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VvZFP11, a Cys2His2-type zinc finger transcription factor, is involved in defense responses in *Vitis vinifera*

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Abstract

In plants, many C2H2-type zinc finger transcription factors function in plant defense responses to biotic and abiotic stresses. Here, we report cloning and characterization of *VvZFP11* which encoded a C2H2-type zinc finger protein (ZFP) in grapevine (*Vitis vinifera*). Sequence analysis shows that *VvZFP11* contained one L-box, two C2H2-type zinc finger motifs and one ERF-associated amphiphilic repression (EAR) motif. The VvZFP11 localized to the nucleus and functional analysis shows that full-length VvZFP11 had no transcriptional activity, but VvZFP11 lacking the EAR motif had a strong transcriptional activity in yeast. In grapevine, expression of *VvZFP11* was induced by salicylic acid and methyl jasmonate and also quickly responded to infection with *Erisiphe necator*. *Arabidopsis thaliana* plants overexpressing *VvZFP11* were more resistant to *Golovinomyces cichoracearum*, and real time quantitative polymerase chain reaction revealed that defense-related genes *AtPR1* and *AtPDF1.2* were up-regulated in the overexpressing lines. These results suggest that *VvZFP11* might play an important role in defense responses in grapevine.

Additional key words: *Erisiphe necator, Golovinomyces cichoracearum,* grapevine, methyl jasmonate, salicylic acid, zinc finger protein.

Introduction

Plants are continuously challenged by various biotic and abiotic stresses; to survive, they have developed complex defense mechanisms that sense stresses and activate protective mechanisms. Transcription factors play important roles in signal recognition and activation of defenses. Transcription factors function as master regulators that bind to specific *cis*-regulatory elements and then activate expression of target genes resulting in various outcomes including stress tolerance (Golldack *et al*. 2011). Many transcription factors, including members of the zinc finger protein (ZFP), AP2/EREBP, bZIP, MYC, NAC, MYB, HSF, AREB/ABF, bHLH, HB, and WRKY families, participate in stress response in plants (Golldack *et al*. 2011, Yu *et al*. 2011, Cabello *et al*. 2014).

Zinc finger proteins comprise a large and abundant family of proteins that function in many aspects of plant growth and development. Based on their individual Cys2-His2 zinc fingers, ZFPs are further divided into different families, including C2HC, C4, C4HC3, C3HC4, C8, C2H2, C6, C2HC5, and CCCH types (C and H represent cysteine and histidine, respectively; Ciftci-Yilmaz and Mittler 2008). One of the most-common types, the C2H2-type ZFPs, also known as TFIIIA-type zinc fingers, possess a conserved motif $X_2CX_{2-4}CX_{12}HX_{2-8}H$ (Muthamilarasan *et al*. 2014). Most C2H2-type ZFPs have confirmed functions in abiotic stress. For example, the *Arabidopsis* ZFPs Zat10 (Nguyen *et al*. 2012), Zat12 (Davletova *et al*. 2005), and Zat7 (Gupta *et al*. 2012) are involved in responses to drought, salt, cold, heat, and oxidative stresses. Also, overexpression of rice ZFP15,

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Abbreviations: DAPI - 4',6-diamidino-2-phenylindole; EAR - ERF-associated amphiphilic repression; hpi - hours post infection; MeJA - methyl jasmonate; GFP - green fluorescent protein; RT-qPCR - reverse transcription quantitative polymerase chain reaction; SA - salicylic acid; ZFP - zinc finger protein.
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ZFP179, ZFP182, ZFP207, ZFP245, and ZFP252 enhances tolerance to cold, salt, and drought stresses (Sun *et al*. 2010*,* Zhang *et al*. 2012, 2014). Furthermore, many ZFPs have been identified in wheat (Li *et al*. 2010a), cotton (Li *et al*. 2010b), soybean (Kim *et al*. 2001), poplar (Hamel *et al*. 2011), tobacco (Uehara *et al*. 2005), tomato (Hichri *et al*. 2014), and potato (Lawrence *et al*. 2014) and shown to be involved in responses to abiotic stresses. However, the role of ZFPs in plant disease resistance remains unclear.

Grapevine is an important commercial fruit crop worldwide and it is highly susceptible to many fungal pathogens such as downy mildew (*Plasmopara viticola*), powdery mildew (*Erysiphe necator*), and grey mould (*Botrytis cinerea*) (Yu *et al*. 2011, 2013). Therefore, understanding the molecular basis of natural resistance in *V. vinifera* can improve its disease resistance.

Our previous gene expression profiling in grapevine under various stress conditions revealed that most of the genes in the C2H2-type zinc finger subfamily are induced to different degrees by abscisic acid, drought, or high salinity. Among these genes, we identified one homolog of *Arabidopsis ZAT11*, designated *VvZFP11*. Transcription of *VvZFP11* also changes in *V. vinifera* cv. Cabernet Sauvignon treated with salicylic acid (SA) and methyl jasmonate (MeJA). This interesting finding prompted us to further investigate the molecular function of *VvZFP11* in defense responses.

Materials and methods

Plant materials and treatments: *Vitis vinifera* L. cv. Cabernet Sauvignon and *Arabidopsis thaliana* (Col-0 ecotype) plants were grown in vermiculite and perlite (1:1, v/v) in plastic pots in a growth chamber at a 12-h photoperiod, an irradiance of 280 μmol m^{-2} s^{-1}, day/night temperatures of 22/16 °C, and an air humidity of 85 %. *E. necator* was collected from two years old Cabernet Sauvignon plants in the field. *E. necator* challenge infection of Cabernet Sauvignon leaves was conducted as previously described (Yu *et al*. 2013). Salicylic acid (10 mM) or MeJA (100 mM) solutions with *Tween 20* (0.05 %, v/v) were sprayed on grapevine leaves of the same age. Grapevine leaves sprayed with *Tween 20* served as control. *Golovinomyces cichoracearum* (UCSC1 isolate) was cultured on *Arabidopsis phytoalexin deficient 4 (pad4*) mutant plants. *G. cichoracearum* challenge infection of the four weeks old *Arabidopsis* wild type was performed as previously described (Wang *et al*. 2007). The genomic DNA was extracted from grapevine leaves as described by Kim *et al*. (1997).

Plasmid construction and generation of transgenic plants: The full-length cDNA of *VvZFP11* was amplified from *V. vinifera* cv. Cabernet Sauvignon by reverse transcription polymerase chain reaction (RT-PCR) using *VvZFP11*-BamHI-F and *VvZFP11*-SacI-R primers (Table 1 Suppl.). The PCR fragment was confirmed by sequencing and was directionally cloned into the pCAMBIA3301 vector, downstream of the CaMV 35S promoter, to create the pCAMBIA3301-*VvZFP11* construct. For *Arabidopsis* transformation, the pCAMBIA3301-*VvZFP11* construct was introduced into *Agrobacterium* strain GV3101 and transferred into *Arabidopsis* by using floral dip transformation (Clough and Bent 1998). Positive transgenic lines were first screened on *Basta* plates and then identified by PCR*,* T3 homozygous transgenic lines were selected for evaluation of disease resistance.

Real-time quantitative PCR assays: The total RNA of Cabernet Sauvignon leaves was extracted with a *Spectrum* plant total RNA kit (*Sigma-Aldrich*, Shanghai, China). The first-strand cDNA was synthesized using a *PrimeScript* 1st strand cDNA synthesis kit (*TaKaRa*, Dalian, China). Real-time quantitative PCR (qPCR) was performed with a *Bio-Rad IQ5* real-time PCR detection system (*Bio-Rad Laboratories*, Hercules, CA, USA). The reaction was conducted following the instructions of an *SYBR Premix Ex Taq II* kit (*TaKaRa*). A real time qPCR amplification volume was 0.025 cm^3, *VvActin* or *β-TUB4* genes were used as internal control. Primers are listed in Table 1 Suppl. The relative expression of the target genes was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). All reactions were run in three replicates for each sample.

Transcription activation assay in yeast: The transcriptional activation of *VvZFP11* was determined as previously described (Yu *et al*. 2011). Fragments of DNA containing the whole or partial open reading frame of *VvZFP11* were inserted into the *Bgl* II/*Pst* I sites of the pGBKT7 vector to create the pGBKT7-*VvZFP11*, pGBKT7-*VvZFP11*ΔA, pGBKT7-*VvZFP11*ΔB, pGBKT7-*VvZFP11*ΔC, pGBKT7-*VvZFP11*ΔD, and pGBKT7-*VvZFP11*ΔE constructs. Full-length GAL4 sequence from pCL-1 was cloned into pGBKT7 to produce pGBKT7-GAL4 as positive control. The pGBKT7 empty vector was used as negative control. All above constructs were transformed into yeast strain AH109 and grown on SD/-Trp, SD/-Trp-His-Ade, or SD/-Trp-His-Ade+X-α-Gal plates. Primer sequences are listed in Table 1 Suppl.

Subcellular localization: The open reading frame sequence of *VvZFP11* without the termination codon was cloned into the *Xba* I/*Kpn I* site of the pBI221-GFP vector generating the pBI221-GFP/*VvZFP11* fusion with

the CaMV 35S promoter. Specific primers containing *Xba* I and *Kpn I* sites are listed in Table 1 Suppl. The recombinant vector was verified by sequencing three times. The fusion construct was transformed into onion epidermal cells by particle bombardment using a *Bio-Rad* biolistic *PDS 1000/He* system. The pBI221-GFP vector was used as control. Transformed materials were incubated in darkness in a growth chamber (24 °C, 16 - 18 h) and 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclear DNA. A *Zeiss LSM 510* confocal laser microscope (*Zeiss*, Oberkochen, Germany) was used for detection of green fluorescent protein (GFP) signals. The results shown are representative of three similar individually acquired images.

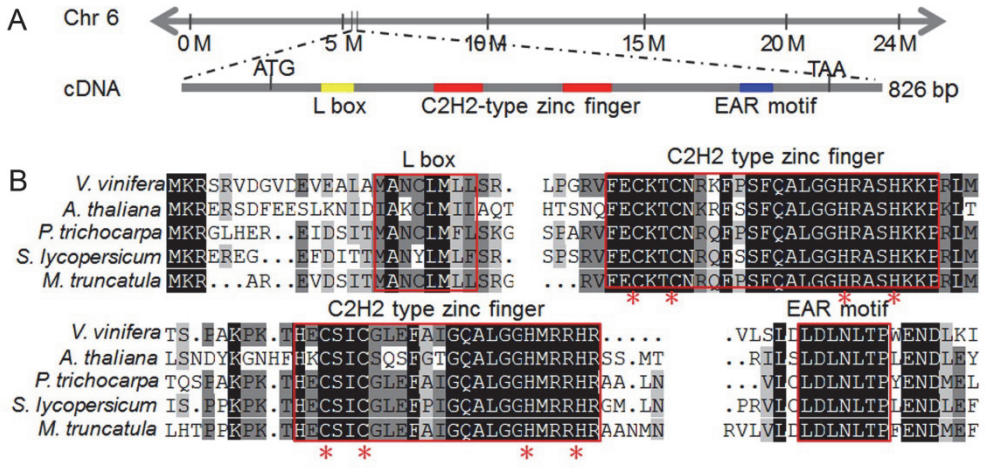

Fig. 1. Sequence analysis of *VvZFP11*. *A* - A schematic representation of the structure of *VvZFP11* and its chromosomal location which was predicted in the *Vitis vinifera* cv. Pinot Noir clone P40024 genomic sequence. VvZFP11 had four conserved motifs: one L-box, two C2H2-type zinc finger motifs, and one EAR motif. *B* - Alignment of the VvZFP11 amino acid sequence with other plant C2H2-type zinc finger proteins. Four predicted domains are labeled: the L-box, two C2H2-type zinc finger motifs, and the EAR motif. The *stars* indicate the conserved amino acid of the C2H2 type zinc finger. Sequences and accession numbers are: *V. vinifera*, XP_002284384; *Arabidopsis thaliana*, NP_181279; *Populus trichocarpa*, XP_002316305; *Solanum lycopersicum*, P_004239776; *Medicago truncatula*, XP_003621801.

Results

Based on the grapevine (*V. vinifera* cv. Pinot Noir) genome sequence (accession No. XM_002284348), we isolated *VvZFP11* from Cabernet Sauvignon leaves by PCR. The full-length cDNA of *VvZFP11* was 826 bp

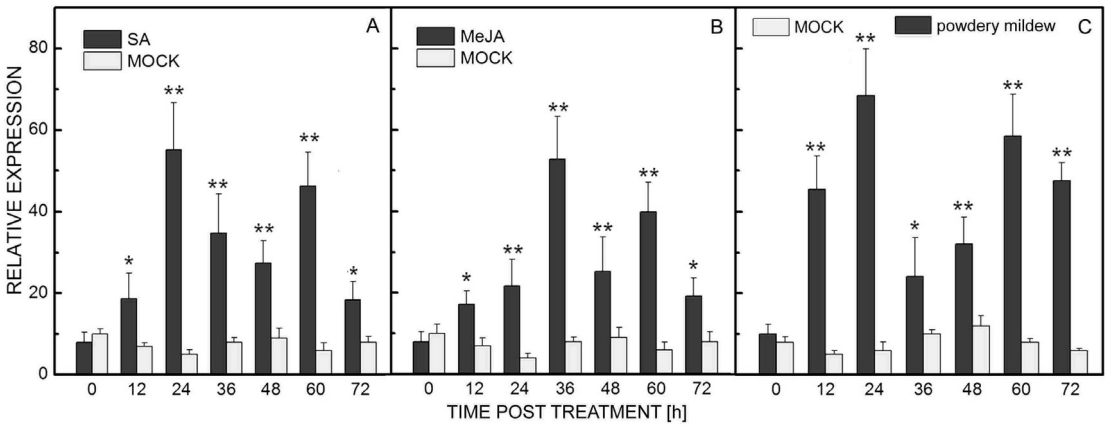

Fig. 2. Expression of *VvZFP11* measured by real time qPCR in *V. vinifera* cv. Cabernet Sauvignon leaves treated with 10 mM salicylic acid (SA) (*A*), 100 mM methyl jasmonate (MeJA) (*B*), or *Erisiphe necator* infection (*C*). Control leaves (MOCK) were sprayed with 0.05 % (v/v) *Tween 20* (*A*,*B*) or sterile water (*C*). The leaves were collected at different time points as indicated. *VvAct* was used as internal control. Means ± SDs of three independent experiments. *Asterisks* indicate statistically significant differences compared to the corresponding control (* - $P< 0.05$, ** - $P< 0.01$, Student's *t*-test).

with an open reading frame of 480 bp with a 167-bp 5' untranslated sequence and a 179-bp 3' untranslated region (Fig. 1*A*). *VvZFP11* encoded a protein of 159 amino acids with a calculated molecular mass of 17.81 kDa and an isoelectric point of 9.15. The deduced amino acid sequence of VvZFP11 contained an L-box at its N-terminus, two C2H2-type zinc finger motifs in the middle, and an EAR motif at its C-terminus (Fig. 1*A*). Comparison of the amino acid sequence of VvZFP11 with other related proteins (Fig. 1*B*) shows than the VvZFP11 protein shared a 57 % identity with *Populus trichocarpa* (acc. No. XP_002316305), 46 % identity with *A. thaliana* (acc. No. NP_181279), 59 % identity with *Solanum lycopersicum* (acc. No. XP_004239776), and 56 % identity with *Medicago truncatula* (acc. No. XP_003621801) proteins.

To test whether expression of *VvZFP11* was induced by SA or MeJA, we used real time qPCR to measure *VvZFP11* transcription in Cabernet Sauvignon leaves treated with these hormones. After SA treatment, expression of *VvZFP11* was induced at 12 h, peaked at 48 h, and remained relatively high up to 72 h (Fig. 2). Likewise, after MeJA treatment, expression of *VvZFP11* increased from 12 to 72 h and showed two peaks, at 36 and 60 h (Fig. 2). These results indicate that *VvZFP11* responded to multiple defense-related signals suggesting that VvZFP11 played a role in induced defense responses in grapevine.

To determine whether pathogen infection induces *VvZFP11* expression, we also measured the abundance of *VvZFP11* transcripts in Cabernet Sauvignon leaves at different time points after inoculation with *E. necator* (Fig. 2). The results show that expression of *VvZFP11* increased at 12 h post infection (hpi), peaked at 24 hpi, and remained at a high level up to 72 h. These results indicate that expression of *VvZFP11* was involved in plant defense responses.

Most ZFPs function as transcription factors in the

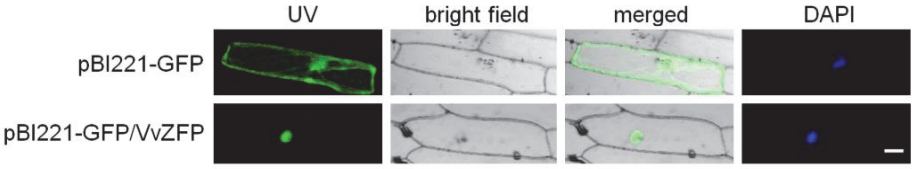

Fig. 3. Subcellular localization of VvZFP11 in onion epidermal cells. At 16 h after transformation, VvZFP11-GFP fusion proteins were detected by confocal laser-scanning microscopy. The nucleus was stained with DAPI. Data are representative of three independent experiments. *Scale bar* = 200 μm.

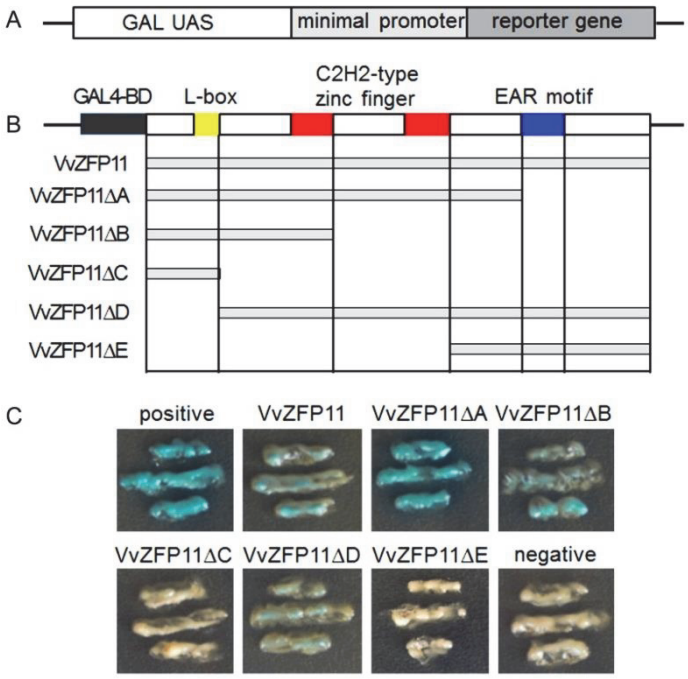

Fig. 4. Transcription activity of VvZFP11. *A* - A diagram of effector constructs. *B* - A schematic of VvZFP11 deletion constructs. *C* - Fusion proteins of pGBKT7-*VvZFP11*, pGBKT7-*VvZFP11*ΔA, pGBKT7-*VvZFP11*ΔB, pGBKT7-*VvZFP11*ΔC, pGBKT7-*VvZFP11*ΔD, pGBKT7-*VvZFP11*ΔE, pCL1-GAL4 (a positive control), and pGBKT7 (a negative control) were expressed in yeast strain AH109. Transformants were incubated on SD/-Trp/-His/-Ade/+X-α-gal to examine their activity.

nucleus. To examine whether VvZFP11 localizes to the nucleus, we fused the *VvZFP11* coding sequence to GFP (Fig. 3) and expressed the fusion protein in onion epidermal cells. Examination of protein fluorescence by confocal laser-scanning microscopy showed VvZFP11-GFP localized exclusively in the nucleus, whereas the control GFP signal localized to both the nucleus and the cytosol of onion epidermal cells (Fig. 3). These results demonstrate that VvZFP11 was a nuclear-localized protein.

To provide evidence that VvZFP11 encodes a transcription factor, we carried out a transcriptional activation assay in yeast. As shown in Fig. 4, a positive control grew well on a histidine-deficient medium and induced Mel1 activity in the presence of X-α-Gal. By contrast, a negative control (pGBKT7 alone) showed no activity (Fig. 4). The full-length VvZFP11 protein could activate transcription of reporter genes, but it showed a weak activity in yeast (Fig. 4). To determine key domains required for transcriptional activation, we tested several truncated fragments of VvZFP11 in yeast. VvZFP11ΔA, which lacks the EAR motif, strongly activated transcription of the reporter gene (Fig. 4). The experiment also shows that the C2H2 zinc finger DNA-binding domain was essential for transcriptional activation activity in yeast, whereas the N-terminal L-box was not (Fig. 4).

To characterize the function of *VvZFP11*, we generated transgenic *Arabidopsis thaliana* plants that overexpressed *VvZFP11* under the control of the CaMV35S promoter. Three independent homozygous transgenic lines were obtained, and the transcript of *VvZFP11* was detected by RT-PCR in the positive plants. In the normal growth conditions, the *VvZFP11*

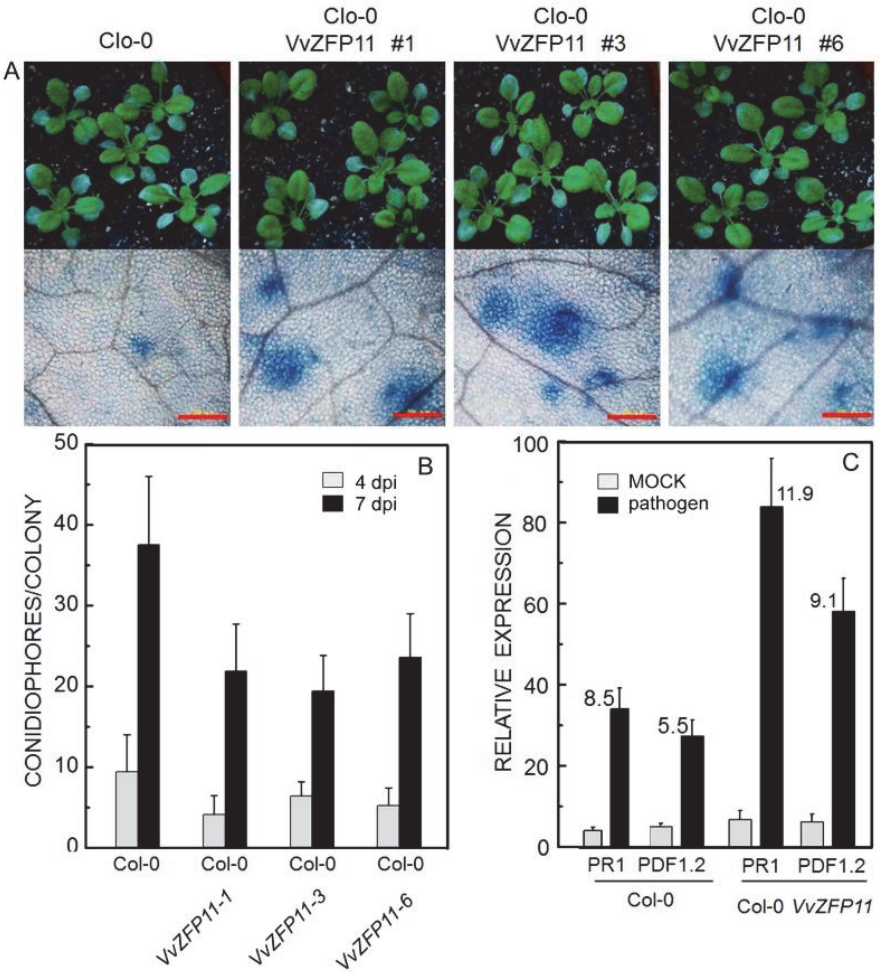

Fig. 5. Overexpression of *VvZFP11* in *Arabidopsis*. *A* - Overexpression of *VvZFP11* enhanced resistance to *Golovinomyces cichoracearum* in *Arabidopsis*. Disease symptoms developed on the leaves of wild type and transgenic lines 7 d post inoculation (dpi) with fungal spores (the *top panels*). The leaves were stained with trypan blue (the *bottom panels*). *Scale bar* = 200 μm. Three independent experiments showed similar results. *B* - The number of conidiophores per colony at 4 and 7 dpi. Means ± SDs, $n = 30$. *C* - A relative expression of defense-related genes in *Arabidopsis* wild-type (Col-0) and transgenic plants. Five-week-old plants were inoculated with *G. cichoracearum*. Relative transcriptions of *AtPR1* and *AtPDF1.2* were measured at 24 h post infection using real time qPCR with *β-TUB4* as internal control. Means ± SDs from three biological replicates.

transgenic plants showed no obvious growth or developmental changes in phenotype. We next tested their response to infection using *Arabidopsis* wild-type plants inoculated with *G. cichoracearum* as control. We found that the wild-type plants were highly susceptible to *G. cichoracearum* (Fig. 5*A*). However, the plants from the three independent transgenic lines were more resistant to the pathogen compared with the wild-type plants (Fig. 5*A*). To evaluate the resistance at the histological level, we stained the infected leaves with trypan blue, which indicated that the *VvZFP11* transgenic plants showed more severe cell death than the wild type (Fig. 5*A*). To measure fungal reproduction and development, the conidiophores in the wild type and the transgenic plants were counted. We found that the transgenic plants supported significantly fewer conidiophores than the wild type plants (Fig. 5*B*).

To provide more evidence about the function of *VvZFP11*, we measured the transcription of defense-related genes in the wild type and the transgenic plants after challenge with pathogens. In wild-type *Arabidopsis*, the transcripts of *PR1* and *PDF1.2* rapidly responded to the pathogen within 24 hpi (Fig. 5*C*). However, after being challenged with the pathogen, the transcription of *PR1* and *PDF1.2* increased more in the *VvZFP11* transgenic plants than in the wild type (Fig. 5*C*). These results suggest that *VvZFP11* overexpression increased induction of defense-related genes *PR1* and *PDF1.2*.

Discussion

In *V. vinifera*, zinc finger-homeodomain family genes were identified. The results show that those genes took part in variety responses to biotic and abiotic insults (Wang *et al*. 2014). In our previous work, we cloned C2H2-type zinc finger subfamily genes from grapevine and examined their expression under various stresses. These results show that expression of *VvZFP11* was induced by abscisic acid, drought, high salinity, SA, and MeJA. An extensive work has examined the roles of some C2H2 zinc-finger transcription factors in stress responses and development (Sun *et al*. 2010, Muthamilarasan *et al*. 2014), but the functions of the grapevine ZFPs involved in defense response are largely unknown. In this study, we characterized the function of *VvZFP11* in response to powdery mildew.

Sequence analysis reveals that the protein encoded by *VvZFP11* showed a high conservation in the zinc finger domains ($X_2CX_{2-4}CX_{12}HX_{2-8}H$) with other C2H2-type ZFPs. This motif recognizes DNA sequences for binding to target genes, so this type of protein generally activates or inhibits transcription (Kazan 2006). *VvZFP11* also possesses two other characteristic features of C2H2-type ZFPs, the L-box and the EAR motif, located in the N-terminal and C-terminal of the protein, respectively (Fig. 1). The EAR motif is also present in ethylene responsive transcription factors (ERF) (Kazan 2006). Transcription factors that contain EAR motifs generally down-regulate transcription of a reporter gene and can also repress activity of other transcription factors (Kazan 2006). To understand whether VvZFP11 functions to activate or repress transcription, we examined its subcellular localization and ability to activate transcription. The results show that VvZFP11 localized in the nucleus and the full-length VvZFP11 had no activation activity in yeast, but the fragment lacking the EAR motif did have activation activity (Fig. 4). These results suggest that VvZFP11 might function to repress transcription.

Gene expression profiling indicates that *VvZFP11* was not involved only in responses to abiotic stresses, but also it was affected by defense signaling molecules SA and MeJA (Fig. 2). We also detected increases in the transcription of *VvZFP11* in response to challenge with powdery mildew (Fig. 2). Overexpression of *VvZFP11* in *Arabidopsis* resulted in an enhanced resistance to powdery mildew and promoted defense-related marker gene expression (Fig. 5). These results show that *VvZFP11* promoted defense gene expression and increased disease resistance. Based on previous findings that proteins containing the EAR motif generally function as transcriptional repressors, it is possible that enhanced resistance of transgenic plants expressing EAR-containing VvZFP11 proteins results from VvZFP11 suppressing another repressor of defense responses. Thus, VvZFP11 suppresses a repressor and prevents its inhibition of defense responses, thus causing activation of plant defenses. Results of other studies have supported this speculation. In *Arabidopsis*, Zat12, Zat10, and Zat7 are EAR-containing C2H2-type zinc finger proteins (Davletova *et al*. 2005, Ciftci-Yilmaz *et al*. 2007, Nguyen *et al*. 2012), and constitutive expression of these proteins enhances tolerance of transgenic plants to abiotic stresses. Further work is needed to elucidate regulatory mechanisms underlying the enhanced disease resistance of transgenic plants expressing *VvZFP11*.

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