can induce *VpWRKY2* expression in grapevine, whereas they cannot induce *VpWRKY1*. Third, weak *VpWRKY2* expression can be detected in roots while *VpWRKY1* cannot be detected in roots of 'Baihe-35-1' under natural field conditions (Supplementary Fig. S7); fourth, VpWRKY1 regulates expression of *AtPR10*, while VpWRKY2 cannot, and VpWRKY2 regulates *AtCOR1* and *AtPDF1.2*, while VpWRKY1 cannot; fifth, VpWRKY2 can enhance cold tolerance of transgenic *Arabidopsis* plants, while VpWRKY1 cannot. There may be additional differences between these two *WRKY* genes, suggesting that they perform different functions in plant defense and development.

In summary, the two transcription factor genes, *VpWRKY1* and *VpWRKY2*, isolated from grapevine powder mildew-resistant Chinese wild *V. pseudoreticulata*, were induced rapidly by *E. necator* infection in 11 grapevine genotypes. The increase in induction of *VpWRKY1* correlates with the level of resistance of the genotypes. Overexpression of *VpWRKY1* and *VpWRKY2* in *Arabidopsis* can increase resistance to powdery mildew and regulate the expression of some defense marker genes in transgenic

Arabidopsis plants. The two genes can also regulate the expression of some grapevine defense marker genes in a transient transformation assay. Results suggest that VpWRKY1 and VpWRKY2 may participate in the resistance of transgenic grapevine to *E. necator*. In addition, these two genes may enhance tolerance of transgenic grapevine to abiotic stresses. Further studies using a stable homologous expression system will confirm the role of these two WRKY transcription factors in grapevine defense mechanisms.

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