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RESEARCH ARTICLE

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CAP superfamily proteins (VdPRYs) manipulate plant immunity and contribute to the virulence of *Verticillium dahliae*

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ABSTRACT

CAP (cysteine-rich secretory proteins, antigen5, pathogenesis-related proteins) superfamily proteins are widely distributed, can be subdivided into 11 subfamilies, and form a unique branch in fungi, named PRY proteins. *Verticillium dahliae* is a soil-borne fungal pathogen of vascular plants that causes plant Verticillium wilt. However, the roles of CAP superfamily proteins in this fungus is unclear. Here, four CAP superfamily members with a conserved domain were identified in *V. dahliae*: VdPRY1, VdPRY2, VdPRY3, and VdPRY4. VdPRY1 and VdPRY3 were found to be key in suppressing plant immune responses. Moreover, these four members are highly expressed during early infection of cotton by *V. dahliae*. Deleting *VdPRY1*, *VdPRY2*, or *VdPRY3* reduced the fungus's ability to cause disease, but *VdPRY4* deletion did not affect virulence. Deletion of any of four members did not impact fungal growth or carbon source use. Yeast two-hybrid experiments suggest that these proteins may function through interactions with each other. This investigation has, for the initial time, elucidated the pivotal roles of *V. dahliae* CAP superfamily proteins in inhibiting plant immunity and exerting virulence during interaction with the host plant.

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1. Introduction

CAP [cysteine-rich secretory protein (CRISP), antigen 5 (Ag5), and pathogenesis-related protein 1 (PR-1)] superfamily proteins are commonly referred to as the sperm envelope glycoprotein (SCP) family, a designation derived from the conserved CAP structural domains that are characteristic of CRISP, Ag5, and PR-1, as well as all other members of the CAP superfamily (Gibbs et al. 2008; Darwiche et al. 2017). Plant PR-1, which accumulates after pathogenic infection and may act as an antifungal agent, has been shown to exert anti-microbial functions (Luo et al. 2022; Wangorsch et al. 2022). Roles of mammalian CRISPs in the response to pathogens, fertilisation, and sperm maturation have been proposed (Koppers et al. 2011; Gonzalez et al. 2021). Ag5 protein is one of the most abundant immunogenic proteins in the venom secretory ducts of stinging insects (Gibbs et al. 2008). Among them, the CAP domain, which is the major taxonomic feature, contains four highly conserved feature motifs; the domain contains α-helix and β foldings, which spatially form an "α-β-α" sandwich structure, and the diversity outside the core region greatly changes the specificity of these proteins (Asojo et al. 2011; Darwiche et al. 2016).

Evolutionary and systematic analyses have shown that the CAP superfamily can be divided into 11 subfamilies, among which the fungal pathogenesis-related (PRY) proteins constitute a distinct phylogenetic branch (Abraham and Chandler 2017; Gaikwad et al. 2020). The CAP superfamily proteins present in *Saccharomyces cerevisiae* contain three members: Pry1 and Pry2, which are secreted glycoproteins, and Pry3, which is a glycosylphosphatidylinositol (GPI)-anchored protein tethered to cell wall (Choudhary and Schneiter 2012). Functional analysis showed that *in-vitro* recombinant Pry1 exists as dimers in solution and binds free and acetylated sterols, and this

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function can be achieved via the CAP structural domain alone (Choudhary et al. 2014; Darwiche et al. 2016, 2018). In addition, CAP superfamily proteins play important roles in regulating fungal pathogenicity. *Fpr 1*, the CAP superfamily gene of *Fusarium oxysporum*, is required for *F. oxysporum* to exert virulence on immunodepressed mice (Prados-Rosales et al. 2012). The deletion mutant of the RBT4 and its homologous protein Rbe1 in human pathogen *Candida albicans* significantly reduced the pathogenicity in animals (Röhm et al. 2013). *Fusarium graminearum* contains four PR-1-like (PR-1 L) members, and the deletion of *FgPR-1 L-4* led to diminished virulence in wheat (Lu and Edwards 2016, 2018). The *Cytospora chrysosperma* CAP superfamily protein CcCAP1, as a virulence-related effector, regulates plant immunity (Han et al. 2021). Three CAP superfamily proteins were identified in *Valsa mali*, among which *VmPR1a* and *VmPR1c* act as important virulence factors affecting fungal pathogenicity (Wang et al. 2021).

Verticillium dahliae (Kleb.) is a native soil-borne filamentous fungus that is one of the few fungi that cause plant diseases by infesting the vascular system (Klosterman et al. 2009). The hosts of *V. dahliae* encompass over 200 species of dicotyledonous plants (Bhat and Subbarao 1999). After accepting signals from host root secretions, *V. dahliae* starts to germinate, produces mycelia, enters the xylem, and degrades the vessel wall, ultimately leading to wilt symptoms in the infected plant (Dhar et al. 2020; Zhang et al. 2022). Identifying and analysing the genes related to virulence or immunity assist in uncovering the molecular pathogenesis of *V. dahliae*. It has been shown that the secreted proteins of *V. dahliae* are the main substances responsible for its biotoxicity, and the *V. dahliae* secretome contains numerous potential carbohydrate-active enzymes, proteins facilitating pathogen-host interaction, and small cysteine-rich secreted proteins (SCRPs) (Chen et al. 2018). Some SCRPs have been documented to play pivotal roles in regulating host immunity and virulence. For example, VdSCP27, VdSCP113, and VdSCP126 can induce *Nicotiana benthamiana* cell death, which relied on both BAK1 and SOBIR1, and knock out of VdSCP27 and VdSCP126 simultaneously resulted in reduced pathogenicity on *N. benthamiana* and *Gossypium hirsutum* (Wang et al. 2020). VdSCP41 is excreted and transported into the host nucleus, where it inhibits the activation of plant immune-related genes by interfering with the activity of the host transcription factors CBP60g and SARD1; VdSCP41 is also essential for the pathogenicity of *V. dahliae* (Qin et al. 2018). Two CFEM domain-containing proteins, VdSCP76 and VdSCP77, are localised on the plant plasma membrane and can mediate the broad-spectrum immune suppression induced by various effectors and deletion of either VdSCP76 or VdSCP77 significantly reduced the pathogenicity of *V. dahliae* on cotton (Wang et al. 2022).

Fungal PRYs could play a role in virulence, but their functions have not been confirmed in *V. dahliae*. Thus, the main goals of the current research were to: (1) identify CAP superfamily members in *V. dahliae*; (2) elucidate the regulatory impact of VdPRYs on plant immune responses; (3) ascertain whether VdPRYs contribute to the pathogen's virulence during infection of host plants; and (4) preliminary investigate potential interrelationships among the CAP members. Ultimately, this study seeks to illuminate the functions of CAP proteins during the interaction of *V. dahliae* and host plants.

2. Materials and methods

2.1. Bioinformatics analysis

Candidate CAP family members were predicted using the HMMER package v3.4 (http://www.hmmer.org), where the HMM profile for the CAP domain (Pfam ID: PF00188) was used to query the *V. dahliae* Vd991 genome under default parameters. Returned hits with *E*-values <1e−10 and Scores >30 were then manually selected. The domain architectures of the CAP superfamily were annotated using the InterPro (http://www.ebi.ac.uk/interpro/) (Apweiler et al. 2000) and Pfam database (http://pfam.xfam.org/) (Mistry et al. 2014). They were displayed using Illustrator for Biological Sequences. The N-terminal signal peptide and transmembrane domains were predicted using SignalP5.0 (https://services.healthtech.dtu.dk/services/SignalP-5.0/) (Almagro Armenteros et al. 2019) and TMHMM 2.0 (https://services.healthtech.dtu.dk/services/TMHMM-2.0/) (Krogh et al. 2001). The gene structure and multiple sequence alignments were determined using ESPript3.0 (https://espript.ibcp.fr/ESPript/) (Gouet et al. 1999). The homologs of the CAP superfamily in different species were searched using BLASTp based on the National Center for

Biological Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). The phylogenetic tree was constructed in the MEGAX program using the maximum-likelihood method with 1,000 bootstrap replicates.

2.2. *Transient expression in* N. benthamiana

The full-length sequences of VdPRYs with FLAG tags connected to the C-terminus were cloned individually into the pGR107 (potato virus X vector) by DNA recombination, and subsequently transformed into the *Agrobacterium tumefaciens* GV3101 strain. The primers used for vector construction are listed in Table S3. To investigate the function of cell death induction, *A. tumefaciens* cells carrying PGR107:VdPRYs were infiltrated into *N. benthamiana* leaves. *Agrobacterium tumefaciens* cells carrying cell death inducing genes were co-infiltrated with the cells carrying PGR107:VdPRYs to investigate the suppression of cell death induction (Wang et al. 2020). Symptom development was monitored from 3 to 7 d after infiltration. Agro-infiltration assays were performed on *N. benthamiana* plants using GFP and Bcl-2-associated X protein (BAX) as negative and positive controls, respectively. Each assay was repeated at least three times.

2.3. Botrytis cinerea *infection by protein infiltration*

Whole leaves of four-week-old *N. benthamiana* were infiltrated with VdPRY proteins via agro-infiltration. The centre of the infiltration area of the leaves was inoculated with bulk *B. cinerea* after 24 h of agro-infiltration. The treated plants were positioned within an incubator set at a temperature of 25 °C and a relative humidity of 80%. The lesion diameters were recorded 48 h following inoculation. In RT-qPCR analyses of *B. cinerea* biomass, the fungus's *actin* gene was utilised as a marker for quantification, and the *N. benthamiana EF-1α* gene served as an endogenous reference standard. Infection of the plants was performed by inoculating three leaves per plant, which was repeated three times. Primers used for expression analysis are listed in Table S3.

2.4. DAB staining

The elicitor activity of VdEG1 was detected using co-infiltration with VdPRY1, VdPRY2, VdPRY3, and VdPRY4 in *N. benthamiana* leaves, respectively. GFP and VdEG1 were used as negative and positive controls, respectively. The leaf phenotypes were collected 60 h after infiltration. The leaf tissues were stained using 3'3-diaminobenzidine (DAB) solution (Bindschedler et al. 2006) and incubated for 8 h at 25 °C. Subsequently, the leaves were cleared by boiling in 95%-(vol/vol) ethanol until discoloration, then the cleared samples were mounted in 50% glycerol for imaging using stereomicroscope.

2.5. Gene expression and fungal biomass analysis

Samples were taken at 48 h post-transient expression of GFP and VdEG1, as well as VdEG1 with VdPRY1, VdPRY2, VdPRY3, or VdPRY4 in *N. benthamiana*, using *EF-1α* as the internal control. To analyse the expression of *VdPRYs* during infection, cotton seedlings were root-dip-inoculated with *V. dahliae*, and the roots were taken at various days post-inoculation (dpi). The *V. dahliae* elongation factor 1α *VdEF-1α* served as a reference gene. Total RNA was extracted using an EASYspin Kit (Aidlab Biotechnologies, Beijing, China), and cDNA was synthesised using *TransScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). The cotton *Gh18S* was used as an internal reference gene for RT-qPCR, and *VdEF-1α* was used as a target to quantify DNA of *V. dahliae* for the fungal biomass analysis. The RT-qPCR process starts with a pre-denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s. The different expression of genes across various samples was assessed by employing the $2^{-\Delta\Delta CT}$ method (Rao et al. 2013). Each experiment was conducted in triplicate, with each including three technical repeats. To enhance clarity of the results, some gene expression profiles were elegantly transformed into heat maps using HemI (Heatmap Illustrator, version 1.0). Statistical significance was measured using an unpaired Student *t*-test. Primer sequences are detailed in Table S3.

2.6. Gene deletion and complement

To construct *VdPRYs* knockout mutants, approximately 1-kb upstream and downstream fragments of the respective *VdPRYs* were amplified from Vd991 genomic DNA and then ligated into the vector pGKO2-*Hyg* (Wang et al. 2016). To obtain the complemented strain, the native promoter, gene sequence,

and terminator of the target gene were amplified from Vd991 genomic DNA and then ligated into the pCOM vector carrying geneticin resistance (Zhou et al. 2013). The primers employed in vector construction are detailed in Table S3. The positive recombinant vectors were introduced into AGL-1 cells. Gene deletion and complementation transformants were produced according to the *A. tumefaciens*-mediated transformation (ATMT) method as delineated by Wang et al. (2016). The mutant strains with gene deletion were selected on potato dextrose agar (PDA, potato, 200 g/L; glucose, 20 g/L; agar 15 g/L) medium with 200 μg/mL cefotaxime and 50 μg/mL hygromycin. Meanwhile, the strains that were genetically complemented were isolated on PDA with 200 μg/mL cefotaxime and 50 μg/mL geneticin (G418).

2.7. Fungal culture and plant growth

The wild-type (WT) *V. dahliae* strain Vd991 was cultured on PDA medium or in liquid Czapek medium ($NaNO_3$, 3 g/L; K_2HPO_4, 1 g/L; $MgSO_4{\cdot}7H_2O$, 0.5 g/L; KCl, 0.5 g/L; $FeSO_4$, 0.01 g/L; sucrose, 30 g/L) for 5 d at 25 °C. *B. cinerea* strain B05.10 was grown on PDA medium at 25 °C. *Arabidopsis thaliana* (Columbia-0), cotton (*G. hirsutum*) Junmian No. 1, and *N. benthamiana* were cultivated in a greenhouse at 25 °C with a relative humidity of 70% under a consistent photoperiod of 14 h light followed by 10 h darkness. To plant sterile seedlings, we selected full-grained seeds and soaked them in 75% alcohol for 8 min; they were then washed with 8% sodium hypochlorite for 50 s. Finally, they were washed with sterile water 4–6 times and the above operation was repeated three times. The appropriate amount of nutrient soil was transferred into a sterilised wide-mouth jar and sown with sterile cotton seeds, which were then grown in a greenhouse at 25 °C and 70% relative humidity with a 14 h light/10 h dark photoperiod cycle.

Morphology evaluation and penetration analysis of the effect of the CAP superfamily on the basic growth characteristics of *V. dahliae* was performed via plate phenotyping. The Vd991, deletion strains, and complemented strain colonies were cultivated in liquid CM medium (casein acids hydrolysate, 6 g/L; yeast extract, 6 g/L; sucrose, 10 g/L) for 5 d, and spores were collected via filtration and centrifugation. The spore concentration was adjusted to 1×10^6 cfu/mL before being readied for use. The effect of CAP superfamily genes on the growth of the strains was explored through different media, including configured CM medium, PDA, and Czapek plates ($NaNO_3$, 3 g/L; K_2HPO_4, 1 g/L; $MgSO_4{\cdot}7H_2O$, 0.5 g/L; KCl, 0.5 g/L; $FeSO_4$, 0.01 g/L; sucrose, 30 g/L or starch, 17 g/L or pectin, 10 g/L or CMC-Na, 10 g/L; agar, 18 g/L). The prepared 1 μL conidial suspensions were inoculated on different medium plates and cultivated at 25 °C for 9 d before measuring the colony growth diameter using vernier calipers and photographing the colony growth phenotype.

2.8. Penetration capacity analysis

To perform simulated penetration experiments, equal amounts of conidia from the respective *V. dahliae* strains were inoculated on MM medium (Glucose 5.0 g/L, NH_4NO_3 1.0 g/L, KH_2PO_4 0.5 g/L, Na_2HPO_4 1.5 g/L, NaCl 1.0 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L) overlaid with cellophane membranes at 25 °C for 9 d. Subsequently, the cellophane membranes were peeled away from the plates, which were then subjected to an additional three days of incubation to assess the growth of *V. dahliae* post-penetration of the cellophane membranes.

2.9. Pathogenicity assay

The conidial suspensions of each strain were collected by filtration, centrifugation, washing, and dilution after shaking in liquid CM medium; the suspensions were then diluted to 1×10^7 spores/mL. The cotton and *Arabidopsis* seedlings were inoculated using the root-dip method (Tian et al. 2023). The Cotton and *Arabidopsis* roots were immersed in conidial suspensions for 40 min or 2 min, respectively. To inoculate tobacco, the bottom of the seedling pot was cut off, and each plant was treated with 30 mL of conidial suspensions by root drenching (Tian et al. 2023). Pathogenicity phenotypes were observed, and samples were collected for biomass analyses after approximately 20, 15, and 10 d of inoculation on cotton, tobacco, and *Arabidopsis*, respectively.

2.10. Yeast two-hybrid assay

The quantitative assessment of interactions within the test groups was conducted in accordance with the

protocol provided in the Yeast Protocols Handbook (Clontech, Mountain View, CA, USA). The coding regions of the genes selected for bait or prey functions were amplified from the cDNA library originating from the *V. dahliae* strain Vd991, utilising the primer pairs specified in Table S3. Subsequently, the cDNA corresponding to the bait and prey genes was inserted into the yeast vectors pGBKT7, harbouring the GAL4-binding domain, and pGADT7, containing the GAL4 activation domain, respectively. To verify the self-activation of PRYs, the successfully constructed bait plasmid pGBKT7-PRYs and empty pGADT7 were transformed into the yeast strain Y2H Gold. The recombinant pGBKT7 and pGADT7 plasmids were co-transformed into Y2H Gold cells and incubated at 30 °C. The existence of both plasmids was ascertained through growth on SD-Leu-Trp (SD-LW). Following a three-day period, single colonies were picked from each of the transformed plates, adjusted to the same concentration, and grown on SD-Leu-Trp-His-Ade (SD-LWHA) to confirm interaction. The interaction between human lamin C (lam) and SV40 large T-antigen (T) served as a negative control, while murine p53 (p53) was a positive control.

3. Results

3.1. Identification of CAP superfamily proteins in V. dahliae

Genomic probing unveiled that the *V. dahliae* Vd991 genome harbours four proteins with CAP domains (Figure 1(a)), which are members of the CAP superfamily (NCBI CD database: cl00133). Phylogenetic analysis has previously indicated that fungal PRY proteins constitute a distinct subfamily within the CAP superfamily; consequently, these four proteins were designated as VdPRY1, VdPRY2, VdPRY3, and VdPRY4. The amino acid sequence length of the four CAP members ranged from 198 to 288 amino acids (Figure 1(a)). Sequence analysis showed that all VdPRYs with a CAP domain (PF00188), and a signal peptide (SP) in the *N* terminus, while they lacked transmembrane domains (Figure 1(a)). Among them, VdPRY1, VdPRY2, and VdPRY4 were found to be acidic, with predicted isoelectric points (pI) less than 5.0. In contrast, VdPRY3 was found to be basic, with a pI above 9.0 (Table S1). *S. cerevisiae* Pry1 is considered a typical fungal CAP protein (Choudhary et al. 2014). CAP domain sequence alignment analysis of the four PRY proteins in *V. dahliae* with the *S. cerevisiae* Pry1 protein revealed that, although the overall similarity was very low (ranging from a minimum of 15.79% to a maximum of 44.03%), the amino acid residues in the CAP domain, as well as the feature motifs forming α-helices and β-sheets, were highly conserved (Figure S1). The four catalytic sites, comprising a pair of glutamic acid (E) and two histidine (H) residues were found to be strictly conserved in VdPRY2 and VdPRY3, while one glutamic acid residue was missing in VdPRY1 and one glutamic acid and one histidine residue were missing in VdPRY4 (Figure S1).

Many fungal species have been found to have more than one CAP protein, with a maximum of 11, depending on the species tested (Teixeira et al. 2012). To further detect the evolutionary relationship of CAP members in pathogenic fungi, 55 homologous protein sequences of 17 species with CAP (or SCP) structural domains were selected (Table S2). Phylogenetic analysis divided the fungal CAP proteins into three main branches (Figure 1(b)), similar to the genetic relationship of CAP protein members in other fungi (Prados-Rosales et al. 2012; Lu and Edwards 2018; Han et al. 2021). Correspondingly, the four VdPRY members were also distributed in three clades. These results imply that the *V. dahliae* CAP proteins are evolutionarily diverging, and some members are highly related to pathogenicity.

3.2. VdPRY members display broad-spectrum cell death suppression ability

To investigate the immune manipulating function of CAP superfamily proteins during *V. dahliae* interaction with host plants, the four VdPRYs were transiently expressed in *N. benthamiana* leaves, respectively. Unlike the effector VdEG1, which can cause necrosis of *N. benthamiana* leaves, the four VdPRYs did not cause necrosis of *N. benthamiana* leaves (Figure S2a). To investigate whether the four VdPRYs have the ability of cell death suppression, they were co-expressed with the programed cell death factor Bcl-2-associated X protein (BAX) (Lacomme and Santa Cruz 1999) and seven other known cell death-inducing proteins of *V. dahliae* (VdNEP1, VdNEP2, VdEG1, VdEG3, VdCUT11, VdSCP113, and VdSCP126), respectively (Santhanam et al. 2013; Gui et al. 2017, 2018; Wang et al. 2020). As shown in Figure 2(a),

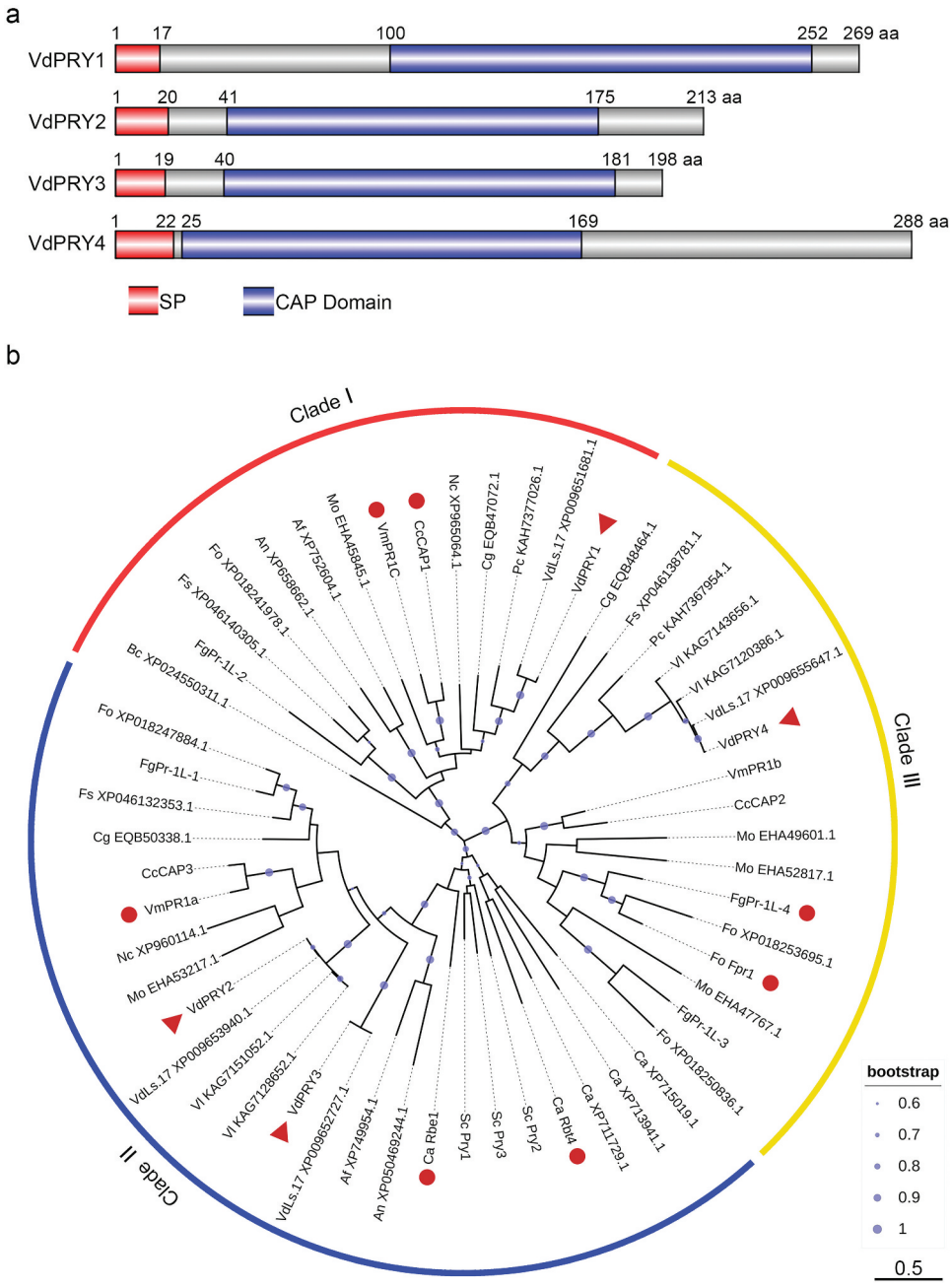

Figure 1. Bioinformatics analysis of CAP superfamily proteins from *Verticillium dahliae* and phylogenetic analysis. (a) Schematic diagram of the protein domains of four members of CAP superfamily proteins. SP, the signal peptide; aa, amino acid. (b) Phylogenetic tree of VdPRYs with other homologous sequences. Four CAP members of *V. dahliae*, VdPRY1, VdPRY2, VdPRY3, and VdPRY4, are marked by a solid red triangle. Species abbreviations for protein names or species are listed in Table S2 of the supplementary material. Scale bar = relative length of each branch.

VdPRY1 and VdPRY3 inhibited cell death induced by the chosen necrosis inducers (PAMPs or effectors), thus displaying broad-spectrum cell death suppression ability. However, VdPRY4 exhibited a relatively narrow cell death suppression ability, and only some effectors (VdNEP2, VdCUT11, VdSCP113, and VdSCP126) were inhibited. Moreover, VdPRY2 had no cell death suppression ability.

Botrytis cinerea infestation experiments indicated that VdPRY1 and VdPRY3 suppress the immune response in *N. benthamiana* leaves, thereby facilitating *B. cinerea* infestation (Figure 2(b) and S2b). In addition, co-expression of VdEG1 with either VdPRY1 or VdPRY3 significantly reduced the ROS accumulation caused by VdEG1 compared to the green fluorescent protein (GFP) control (Figure 2(c)). VdEG1 has

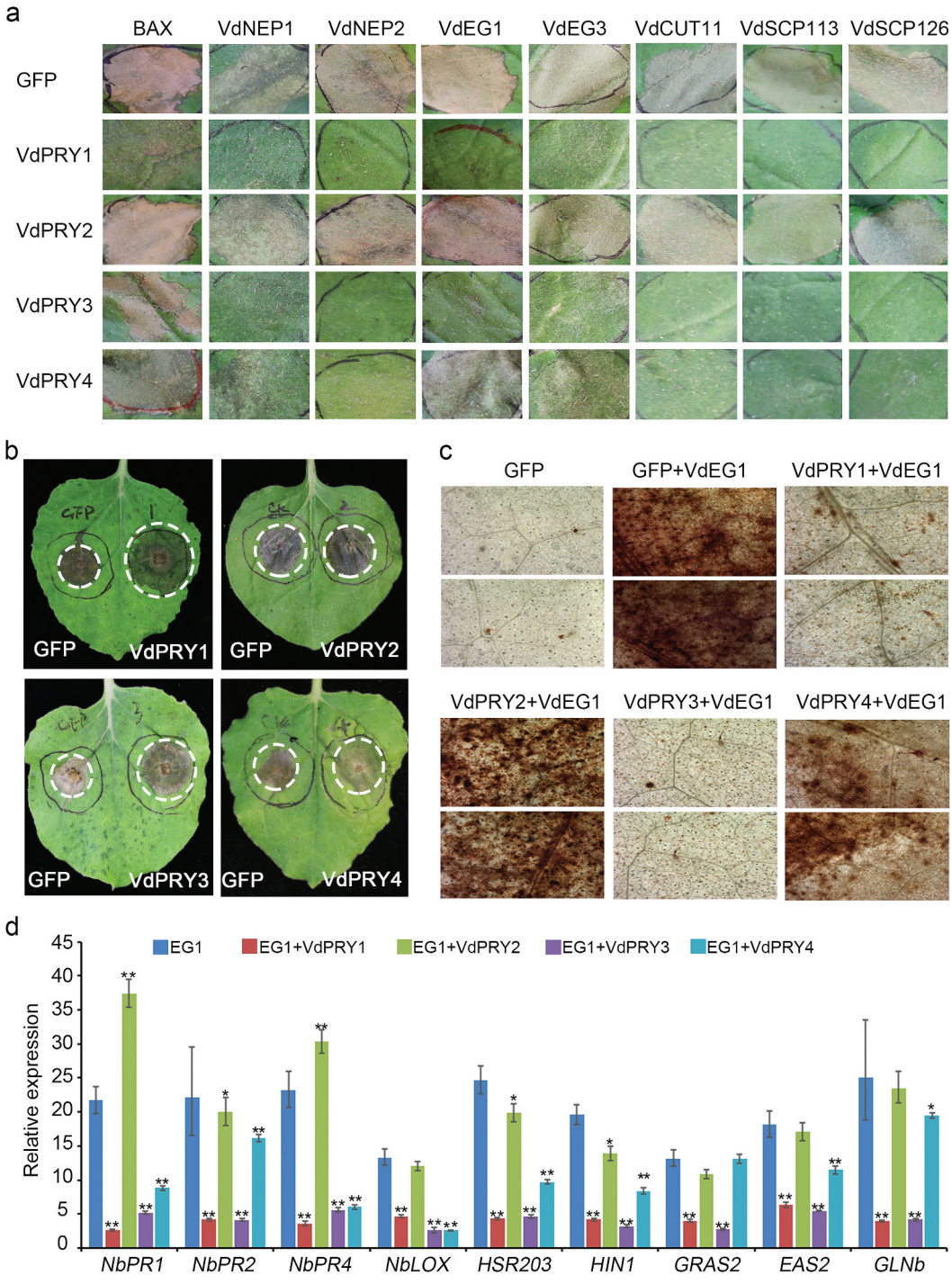

Figure 2. Inhibition of plant immune response by CAP superfamily proteins. (a) Identification of the broad-spectrum cell death-suppression of CAP superfamily proteins. (b) Disease symptoms of *Nicotiana benthamiana* plants that express VdPRYs for 2 d induced by *Botrytis cinerea*. GFP was used as the control. (c) Reactive oxygen species (ROS) accumulation detection. Expression of VdEG1 and GFP was set as positive and negative control, respectively. (d) Disease resistance-related gene expression assay. Statistical significance was tested using an unpaired Student's *t*-test. ** and * represent significant differences at $p < 0.01$ and $p < 0.05$, respectively.

been reported to act as a typical PAMP to trigger the upregulation of host resistance-related genes (Gui et al. 2017). Correspondingly, the upregulation of defence-related genes (*EAS2*, *GRAS2*, *NbGLNb*, *NbHIN1*, *NbHSR203*, *NbLOX*, *NbPR1*, *NbPR2*, and *NbPR4*) induced by VdEG1 in *N. benthamiana* leaves were also significantly suppressed after co-expression with VdPRY1 and VdPRY3 (Figure 2(d)). On the

contrary, neither *B. cinerea* extension, ROS accumulation, nor upregulation of defence-related genes induced by VdEG1 in *N. benthamiana* obviously changed when co-expressed with VdPRY2 or VdPRY4 compared to the GFP control (Figure 2(a–c)). These results further confirmed that the plant immunity-inhibition function of the four VdPRYs differed, with VdPRY1 and VdPRY3 having broad-spectrum plant immunity-inhibition function, but not VdPRY2 and VdPRY4.

3.3. VdPRY members were not required for morphological development and carbon source utilization in V. dahliae

To establish whether the four VdPRYs are involved in morphological development and carbon source utilisation in *V. dahliae*, single deletion strains (Δ*VdPRY1*, Δ*VdPRY2*, Δ*VdPRY3*, and Δ*VdPRY4*) and the corresponding complementary strains ($EC^{\Delta VdPRY1}$, $EC^{\Delta VdPRY2}$, $EC^{\Delta VdPRY3}$, and $EC^{\Delta VdPRY4}$) were obtained by homologous recombination methods (Figure S3a). The positive transformants were verified by multiple diagnostic PCR assays (Figure S3b).

The growth phenotypes of the wild type (Vd991), deletion strains (Δ*VdPRY1*, Δ*VdPRY2*, Δ*VdPRY3*, and Δ*VdPRY4*), and complementary strains ($EC^{\Delta VdPRY1}$, $EC^{\Delta VdPRY2}$, $EC^{\Delta VdPRY3}$, and $EC^{\Delta VdPRY4}$) were firstly observed on PDA and CM plates, respectively. The results showed that a single deletion of any VdPRY member did not affect the colonial morphology and radial growth rate of *V. dahliae* on these basic media (Figure 3(a,b)). In addition, the ability of the four VdPRY deletion mutants to use different carbon sources was also analysed. The growth phenotype and growth rate of the four deletion mutants showed no significant difference to the Vd991 strain on media with sucrose, starch, pectin, and cellulose as carbon sources (Figure 3(c,d)). In conclusion, deletion of CAP superfamily genes did not alter the basic growth capacity and carbon source utilisation of *V. dahliae*.

3.4. VdPRY members differentially contribute to the virulence of V. dahliae

To determine the virulent role of the four VdPRY members in the pathogenesis of *V. dahliae*, the expression patterns of the four corresponding genes were detected during infection of cotton seedlings. The RT-qPCR results showed that the four genes (*VdPRY1*, *VdPRY2*, *VdPRY3*, and *VdPRY4*) were significantly induced during the early stages of infection, reaching their maximum at 36 h post-inoculation (hpi) for *VdPRY1*, *VdPRY2*, and *VdPRY4* and at 48 hpi for *VdPRY3*, with subsequently declining expression levels (Figure 4(a)). These results indicate that the four VdPRY members are highly responsive to plant inoculation and may be associated with *V. dahliae* virulence. Furthermore, the penetration ability of the four deletion strains was also estimated on MM medium overlaid by cellophane membranes. It was found that the penetration ability of the four VdPRY deletion mutants did not affect *V. dahliae* hyphal penetration and growth (Figure S4).

To verify the contribution of the four VdPRYs to virulence, independent VdPRY gene deletion strains, Vd991, and complementary strains were inoculated in the susceptible cotton (*G. hirsutum*) Junmian No. 1 using the root dipping method. Cotton inoculated with Vd991 showed typical symptoms of Verticillium wilt, including leaf wilt, necrosis, and vascular bundle browning (Figure 4(b)). On the contrary, the pathogenicity of Δ*VdPRY1*, Δ*VdPRY2*, and Δ*VdPRY3* was significantly reduced compared with Vd991, and only few leaves showed slight wilt symptoms (Figure 4(b)). However, there was no obvious difference in virulence between the Δ*VdPRY4* mutant and Vd991 (Figure 4(b)). Fungal biomass analysis showed that inoculation of Δ*VdPRY1*, Δ*VdPRY2*, and Δ*VdPRY3* mutants resulted in a significant reduction in *V. dahliae* biomass in cotton, while the fungal biomass in ΔVd*PRY4*-inoculated cotton was comparable to that inoculated with Vd991 (Figure 4(c)). Correspondingly, the wilt symptoms and fungal biomass of the Δ*VdPRY1*, Δ*VdPRY2*, and Δ*VdPRY3* deletion strains were recovered in their complemented strains (Figure 4(b,c)). The virulence of Vd991 and deletion strains against *N. benthamiana* and *A. thaliana* was analysed (Figure S5) and similarly, Δ*VdPRY1*, Δ*VdPRY2*, and Δ*VdPRY3* had significantly reduced pathogenicity on *N. benthamiana* and *A. thaliana*, while Δ*VdPRY4* exhibited no significant virulence change compared with Vd991. In conclusion, CAP superfamily members are integral to the virulence attributes of *V. dahliae*, but individual members differentially contribute to this virulence.

3.5. Interactive relationship analysis of CAP superfamily members in V. dahliae

The CAP domain of the yeast Pry1 can form dimers in solution and bind cholesterol *in vitro* (Darwiche et al.

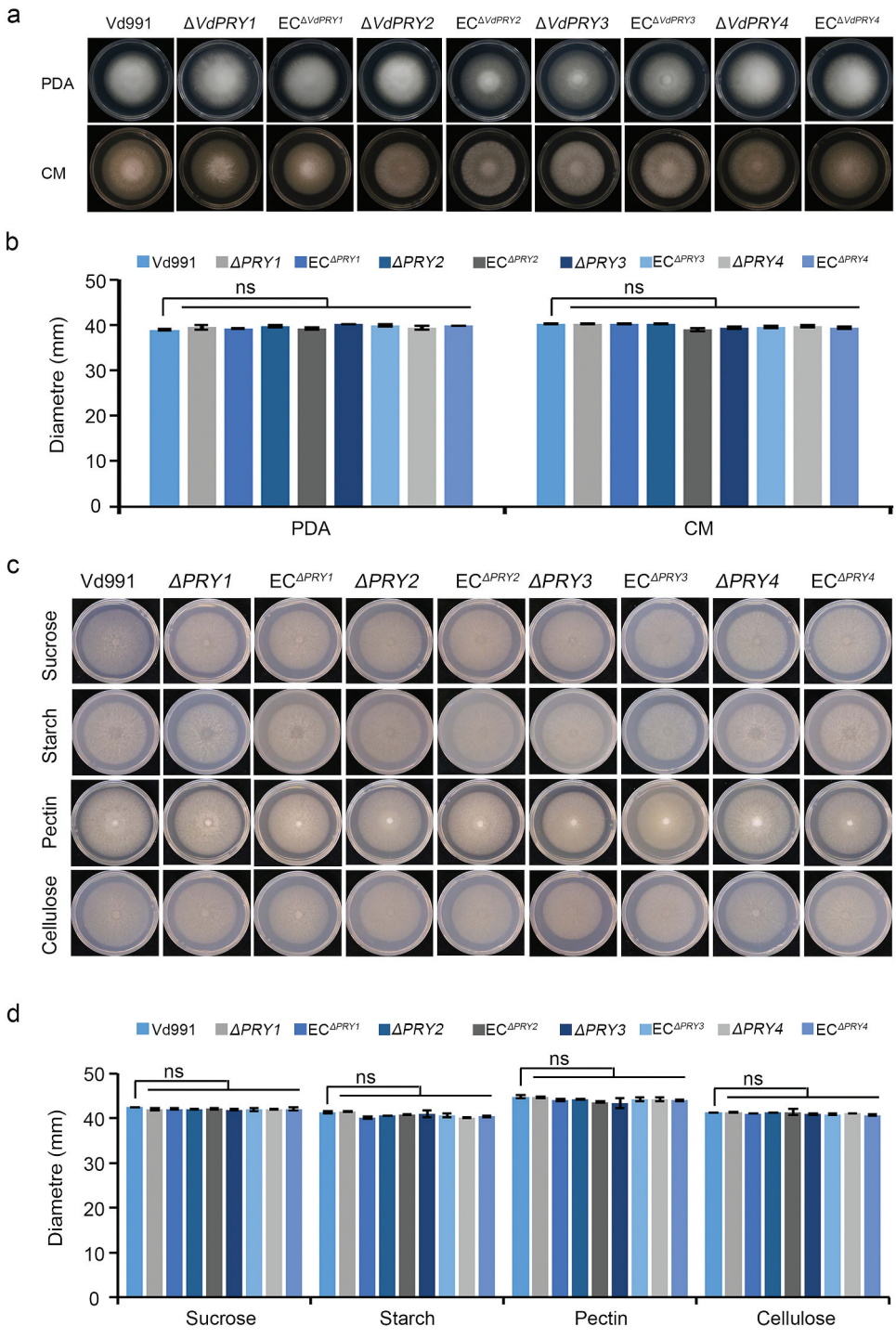

Figure 3. The CAP superfamily proteins are dispensable in the morphology development and carbon source utilisation of *Verticillium dahliae*. (a) Colony morphology of Vd991, Δ*VdPRYs* (Δ*VdPRY1*, Δ*VdPRY2*, Δ*VdPRY3*, and Δ*VdPRY4*), and $EC^{\Delta VdPRYs}$ ($EC^{\Delta VdPRY1}$, $EC^{\Delta VdPRY2}$, $EC^{\Delta VdPRY3}$, and $EC^{\Delta VdPRY4}$) strains on PDA and CM plates. (b) Colony growth phenotype diameter measurement of Vd991, Δ*VdPRYs*, and $EC^{\Delta VdPRYs}$ strains on PDA and CM media. (c) Growth phenotypes of Vd991, Δ*VdPRYs*, and $EC^{\Delta VdPRYs}$ strains on the based Capzek media. (d) Colony growth phenotype diameter measurement of Vd991, Δ*VdPRYs*, and $EC^{\Delta VdPRYs}$ strains based on Capzek media. The above images were captured and the above diameter was measured using vernier calipers with at least three replicates per group after 9 d of incubation at 25 °C. Statistical significance was tested using an unpaired Student's *t*-test. ns, no significance.

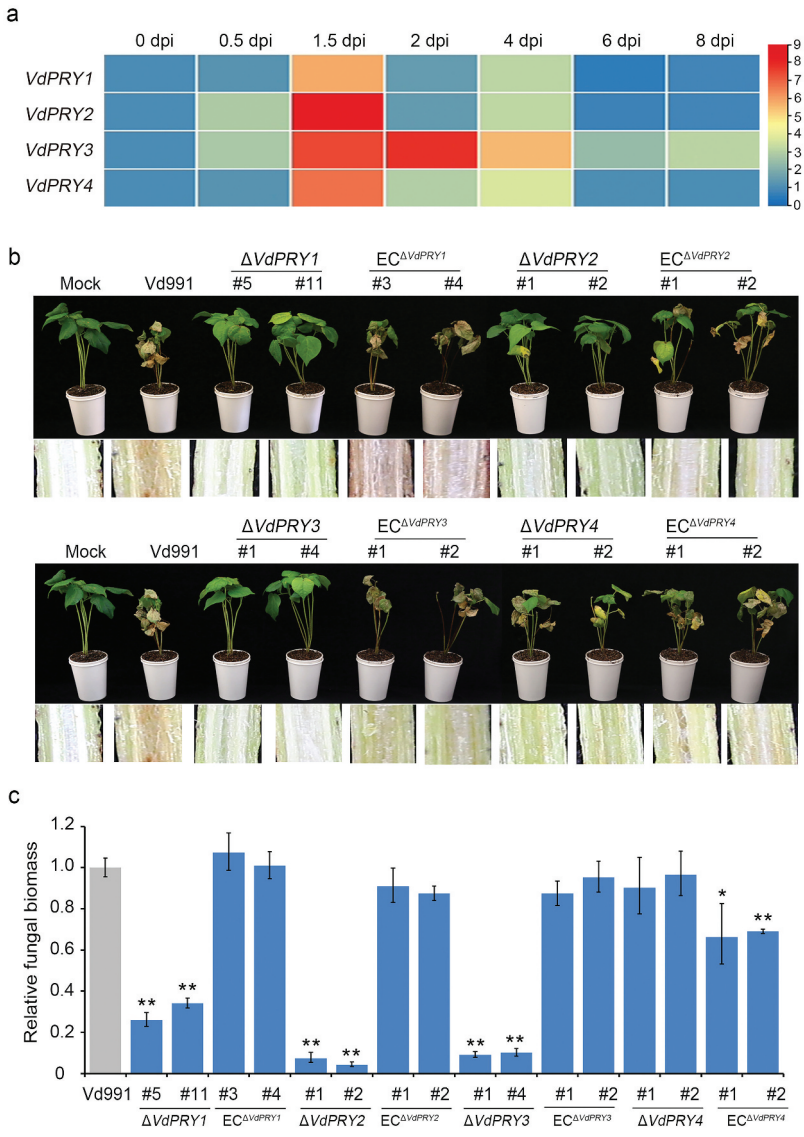

Figure 4. CAP superfamily gene expression patterns and contribution to pathogenicity of *Verticillium dahliae*. (a) Heat map of expression pattern of CAP superfamily genes. The heat map of gene expression patterns was normalized by the value Log_2 (fold-change). (b) Cotton seedlings disease symptoms after inoculated with the Vd991, mutants (Δ*VdPRY1*, Δ*VdPRY2*, Δ*VdPRY3*, Δ*VdPRY4*), and complementary strains ($EC^{\Delta VdPRY1}$, $EC^{\Delta VdPRY2}$, $EC^{\Delta VdPRY3}$, $EC^{\Delta VdPRY4}$). Discoloration of stem xylem sections resulting from *V. dahliae* colonization is shown at the bottom. (c) Fungal biomass in the cotton seedlings infected with Vd991, mutants, and complementary strains. ** and * represent significant differences at $p < 0.01$ and $p < 0.05$ using Student's *t-test*, respectively.

2016). In addition, some other CAP proteins, including Glipr-1, GAPR-1, and Na-ASP-2, crystallise with a monomer in the asymmetric unit, while they dimerise in solution (Asojo et al. 2005, 2011; van Galen et al. 2012). To further detect whether the four CAP members could form dimers or not, yeast two-hybrid experiments were used to detect interactions between the VdPRY members. The yeast two-hybrid assay verified that *VdPRY1*, *VdPRY2*, *VdPRY3*, and *VdPRY4* were not self-activating in the yeast hybridisation system (Figure S6). This result indicated that VdPRY1 can interact with itself and with VdPRY2 and VdPRY4. Similarly, VdPRY2 and VdPRY4 could both interact with themselves and the other three members. VdPRY3 could not interact with itself, but with the other three members (Figure 5(a)). The involvement of CAP domains in forming dimers was further analysed, and the results confirmed that the CAP domain of each member can mediate interactions with themselves and other members of the CAP domain (Figure 5(b)). Thus, *V. dahliae* CAP superfamily members possess the ability to interact with other

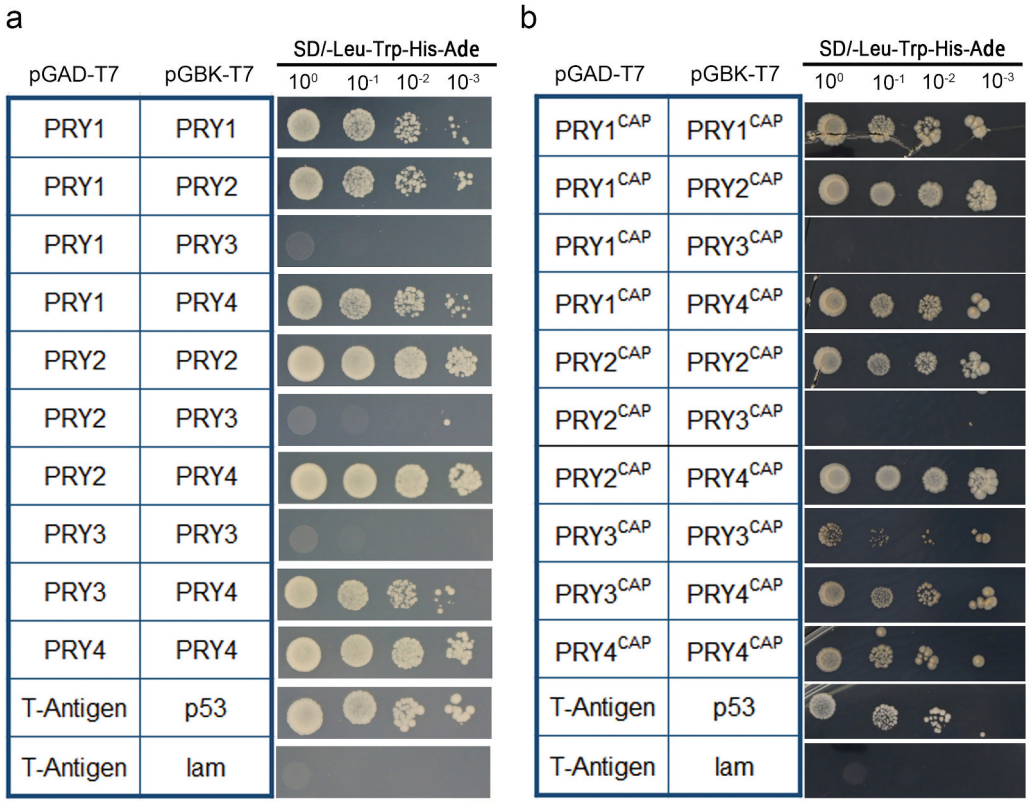

Figure 5. Interaction between CAP superfamily members. Identification of the physical interaction of the coding sequence-deleted SP domain (PRYs) (a) or CAP domain (PRYs^{CAP}) (b) of CAP superfamily members in *Verticillium dahliae* via yeast two-hybrid assays.

members, such as CAP proteins from other fungi. CAP superfamily members may thus form homologous or heterologous complexes, and CAP domains are necessary for the formation of complexes.

4. Discussion

CAP proteins constitute a large protein superfamily with members found in all kingdoms of life (Gibbs et al. 2008). With continuous evolution, the sequence and functional differences between CAP proteins of different species, and even between members of the same species, have gradually increased, but most of them have retained the core CAP domain, which is the main basis for CAP protein classification (Abraham and Chandler 2017). The genomic annotation showed that *V. dahliae* contains a CAP protein superfamily consisting of four members, which were identified as VdPRY1, VdPRY2, VdPRY3, and VdPRY4 (Figure 1(a)). Despite the low homology among the four members in the protein sequence and the lack or mutation of conserved amino acid sites in some members, members can form conserved secondary structures and share the conserved functional domain of the CAP superfamily (Figures 1(a) and S1). The four members play different roles in regulating plant immunity and fungal virulence: VdPRY1 and VdPRY3 had broad-spectrum ability to inhibit plant immunity, while separate knockout of VdPRY1, VdPRY2, and VdPRY3 significantly reduced the virulence on the host plant without affecting *V. dahliae* morphology development and carbon sources utilisation (Figures 2–4).

In non-mammals, the roles of CAP proteins in regulating the immune system have been widely studied, including the venom allergen Antigen 5 (Ag5) from insects, pathogenesis related-1 (PR-1) from plants, or proteins containing bacterial SCP domains from nematodes (Gibbs et al. 2008). In fungi, the virulent CAP protein, CcCAP1 of *C. chrysosperma*, can be localised in the plant cell nucleus to inhibit the plant immune response, and the CAP domain was sufficient for the immune inhibiting activity (Han et al. 2021). In this study, we found that the CAP superfamily members VdPRY1 and VdPRY3 exhibited significant inhibitory effects on plant cell necrosis induced by other

PAMPs or effectors (Figure 2). Many CAP superfamily proteins are important virulence factors in different fungal pathogens, including *C. chrysosperma*, *F. graminearum*, and *V. mali*. Correspondingly, their expression is usually significantly upregulated in the early stage of infection (Lu and Edwards 2018; Han et al. 2021; Wang et al. 2021). In this study, expression pattern analysis of the four VdPRYs revealed that all of them were highly induced in the early stage of infection of cotton (Figure 4(a)). Separate deletion of *VdPRY1*, *VdPRY2*, and *VdPRY3* led to a significant decrease in virulence of *V. dahliae* towards the cotton, *N. benthamiana* and *A. thaliana* (Figure 4(b)). While the deletion of any of these genes did not affect the growth phenotype of *V. dahliae* (Figure 3).

Bioinformatics analysis revealed that the whole genome of *V. dahliae* strain Vd991 encodes 123 SCRPs, and it was found that VdSCP27, VdSCP113, VdSCP126, VdSCP41, and VdSCP76 play important roles in the regulation of plant immunity and fungal virulence (Chen et al. 2018; Qin et al. 2018; Wang et al. 2020, 2022). In addition, it has been shown that the secreted proteome of other pathogenic fungi contains a large number of SCRPs, such as Six1 in *F. oxysporum* and Avr-Pita in *Magnaporthe oryzae*, and these SCRPs are usually involved in the recognition of disease resistance genes (Orbach et al. 2000; Rep et al. 2004). During extensive research on the interactions between pathogens and their hosts, it has been observed that the SCRPs from pathogenic fungi often function as effector. These proteins are involved in recognising and invading the host, as well as triggering the host's immune response. However, in the absence of host resistance genes, SCRPs can also serve as virulence factors that contribute to the pathogenicity of the fungi. Whether VdPRYs, as a small molecule protein containing an N-terminal secretion signal peptide, can act as effectors to modulate plant immunity, and the specific mechanism underlying this process, needs to be further investigated.

The presence of conserved structural domains in proteins implies that there may be a conserved biological function for these proteins, but over the course of continuous evolution, functional differentiation usually occurs as well. Many studies have shown that CAP proteins of the same fungus generally contain multiple members, with significant functional differentiation (Choudhary and Schneiter 2012; Röhm et al. 2013; Lu and Edwards 2018; Wang et al. 2021). For example, of three members of *S. cerevisiae* CAP superfamily proteins, only Pry1 and Pry2 have the binding and exporting cholesterol function (Choudhary and Schneiter 2012). Similarly, three CAP superfamily genes were identified in *V. mali*, and VmPR1a and VmPR1c are virulence factors (Wang et al. 2021). Functional differentiation also occurred in this study. Similar to previous reports, phylogenetic tree analysis supported the functional or evolutional division of the four *V. dahliae* CAP superfamily protein members (Figure 1(a)) (Lu and Edwards 2018; Han et al. 2021; Wang et al. 2021). Phylogenetic tree analysis showed that although VdPRY1 and VdPRY3 belong to different evolutionary branches (Figure 1(b)), both have immunosuppressive function and regulate fungal virulence (Figures 2 and 4). Among them, VdPRY2 is in a separate evolutionary branch with the sterol-binding homologous genes *pry1* and *pry2* from *S. cerevisiae* and *Rbt4* and *Rbe1* from *C. albicans*; *Rbt4* and *Rbe1* also contribute to pathogen virulence (Choudhary and Schneiter 2012; Röhm et al. 2013; Schneiter and Di Pietro 2013). Therefore, we speculate that similar to these homologous genes of *S. cerevisiae* and *C. albicans*, VdPRY2 has sterol binding function and thus contributes to virulence; however, this needs to be further verified. In addition, the non-virulent member VdPRY4 was located in the separate evolutionary clade III with the virulence factors Fg-1 L-4 and FoFpr1 (Figure 1(b)). The CAP structural domains generally contain two conserved histidine and glutamate amino acid residues, and has been suggested that conserved amino acid residues play an important role in binding Mg^{2+}, Zn^{2+}, and Ca^{2+} ions (Shikamoto et al. 2005; Asojo et al. 2011; Darwiche et al. 2016; Wilbers et al. 2018). In *F. oxysporum*, the fpr1 gene is required for virulence and its function depends on the integrity of the proposed active site of PR-1-like proteins (Prados-Rosales et al. 2012). In this study, one conserved glutamic acid and one histidine were lacking in VdPRY4 (Figure S1). We thus speculate that the functional divergence of VdPRY4, including virulence loss and reduced immune manipulation compared to other CAP superfamily members in *V. dahliae* may be due to the absence of the active site.

Previous studies showed that some CAP proteins have dimerisation characteristics (Asojo 2011;

Darwiche et al. 2016; Li et al. 2017). In this study, yeast two-hybrid assays confirmed these characteristics also for the four CAP superfamily proteins of *V. dahliae*. In addition to dimerisation of individual proteins, interactions between these proteins occurred as well, which could only be accomplished via the CAP domain (Figure 5). It has been shown that Golgi-Associated plant Pathogenesis-Related protein 1 (GAPR-1) can interact with other GAPR-1 to form homodimers *in vitro* and *in vivo*, and mutation of conserved glutamate and histidine residues in GAPR-1 results in a greatly increased dimer population (Serrano et al. 2004; van Galen et al. 2012). In *V. dahliae*, the CAP superfamily proteins VdPRY2 and VdPRY3 contained four active sites, while a glutamic acid was missing in VdPRY1, and one glutamic acid and one histidine were missing in VdPRY4 (Figure S1). *In-vitro* yeast two-hybrid assays revealed that VdPRY1 could interact with VdPRY2 and VdPRY4, in addition to other VdPRY1s, and VdPRY4 could interact with the other three members in addition to other VdPRY4s (Figure 5). However, the key sites of interaction between the four VdPRYs need to be further explored. Consequently, it is imperative to ascertain through additional experimentation whether members of the CAP superfamily can assemble into homologous or heterologous complexes, and if the structural domains of CAP are essential for the creation of such complexes. Furthermore, it remains to be confirmed whether proteins from the CAP superfamily of *V. dahliae* can engage in interactions with CAP superfamily proteins from other species. In addition, direct interactions between the VdPRYs suggest their functional complementarity or the existence of more complex mechanisms in regulating host immunity and virulence.

5. Conclusions

Overall, our study for the first time demonstrated that four CAP superfamily proteins in *V. dahliae* could manipulate plant immunity and contribute to virulence. The four CAP proteins (VdPRY1, VdPRY2, VdPRY3, and VdPRY4) were all secreted proteins. VdPRY1 and VdPRY3 played important roles in inhibiting the plant immune response. Furthermore, VdPRY1, VdPRY2, and VdPRY3 are indispensable in maintaining the virulence of *V. dahliae*. The four VdPRYs also possess dimerisation properties with themselves and other members of the CAP superfamily. Further experiments are needed on the specific mechanisms involved in regulating immunity and fungal virulence, as well as on the mechanisms by which interactions between members occur.

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References

Abraham A, Chandler DE. 2017. Tracing the evolutionary history of the CAP superfamily of proteins using amino acid sequence homology and conservation of splice sites. J Mol Evol. 85(3–4):137–157. doi: 10.1007/s00239-017-9813-9.

Almagro Armenteros JJ, Tsirigos KD, Sønderby CK, Petersen TN, Winther O, Brunak S, von Heijne G, Nielsen H. 2019. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol. 37(4):420–423. doi: 10.1038/s41587-019-0036-z.

Apweiler R, Attwood TK, Bairoch A, Bateman A, Birney E, Biswas M, Bucher P, Cerutti L, Corpet F, Croning MD, et al. 2000. InterPro—an integrated documentation resource for protein families, domains and functional sites. Bioinformatics. 16(12):1145–1150. doi: 10.1093/bioinformatics/16.12.1145.

Asojo OA. 2011. Structure of a two-cap-domain protein from the human hookworm parasite *Necator americanus*. Acta Crystallogr D Biol Crystallogr. 67(5):455–462. doi: 10.1107/S0907444911008560.

Asojo OA, Goud G, Dhar K, Loukas A, Zhan B, Deumic V, Liu S, Borgstahl GE, Hotez PJ. 2005. X-ray structure of Na-ASP-2, a pathogenesis-related-1 protein from the nematode parasite, *Necator americanus*, and a vaccine antigen for human hookworm infection. J Mol Biol. 346(3):801–814. doi: 10.1016/j.jmb.2004.12.023.

Asojo OA, Koski RA, Bonafé N. 2011. Structural studies of human glioma pathogenesis-related protein 1. Acta

Crystallogr D Biol Crystallogr. 67(10):847–855. doi: 10.1107/S0907444911028198.

Bhat RG, Subbarao KV. 1999. Host range specificity in *Verticillium dahliae*. Phytopathology. 89(12):1218–1225. doi: 10.1094/PHYTO.1999.89.12.1218.

Bindschedler LV, Dewdney J, Blee KA, Stone JM, Asai T, Plotnikov J, Denoux C, Hayes T, Gerrish C, Davies DR, et al. 2006. Peroxidase-dependent apoplastic oxidative burst in *Arabidopsis* required for pathogen resistance. Plant J. 47 (6):851–863. doi: 10.1111/j.1365-313X.2006.02837.x.

Chen JY, Liu C, Gui YJ, Si KW, Zhang DD, Wang J, Short DPG, Huang JQ, Li NY, Liang Y, et al. 2018. Comparative genomics reveals cotton-specific virulence factors in flexible genomic regions in *Verticillium dahliae* and evidence of horizontal gene transfer from *Fusarium*. New Phytol. 217(2):756–770. doi: 10.1111/nph.14861.

Choudhary V, Darwiche R, Gfeller D, Zoete V, Michielin O, Schneiter R. 2014. The caveolin-binding motif of the pathogen-related yeast protein Pry1, a member of the CAP protein superfamily, is required for *in vivo* export of cholesteryl acetate. J Lipid Res. 55(5):883–894. doi: 10.1194/jlr.M047126.

Choudhary V, Schneiter R. 2012. Pathogen-related yeast (PRY) proteins and members of the CAP superfamily are secreted sterol-binding proteins. Proc Natl Acad Sci USA. 109 (42):16882–16887. doi: 10.1073/pnas.1209086109.

Darwiche R, El Atab O, Cottier S, Schneiter R. 2018. The function of yeast CAP family proteins in lipid export, mating, and pathogen defense. FEBS Lett. 592(8):1304–1311. doi: 10.1002/1873-3468.12909.

Darwiche R, Kelleher A, Hudspeth EM, Schneiter R, Asojo OA. 2016. Structural and functional characterization of the CAP domain of pathogen-related yeast 1 (Pry1) protein. Sci Rep. 6(1):28838. doi: 10.1038/srep28838.

Darwiche R, Mène-Saffrané L, Gfeller D, Asojo OA, Schneiter R. 2017. The pathogen-related yeast protein Pry1, a member of the CAP protein superfamily, is a fatty acid-binding protein. J Biol Chem. 292(20):8304–8314. doi: 10.1074/jbc.M117.781880.

Dhar N, Chen JY, Subbarao KV, Klosterman SJ. 2020. Hormone signaling and its interplay with development and defense responses in *Verticillium*-plant interactions. Front Plant Sci. 11:584997. doi: 10.3389/fpls.2020.584997.

Gaikwad AS, Hu J, Chapple DG, O'Bryan MK. 2020. The functions of CAP superfamily proteins in mammalian fertility and disease. Hum Reprod Update. 26(5):689–723. doi: 10.1093/humupd/dmaa016.

Gibbs GM, Roelants K, O'Bryan MK. 2008. The CAP superfamily: cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins-roles in reproduction, cancer, and immune defense. Endocr Rev. 29(7):865–897. doi: 10.1210/er.2008-0032.

Gonzalez SN, Sulzyk V, Weigel Muñoz M, Cuasnicu PS. 2021. Cysteine-rich secretory proteins (CRISP) are key players in mammalian fertilization and fertility. Front Cell Dev Biol. 9:800351. doi: 10.3389/fcell.2021.800351.

Gouet P, Courcelle E, Stuart DI, Métoz F. 1999. Espript: analysis of multiple sequence alignments in PostScript. Bioinformatics. 15(4):305–308. doi: 10.1093/bioinformatics/15.4.305.

Gui YJ, Chen JY, Zhang DD, Li NY, Li TG, Zhang WQ, Wang XY, Short DPG, Li L, Guo W, et al. 2017. *Verticillium dahliae* manipulates plant immunity by glycoside hydrolase 12 proteins in conjunction with carbohydrate-binding module 1. Environ Microbiol. 19(5):1914–1932. doi: 10.1111/1462-2920.13695.

Gui YJ, Zhang WQ, Zhang DD, Zhou L, Short DPG, Wang J, Ma XF, Li TG, Kong ZQ, Wang BL, et al. 2018. A *Verticillium dahliae* extracellular cutinase modulates plant immune responses. Mol Plant Microbe Interact. 31(2):260–273. doi: 10.1094/MPMI-06-17-0136-R.

Han Z, Xiong D, Xu Z, Liu T, Tian C. 2021. The *Cytospora chrysosperma* virulence effector CcCAP1 mainly localizes to the plant nucleus to suppress plant immune responses. mSphere. 6(1):e00883-20. doi: 10.1128/mSphere.00883-20.

Klosterman SJ, Atallah ZK, Vallad GE, Subbarao KV. 2009. Diversity, pathogenicity, and management of *Verticillium* species. Annu Rev Phytopathol. 47(1):39–62. doi: 10.1146/annurev-phyto-080508-081748.

Koppers AJ, Reddy T, O'Bryan MK. 2011. The role of cysteine-rich secretory proteins in male fertility. Asian J Androl. 13(1):111–117. doi: 10.1038/aja.2010.77.

Krogh A, Larsson B, von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 305(3):567–580. doi: 10.1006/jmbi.2000.4315.

Lacomme C, Santa Cruz S. 1999. Bax-induced cell death in tobacco is similar to the hypersensitive response. Proc Natl Acad Sci USA. 96(14):7956–7961. doi: 10.1073/pnas.96.14.7956.

Li Y, Zhao Y, Su M, Glover K, Chakravarthy S, Colbert CL, Levine B, Sinha SC. 2017. Structural insights into the interaction of the conserved mammalian proteins GAPR-1 and Beclin 1, a key autophagy protein. Acta Crystallogr D Struct Biol. 73(Pt 9):775–792. doi: 10.1107/S2059798317011822.

Lu S, Edwards MC. 2016. Genome-wide analysis of small secreted cysteine-rich proteins identifies candidate effector proteins potentially involved in *Fusarium graminearum*-wheat interactions. Phytopathology. 106 (2):166–176. doi: 10.1094/PHYTO-09-15-0215-R.

Lu S, Edwards MC. 2018. Molecular characterization and functional analysis of PR-1-Like proteins identified from the wheat head blight fungus *Fusarium graminearum*. Phytopathology. 108(4):510–520. doi: 10.1094/PHYTO-08-17-0268-R.

Luo X, Tian T, Feng L, Yang X, Li L, Tan X, Wu W, Li Z, Treves H, Serneels F, et al. 2022. Pathogenesis-related protein 1 suppresses oomycete pathogen by targeting against AMPK kinase complex. J Adv Res. 43:13–26. doi: 10.1016/j.jare.2022.02.002.

Mistry J, Chuguransky S, Williams L, Qureshi M, Salazar GA, Sonnhammer ELL, Tosatto SCE, Paladin L, Raj S, Richardson LJ, et al. 2014. Pfam: the protein families

database in 2021. Nucl Acids Res. 42(D1):D222–D230. doi: 10.1093/nar/gkt1223.

Orbach MJ, Farrall L, Sweigard JA, Chumley FG, Valent B. 2000. A telomeric avirulence gene determines efficacy for the rice blast resistance gene Pi-ta. Plant Cell. 12(11):2019–2032. doi: 10.1105/tpc.12.11.2019.

Prados-Rosales RC, Roldán-Rodríguez R, Serena C, López-Berges MS, Guarro J, Martínez-Del-Pozo Á, Di Pietro A. 2012. A PR-1-like protein of *Fusarium oxysporum* functions in virulence on mammalian hosts. J Biol Chem. 287 (26):21970–21979. doi: 10.1074/jbc.M112.364034.

Qin J, Wang K, Sun L, Xing H, Wang S, Li L, Chen S, Guo HS, Zhang J. 2018. The plant-specific transcription factors CBP60g and SARD1 are targeted by a *Verticillium* secretory protein VdSCP41 to modulate immunity. eLife. 7:e34902. doi: 10.7554/eLife.34902.

Rao X, Huang X, Zhou Z, Lin X. 2013. An improvement of the 2ˆ (–delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. Biostat Bioinforma Biomath. 3(3):71–85.

Rep M, Van Der Does HC, Meijer M, Van Wijk R, Houterman PM, Dekker HL, De Koster CG, Cornelissen BJC. 2004. A small, cysteine-rich protein secreted by *Fusarium oxysporum* during colonization of xylem vessels is required for I-3-mediated resistance in tomato. Mol Microbiol. 53 (5):1373–1383. doi: 10.1111/j.1365-2958.2004.04177.x.

Röhm M, Lindemann E, Hiller E, Ermert D, Lemuth K, Trkulja D, Sogukpinar O, Brunner H, Rupp S, Urban CF, et al. 2013. A family of secreted pathogenesis-related proteins in *Candida albicans*. Mol Microbiol. 87(1):132–151. doi: 10.1111/mmi.12087.

Santhanam P, van Esse HP, Albert I, Faino L, Nürnberger T, Thomma BP. 2013. Evidence for functional diversification within a fungal NEP1-like protein family. Mol Plant Microbe Interact. 26(3):278–286. doi: 10.1094/MPMI-09-12-0222-R.

Schneiter R, Di Pietro A. 2013. The CAP protein superfamily: function in sterol export and fungal virulence. Biomol Concepts. 4(5):519–525. doi: 10.1515/bmc-2013-0021.

Serrano RL, Kuhn A, Hendricks A, Helms JB, Sinning I, Groves MR. 2004. Structural analysis of the human Golgi-associated plant pathogenesis related protein GAPR-1 implicates dimerization as a regulatory mechanism. J Mol Biol. 339(1):173–183. doi: 10.1016/j.jmb.2004.03.015.

Shikamoto Y, Suto K, Yamazaki Y, Morita T, Mizuno H. 2005. Crystal structure of a CRISP family Ca^{2+}-channel blocker derived from snake venom. J Mol Biol. 350(4):735–743. doi: 10.1016/j.jmb.2005.05.020.

Teixeira PJ, Thomazella DP, Vidal RO, Do Prado PF, Reis O, Baroni RM, Franco SF, Mieczkowski P, Pereira GA, Mondego JM. 2012. The fungal pathogen *Moniliophthora perniciosa* has genes similar to plant PR-1 that are highly expressed during its interaction with cacao. PLoS One. 7(9): e45929. doi: 10.1371/journal.pone.0045929.

Tian L, Zhuang J, Li JJ, Zhu H, Klosterman SJ, Dai XF, Chen JY, Subbarao KV, Zhang DD. 2023. Thioredoxin VdTrx1, an unconventional secreted protein, is a virulence factor in *Verticillium dahliae*. Front Microbiol. 14:1130468. doi: 10.3389/fmicb.2023.1130468.

van Galen J, Olrichs NK, Schouten A, Serrano RL, Nolte-'t Hoen EN, Eerland R, Kaloyanova D, Gros P, Helms JB. 2012. Interaction of GAPR-1 with lipid bilayers is regulated by alternative homodimerization. Biochim Biophys Acta. 1818 (9):2175–2183. doi: 10.1016/j.bbamem.2012.04.016.

Wang CL, Yin ZY, Nie JJ, Lin YH, Huang LL. 2021. Identification and virulence analysis of CAP superfamily genes in *Valsa mali*. Sci Agr Sin. 54(16):3440–3450. doi: 10.3864/j.issn.0578-1752.2021.16.007.

Wang D, Tian L, Zhang DD, Song J, Song SS, Yin CM, Zhou L, Liu Y, Wang BL, Kong ZQ, et al. 2020. Functional analyses of small secreted cysteine-rich proteins identified candidate effectors in *Verticillium dahliae*. Mol Plant Pathol. 21 (5):667–685. doi: 10.1111/mpp.12921.

Wang D, Zhang DD, Song J, Li JJ, Wang J, Li R, Klosterman SJ, Kong ZQ, Lin FZ, Dai XF, et al. 2022. *Verticillium dahliae* CFEM proteins manipulate host immunity and differentially contribute to virulence. BMC Biol. 20(1):55. doi: 10.1186/s12915-022-01254-x.

Wang S, Xing H, Hua C, Guo HS, Zhang J. 2016. An improved single-step cloning strategy simplifies the *Agrobacterium tumefaciens*-mediated transformation (ATMT)-based gene-disruption method for *Verticillium dahliae*. Phytopathology. 106(6):645–652. doi: 10.1094/PHYTO-10-15-0280-R.

Wangorsch A, Scheurer S, Blanca M, Blanca-Lopez N, Somoza ML, Martín-Pedraza L. 2022. Allergenic properties and molecular characteristics of PR-1 proteins. Front Allergy. 3:824717. doi: 10.3389/falgy.2022.824717.

Wilbers RHP, Schneiter R, Holterman MHM, Drurey C, Smant G, Asojo OA, Maizels RM, Lozano-Torres JL, Lok JB. 2018. Secreted venom allergen-like proteins of helminths: conserved modulators of host responses in animals and plants. PLoS Pathog. 14(10):e1007300. doi: 10.1371/journal.ppat.1007300.

Zhang Y, Zhou J, Zhao L, Feng Z, Wei F, Bai H, Feng H, Zhu H. 2022. A review of the pathogenicity mechanism of *Verticillium dahliae* in cotton. J Cotton Res. 5(1):3. doi: 10.1186/s42397-021-00111-6.

Zhou L, Zhao J, Guo W, Zhang T. 2013. Functional analysis of autophagy genes via *Agrobacterium*-mediated transformation in the vascular wilt fungus *Verticillium dahliae*. J Genet Genomics. 40(8):421–431. doi: 10.1016/j.jgg.2013.04.006.