

RESEARCH PAPER

VpRFP1, a novel C4C4-type RING finger protein gene from Chinese wild *Vitis pseudoreticulata*, functions as a transcriptional activator in defence response of grapevine

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Abstract

RING finger proteins comprise a large family and play important roles in regulation of growth and development, hormone signalling, and responses to biotic and abiotic stresses in plants. In this study, the identification and functional characterization of a C4C4-type RING finger protein gene from the Chinese wild grapevine *Vitis pseudoreticulata* (designated VpRFP1) are reported. VpRFP1 was initially identified as an expressed sequence tag (EST) from a cDNA library constructed from leaves of *V. pseudoreticulata* inoculated with the grapevine powdery mildew *Uncinula necator*. Sequence analysis of the deduced VpRFP1 protein based on the full-length cDNA revealed an N-terminal nuclear localization signal (NLS) and a C-terminal C4C4-type RING finger motif with the consensus sequence Cys-X₂-Cys-X₁₃-Cys-X₁-Cys-X₄-Cys-X₂-Cys-X₁₀-Cys-X₂-Cys. Upon inoculation with *U. necator*, expression of VpRFP1 was rapidly induced to higher levels in mildew-resistant *V. pseudoreticulata* plants. In contrast, expression of VpRFP1 was down-regulated in mildew-susceptible *V. vinifera* plants. Western blotting using an antibody raised against VpRFP1 showed that VpRFP1 was also induced to higher levels in *V. pseudoreticulata* plants at 12–48 hours post-inoculation (hpi). However, there was only slight increase in VpRFP1 in *V. vinifera* plants in the same time frame, even though a more significant increase was observed at 96–144 hpi in these plants. Results from transactivation assays in yeast showed that the RING finger motif of VpRFP1 exhibited some activity of transcriptional activation; however, no activity was seen with the full-length VpRFP1. Overexpression of VpRFP1 in *Arabidopsis* plants was found to enhance resistance to *Arabidopsis* powdery mildew *Golovinomyces cichoracearum*, which seemed to be correlated with increased transcript levels of *AtPR1* and *AtPR2* in the pathogen-infected tissues. In addition, the *Arabidopsis* transgenic lines showed enhanced resistance to a virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000. Taken together, the results suggested that VpRFP1 may be a transcriptional activator of defence-related genes in grapevines.

Key words: C4C4-type RING finger, Chinese wild *Vitis pseudoreticulata*, disease resistance, powdery mildew, VpRFP1.

Introduction

The capacity of plants to protect themselves against pathogens depends on the detection mechanisms that recognize pathogen-derived molecules to activate host defence responses. Sophisticated plant defence responses, such as hypersensitive

cell death, oxidative burst, and synthesis of pathogen-related (PR) proteins, are activated upon invasion by pathogens or changes in environmental conditions (Wang *et al.*, 2005; Koga *et al.*, 2006; Shabab *et al.*, 2008). Subsequent to these events,

Abbreviations: EST, expressed sequence tag; GST, glutathione-S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; NLS, nuclear localization signal; PR, pathogenesis-related; RING, really interesting new gene; RT-PCR, reverse transcription-PCR; SAR, systemic acquired resistance; SD, synthetic dextrose; YPAD, yeast extracts peptone adenine dextrose.

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plants may develop a long-lasting and broad-spectrum resistance known as systemic acquired resistance (SAR) (Wang *et al.*, 2005; Park *et al.*, 2007). Plant transcription factors (TFs) play crucial roles in the plant defence response to pathogen infection (Jones *et al.*, 2006; Mozoruk *et al.*, 2006). TFs contribute to the regulation of the plant defence response, including the up-regulation of the PR genes via recognition of specific DNA sequences in the promoter region (Rushton and Somssich, 1998). So far, at least six major families of plant TFs, including R2R3 MYB (Stracke *et al.*, 2001), ERF (Andriankaja *et al.*, 2007), TGA bZIP (Jakoby *et al.*, 2002), WRKY (Zhang and Wang, 2005), NPR1 (Zhang *et al.*, 1999), and Whirly (Desveaux *et al.*, 2004, 2005), have been reported to participate in the regulation of plant defence responses.

Zinc finger proteins are the most abundant proteins in plants, which may be essential for fundamental plant growth and development (Takatsuji, 1998). As a member of TFs, zinc finger proteins can be grouped into the subfamilies of TFIIIA, WRKY, Dof, GATA, RING finger, and PHD (Takatsuji, 1998). RING finger proteins are defined by the consensus sequence containing cysteine (Cys) and histidine (His) residues Cys-X₂-Cys-X₉₋₃₉-Cys-X₁₋₃-His-X₂₋₃-Cys/His-X₂-Cys-X₄₋₄₈-Cys-X₂-Cys, where X is any amino acid and the number of X residues varies in different fingers (Borden and Freemont, 1996). Based on the variation in composition of the eight zinc-coordinating Cys (C) and His (H) residues and the distance between the metal ligands, eight types of RING domain have been identified in *Arabidopsis thaliana* (Stone *et al.*, 2005). The eight types include two canonical RING types: RING-HC (C3HC4) and RING-H2 (C3H2C3), and six modified RING domain types, namely RING-v (C4HC3), RING-C2 (C4C4), RING-D (C3HDC3), RING-S/T (C3H2SC2 or CSCHCTC2), RING-G (C3HGC3) (Albert *et al.*, 2002; Hewitt *et al.*, 2002; Dasgupta *et al.*, 2004; Stone *et al.*, 2005), and C5HC2 (Song *et al.*, 2007). The RING finger domain has also been documented with E3 ligase function, which plays a crucial role in ubiquitin-dependent protein degradation (Smalle and Vierstra, 2004).

Genetic and biochemical studies have identified a number of RING finger proteins that play key roles in various biological processes. Initial functional evaluations have indicated that RING finger proteins regulate photomorphogenesis. For example, the regulatory protein COP1 was found to represses photomorphogenesis in darkness (Deng *et al.*, 1991, 1992; Ang and Deng, 1994). Previous studies also showed that RING finger proteins are associated with plant growth and development (Xu and Li, 2003; Disch *et al.*, 2006; Zhang *et al.*, 2008). Moreover, other studies also demonstrated that some RING finger proteins are directly involved in regulation of hormone signalling pathways by targeting of specific proteins for degradation (Zhang *et al.*, 2005, 2007; Bu *et al.*, 2009). In particular, some reports indicated that an abundance of RING finger proteins play key roles in regulating defence responses against abiotic and biotic stresses. HOS1 is a functional RING finger protein that has ubiquitin E3 ligase activity, is a negative regulator of cold signal transduction, physically interacts with *ICE1*, and is a TF activating the expression of *CBF* genes (Lee *et al.*, 2001; Dong *et al.*, 2006).

RING finger proteins also seem to be involved in pathogen response or plant defence. *Arabidopsis* ATL2 is induced rapidly and momentarily by chitin and cellulose treatments (Salinas-Mondragon *et al.*, 1999), and the *eca* mutants with constitutive expression of the ATL2 gene exhibited up-regulated expression of defence-related genes and salicylic acid (SA)- and jasmonic acid (JA)-responsive genes (Serrano and Guzman, 2004). A T-DNA insertion mutant of *Arabidopsis* ATL9 results in increased susceptibility to powdery mildew (Ramonell *et al.*, 2005). *Arabidopsis* RIN2 and RIN3 and tobacco ACRE132 regulate expression of disease resistance genes specifically involved in the hypersensitive response (Kawasaki *et al.*, 2005).

Grapevine (*Vitis vinifera* L.) is economically the most important fruit species worldwide. *Vitis vinifera* is currently the major species cultivated for its high quality in producing wine, juice, table grapes, and dried fruit. However, it is susceptible to many fungal diseases including powdery mildew [*Uncinula necator* (Schw.) Burr. or *Erysiphe cichoracearum*], anthracnose [*Elsinoe ampelina* (de Bary) Shear], and downy mildew [*Plasmopara viticola* (Berk. & Curt.) Berl. & De Toni] (Pavloušek, 2007). Fungal pathogens are a major problem in grapevine globally; they cause huge losses in yield and significant reduction in berry and wine quality. China is one of the origins of *Vitis* species, and some Chinese wild *Vitis* species possess desirable disease resistance to various pathogens, such as extremely high resistance to anthracnose and ripe rot [*Glomerella cingulata* (Ston.) Spauld et Schrenk], high resistance to powdery mildew, and resistance to crown gall (*Agrobacterium vitis*) (Chai *et al.*, 1997; He, 1999). Baihe-35-1 is a unique accession of Chinese wild *Vitis pseudoreticulata* W. T. Wang, which possesses a high resistance to multiple fungi, particularly to *U. necator* (Wang *et al.*, 1995).

To reveal the molecular mechanisms involved in the defence response to pathogen infection in Chinese wild *V. pseudoreticulata*, pathogen-induced genes have previously been isolated from a cDNA library of *V. pseudoreticulata* leaves inoculated with *U. necator* (Xu *et al.*, 2009). Among them, one was predicted to encode a putative RING finger protein, and its full-length cDNA (GenBank accession no. FJ356672) was obtained based on one expressed sequence tag (EST) sequence (GenBank accession no. DQ354158). This gene was designated as *VpRFP1* (*V. pseudoreticulata* RING-finger protein 1). In this study, the *VpRFP1* gene from Chinese wild *V. pseudoreticulata* accession Baihe-35-1 was cloned and its expression patterns were investigated. It was shown that ectopic overexpression of *VpRFP1* in transgenic *Arabidopsis* resulted in enhanced resistance against both the powdery mildew pathogen *Golovinomyces cichoracearum* and the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000.

Materials and methods

Plant materials

Grapevine (Chinese wild *V. pseudoreticulata* accession Baihe-35-1, *V. vinifera* cv. Carignane) leaves were obtained from the Grape

Repository of Northwest A&F University, Yangling, Shaanxi, PR China. *Arabidopsis thaliana* plants (ecotype Columbia, Col-0) were grown in a soil mix of peat moss, perlite and vermiculite (3:1:1, v/v/v) under a 12/12 h day/night cycle at 24 °C with 60% humidity.

Pathogen inoculations

The pathogen *U. necator*, collected from leaves of field-grown *V. vinifera* cv. Cabernet Sauvignon, was maintained in greenhouse-grown *V. vinifera* cv. Carignane plantlets. The pathogens were collected and suspended in sterile water with a concentration of 5×10^5 sporangia ml^{-1} . The spore suspension were sprayed onto the abaxial leaf surface of attached grapevine leaves. The inoculated leaves were enclosed in plastic bags to maintain high humidity. After inoculation for 0, 12, 24, 48, 72, 96, 120, and 144 h, leaves were sampled, immediately frozen in liquid nitrogen, and stored at -80 °C for use.

The disease assay with *G. cichoracearum* isolate UCSC1 was obtained from Dingzhong Tang and conducted as previously described (Wilson *et al.*, 2001). *VpRFP1*-overexpressing *Arabidopsis* were inoculated by spraying leaves with the pathogen conidial suspension (5×10^5 conidia ml^{-1}). The inoculated plants were placed in a plant-growth box (25 °C with 16 h of illumination per day and 100% relative humidity). After inoculation for 0, 24, 48, 72, and 96 h, the leaves were sampled.

Pseudomonas syringae pv. *tomato* DC3000 was provided by Dingzhong Tang, and grown at 28 °C in King's B medium (supplemented with 100 mg l^{-1} rifampicin) overnight, then diluted to $\sim 10^7$ cfu ml^{-1} with 10 mM MgCl_2 solution. Approximately 10 μl of bacterial suspension was infiltrated into the abaxial side of 4–5 leaves per plant using a 1 ml needleless syringe. Quantification of bacterial growth was performed as described (Wang *et al.*, 2007). Trypan blue staining was used to detect cell death as described (Yang *et al.*, 2009).

DNA sequencing and sequence analysis

The open reading frame (ORF) of *VpRFP1* was cloned into the pMD-19 vector (TaKaRa, Dalian, China) and sequenced (SunBio-tech Company, Beijing, China). DNA sequences were analysed using BLASTN and BLASTX in the National Center for Biotechnology Information (NCBI) databases (<http://www.ncbi.nlm.nih.gov>). The protein conserved domain was analysed using Smart (http://smart.emblheidelberg.de/smart/change_mode.pl) and ExPASy (<http://au.expasy.org/tools/>). The deduced amino acid sequence was aligned and the phylogenetic tree was generated by ClustalW (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

Preparation of fusion protein and polyclonal antibodies

The *VpRFP1* gene from Chinese wild *V. pseudoreticulata* was PCR cloned in-frame into the pGEX-4T-1 vector. The construct was verified by DNA sequencing and transformed into *Escherichia coli* BL21. Expression of the fusion protein glutathione *S*-transferase (GST)-*VpRFP1* was induced by isopropyl- β -D-thiogalactopyranoside (IPTG). The purification of the fusion protein was performed by electro dialysis assay, and New Zealand rabbits were immunized with the purified product to obtain polyclonal antibodies. The polyclonal antisera were used for western blot and subcellular immunogold labelling.

Gene expression analysis by real-time quantitative PCR and semi-quantitative PCR

Total RNA was extracted from grapevine leaves as previously described (Asif *et al.*, 2000). First-strand cDNA was synthesized from 2 μg of DNase-treated total RNA using PrimeScript™ RTase (TaKaRa). Semi-quantitative RT-PCR was performed by using a PrimeScript™ RT-PCR Kit (TaKaRa). Control reactions to normalize RT-PCR were done with primers derived from grapevine

GAPDH (glyceraldehyde phosphate dehydrogenase) sequences. PCRs on serial dilutions of cDNA were performed at 57 °C for 29 cycles to define semi-quantitative conditions that resulted in amplification linear to RNA amounts. RT-PCR products were separated on 1.2% agarose gels. The experiments were performed three times with similar results. The special primers used for PCR are listed in Supplementary Table S2 available at JXB online.

For real-time PCR, reactions were carried out on the Bio-Rad IQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). PCRs were carried out in triplicate in a reaction buffer containing $1 \times$ SYBR® Premix Ex Taq™ (TaKaRa), 0.2 μM of forward and reverse primers, and 10 ng of reverse-transcribed RNA in a final volume of 25 μl . Thermal cycling conditions were: 30 s at 95 °C followed by 40 cycles of 15 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C. The specificity of the individual PCR amplifications was checked using a heat dissociation curve from 55 °C to 95 °C following the final cycle of the PCR. The results obtained for each gene of interest at each time point were normalized against the grapevine *GAPDH* gene, which was used as a reference gene. Mean values and SDs were obtained from three duplicate experiments and are representative of three independent experiments. Primers used for real-time quantitative PCR are listed in Supplementary Table S2 at JXB online.

Plant protein extraction and protein gel blot analysis

Total protein was extracted from infected leaves as described (Wang *et al.*, 2003, 2006). The concentration of the protein extract was determined with the Bradford protein assay (Bradford, 1976). SDS-PAGE was carried out according to standard procedures with 25 μg of total proteins. Proteins were electrotransferred onto PVDF membranes (Bio-Rad). The membrane was blocked for 3 h in blocking buffer [phosphate-buffered saline (PBS), 0.1% (w/v) Tween-20, and 5% (w/v) skim milk] before incubating overnight in a 1:1000 dilution of polyclonal antiserum prepared in blocking buffer. Detection of *VpRFP1* was achieved with goat anti-rabbit IgG secondary antibody and the BCIP/NBT chemiluminescent system according to the manufacturer's instructions (Sigma-Aldrich, Beijing, China). The experiment was performed twice with similar results.

Analysis of *VpRFP1* transcriptional activity

The deletion constructs of *VpRFP1* for the transactivation assay were generated by fusing the *VpRFP1* gene downstream of the GAL4 DNA-binding domain (BD). Fifteen *VpRFP1* gene regions were PCR amplified to introduce a *Bgl*II site at the 5' end and a *Pst*I site at the 3' end. The fragments were digested with *Bgl*II and *Pst*I, and cloned into the same sites of pGBKT7. The gene-specific primers are listed in Supplementary Table S1 at JXB online. The pGBKT7 vector which expressed GAL4 BD alone served as the negative control, and the GAL4-*VpERF1* fusion construct (designated as pGBKT7-*VpERF1*) was used as a positive control.

The 14 deletion constructs of *VpRFP1* gene fused to the vector pGBKT7 for the yeast GAL4 BD were generated. The fusion constructs were transformed into the *Saccharomyces cerevisiae* strain AH109 and cultured on YPDA plates at 28 °C for 3 d. The well-isolated colonies were transferred from YPDA plates to SD/-Ade/-His/-Trp/X- α -Gal plates to test the transcriptional activation activity according to their growth and the activity of β -galactosidase by observing the blue colour produced.

Subcellular immunogold labelling

Ultrathin sections were prepared from Lowicryl K₄M-embedded specimens according to the method previously described (Peng *et al.*, 2003). The ultrathin sections were first blocked for 30 min at room temperature by floating the grids on droplets of PBS containing 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 3 mM KCl, and 500 mM NaCl (pH 7.4) supplemented with 50 mM glycine and

continuously blocked with PBS supplemented with 0.1% (w/v) gelatin, 0.5% (w/v) bovine serum albumin (BSA), and 0.1% (v/v) Tween-20 (pH 7.4, PBGT). Without rinsing, the sections were incubated with New Zealand rabbit antiserum directed against VpRFP1 (all diluted 1:100 in PBGT buffer) overnight at 4 °C. Following extensive washes with PBGT buffer, the sections were incubated with secondary antibody (goat anti-rabbit IgG antibody conjugated with 10 nm gold) at a 1:100 dilution in PBGT buffer for 2 h at room temperature. The sections were rinsed consecutively with PBGT and double-distilled water, followed by staining with 2% uranyl acetate in 50% ethanol for 25 min at 25 °C and with alkaline lead citrate for 15 min. After washing extensively with double-distilled water, the ultrathin sections were examined with an electron microscope.

The specificity and reliability of the immunogold labelling were tested by two negative controls. In the first one, the antiserum was omitted to test possible unspecific labelling of the goat anti-rabbit IgG antibody-gold conjugate. In the second, rabbit pre-immune serum was used instead of the rabbit antiserum before immunogold labelling to test the specificity of the antiserum. At least three repetitions for each control were conducted for each sample.

Plant expression vector construction and Arabidopsis transformation

To generate the *VpRFP1*-overexpressing gene construct, the *VpRFP1* coding region including the termination codon was amplified using the following primers: *VpRFP1* forward primer: 5'-GGGGTTCGACATGATTACCGATTTCGATCAC-3' (*Sall* site underlined), and *VpRFP1* reverse primer: 5'-GGGGGTACCC-TAAGACCTTGCAATCATGC-3' (*KpnI* site underlined). The PCR products were inserted in the pMD-19-T vector (TaKaRa) and identified by DNA sequencing. The digested *VpRFP1* fragments were subcloned in the same sites of the plant expression vector pWR11 (Lei *et al.*, 2009) in which the *VpRFP1* was driven by the enhanced *Cauliflower mosaic virus* (CaMV) 35S promoter and possesses hygromycin resistance in plants. The recombinant construct harbouring *VpRFP1* was introduced into *Agrobacterium tumefaciens* strain EHA105 via electroporation. *Arabidopsis* transformation of the *VpRFP1* gene was carried out according to the floral dipping method (Clough and Bent, 1998). For selection of *Arabidopsis* transgenic lines, seeds were surface-sterilized with 70% ethanol for 2 min, then 2% sodium hypochlorite (NaOCl) solution for 6 min and rinsed five times in sterile water. Seeds were plated on basal MS salt medium containing 1% sucrose and 20 mg l⁻¹ hygromycin. Plates were kept at 4 °C for 2–4 d in darkness to synchronize germination, then transferred to growth chambers, and maintained under the environmental conditions described above for plant growth in pots. Fourteen-day-old seedlings were transferred to the soil mix. Wild-type plants and transgenic *Arabidopsis* T₃ lines were used for further analysis.

Results

Isolation and sequence analysis of a novel RING finger protein (VpRFP1) gene from Chinese wild V. pseudoreticulata

Based on the EST sequence (DQ354158) obtained previously (Xu *et al.*, 2009), the full-length cDNA sequence (FJ356672) has now been obtained. The *VpRFP1* cDNA of 1415 nt contains a 170 nt untranslated region (UTR) at the 5' end and a 192 nt 3'-UTR. The genomic sequence of the *VpRFP1* gene was also cloned directly from the *V. pseudoreticulata* genomic DNA (GeneBank accession no. GU446678), *VpRFP1* is located on chromosome 17 of the published Pinot Noir whole genome (Jaillon *et al.*, 2007). Alignment of the

genomic DNA sequence with the *VpRFP1* cDNA indicated that the *VpRFP1* gene contained two introns and three exons. The predicted ORF encodes a RING finger protein of 350 amino acids, which has a theoretical pI value of 5.83 and a deduced molecular mass of 38 005 Da. The deduced amino acid sequence of *VpRFP1* contained a nuclear localization signal (NLS) at its N-terminus and the RING finger motif at its C-terminus. Compared with other species, the RING finger motifs belong to a novel RING finger variant of the C4C4 type, with the consensus sequence Cys-X₂-Cys-X₁₃-Cys-X₁-Cys-X₄-Cys-X₂-Cys-X₁₀-Cys-X₂-Cys (X indicates any amino acid, and subscript number indicate the number of amino acids) (Fig. 1A). *AtPEX10* is a typical C3HC4-type RING finger (Schumann *et al.*, 2007) which possesses the conserved sequence C-X₂-C-X₉₋₃₉-C-X₁₋₃-H-X₂₋₃-C-X₂-C-X₄₋₄₈-C-X₂-C (Stone *et al.*, 2005) (Fig. 1A). Comparative analysis of these two motifs revealed that both are remarkably similar, but with the histidine of the C3HC4 type replaced by a cysteine residue.

Comparisons of the amino acid sequences among *VpRFP1* and other putative proteins are presented in Fig. 1B. The *VpRFP1* protein shared 50% identical amino acids with a *Solanum lycopersicum* (formerly *Lycopersicon esculentum*) hypothetical protein (accession no. ABI34275), 46% homology with an *A. thaliana* RING finger family protein (accession no. AAY57618), and 38% homology with an *Oryza sativa* hypothetical protein (accession no. EAZ23574). Phylogenetic tree analysis showed that they were classified into three subfamilies. *Vitis pseudoreticulata* (accession no. FJ356672), *V. vinifera* (accession no. XP_002281744), and *S. lycopersium* (accession no. ABI34275) were part of the same subfamily (Fig. 1C).

Expression patterns of VpRFP1 in grapevine resistance responses

To investigate whether the expression pattern of *RFP1* was similar among different *Vitis* species, the expression levels of *RFP1* in a resistant grapevine (accession Baihe-35-1 of *V. pseudoreticulata*) and a susceptible variety (*V. vinifera* cv. Carignane) inoculated with *U. necator* were determined using real-time PCR and western blot. The results showed that the expression of *RFP1* was induced by *U. necator* at the transcriptional level. For resistant grapevine accession Baihe-35-1 of *V. pseudoreticulata*, the up-regulated expression of *VpRFP1* was detected at 12 hours post-inoculation (hpi) (Fig. 2A), peaked at 24 hpi, and returned to low baseline levels at 48 hpi. In contrast, the susceptible *V. vinifera* cv. Carignane displayed a high transcription level at 0 h, but this decreased immediately after inoculation, and reached the lowest levels at 24 hpi and 48 hpi, respectively.

To further determine whether *RFP1* was induced by the pathogen at the translational level, western blot assay was carried out using a specific antiserum. The result showed that the expression of *RFP1* at the translational level was induced by *U. necator* both in the resistant accession of *V. pseudoreticulata* and in the susceptible *V. vinifera* cv. Carignane (Fig. 2B). The expression of *RFP1* in *V. vinifera*

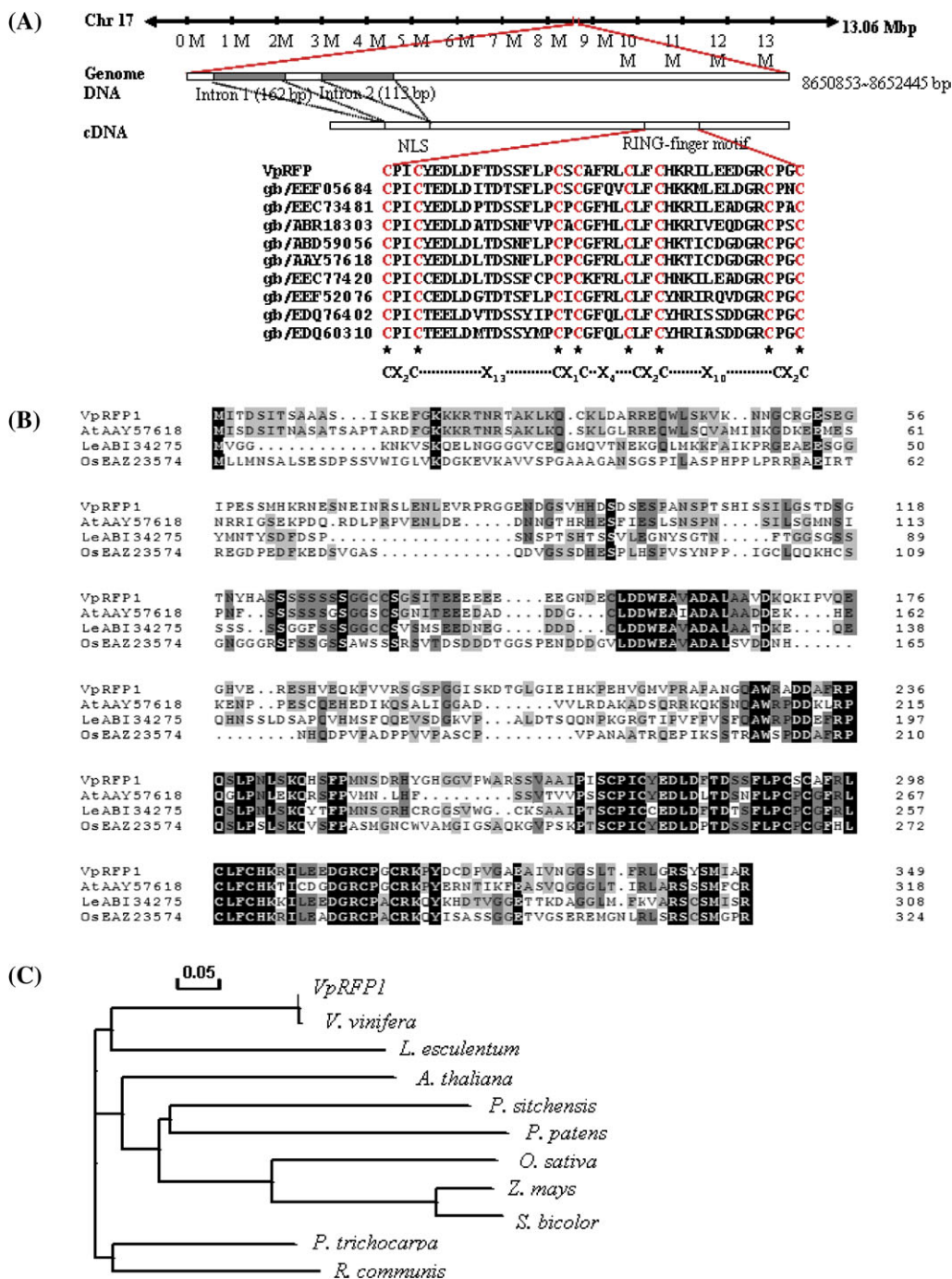


Fig. 1. Sequence analysis of the *VpRFP1*. (A) Schematic representation of the primary structure of the putative *VpRFP1* gene and major motif. (B) Amino acid sequence alignment of VpRFP1 and closely related proteins from *Arabidopsis thaliana* (GenBank accession no. AAY57618), *Solanum lycopersicum* (formerly *Lycopersicon esculentum*; GenBank accession no. ABI34275), and *Oryza sativa* (GenBank accession no. EAZ23574). Identical amino acids are shaded in black, and conserved amino acid changes are shaded in grey. (C) Phylogenetic analysis of VpRFP1 and closely related proteins. The tree was generated based on the deduced amino acids from 10 plant species using the ClustalW method of the MegAlign program: *Arabidopsis thaliana* (GenBank accession no. AAY57618), *Lycopersicon esculentum* (GenBank accession no. ABI34275), *Picea sitchensis* (GenBank accession no. ABR18303), *Oryza sativa* (GenBank accession no. EAZ23574), *Zea mays* (GenBank accession no. NP_001143878), *Populus trichocarpa* (GenBank accession no. XP_002321557), *Sorghum bicolor* (GenBank accession no. XP_002447976), *Ricinus communis* (GenBank accession no. XP_002511474), *Physcomitrella patens* (GenBank accession no. XP_001758896), and *Vitis vinifera* (GenBank accession no. XP_002281744).

cv. Carignane presented a gradually increasing trend after inoculation, and reached maximum expression at 144 hpi. In contrast, the expression of VpRFP1 in the resistant accession

Baihe-35-1 of *V. pseudoreticulata* was transiently changeable, and high peaks occurred at 48 hpi and 96 hpi, and low peaks at 72 hpi and 120 hpi, respectively.

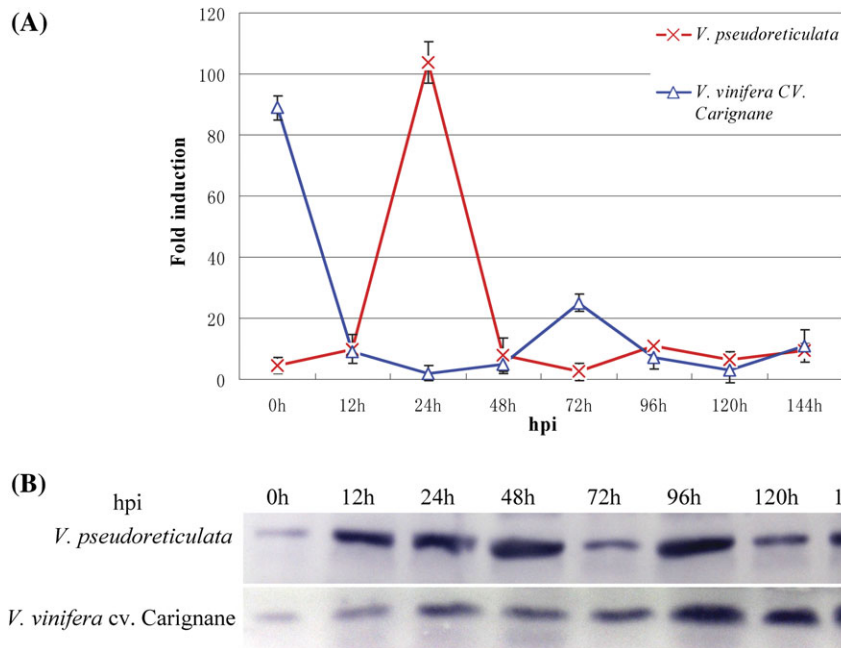


Fig. 2. Expression of *RFP1* in grapevine leaves that were inoculated with the pathogen *U. necator*. Leaves of plantlets of *V. pseudoreticulata* accession Baihe-35-1 and *V. vinifera* cv. Carignane were inoculated with *U. necator* (5×10^5 spores ml^{-1}). Control leaves were sprayed with sterile water. Leaves were collected at different time points as indicated. Hpi, hours post-inoculation with fungal spores. (A) Analysis of *VpRFP1* expression at the transcript level by real-time PCR. *VpGAPDH* was used as the internal control. Data are the mean \pm SE ($n=3$) of a representative experiment. (B) Analysis of *VpRFP1* expression at the translational level by western blot. A 28 μg aliquot of total protein was used for each line, separated by SDS-PAGE, transferred onto a PVDF membrane, and immunodetected with a *VpRFP1*-specific antiserum.

RFP1 is induced by *U. necator* in both *V. pseudoreticulata* and *V. vinifera* cv. Carignane; the expression pattern of *RFP1*, however, was different between the two grapevine genotypes. This prompted the further elucidation of the function of *VpRFP1* in response to pathogen attack.

Immunogold labelling localization of *VpRFP1*

VpRFP1 fused with a GST tag was induced in *E. coli* BL21 (Supplementary Fig. S1A at JXB online), and then purified by an electro dialysis procedure (Supplementary Fig. S1B). The purified protein was used to immunize New Zealand rabbits to obtain polyclonal antiserum. Immunogold labelling with *VpRFP1* antiserum showed that gold particles were mainly localized in the nucleus (Fig. 3). No substantial signal was detected in controls (Fig. 3A, B), confirming the specificity of the immunolocalization. Non-infected leaves treated with the specific antiserum against *VpRFP1* showed few gold particles in the nucleus and fewer in the cytoplasm (Fig. 3C). At 24 hpi, pathogen-infected samples showed a high density of gold particles in the nucleus (Fig. 3D), while similar signals were found in the cytoplasm of non-infected samples.

Transcriptional activation activity of *VpRFP1* in yeast

To examine whether *VpRFP1* protein could function as a transcriptional activator in yeast, a series of deletion constructs of the *VpRFP1* gene were fused to the GAL4 BD

domain. The constructs were then transformed into yeast (strain AH109), and screened on SD medium. The *VpERF1* cloned from Chinese wild grapevine has been previously confirmed to be active in yeast (unpublished data). Therefore, *VpERF1::GAL4* was used as the positive control, and the empty vector pGBKT7 as the negative control.

AH109 competent cells harbouring the recombinant plasmid or the empty vector pGBKT7 were able to grow on SD/-Trp medium, indicating that all recombinant plasmids or the empty vector pGBKT7 were transferred into AH109 (Fig. 4BII). All yeast cells carrying constructs pGBKT7-A, pGBKT7-B, pGBKT7-C, pGBKT7-E, pGBKT7-F, pGBKT7-G, pGBKT7-H, pGBKT7-I, pGBKT7-K, pGBKT7-L, pGBKT7-M, pGBKT7-N, and pGBKT7-O could not grow on the SD/-Trp/-Ade/-His medium, or yield the colour reaction on the SD/-Trp/-Ade/-His/+X- α -gal medium. Cells expressing the constructs pGBKT7-D and pGBKT7-J, and the positive control caused yeast growth on the SD/-Trp/-Ade/-His medium, and induced the blue colour on the SD/-Trp/-Ade/-His/+X- α -gal medium (Fig. 4BIII, IV), indicating that portions D and J of *VpRFP1* have potential transcriptional activity in yeast. The effector constructs pGBKT7-D and pGBKT7-J (Fig. 4A) shared the C-terminal region with the RING finger motif, and displayed obvious transcriptional activities. This result suggested that the C-terminal region with the RING finger motif plays a significant role in the transcriptional activity. Interestingly, the full-length *VpRFP1* has no transcriptional activity in yeast.

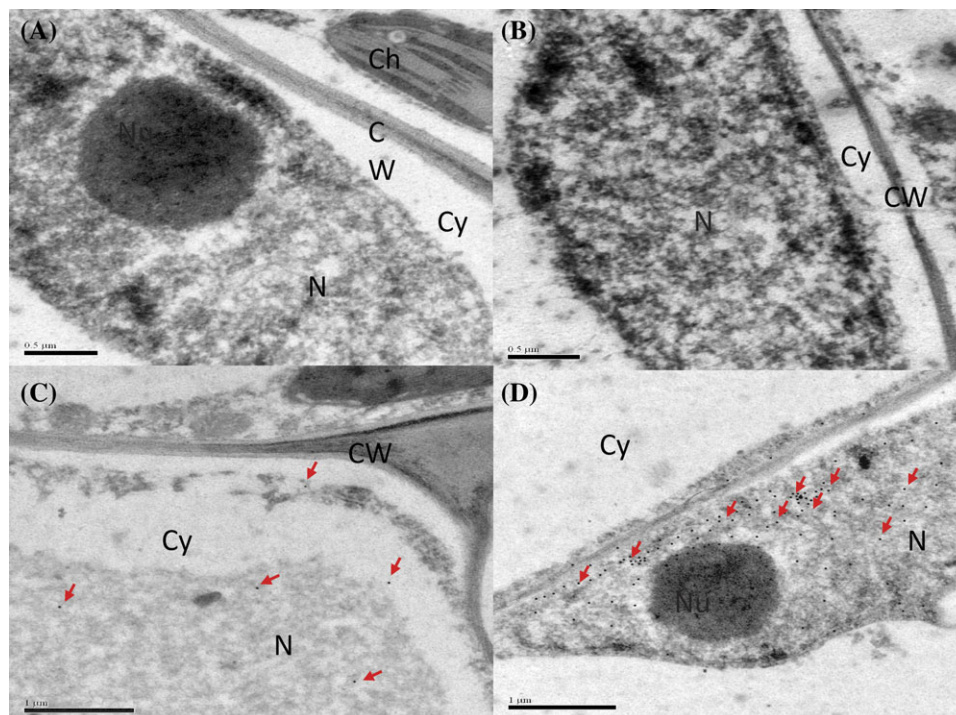


Fig. 3. Immunogold localization of VpRFP1 in *Vitis pseudoreticulata* leaves. (A) With use of the rabbit pre-immune serum instead of the specific rabbit antiserum as control, no substantial signal was detected. (B) The absence of the antiserum to test the non-specific labelling of goat anti-rabbit IgG antibody–gold conjugate; no substantial signal was detected. (C) Before inoculation with pathogen, a few gold particles were detected in the nucleus and cytoplasm. (D) At 24 h after inoculation with pathogen, more gold particles were detected in the nucleus. N, nucleus; Nu, nucleolus; Cy, cytoplasm; CW, cell wall.

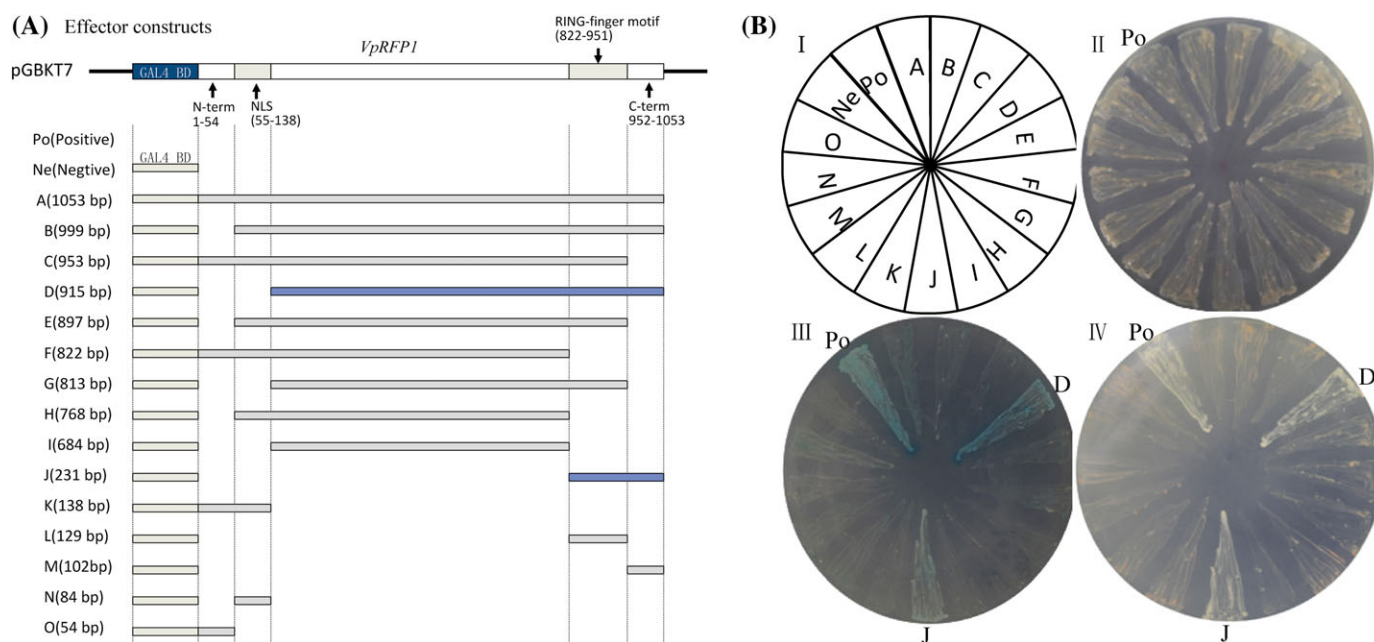


Fig. 4. Functional analysis of the potential transcriptional activation of the *VpRFP1* gene in yeast. (A) Diagram of the effector constructs. (B) Transcriptional activation assay of the different fragments of the *VpRFP1* gene in yeast. The yeast strain AH109 harbouring plasmids that encoded GAL4-VpRFP1 and different fragments were grown on: II, SD/-Trp medium; III, SD/-Trp/-Ade/-His medium; IV, SD/-Trp/-Ade/-His/+X-α-gal medium. Plates were incubated at 28 °C for 3 d. For the positive control, yeast cells harbouring pGBK-T7-VpERF1 was used as a positive control. The empty vector pGBKT7 expressing GAL4 BD alone served as a negative control.

Overexpression of *VpRFP1* in *Arabidopsis* enhanced resistance to pathogens

To further study the biological role of *VpRFP1* in defence responses, functional analyses of *VpRFP1* were performed in transgenic *Arabidopsis* plants transformed with the overexpression construct pWR11/*VpRFP1* (Fig. 5A). Upon inoculation, *VpRFP1* transgenic plants were more resistant against the pathogen than the wild type (Fig. 5B, C). At 8 days post-inoculation (dpi) with *G. cichoracearum*, wild-type leaves showed some disease symptoms, while transgenic plants remained disease free with no visible fungal colonies. The microscopic images showed that the transgenic *Arabidopsis* plant inhibited the spread of *G. cichoracearum* (Fig. 5D). At 12 dpi, the disease symptoms in wild-type leaves became more severe than in the transgenic plants, and only a few symptoms occurred in transgenic plant leaves (Fig. 5C). As is shown (Fig. 5E), *VpRFP1* was constitutively expressed in the T₃ generation, but was also affected by *G. cichoracearum* inoculation. Interestingly, *PR1* and *PR2* displayed the same expression

pattern as *VpRFP1*, whose expression increased gradually, and began to decrease after reaching a peak at 72 hpi. In contrast, the transcripts of *PR3* and *PDF1.2* were suppressed at 24 hpi, and reached maximum expression at 96 hpi.

Experiments were also carried out to examine whether or not the transgenic plants were resistant to *P. syringae* pv. *tomato* DC3000 infection. The result indicated that none of the *VpRFP1* transgenic plants exhibited spreading maceration in the infected leaves, and displayed clearly enhanced resistance (Fig. 6A). The growth of *P. syringae* pv. *tomato* DC3000 in inoculated plants was measured and it was found that the bacterial titres in transgenic lines were significantly lower than in the wild-type plants at 3 dpi (Fig. 6B). Macroscopic and microscopic analyses were also performed to detect the cell death. The results indicated that at the macroscopic level, cell death in infected mature leaves significantly increased in the transgenic lines compared with the wild-type plants (Fig. 6C), and at the microscopic level, the extent of visible lesions also increased significantly in the transgenic lines (Fig. 6D).

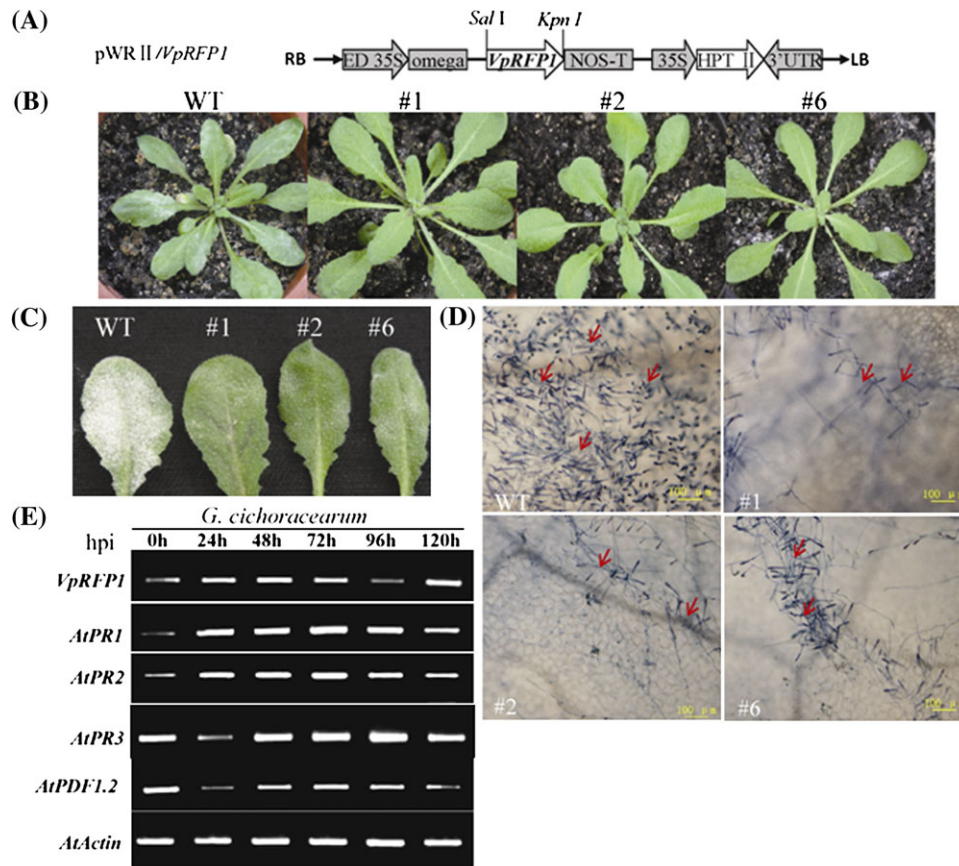


Fig. 5. Enhanced disease resistance and elevated expression levels of defence-related genes in transgenic *Arabidopsis* plants. (A) The schematic representation of the pWR11/*VpRFP1* construct used for *Arabidopsis* transformation. RB, right border; LB, left border; ED 35S, CaMV 35S promoter; omega, the 5'-leader sequence of tobacco mosaic virus (TMV); NOS-T, nopaline synthase terminator; 35S, CaMV 35S promoter; HPTII, hygromycin phosphotransferase; 3'UTR, 3'-untranslated region. (B) Disease symptoms developed on the leaves of wild-type and transgenic lines 8 d after inoculation with fungal spores. (C) Disease symptoms developed on the leaves of wild-type and transgenic lines 12 d after inoculation. (D) Microscopic visualization of fungal growth on leaves of 3-week-old plants at 8 dpi with *G. cichoracearum*. Leaves were stained with trypan blue. Scale bar=100 μ m. Three independent experiments showed similar results. (E) RT-PCR analysis of defence-related genes in transgenic T₃ lines, and transgenic plant #1 is indicated. Amplification of actin is shown for the equal loading of RNA. Hpi, hours post-inoculation.

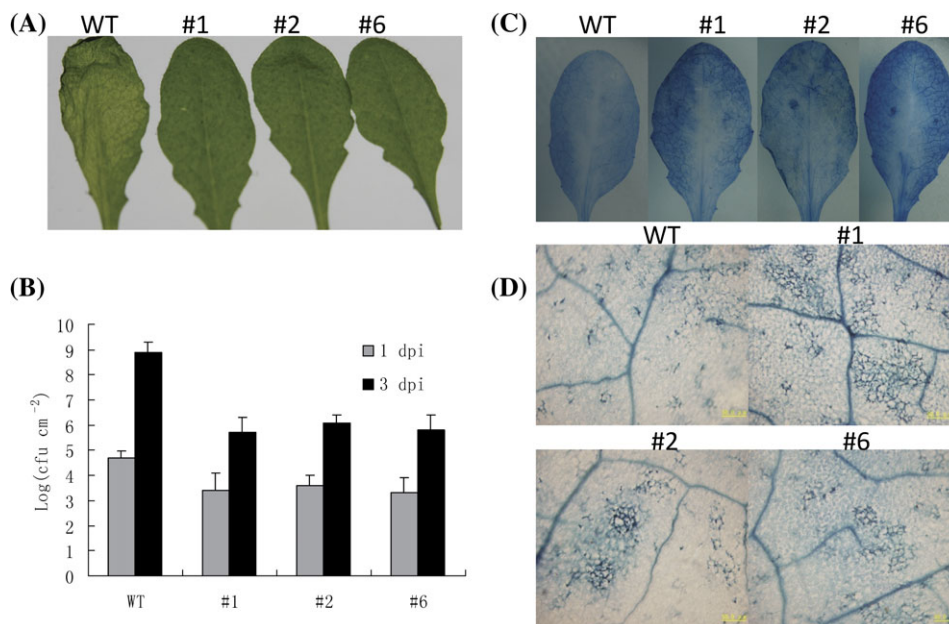


Fig. 6. Reactions of the *VpRFP1* transgenic *Arabidopsis* plants to *P. syringae* pv. *tomato* DC3000 infection. (A) Disease symptoms developed on the leaves of wild-type and transgenic lines 2 days post-inoculation with *P. syringae* pv. *tomato* DC3000. (B) The numbers of bacterial cells in the leaf tissues were measured at different time points after infiltration with bacterial suspension. Data are the means \pm SD from three independent experiments. (C) The transgenic *Arabidopsis* and the wild-type leaves stained with trypan blue 2 d post-inoculation with *P. syringae* pv. *tomato* DC3000; (D) Trypan blue-stained leaves were viewed under a light microscope for plant cell death 2 d after inoculation with *P. syringae* pv. *tomato* DC3000.

Discussion

Based on previous findings from studies of other plant C4C4-type RING finger (RING finger-C2) genes (Albert *et al.*, 2002; Stone *et al.*, 2005), it was hypothesized that *VpRFP1* isolated from the resistant accession Baihe-35-1 of Chinese wild *V. pseudoreticulata* may be a novel C4C4-type RING finger protein involved in grapevine–powdery mildew interaction. The gene was found to show strong evidence for transcriptional change in the susceptible *V. vinifera* and a significantly different response in the disease-resistant *V. pseudoreticulata* (Fig. 2). This rapid and strong transcriptome response to *U. necator* infection is consistent with recent findings of the C3HC4-type RING finger protein in pepper–pathogen interaction (Hong *et al.*, 2007). It is possible that this induced expression pattern of defence-related transcripts plays a critical role in the regulation of early defence signalling pathways in the leaves of disease-resistant *V. pseudoreticulata*. Further investigations will be required to provide evidence for the relationship between the expression pattern of this gene and *U. necator* resistance.

The translocation of TFs from the cytoplasm into the nucleus is considered to be an important step in post-translational control (Laskey and Dingwall, 1993), and requires an NLS for selective transportation into the nucleus (Raikhel, 1992). The present data suggest that the *VpRFP1* amino acid sequence from residues 18 to 46 contained a putative NLS, thus suggesting that *VpRFP1* can function as a TF and may be targeted to the nucleus. Evidence from immunolocalization analyses indicated that *VpRFP1* was not only located in the nucleus (Fig. 3), but

was also induced by the pathogen. It is interesting to note that the full-length *VpRFP1* and most of the deletion mutants showed no transcriptional activation activity, whereas the RING finger motif connected to the C-terminal region of *VpRFP1* (pGBKT7-D and pGBKT7-J) presented transactivation activity in yeast (Fig. 4). A possible explanation is that the transcriptional activation activity of *VpRFP1* may require an environmental stimulus to become activated, such as pathogen induction. This induced activation seems to be coordinated with the phosphorylation state of the TF gene involved in response to extracellular signals via the modulation of the activities of protein kinases and protein phosphatases (Whitmarsh and Davis, 2000). It has also been documented that the activation function of TFs *in planta* was considerably different from that in yeast systems (Sprenger-Haussels and Weisshaar, 2000; Heinekamp *et al.*, 2002). Therefore, another possible explanation is that the novel *VpRFP1* could function as a transcriptional repressor in yeast, but as a transcriptional activator in grapevine.

Functional analyses revealed that the expression of the novel *VpRFP1* was regulated by *U. necator* at both the transcriptional and translational levels, and also enhanced the resistance to *G. cichoracearum* and *P. syringae* pv. *tomato* DC3000 in transgenic *Arabidopsis* plants. Further functional characterization of *VpRFP1* in interacting with other proteins, such as ubiquitin ligases activity *in vitro* and the degradation substrate, is required to provide more comprehensive information about the disease-resistant specificities of the novel C4C4-type RING finger protein *VpRFP1*, and will also be helpful in understanding the

molecular mechanism of disease resistance in Chinese wild *V. pseudoreticulata* species.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. *VpRFP1* expression in *E. coli* and purification of GST–VpRFP1 fusion protein.

Table S1. Primers used for amplifying the target gene.

Table S2. Primers used for RT-PCR and real-time PCR analysis of target gene expression.

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