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Research article

Grape (*Vitis vinifera*) VvDOF3 functions as a transcription activator and enhances powdery mildew resistance

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ABSTRACT

DOF proteins are plant-specific transcription factors that play vital roles in plant development and defense responses. However, DOFs have primarily been investigated in model plants, and fairly limited research has been performed on grape (*Vitis vinifera*). In this study, we isolated and characterized a C_2–C_2 zinc finger structural DOF gene, *VvDOF3*, from the grape cultivar Jingxiu. The VvDOF3 protein showed nuclear localization and transcriptional activation ability, indicating that it functions as a transcription factor. The *VvDOF3* gene was rapidly induced by exogenous salicylic acid (SA), jasmonic acid (JA), and powdery mildew infection. Overexpression of *VvDOF3* in *Arabidopsis thaliana* enhanced resistance to *Golovinomyces cichoracearum*. Expression of the SA-responsive defense-related gene *PR1* and the concentration of SA were up-regulated in transgenic *Arabidopsis* plants overexpressing *VvDOF3*. Together, these data suggest that *VvDOF3* functions as a transcription factor in grape and enhances powdery mildew resistance through the SA signaling pathway.

1. Introduction

Plants exhibit varying degrees of resistance to infection by various pathogens. Based on the characteristics of their life cycles, plant pathogens are categorized as either biotrophs or necrotrophs. Biotrophs are parasites that feed on living tissues, while necrotrophs kill host tissues for nutrition (Pieterse et al., 2009). *Golovinomyces cichoracearum*, the causal pathogen of powdery mildew in several plant species, is a biotroph, whereas *Botrytis cinerea* is a typical necrotrophic pathogen.

Many pathogens, however, do not exhibit the distinct traits of biotrophs or necrotrophs; these pathogens depend on living tissues during the initial stages of infection and eventually kill the host. Plants exhibit two different modes of immunity to pathogens. In the first mode, plant pattern-recognition receptors (PRRs) recognize microbial-/pathogen-associated molecular patterns (MAMPs/PAMPs), resulting in PAMP-triggered immunity (PTI). In the second mode, plant disease resistance (R) genes encoding nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins initiate effector-triggered susceptibility (ETI) (Leipe et al., 2004). The NBS-LRR-mediated disease resistance is effective against biotrophic or hemi-biotrophic pathogens but not against necrotrophic pathogens (Glazebrook, 2005).

Salicylic acid (SA) and jasmonic acid (JA) signaling pathways are involved in plant defense. Pathogen infection results in the accumulation of SA, which activates pathogenesis-related (PR) genes, thus inducing systemic acquired resistance (SAR) (Durrant and Dong, 2004). Plant defense response is induced by an insect, and mechanical injury is mediated by the JA signaling pathway (Liu et al., 2016). The SA and JA signaling pathways do not function independently, but rather interact with each other to induce pathogen resistance. The JA signaling mutants *mpk4* and *coi1* show enhanced SA accumulation and signaling (Kloek et al., 2001; Lindhart et al., 2000). By contrast, blocking SA accumulation in infected plants improves JA signaling (Spoel et al., 2003). Many studies have shown that SA and JA signal transduction pathways share several key controlling factors, such as NPR1, WRKY, EDS1, MPK4, and GRX480.

Several DNA-binding one zinc finger (DOF) domain-containing proteins exhibit the characteristics of a plant-specific transcription factor (Yanagisawa, 1995). The DOF proteins harbor a highly conserved DNA-binding domain and a single C_2–C_2 zinc finger located in the N-terminal regions, and amino acids in the C-terminal region are associated with specific biological functions. The *DOF* genes are involved in plant growth, development, and defense response. In apple (*Malus domestica*), *MdDOF15* and *MdDOF48* are up-regulated specifically during pollen tube growth, whereas *MdDOF18* is up-regulated during flower development. Additionally, the expression level of these genes is up-regulated by heat stress, suggesting that the DOF transcriptional factors play an important role in heat stress response (Yang et al., 2018). In

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strawberry (*Fragaria* × *ananassa*), *FaDOF2* shows a high transcript level in ripe strawberry receptacles, indicating that *FaDOF2* plays a subsidiary regulatory role in the volatile phenylpropanoid pathway (Molina-Hidalgo et al., 2017). In banana (*Musa acuminata*), *MaDOF23* functions as a transcriptional repressor and interacts with the transcriptional activator MaERF9 to control fruit ripening (Feng et al., 2016).

Grape (*Vitis vinifera*) has a high nutritional value as well as significant health benefits (Guo et al., 2019a,b). Nevertheless, Eurasian grape varieties exhibit low resistance to powdery mildew, a fungal disease that causes substantial reductions in yield and loss of quality of the fruit (Guo et al., 2019a,b). Powdery mildew affects the leaves, branches, and fruits, and young tissues are the most sensitive to it. Many genes related to powdery mildew resistance have been investigated in the Chinese wild grape (*Vitis pseudoreticulata*); knockdown of *VvMLO7* and *VvMLO6* reduced the sensitivity of grape cultivars to powdery mildew (Pessina et al., 2016). Authentication of powdery mildew resistance loci, *Ren6* and *Ren7*, is of great significance to induce powdery mildew resistance in grape (Pap et al., 2016). (Yu et al., 2013) showed that the EIRP1 E3 ligase positively regulates the resistance of Chinese wild grape to *G. cichoracearum* and *Pseudomonas syringae* pv *tomato* DC3000 by mediating proteolysis of the negative regulator VpWRKY11 via degradation by the 26S proteasome. However, little information is available on powdery mildew resistance in cultivated grape (*Vitis vinifera* L.).

In this study, we isolated a *DOF* gene, *VvDOF3* (VIT_09s0002g02490), from the grape cultivar Jingxiu and characterized its expression and protein structure. The VvDOF3 protein contained a DOF motif. Overexpression of *VvDOF3* in *Arabidopsis thaliana* enhanced resistance to the powdery mildew pathogen, *G. cichoracearum*.

2. Methods

2.1. Plant material and treatment

Plants of the grape cultivar Jingxiu were grown on the research farm of the Henan University of Science and Technology. Plants of the *Arabidopsis* ecotype Columbia were grown in a greenhouse at 25 °C under a 16 h light/8 h dark photoperiod and 100 $\mu mol\,m^{-2}\,s^{-1}$ light intensity.

Leaves of grape plants were sprayed with SA (10 mM) or methyl jasmonate (MeJA) (100 mM) solutions containing Tween 20 (0.05%, v/v). Grape leaves were collected at 0, 12, 24, 36, 48, 60, and 72 h post-inoculation (hpi) and immediately frozen in liquid nitrogen. Leaves sprayed with sterile water were used as a control. Inoculation of grape and *Arabidopsis* leaves with pathogenic (PA) bacteria was performed as described previously (Xu et al., 2010). All experiments were repeated three times.

2.2. Sequence comparison and phylogenetic analysis

The genome reference is Ensembl Plants (http://plants.ensembl.org/index.html). Amino acid sequence alignment was performed using ClustalX. Phylogenetic tree analysis was conducted using MEGA7. Chromosomal assignment for *VvDOF3* was confirmed by MG2C (http://mg2c.iask.in/mg2c_v2.0). PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) was used to predict the *cis*-acting elements in gene promoters. The nuclear localization sequence (NLS) was predicted at http://linux1.softberry.com/cgi-bin/programs/proloc/protcomppl.pl.

2.3. VvDOF3 cloning, vector construction, and transformation

The cDNA fragment encoding *VvDOF3* was amplified using gene-specific primers (Table S1). A translational fusion of VvDOF3 with green fluorescent protein (GFP) was generated by cloning the *VvDOF3* PCR product into the overexpression vector *pCambia2300-GFP*. The resulting plasmid was introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation using the GV3101 strain with the floral dip method (Clough and Bent, 1998). Transgenic seeds were germinated in a tissue culture chamber under optimum conditions (25 °C and 16 h light/8 h dark photoperiod) on Murashige and Skoog (MS) medium. Transgenic seedlings were transferred to a cultivation room for inoculation with the powdery mildew pathogen.

2.4. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from fresh tissues of grape plants using an RNAprep Pure Plant Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. To remove any traces of genomic DNA contamination, total RNA was treated with RNase-free DNase (TaKaRA, Dalian, China). Subsequently, cDNA was synthesized and used as a template for qRT-PCR on a Bio-Rad IQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with SYBR Premix Ex Taq II (TaKaRA, Dalian, China) and gene-specific primers (Table S1). The qRT-PCR was performed in a 25 μl volume, and *Actin* was used as the reference gene. The relative expression of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All reactions were performed in triplicate.

2.5. Transcriptional activation assay of VvDOF3 in yeast

The *VvDOF3* gene was cloned into *pGBKT7* using *Nde*I and *Bam*HI restriction endonucleases to construct the bait plasmid *pGBKT7-VvDOF3*. The primers used for cloning are listed in Table S1. The empty *pGBKT7* vector was used as a negative control, whereas the *pGBKT7-GAL4* vector was used as a positive control. All plasmids were transformed into the yeast strain AH109. After transformation, the yeast cells were grown on a selective medium lacking tryptophan (SD/−Trp), and the positive clone was plated on SD/−Trp/−Ade/−His/+X-α-Gal medium to continuous cultivation.

2.6. Quantification of SA and JA levels and number of conidia

The concentrations of SA and JA in transgenic *Arabidopsis* plants were determined as described previously (Yu et al., 2019). Subsequently, SA and JA were extracted from the leaves of *Arabidopsis* plants according to the methods described by (Bowling et al., 1994) and (Meyer et al., 1984), respectively, and quantified by high-performance liquid chromatography (HPLC) analysis.

The number of conidia were counted under a microscope, as described previously (Yu et al., 2013).

2.7. Statistical analysis

The statistical significance of the data was determined using the Student's *t*-test. Differences with $p \leq 0.05$ were considered statistically significant.

3. Results

3.1. Isolation and characterization of VvDOF3

A cDNA fragment encoding *VvDOF3* was isolated from cultivated grape variety Jingxiu using gene-specific primers. The *VvDOF3* gene is located on chromosome 9 of the wine grape genome and is 1438 base pairs (bp) long. The deduced amino acid sequence of VvDOF3 is 216 amino acids, with a molecular weight of 23.38 kDa and a predicted theoretical isoelectric point of 9.10. Sequence analysis showed that the deduced amino acid sequence of *VvDOF3* contained one Cys2/Cys2-type zinc finger motif (position 58–112 amino acids) in the N-terminal

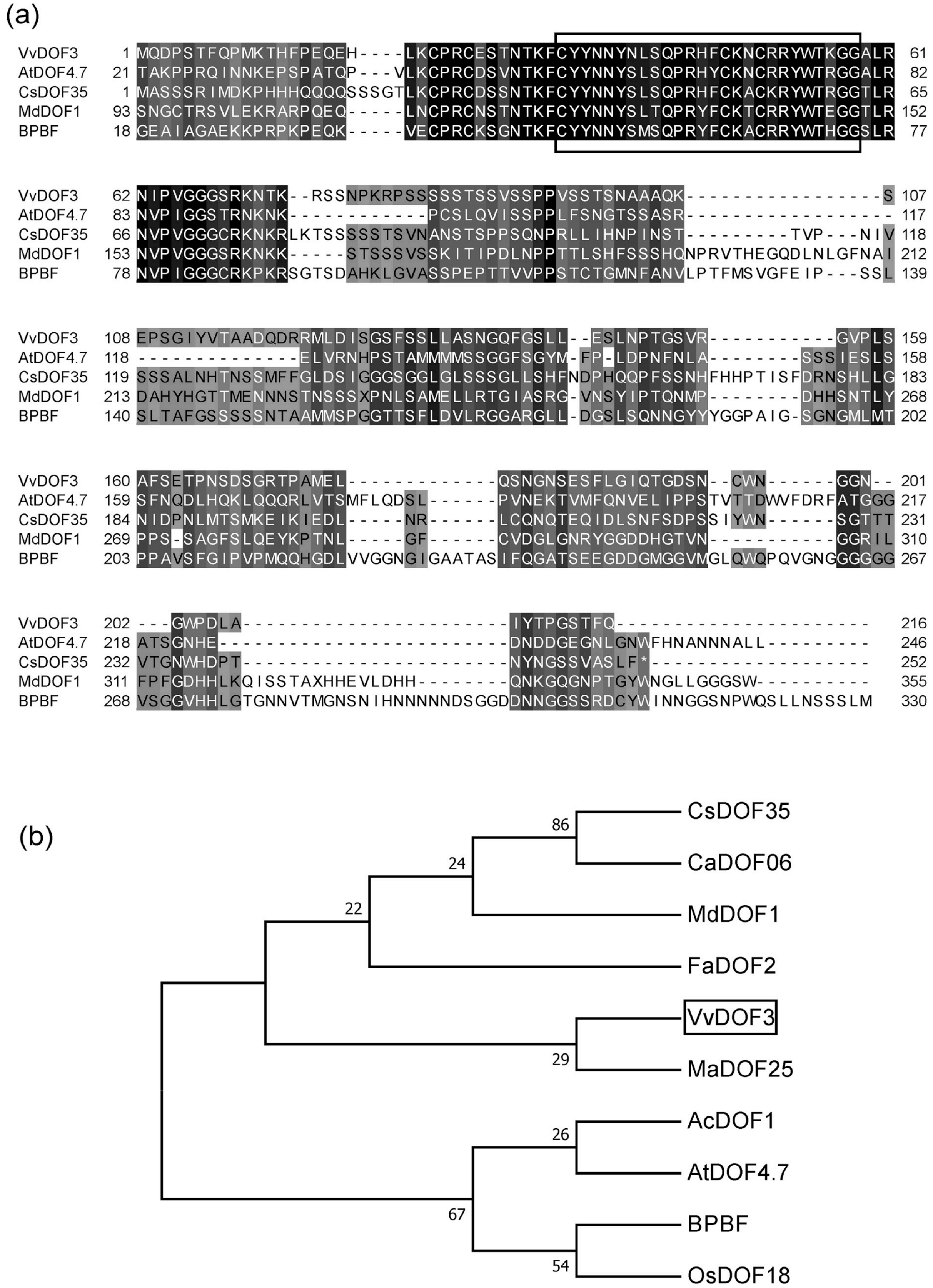

Fig. 1. Characterization of the *VvDOF3* gene structure and VvDOF3 amino acid sequence. (a) amino acid sequence alignment of VvDOF3 and closely related DOF3 proteins from other species; the black box is the zinc finger motif; (b) phylogenetic analysis of VvDOF3 and AcDof1(XP_020106276.1), FaDOF2(KP100112), BPBF (AAS19857.1), CsDof35(Csa6M517950.1), CaDof06(CA02g15190), MdDof1(MDP0000183534), OsDOF18(Os04g0678400), and MaDof25(GSMUA_Achr1T20550_001), AtDof4.7(Q84K52.1).

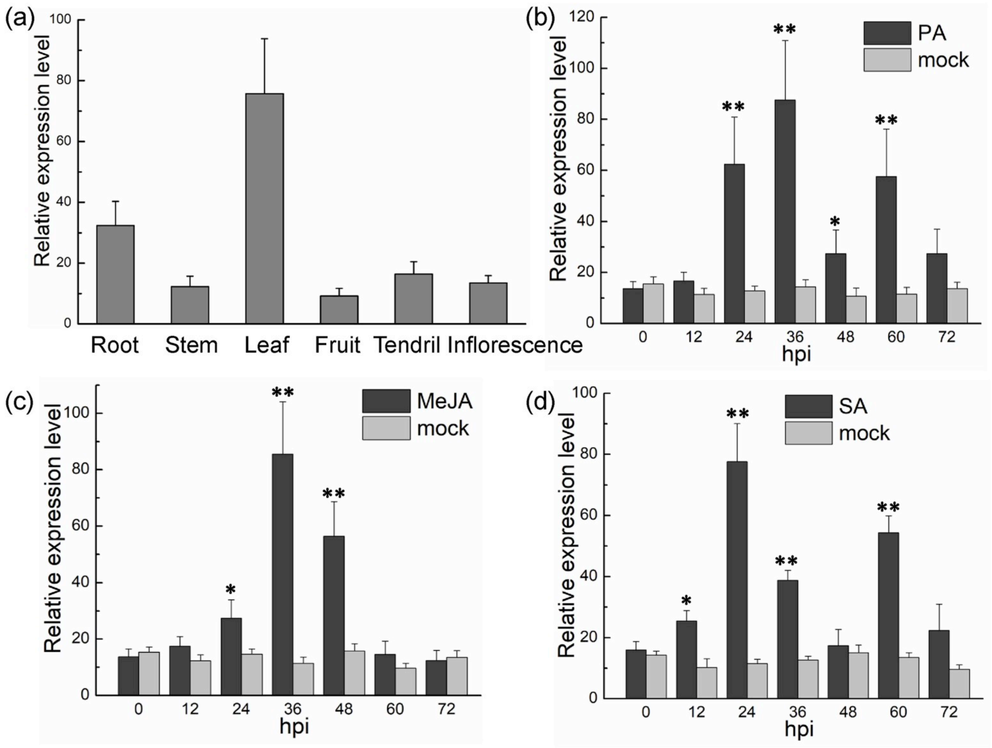

Fig. 2. Expression analysis of *VvDOF3*. (a) expression profile of *VvDOF3* in different grape tissues; (b–d) expression profile of *VvDOF3* in grape leaves after: (b) *G. cichoracearum* inoculation; (c) MeJA spray; and (d) SA spray. Data represent mean ± standard deviation (SD). Asterisks indicate statistically significant differences (*, $p \leq 0.05$; **, $p < 0.01$; Student's *t*-test).

region; the consensus pattern of the zinc finger motif is C-x(2)-C-x(7)-[CS]-x(13)-C-x(2)-C-x-R-x-W-T-x-G-G (Fig. 1a). The VvDOF3 protein shared 29% sequence similarity with MaDOF25 (GSMUA_Achr1T20550_001). The promoter sequence of *VvDOF3* was extracted using Tbtools. Analysis of the *VvDOF3* promoter using PlantCARE revealed two MeJA-responsive *cis*-acting regulatory elements (TGACG and CGTCA), three light-responsive elements (GA, GAG, and TCT motifs), and three TC-rich repeats involved in defense and stress response (Table S2).

3.2. Expression analysis of VvDOF3

The expression of *VvDOF3* in root, stem, leaf, fruit, tendril, and inflorescence tissues was detected by qRT-PCR. The expression level of *VvDOF3* was highest in the leaves compared with the other tissues (Fig. 2a). To further investigate the expression pattern of *VvDOF3*, grape leaves were inoculated with *G. cichoracearum* or sprayed with MeJA or SA. The expression of *VvDOF3* significantly increased at 24, 36, and 60 hpi (Fig. 2b). In response to MeJA spray, the expression of *VvDOF3* increased at 24 h, reaching a peak at 36 h, and then rapidly decreased after 48 h (Fig. 2c). In response to SA, the expression of *VvDOF3* sharply increased at 24 h, then gradually decreased until 48 h, and showed another increase at 60 h (Fig. 2d). *VvDOF3* showed a similar expression pattern between pathogen inoculation and SA treatment, but an opposite expression pattern between MeJA and SA treatments (Fig. 2b–d).

3.3. VvDOF3 exhibits nuclear localization and transcriptional activation activity

To determine whether VvDOF3 functions as a transcription factor, the *pBI221-GFP-VvDOF3* and *pBI221-GFP* vectors were introduced into onion epidermal cells. In cells transformed by *pBI221-GFP*, GFP fluorescence was detected in the entire cell, whereas cells transformed by *pBI221-GFP-VvDOF3* showed GFP fluorescence in the nucleus only (Fig. 3a). To examine the transcriptional activation activity of VvDOF3, a transcriptional activation assay was conducted in yeast. The *pGBKT7* empty vector (negative control), *pGBKT7-GAL4* (positive control), and *pGBKT7-VvDOF3* were transformed into yeast. All transformants grew well on SD/−Trp media. The yeast cells transformed with *pGBKT7-GAL4* and *pGBKT7-VvDOF3* grew well on the SD/−Trp/−Ade/−His media and stained blue in the presence of X-α-Gal. However, cells transformed by *pGBKT7* showed minimal growth on the SD/−Trp/−Ade/−His media (Fig. 3b).

3.4. Overexpression of VvDOF3 in Arabidopsis

To further analyze the role of *VvDOF3* in plant defense, transgenic *Arabidopsis* plants overexpressing *VvDOF3* were generated. Three transgenic lines were obtained by screening for antibiotic resistance and PCR amplification. The transgenic lines showed increased resistance to *G. cichoracearum* compared with wild-type (WT) plants (Fig. 4a). The *VvDOF3* overexpression lines (OE#2, OE#7, and OE#15) showed fewer disease symptoms than WT plants. We also quantified the number of conidiophores on *G. cichoracearum*-inoculated transgenic and WT plants. All transgenic lines and WT plants showed a lower number of conidiophores at 4 days post-inoculation (dpi) than at 7 dpi, and the conidiophore number was lower in transgenic plants than in WT plants (Fig. 4b).

Next, the expression level of genes involved in SA signaling (*PR1*) and JA signaling (*PDF1.2*) pathways was examined in WT and transgenic plants by qRT-PCR. The expression of *PR1* showed a significant difference between the WT and the transgenic plants (Fig. 4c). However, the expression of *PDF1.2* showed a less significant difference between the WT and the transgenic lines. The concentration of SA was higher than that of JA in the transgenic and WT plants inoculated with *G. cichoracearum* (Fig. 4d). Additionally, the SA concentration was significantly different between the transgenic and the WT plants (Fig. 4d).

4. Discussion

The results of qRT-PCR analysis showed diverse expression patterns of *VvDOF3* in various grape tissues, with higher expression in leaves and

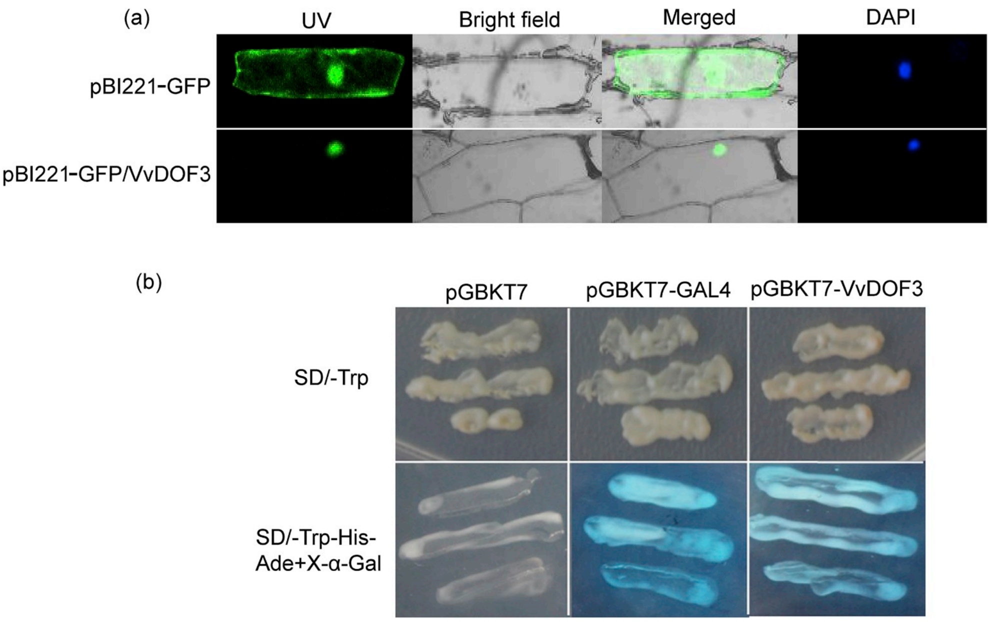

Fig. 3. Analysis of the localization and activity of the VvDOF3 protein. (a) subcellular localization of VvDOF3 in onion epidermal cells. The nucleus was stained with DAPI. Three independent experiments were performed, and representative images are shown; (b) transcriptional activation assay of the different fragments of the *VvDOF3* gene in yeast. Transformed yeast cells were grown on SD/−Trp and SD/−Trp/−Ade/−His/+X-α-Gal media. The *pGBKT7* empty vector served as a negative control, and the *pGBKT7-GAL4* vector was used as a positive control.

roots compared with other tissues (Fig. 2a). Previously, 46 *DcDOF* genes were identified in carrot (*Daucus carota*), some of which showed distinct tissue-specific expression patterns; *DcDOF-A-1* and *DcDOF-C1-1* are highly expressed in petioles, whereas *DcDOF-B2-2* and *DcDOF-D1-7* are highly expressed in leaves (Huang et al., 2016). In *Arabidopsis*, the *AtDOF2.4* gene is expressed in the vasculature of immature portions of leaves, whereas *AtDOF2.1*, *AtDOF4.6*, and *AtDOF5.3* are expressed in the root vascular cells (Gardiner et al., 2010; Konishi and Yanagisawa, 2007). In wheat (*Triticum aestivum*), the *TaDOF* family members are predominately expressed in the vegetative organs, and *TaDOF1* shows significantly higher expression in the stem than in other tissues (Shaw et al., 2009). In this study, the expression of *VvDOF3* in grape leaves was up-regulated after exogenous SA or MeJA application or after inoculation with the powdery mildew pathogen, *G. cichoracearum* (Fig. 2b). These results suggest that the *VvDOF3* gene confers grape plants with the ability to respond to exogenous hormone application and powdery mildew infection.

According to previous studies, DOF transcription factors respond to various abiotic stresses. In potato (*Solanum tuberosum*), *StDOF18* and *StDOF5* are up-regulated by abscisic acid (ABA) treatment, whereas *StDOF28* and *StDOF9* are up-regulated in response to salt treatment (Venkatesh and Park, 2015). In pineapple (*Ananas comosus*), *AcoDOF2*, *AcoDOF8*, *AcoDOF11*, *AcoDOF12*, and *AcoDOF24* are up-regulated in response to salt treatment, whereas *AcoDOF1* shows high expression under drought stress (Azam et al., 2018). In walnut, *Juglans regia*, the JrDOF3 transcription factor confers an improvement in high-temperature tolerance (Yang et al., 2018).

Amino acid sequence analyses showed that VvDOF3 contained a putative NLS. This suggested that VvDOF3 may function as a transcription factor. To test this hypothesis, subcellular localization and

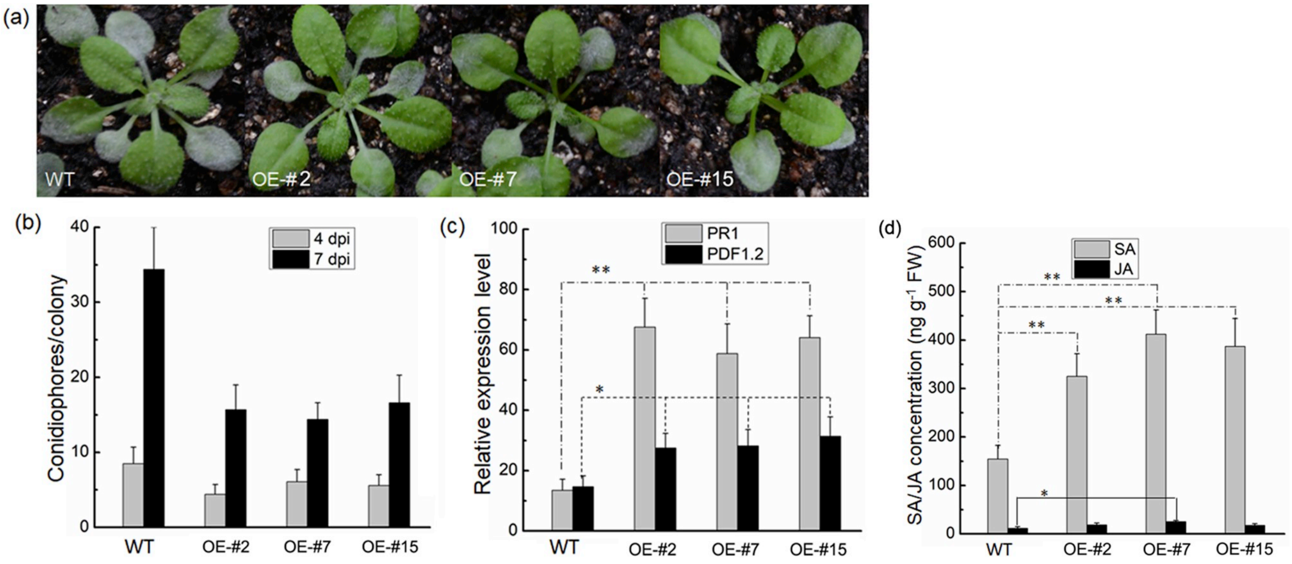

Fig. 4. Enhanced disease resistance and elevated expression levels of defense-related genes in transgenic *Arabidopsis* plants overexpressing *VvDOF3*. (a) disease symptoms developed on the leaves of wild-type (WT) plants and transgenic overexpression (OE) lines after inoculation with *G. cichoracearum*; (b) number of conidiophores on WT plants and transgenic OE lines at 4 and 7 dpi; (c) expression of pathogenesis-related genes (*PR1* and *PDF1.2*) in WT and transgenic plants inoculated with *G. cichoracearum*; (d) concentration of endogenous SA and JA in WT and transgenic *Arabidopsis* plants after *G. cichoracearum* inoculation. Data represent mean ± standard deviation (SD). Asterisks indicate statistically significant differences (*, $p \leq 0.05$; **, $p < 0.01$; Student's *t*-test).

transcription activations were performed. The results showed that *VvDOF3* was localized in the nucleus and showed transcription activation activity in yeast. Based on bioinformatics analysis and experimental results, we can conclude that *VvDOF3* is a transcriptional activator. Previous studies have shown that DOF proteins function as transcriptional activators in different plants. In tomato (*Solanum lycopersicum*), the DOF motif-containing protein SlDOF10 localizes in the nucleus and shows transcriptional activation activity in developed vascular tissue (Rojas-Gracia et al., 2019). In *Arabidopsis*, AtDOF4.2 localizes in the nucleus and shows transcriptional activation activity by directly binding to and activating the promoter of the cell wall loosening-related gene *AtEXPA9* on the seed epidermis (Zou et al., 2013). In tobacco (*Nicotiana tabacum*), the *DOF2.1* transcription factor is downstream of TMO5/LHW, depending on the cytokinin response, and controls vascular proliferation (Smet et al., 2019). These data suggest that VvDOF3 functions as a transcription factor and regulates the expression of downstream genes involved in stress response and plant growth and development.

Three transgenic *Arabidopsis* lines overexpressing *VvDOF3* showed an enhanced defense response to powdery mildew compared with WT plants. The transgenic lines had a lower conidiophore count than the WT at 7 dpi; however, it is particularly interesting that the conidiophore count was similar between WT and transgenic lines at 4 dpi (Fig. 4a and b). This is probably because the appearance of the typical disease symptoms of powdery mildew takes time after the pathogen enters the host plasma membrane (Lipka et al., 2008). Gene expression analysis showed that the expression of the key gene involved in the SA signaling pathway (*PR1*) was high, while that of the key gene involved in the JA signaling pathway (*PDF1.2*) was low. The endogenous SA level is evoked when the plants are invaded by pathogens; subsequently, it increases and changes the redox state of the cells, resulting in a reduction in disulfide bonds in NPR1, as well as the release of monomers from reduced NPR1 oligomers into the nucleus, thus activating the expression of a series of *PR* genes (Kinkema et al., 2007). Furthermore, the high concentration of endogenous SA in transgenic *Arabidopsis* plants indicated that VvDOF3 enhanced the resistance to powdery mildew through the SA signaling pathway.

In this study, *VvDOF3* showed a similar expression pattern between pathogen inoculation and SA treatment but an opposite expression pattern between MeJA and SA treatments (Fig. 2b–d). A possible explanation for this observation is that *VvDOF3* confers enhanced disease resistance by exhibiting antagonistic functions in the SA and JA signaling pathways. SA primarily mediates resistance to biotrophs and necrotrophs, whereas JA mediates mostly resistance to necrotrophs and mechanical injury. Generally, the production of endogenous phytohormones, including SA, JA, and ethylene, is coupled with plant response to biotic or abiotic stress. These endogenous phytohormones modulate the downstream signaling pathways and expression of defense-associated genes (Bari and Jones, 2009). In *Arabidopsis*, the results of multiple microarray co-expression analyses showed that WRKY46 is involved in the activation of SA signaling by regulating *PBS3* gene expression (van Verk et al., 2011). The basic helix-loop-helix (bHLH) transcription factor ILR3 represses reactive oxygen species (ROS) production and the SA signaling pathway following infection by Alfalfa mosaic virus (Aparicio and Pallás, 2017).

In this study, we cloned the *VvDOF3* gene and showed that *VvDOF3* acts as a transcription factor, which plays a positive regulatory role in enhancing the powdery mildew resistance of cultivated grapes through the SA signaling pathway. However, the regulatory mechanisms of the *VvDOF3* downstream genes need further investigation.

5. Conclusions

Overall, this study explored the role of *VvDOF3* in powdery mildew resistance in cultivated grape. The *VvDOF3* gene is located on chromosome 9 of the published wine grape genome and contains one Cys2/Cys2-type zinc finger motif in its N-terminal region. The *VvDOF3* gene showed diverse expression patterns in various tissues and in response to *G. cichoracearum*, MeJA, and SA treatments. The nuclear localization and transcriptional activation activity of VvDOF3 showed that it functions as a transcriptional activator. The transgenic *Arabidopsis* showed enhanced defense response to powdery mildew compared with the WT plants. The high expression of *PR1* and the elevated concentration of endogenous SA in the transgenic *Arabidopsis* lines inoculated with *G. cichoracearum* further supported the hypothesis that VvDOF3 enhances resistance to powdery mildew via the SA signaling pathway.

Author contributions

Yi-He Yu and Lu Bian conceived and designed the experiment, analyzed and interpreted the data and wrote the manuscript.

Yu-Tong Wan, Ze-Ling Jiao, Ke-Ke Yu, Guo-Hai Zhang, Da-Long Guo participated in different tissues of grape sample collection and cultivation of plant materials.

All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.010.

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