RESEARCH PAPER

**Verticillium dahliae** PevD1, an Alt a 1-like protein, targets cotton PR5-like protein and promotes fungal infection

Yi Zhang¹,†, Yuhan Gao¹,†, Yingbo Liang¹, Yijie Dong¹, Xiufen Yang¹,²,* and Dewen Qiu¹,³,*

¹ The State Key Laboratory for Biology of Plant Disease and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China
² Key Laboratory of Control of Biological Hazard Factors (Plant Origin) for Agri-product Quality and Safety, Ministry of Agriculture, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China
³ Key Laboratory of Integrated Pest Management in Crops, Ministry of Agriculture, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China

† These authors contributed equally to this work.
* Correspondence: yangxiufen@caas.cn or qiudewen@caas.cn

Received 2 July 2018; Editorial decision 26 September 2018; Accepted 28 September 2018

Editor: Katherine Denby, York University, UK

Abstract

Alt a 1 family proteins (AA1s) have only been observed in the Dothideomycetes and Sordariomycetes classes of fungi, and their biological functions have remained poorly understood. *Verticillium dahliae*, a soil-borne pathogen that causes plant wilt disease, secretes hundreds of proteins during the process of pathogenic infection, including the AA1 member PevD1. In this study, we found that the *pevd1* transcript was present in all of the hosts studied (cotton, Arabidopsis, tomato, and tobacco) and showed elevated expression throughout the infection process. Furthermore, *pevd1* knockout mutants displayed attenuated pathogenicity compared with the wild-type (WT) strain and complemented strains in hosts. A partner protein of PevD1, pathogenesis-related protein 5 (PR5)-like protein GhPR5, was isolated from cotton (*Gossypium hirsutum*) plants by co-purification assays, and the PevD1–GhPR5 interaction was determined to be localized in the C-terminus (*PevD1b*, amino acids residues 113–155) by pull-down and yeast two-hybrid techniques. Re-introduction of the *pevd1b* gene into a *pevd1* knockout mutant resulted in restoration of the virulence phenotype to WT levels. In addition, PevD1b, which is similar to PevD1, decreased the antifungal activity of GhPR5 *in vitro*. Our findings reveal an infection strategy in which *V. dahliae* secretes PevD1 to inhibit GhPR5 antifungal activity in order to overcome the host defence system.

**Keywords:** Alt a 1, *Gossypium hirsutum*, interaction, pathogenesis-related protein 5 (PR5), PevD1, *Verticillium dahliae*.

Introduction

Plants have evolved two major general defence systems to respond to attacks by pathogens. The primary defence system is that plants respond to pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs), a process termed PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). PTI causes rapid defence reactions, such as ion fluxes, cell wall reinforcement, the production of reactive oxygen species (ROS), and the expression of defence-related genes (Zipfel, 2008, 2009). However, successful pathogens can interfere with PTI by delivering effectors, evading recognition, or suppressing signalling pathways. In turn, the hosts use resistance proteins to recognize the effectors, leading to the secondary defence system of effector-triggered immunity (ETI) (Jones and Dangl, 2006; Zipfel, 2008). ETI commonly results in local cell death, known as the hypersensitive response (HR).

During the recognition of pathogens, most of the inducible defence-related proteins are associated with pathogenesis-related proteins (PRs), which have been divided into 17
families and play important roles in plant defence systems (van Loon et al., 2006). Of these, the PR5 family proteins, which are also referred to as thaumatin-like proteins, have been reported in many species upon infection with pathogens and have been associated with antimicrobial activity. Some PR5s have been shown to have antifungal activity in vitro against *Verticillium dahliae* (Abad et al., 1996; Pressley, 1997; Min et al., 2004; Tjamos et al., 2005; van Loon et al., 2006). The tobacco PR5 protein osmotin is inducible by both osmotic stress and pathogens, and its homologs in tomato and potato have been shown to possess anti-oomycete activity in vitro against Phytophthora infestans, and overexpression of tobacco PR5 in tobacco and potato plants can enhance disease resistance against this pathogen. ATLP3, a PR5 protein identified from Arabidopsis, displays in vitro antifungal activity against *V. dahliae, V. albo-atrum,* and *Fusarium oxysporum* (Hu and Reddy, 1997). Overexpression of a rice thaumatin-like protein results in an enhanced resistance to *Alternaria alternata* in tobacco (Velazhahan and Muthukrishnan, 2004).

*Verticillium dahliae,* which is the causal agent of cotton wilt disease, is a pathogenic fungus with a very wide host range of over 200 dicotyledonous plant species (Fradin and Thomma, 2006). It produces various effector molecules to modulate its infection, although not much is known regarding the components that are crucial for its pathogenicity. In general, cell wall-degrading enzymes (CWDEs), toxins, and elicitor-like molecules have been the focus of most studies. *Verticillium dahliae* secretes CWDEs that act as a virulence factor in its interactions with plants, among which pectinolytic enzymes are the best-characterized and are thought to have important roles in pathogenicity (Dobinson et al., 2004). Some of these enzymes have been shown to cause wilt symptoms and promote the necrosis of plant tissues (Cooper and Wood, 1980). In a recent study, a *V. dahliae* pectate lyase, VdPEL1, was shown to induce plant cell death and contribute to virulence (Yang et al., 2018). Several CWDEs, such as ethylene-inducing xylanase (EIX) (Enkerli et al., 1999) and xyloligucan-specific endoglucanase (XEG1) (Ma et al., 2015), have also been recognized as PAMPs. In addition, *V. dahliae* produces phytotoxins and elicitor proteins that induce host cell death (Pegg, 1965; Wang et al., 2012), suggesting that they may also be associated with pathogenicity. A high-molecular-weight protein–lipopolysaccharide (PLP) complex has been reported to be associated with disease symptoms in susceptible host plants and to be required for pathogenicity (Buchner et al., 1982). Furthermore, many elicitor-like proteins isolated from *V. dahliae,* such as PevD1 (Wang et al., 2012), VdCP1 (Zhang et al., 2017), and VdNEP (Wang et al., 2004), can induce plant defence responses and cell death.

The Alt a 1 family of proteins (AA1s; Pfam family PF16541) are only present in the Dothideomycetes and Sordariomycetes classes of fungi (Chruszcz et al., 2012). The AA1 produced by the first member ‘Alt a 1 (AA1),’ identified from *A. alternata,* is the source of the name of this protein family (Cramer and Lawrence, 2003). Studies have shown that AA1 is involved in *Alternaria* spore germination and that it is localized exclusively in the spore cell wall (Mitakakis et al., 2001; Twaroch et al., 2012). Alt b 1 (Ab1), a homolog of AA1, has been identified from *A. brassicicola.* High expression of *Ab1* mRNA has been detected during the infection process of Arabidopsis (Cramer and Lawrence, 2004). An AA1 member from *Magnaporthe oryzae* has been demonstrated to bind to the plant plasma membrane and to induce necrosis, the accumulation of ROS, and the expression of several defence-related genes, suggesting that it has a PAMP role (Zhang et al., 2017b). To date, numerous AA1 genes have been identified from over 70 species, although the biological functions of AA1 proteins with respect to plant–fungus interactions remain unclear.

PevD1 has previously been isolated from *V. dahliae* culture filtrates and shown to exhibit an elicitor-like activity by inducing defence responses in tobacco plants (Wang et al., 2012). PevD1-induced disease resistance in tobacco has been demonstrated to be modulated by an asparagine-rich protein, NbNRP1 (Liang et al., 2018). Interestingly, overexpression of PevD1 in Arabidopsis has been reported to promote early flowering by interacting with AtNRP1 and plants infected with *V. dahliae* flower earlier than uninfected plants (Zhou et al., 2017), implying that PevD1 has a role in mediating plant development during *V. dahliae* infection. However, even though it is one of the most abundant proteins among the many secreted by *V. dahliae,* the biological role of PevD1 in the infection process, and particularly its possible function in pathogenicity, remains unclear. In this study, we determined that PevD1 is an Aa1-like protein, and an interacting protein named GhPR5 was identified from cotton plants. Our findings show that *V. dahliae* secretes PevD1 to inhibit the antifungal activity of GhPR5 as a strategy to resist the plant defence system and to promote fungal infection.

### Materials and methods

**Plant material, culturing of strains, inoculation, and pathogenicity assays**

Plants of cotton (*Gossypium hirsutum*), *Arabidopsis thaliana,* tobacco (*Nicotiana benthamiana*), and tomato (*Solanum lygoperiscum*) were grown as previously described (Frias et al., 2011; Wang et al., 2012; Zhang et al., 2015). Strains of *Verticillium dahliae* and *Botrytis cinerea* were grown on potato dextrose agar (PDA) medium at 25 °C, and *V. dahliae* was cultured in liquid Czapek Dox medium for 5 d at 25 °C for spore production (Wang et al., 2012; Zhang et al., 2015). Ungermened conidia were used as controls in fungal gene expression analysis. *Agrobacterium tumefaciens* AGL-1 was cultured on liquid LB medium (rifampicin 50 μg ml⁻¹) at 28 °C (Liu et al., 2013).

Pathogenicity assays for *V. dahliae* strains were conducted using 2-week-old cotton plants of cv. Guoxin (more tolerant) and Chuangxin (less tolerant), or 4-week-old Arabidopsis, tobacco, and tomato seedlings (Zhang et al., 2017a). For each strain, conidial suspensions at 5 × 10⁶ ml⁻¹ in sterile water were prepared. Plant seedlings were grown in soil in individual pots, and 15 pots were prepared for each strain, with this set-up replicated three times. Samples of the conidial suspension (15 ml) were aliquoted into 15 separate trays, and each pot was placed in a tray until complete absorption of the liquid had occurred. The inoculated seedlings were then grown for 2–3 weeks at 28 °C, with a day/night period of 16/8 h, and disease symptoms were observed at 21 d post-inoculation on cotton or 15 d post-inoculation on tobacco and tomato. Vascular discoloration was observed by cutting the shoots at 21 d post-inoculation on cotton. For fungal biomass quantification, roots of three inoculated plants were harvested at 21 d after inoculation and ground to powder for genomic DNA extraction. The fungal biomass was determined by qPCR as described previously (Gu et al., 2018), with three biological replicates, and triplicate reactions were performed for each of the biological replicates. *Verticillium EF-1α* was used to quantify fungal colonization, and the cotton 18S gene served as the internal control (Supplementary Table S1 at JXB online).
Quantitative real-time PCR

Roots of host plants (2-week-old cotton and 4-week-old Arabidopsis, tobacco, and tomato) were inoculated with a spore suspension as described above. At the same time, spore suspensions were spread on plates containing PDA medium, covered with cellophane, and then cultured. Subsequently, samples (100 mg of roots or the cellophane covering the plates) were collected at the indicated time points to assess the expression levels of pevd1. Total RNA from V. dahliae and V. dahliae-inoculated plants was extracted with an EZNA Fungal RNA Kit (R66840, Omega) and plant RNA kits (ET121 and ER301, TransGen Biotech), respectively. First-strand cDNA was synthesized from the extracted RNA using a cDNA Synthesis kit (AT341, TransGen Biotech). The synthesized cDNA was then used as a template for qPCR, which was performed under the following conditions: an initial 50 °C incubation step for 2 min and a 94 °C denaturation step for 10 min, followed by 40 cycles of 94 °C for 5 s and 60 °C for 34 s. Amplification was performed using an ABI7500 Real-Time PCR system with a qPCR kit (AQ111, TransGen Biotech). *Verticillium* elongation factor 1-α (EF-1α), the cotton 18S gene, *Arabidopsis* Actin2 gene, tobacco EF-1α, and tomato Actin gene were used as internal references to normalize the amount of RNA in different reactions (for primers see Supplementary Table S1). For each gene, qPCR assays were repeated three times, each with three biological replicates. Relative mRNA quantities were calculated from the average values using the ΔΔCt method (Schmittgen and Livak, 2008).

Deletion and complementation of pevd1 and its truncated fragments

*Verticillium dahliae* mutant (Δpevd1) and complemented strains (Δpevd1-Res, Δpevd1-Res1a and Δpevd1-Res1b) were constructed using an *A. tumefaciens*-mediated transformation method (Liu et al., 2013). All primers used are shown in Supplementary Table S1. The *Agrobacterium* strain AGL-1 was used to transform the recombinant plasmids into *V. dahliae* spores.

Expression and purification of proteins and preparation of polyclonal antibodies

PevD1 (or its truncated fragments) without the predicted signal peptide (SP) and termination codon was inserted into the *EcoR*I and *Xho*I sites of the plasmid *pPICZaA*. The recombinant plasmid *pPICZaA-PEVD1* was linearized with the restriction enzyme *Pme*I and was subsequently transformed into *Pichia pastoris* KM71H for expression (Zhang et al., 2017b). The GhPR5 gene without the predicted SP and the vacuole-targeting sequence was inserted into the *BanHI* and *Xho*I sites of the plasmid *pRSeta* (Invitrogen). The recombinant plasmid *pRSeta-GhPR5* was transformed into *E. coli* BL21(DE3)/pLysS for expression. The recombinant *GhPR5* protein was expressed in inclusion bodies, and the purification and refolding of *GhPR5* was carried out as previously described (González et al., 2017; Zhang et al., 2017). Proteins were detected by SDS-PAGE (RTD6110, Real-Times, Beijing), and were frozen at −80 °C in small aliquots until use. The protein concentration was measured using a BCA™ Protein Assay Kit (R66840, Omega) and plant RNA kits (ET121 and ER301, TransGen Biotech). The purified *GhPR5* was used as an antigen for antibody production. Antibodies were produced in rabbits by the HuaAn Biotechnology Company.

Treatment of *V. dahliae* strains with GhPR5

The *V. dahliae* strains WT, *apd1*, *apd1-Res*, *apd1-Res1a*, and *apd1-Res1b* were tested for their response to GhPR5. Following the method of previous studies (Sella et al., 2014; Quarantin et al., 2016), microtiter plate wells were filled with 200 μl potato dextrose broth (PDB, pH 6.85) containing purified GhPR5 (2 μM) or GhPR5 plus PevD1 (or plus PevD1a/PevD1b, 2 μM). Controls were carried out with buffer instead of the protein solution. A final concentration of 106 spores of *V. dahliae* strains were added into the PDB, and 68 μg ml−1 of resazurin dye (R7017, Sigma-Aldrich) was added to each well to measure fungal growth. The plates were incubated at 25 °C in the dark and the absorbance was measured at 578 nm over a time-course to evaluate *V. dahliae* growth inhibition. The same experiment was also carried out to assess the activity of refolding GhPR5, with 0.2 U of β-1,3-glucanase (67138, Sigma-Aldrich) and BSA (2 μM) used as positive and negative controls, respectively. Measurements were repeated at least twice, each with three biological replicates. In addition, *V. dahliae* spores treated with GhPR5 or GhPR5 plus PevD1 (or PevD1a/PevD1b) for 12 h were used for pathogenicity assays as described above.

Yeast two-hybrid assays

To confirm the protein interactions, experiments were performed using the Matchmaker™ Gold Yeast Two-Hybrid (Y2H) System as described previously (Zhou et al., 2017). The coding sequences of PevD1 (without the SP or its truncated fragments) and GhPR5 (without the SP) were cloned into the bait vector pGBK17 (BD–*pevd1*) and the prey vector pGADT7 (AD–*GhPR5*), respectively. The bait and prey vectors were co-transformed into the Gold Yeast strain, and the subsequent assays were performed according to the manufacturer’s instructions. The transformants were selected on synthetic dextrose medium without Trp and Leu (SD/−Trp/−Leu); double dropout, DDO). Interaction were indicated by the ability of transformants to grow on SD medium lacking Trp, Leu, His, and Ade (SD/−Trp/−Leu/−His/−Ade; quadruple dropout, QDDO).

Transient expression, pull-down assays, and western blot analysis

The genes *pevd1* and *pevd1-ASP* were inserted into the *EcoRI* and *Kpn*I sites of plasmid *pYBA1132*, which contains a GFP tag (green fluorescent protein; Yan et al., 2012). *Agrobacterium* (*GV3101*)-mediated transient expression was performed using leaf infiltration as described previously (Kettles et al., 2017), and this experiment was performed three times with at least six leaves infiltrated each time. Proteins were subsequently extracted from tobacco leaves as previously described (Zhu et al., 2017). For pull-down assays, 10 g of roots or leaves from 2-week-old cotton inoculated with *V. dahliae* spore suspension were collected at 4-d post-inoculation, then frozen with liquid nitrogen and ground in a mortar. Then, 35 ml of protein extraction buffer [50 mM Tris, 200 mM NaCl, 1% Triton X-100, 1 mM PMSF, 0.2% protease inhibitor cocktail (P9599, Sigma), pH 8.0] was added to the tissue powder, and the mixture was transferred to a 50-ml tube and incubated at 4 °C for 2 h with slight shaking. After centrifuging at 5000 g for 5 min, the supernatant was passed through filter paper and then concentrated by using a 3-kD ultra-filtration device. The protein concentrate was mixed with PevD1 (or two different proteins were mixed) and incubated for 2 h at 4 °C with slight shaking, then 20 μl Anti-HA Affinity Gel beads (E5779, Sigma-Aldrich) was added into the protein mixture and the manufacturer’s instructions were followed. Proteins were separated by SDS-PAGE and transferred onto membranes (PVDF), after which the blots were analysed using the appropriate antibodies. The pixel intensities on images of the western blots were quantified using the ImageJ software. The pull-down and western blot assays were repeated at least twice.

Determination of the endogenous levels of salicylic acid

Cotton root tissues inoculated with either a *V. dahliae* spore suspension or water were collected at 4-d post-inoculation, weighed, and frozen in liquid nitrogen. The endogenous salicylic acid (SA) content of the samples was extracted and measured according to previous studies (Bowling et al., 1994; Lv et al., 2016). The assay was carried out at least three times.

Bioinformatics analysis

Amino acid sequence alignment was performed using the MUSCLE software (http://www.drive5.com/muscle/) and processed with AL2CO (Pei and Grishin, 2001) to generate conservation indices for every amino acid. The phylogenetic tree was reconstructed with MEGA 6.0 (Tamura et al., 2017).
et al., 2013) using the maximum-likelihood method. The crystal structures previously determined for the PevD1 protein (Zhou et al., 2017) and predicted for the GhpPR5 protein using SWISS-MODEL (https://swissmodel.expasy.org/interactive) were used. The protein structure was analysed using the PyMOL Molecular Graphics System, v.2.0, and Poisson–Boltzmann electrostatic potentials were computed with the APBS plugin in PyMOL. Protein–protein docking model structures for the PevD1–GhpPR5 complex were obtained using ClusPro 2.0 (https://cluspro.bu.edu/login.php).

Results

PevD1 is an Alt a 1-like protein

PevD1 (VDAG_02735) has previously been identified as an abundant protein in the culture medium of V. dahliae (Wang et al., 2012). The protein contains 155 amino acids and has a predicted SP at the cleavage site between amino acids 18 and 19 (SignalP 4.1 server; http://www.cbs.dtu.dk/services/SignalP/), indicating that PevD1 may be a secretory protein. The mature protein (without the SP) has a predicted molecular mass of 14,523.1 Da and a pI of 4.18. Our amino acid sequence analysis revealed that it has a low molecular weight, four conserved cysteine residues (C70, C84, C125, and C135), and is homologous to three characterized protein members of the AA1 family, namely Alt a 1 (Chruszcz et al., 2012), Alt b 1 (Cramer and Lawrence, 2003), and MoHrpl1 (Zhang et al., 2017), as well as several typical protein members from different species (Fig. 1A). Phylogenetic analysis indicated that homologs of PevD1 are present across a number of important plant pathogens (Fig. 1B). As AA1s are widely distributed and well conserved among the Dothideomycetes and Sordariomycetes classes of fungi, conserved protein region(s) are present in this protein family. To identify such regions in PevD1, an alignment of 36 AA1 proteins was analysed to identify regions of the sequences that are strongly conserved among these family members (Fig. 1C). The results identified three conserved regions in AA1s in the crystal structure of PevD1 (Zhou et al., 2017) (Fig. 1D). PevD1 was highly similar to Aa1 (Chruszcz et al., 2012) (Fig. 1E). Together, these data indicated that PevD1 is a member of AA1 family.

PevD1 contributes to the pathogenicity of V. dahliae

To characterize the function of PevD1 during infection, the full-length protein or PevD1-ΔSP (without SP) was transiently expressed in N. benthamiana by agro-infiltration. The results showed that PevD1 induced distinct necrosis in leaves while the control did not (Fig. 2A) and PevD1-ΔSP was only able to induce slight necrosis, suggesting that it was specifically induced by PevD1. Western blot analysis with anti-GFP antibodies showed that PevD1-GFP and PevD1-ΔSP-GFP were successfully expressed in plant leaves (Fig. 2B).

To investigate whether PevD1 has an important role in the interaction between V. dahliae and the host, a qPCR analysis was carried out to determine the mRNA levels of pevd1 at various stages of infection in hosts and during growth on PDA medium. The mRNA levels of pevd1 increased continuously during the infection process compared to ungerminated conidia in all the hosts studied, and were significantly higher compared with the expression level observed in cells grown on PDA (Fig. 2C). These results indicated that pevd1 was expressed during infection, especially in the later stages, and suggested that PevD1 plays an important role in the infection process. To further test this hypothesis, Δpevd1 and Δpevd1-Res strains were generated (Supplementary Fig. S1). The phenotypes of these strains with respect to their mycelium/spore morphology, growth rate/colony morphology, and sporulation capacity did not differ from those of the wild-type (WT) strain (Fig. 2D–F). However, the Δpevd1 strains exhibited reduced virulence and the Δpevd1-Res strains exhibited restored virulence compared with the WT strain in cotton (Fig. 2G). Quantification in cotton plants using qPCR showed that inoculation with the Δpevd1 strains resulted in significantly less in planta fungal biomass compared with plants inoculated with the WT and Δpevd1-Res strains (Fig. 2G). In addition, tobacco and tomato plants inoculated with these V. dahliae strains showed similar symptom phenotypes to cotton (Supplementary Fig. S2), suggesting that PevD1 is required for full virulence of V. dahliae on hosts.

Cotton PR5-like protein co-purifies with PevD1 as determined by in vitro affinity pull-down

Given PevD1 was shown to be a secretory protein that contributes to virulence, unknown target(s) for it must be present in host cells. To identify cotton protein(s) targeted by PevD1, a pull-down assay was performed. Total leaf and root proteins were extracted from cotton plants at 4 d after inoculation with V. dahliae. PevD1 (expressed in P. pastoris, Supplementary Fig. S3) was mixed with cotton leaf or root extracts, followed by purification with a HA Affinity Gel. After elution, a 25-kD protein band was co-purified with PevD1 from both cotton leaf and root extracts, whereas no additional band appeared in the control assays with extracts alone (Fig. 3). The band was cut from the gel and analysed by mass spectrometry (MS) for protein identification, which revealed a match with the G. hirsutum osmotin-like protein (UniProt No. Q2HPG3), which we designated as GhpPR5.

Osmotin, named on the basis of its induction by osmotic stress (Singh et al., 1985), belongs to the PR5 family. Osmotins and other PR5s have been shown to exhibit antifungal activity against a broad range of plant pathogens (Abad et al., 1996; Min et al., 2004; Tjamos et al., 2005; van Loon et al., 2006; Anil Kumar et al., 2015). GhpPR5, an osmotin-like protein, contains 242 amino acids and has a predicted N-terminal SP to secrete it into the extracellular space and a vacuole-targeting C-terminal signal to transport it to vacuoles. The amino acid sequence of GhpPR5 exhibited five conserved amino acid residues that were also present in the antifungal enzymatic-activity region of PR5s, showing that it is homologous to PR5s (Fig. 4A). The structure of GhpPR5 exhibited the highest identification score with osmotin from tobacco (Fig. 4B). Protein–protein docking calculations were carried out using the experimental X-ray structure of PevD1 (PDB ID: 5XMZ) and the predicted structure of GhpPR5. The best complex candidates indicated the formation of PevD1–GhpPR5 aggregates in the electrostatically
PevD1 promotes *Verticillium dahliae* infection in cotton

**Fig. 1.** Characteristics of the Alt A1 protein member PevD1. (A) Sequence alignment of selected PevD1 homologs was performed using MUSCLE. Identical amino acid residues are highlighted in red, similar residues are indicated by magenta (80–100% identity of amino acid residues) and light blue (60–80% identity of amino acid residue). The GenBank accession numbers of *Verticillium dahliae* PevD1 and its homologs are indicated. (B) The evolutionary relationships between PevD1 and its homologs from other fungi was determined using the maximum-likelihood algorithm. (C) Conservation indices (grey bars) were calculated using AL2CO for every PevD1 residue from the alignment of 36 complete AA1 proteins. Zero corresponds to the average conservation index for the whole protein. Regions 1–3 indicate where the conservation indices that are well above average. (D) The structure of PevD1 (PDB ID: 5XMZ) was shown as a diagram and surface views. The three conserved regions (1–3) are shown in the structure of PevD1, and correspond to magenta, blue, and yellow. (E) Structures of PevD1 (red) and Alt a 1 (green, PDB ID: 3VOR) were aligned using PyMOL. (This figure is available in colour at *JXB* online.)
Fig. 2. Expression patterns and biological function of PevD1. (A) PevD1-induced necrosis in Nicotiana benthamiana. Leaves of were infiltrated with Agrobacterium tumefaciens containing the empty vector pYBA1132, pYBA1132-pevd1, or pYBA1132-pevd1-ASP. The images were taken 3 d after agro-infiltration. (B) Western blot analysis (anti-GFP) of the transiently expressed proteins obtained from the three agro-infiltrated leaves. Coomassie brilliant blue (CBB) is shown as a total protein loading control. (C) Expression profiles of pevd1 compared to ungerminated spores (0 h), as determined by qPCR. The expression levels of pevd1 in Verticillium dahliae-inoculated plants (or PDA plates, potato dextrose agar) are shown as mRNA levels relative to those in ungerminated spores (0 h, set as 1). Data are means (±SD) of three replicates. Significant differences were determined using Student’s t-test: *P<0.05, **P<0.01. (D–F) The phenotypes of the Δpevd1 and Δpevd1-Res strains with respect to their mycelium/spore morphology, growth rate, and sporulation capacity compared with the wild-type (WT) strain. Data are means (±SD) and each assay was replicated three times. No significant differences compared with the WT were found (Student’s t-test). (G) Disease symptoms in cotton plants (G. hirsutum cv. Chuangxin) at 21 d post-inoculation (top) and discoloration of shoot longitudinal sections (bottom), together with qPCR analysis of fungal biomass from 100 mg of ground roots, expressed relative to that of the wild-type. Data are means (±SD) of three replicates and different letters indicate significant differences as determined by ANOVA and post hoc Tukey’s test: P<0.01.
To further determine whether PevD1b contributes to full virulence in the V. dahliae–plant interaction, we generated the complemented strains Δpevd1-Res1a (re-introduction of the pevd1a gene into the pevd1 knockout mutant) and Δpevd1-Res1b (re-introduction of the pevd1b gene into the pevd1 knockout mutant; Fig. 5C, Supplementary Fig. S5). We found that the Δpevd1-Res1a strain displayed decreased virulence and the Δpevd1-Res1b strain showed similar virulence compared with the WT strain (Fig. 5F). Quantification of fungal biomass in cotton plants as determined by qPCR showed that inoculation with the Δpevd1-Res1b strains resulted in significantly more in planta fungal biomass compared with plants inoculated with the Δpevd1.1 and Δpevd1-Res1a strains (Fig. 5F).

GhPR5 plays important roles in the interactions between V. dahliae and its hosts

GhPR5 was identified as a target protein of PevD1 and it can interact with PevD1, suggesting that it has a significant role in the plant defence system against V. dahliae. Pathogenicity tests were first carried out on two different cotton cultivars with differing tolerance to V. dahliae, cv. Guoxin (more tolerant) and Chuangxin (less tolerant). Guoxin showed less severe disease symptoms and significantly less in planta fungal biomass than Chuangxin (Fig. 6A). We next tested whether the severity of the disease was related to the activation of cotton defence responses. The SA pathway is known to be important for plants to protect themselves against V. dahliae, and the downstream protein PR5 of the SA pathway is a determinant for resistance to V. dahliae (Tjamos et al., 2005). We therefore determined the SA contents in the roots of the two cultivars and found that the concentrations were significantly higher at 4 d after inoculation compared with controls, and the SA concentration in Guoxin was significantly higher than that observed in Chuangxin (Fig. 6B). We also used qPCR to examine the level of GhPR5 expression and found that the relative increase in GhPR5 mRNA levels in Guoxin was more than that observed in Chuangxin (Fig. 6C). In addition, mRNA levels for PR5s were observed to increase in every host studied during V. dahliae infection (Supplementary Fig. S6). Given that the mRNA background level of GhPR5 may have been different between the two cultivars, western blot assays were carried out to determine the GhPR5 protein levels. The results confirmed that the production of GhPR5 protein was induced by V. dahliae in both cultivars (Fig. 6D). The signal intensity of GhPR5 induced by V. dahliae in Guoxin was significantly higher than that in Chuangxin, which was consistent with the results for SA content. These data suggest that GhPR5 is an important determinant for cotton resistance against V. dahliae.

PevD1 protects V. dahliae against the antifungal activity of GhPR5

We tested the sensitivity of different V. dahliae strains to the presence of GhPR5 and found that the growth of Δpevd1.1 and Δpevd1-Res1a were significantly inhibited compared to the WT, Δpevd1-Res1, and Δpevd1-Res1b strains (Fig. 7A), suggesting that PevD1b plays a crucial role in protecting V. dahliae against GhPR5. A growth-inhibition assay of the WT strain...
was also performed using different protein PevD1 constructs, which indicated that PevD1b but not PevD1a decreased the antifungal activity of GhPR5, although the inhibition was not complete (Fig. 7B). We also examined the effect of a pre-co-incubation with GhPR5 on \( V. dahliae \) virulence in which cotton plants were inoculated with \( V. dahliae \) WT conidia that had been germinated with GhPR5 (2 \( \mu \)M) or with GhPR5 plus a PevD1 protein for 12 h. The virulence of \( V. dahliae \) incubated with GhPR5 alone was significantly reduced compared to that of \( V. dahliae \) incubated with GhPR5 and PevD1 or PevD1b (Fig. 7C), indicating that PevD1/PevD1b could protect \( V. dahliae \) by inhibiting the antifungal activity of GhPR5.

**Discussion**

Among the vast array of proteins secreted by \( V. dahliae \), one of the most abundant is PevD1 (Wang et al., 2012). Our previous studies indicated that PevD1 could induce plant defence responses (Wang et al., 2012) and interact with NRP1 proteins, which mediated early flowering in Arabidopsis (Zhou et al.,...
Fig. 5. PevD1b interacts with GhPR5 and is sufficient for fungal virulence. (A) The sensitivity of the Verticillium dahliae wild-type (WT) strain in the presence of GhPR5. Growth was determined spectrophotometrically by measuring the absorbance of the resazurin vital dye at 578 nm. BSA at the same concentration was used as a negative control and glucanase was used as a positive control. Data are means (±SD) of three replicates and significant differences were determined using Student’s t-test: **P<0.01. (B) The physical interaction of PevD1 and GhPR5 in vitro was verified by a pull-down assay. PevD1 and a HA tag were incubated in binding buffer containing HA beads with or without GhPR5 with a His tag; the beads were then washed five times and eluted for western blot analyses. The HA and His tags did not appear due to their low molecular masses. (C) Schematic diagram of the generation of the truncated complemented mutants fused with a signal peptide (SP). (D) Verification of a physical interaction between PevD1b and GhPR5 in vitro by pull-down assay. The HA and His tags did not appear due to their low molecular masses. (E) Yeast two-hybrid analysis of the interaction between PevD1 and GhPR5. Double dropout (DDO, SD/–Trp/–Leu,) and quadruple dropout (QDO SD/–Trp/–Leu/–Ade/–His,) were used as non-selective and selective media, respectively. (F) Disease symptoms in cotton plants (G. hirsutum cv. Chuangxin) at 21 d post-inoculation (top) and discoloration of shoot longitudinal sections (bottom), together with qPCR analysis of fungal biomass from 100 mg of ground roots, expressed relative to that of the wild-type. Data are means (±SD) of three replicates and different letters indicate significant differences as determined by ANOVA and post hoc Tukey’s test: P<0.01. (This figure is available in colour at JXB online.)
Zhang et al. (2017) and disease resistance in tobacco (Liang et al., 2018). As the only Aa1-like protein in *V. dahliae*, the pathogenic function of PevD1 during infection has remained elusive. In the present study, we first found that PevD1 contributes to *V. dahliae* pathogenicity in cotton via its interaction with the protein GhPR5. The mRNA levels of *pevd1* showed up-regulation during the early stages of infection in a variety of hosts (Fig. 2C). Although the phenotypes of the Δ*pevd1* strains were not discernibly different from those of the WT strain with respect to their growth rate, sporulation capacity, and mycelium/spore morphology (Fig. 2D–F), they displayed significant decreases in their virulence against hosts, and complementation of *pevd1* resulted in restoration of virulence to the levels observed for the WT strain (Fig. 2G).

To date, few studies have investigated whether AA1 family proteins have roles in plant pathogenesis. Mitakakis et al. (2001) showed that high amounts of Aa1 are released in germinating compared to ungerminated conidia, corresponding to our qPCR results for *pevd1* (Fig. 2C). These data suggest that PevD1 plays an important role in the pathogenicity of *V. dahliae*.

We formed a hypothesis that PevD1 can act as an effector to contribute to *V. dahliae* infection, and thus we attempted to identify the interacting protein partner of PevD1 in cotton using pull-down assays. A PR5-like protein was isolated and was designated as GhPR5. Many investigations have shown that PR5s can be induced by pathogen attacks (e.g. Anil Kumar et al., 2015). One of the best-characterized properties of PR5s is their antimicrobial activity against a wide variety of organisms, including *B. cinerea*, *P. infestans*, *V. dahliae*, *V. alboatrum*, and *F. oxysporum*, and overexpressing a PR5-like protein in plants can enhance their resistance to pathogenic fungi (Anil Kumar et al., 2015). AtPR5 in Arabidopsis has been reported to show a stronger induction after inoculation with *V. dahliae* and is considered as a determinant of resistance (Tjamos et al., 2005).

In our present study, the results showed that GhPR5 was markedly induced by *V. dahliae* infection (Fig. 5A) and possessed anti-fungal activity in vitro (Fig. 6D). Several PR5 proteins with antifungal activity have been reported to exhibit β-1,3-glucanase activity (Grenier et al., 1999; Menu-Bouaouiche, 2003; van Loon et al., 2006), although there is no clear evidence...
PevD1 promotes *Verticillium dahliae* infection in cotton

regarding the correlation between the two activities. Whether GhPR5 possesses β-1,3-glucanase activity requires further investigation.

Chitinase is a PR protein produced in plant cells after a pathogen infection (van Loon et al., 2006) and is able to degrade fungal cell walls into chitin oligomers that can be recognized by plant cell LysM receptors (Miya et al., 2007), thus triggering the plant immunity system and protecting them from infection. Two cerato-platanin family proteins from *Fusarium graminearum* and *V. dahliae* have been observed to bind to chitin and to protect cell wall polysaccharides from chitinase degradation (Quarantin et al., 2016; Zhang et al., 2017a). MoHrip1 and Aa1, homologs of PevD1, have been shown to partially localize to the cell wall (Twaroch et al., 2012; Zhang et al., 2017b), and therefore we suggest that PevD1 may become enriched in cell walls to protect them from being degraded by GhPR5. As shown in the present study, GhPR5 was more active against Δpevd1 mutants than against the WT strain (Fig. 7), and the anti-*V. dahliae* activity of GhPR5 was decreased *in vitro* by the addition of PevD1, which corresponded to the results of assays showing that PevD1 contributed to virulence (Fig. 2G).

Fungal cell walls contain a crucial β-1,3-glucan structural component (Fesel and Zuccaro, 2016) that can act as an elicitor of plant immune responses, including SA-dependent defence (Klarzynski et al., 2000). PevD1 may contribute to virulence by protecting the cell wall, thus allowing fungal growth to be maintained and preventing the recognition of degraded β-1,3-glucan by the plant.

PR5s have been shown to be common host proteins involved in defence responses and possess antifungal activity against many plant pathogens. In addition to PevD1, several effectors have been previously described to interact with PR5s, such as BEC1054 from *Blumeria graminis* (Pennington et al., 2016) and BcIEB1 from *B. cinerea* (González et al., 2017); thus,

![Fig. 7. PevD1b protects *Verticillium dahliae* against the antifungal activity of GhPR5.](image-url)

(A) The sensitivity of different *V. dahliae* strains to the presence of GhPR5: WT, wild-type. Growth was determined spectrophotometrically by measuring the absorbance of the resazurin vital dye at 578 nm. Data are means (±SD) of three replicates and different letters indicate significant differences as determined by ANOVA and post hoc Tukey’s test: *P* < 0.01. (B) The sensitivity of the *V. dahliae* WT strain to the presence of GhPR5 with or without PevD1 (and its truncated fragments). Data are means (±SD) of three replicates and different letters indicate significant differences as determined by ANOVA and post hoc Tukey’s test: *P* < 0.01. (C) Representative images showing symptoms in cotton plants (*G. hirsutum* cv. Chuangxin) at 21 d after being inoculated with the WT strain pre-incubated with different protein solutions, together with qPCR analysis of fungal biomass from 100 mg of ground roots. Data are means (±SD) of three replicates and different letters indicate significant differences as determined by ANOVA and post hoc Tukey’s test: *P* < 0.01. (This figure is available in colour at *JXB* online.)
infection. (This process is illustrated in Fig. 8.) V. dahliae, recognized by the cell via unknown receptors and initiate cellular signalling

PeVd1 leads to an interaction with GhPR5 and protects the fungus against cellular signalling and results in the production of GhPR5. Secretion of PeVd1 can cause necrosis in tobacco (Frías et al., 2016), its antifungal activity of GhPR5 in cotton and promoted PR5s may be targets to be attacked by pathogens. Although BcIEB1 can cause necrosis in tobacco (Frías et al., 2016), its antifungal activity of GhPR5 in cotton and promoted

Fig. 8. Proposed model of PeVd1 function during Verticillium dahliae–host interaction. Upon infection, a receptor localized in the plant plasma membrane recognizes an unknown molecule of V. dahliae, which activates cellular signalling and results in the production of GhPR5. Secretion of PeVd1 leads to an interaction with GhPR5 and protects the fungus against its antifungal activity. In addition, non-interacting fragments of PeVd1 are recognized by the cell via unknown receptors and initiate cellular signalling that induc necrosis to provide nutrients for V. dahliae infection. (This figure is available in colour at JXB online.)

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Deletion and complementation of pevd1.

Fig. S2. Example images showing the disease symptoms at 21 d post-inoculation in tobacco and tomato plants inoculated with V. dahliae wild-type, ∆pevd1, and ∆pevd1-Res1 strains compared with a mock inoculation.

Fig. S3. The expression and purification of PeVd1 and its truncated fragments.

Fig. S4. The expression and purification of GhPR5 and tag peptides.

Fig. S5. Complementation of pevd1 fragments.

Fig. S6. Expression levels of PR5s in Arabidopsis, tobacco, and tomato plants inoculated with V. dahliae.

Fig. S7. GhPR5 does not interfere with the necrosis-inducing activity of PeVd1.

Fig. S8. Assays of the necrosis-inducing ability of PeVd1.

Table S1. List of primers used in this study.

Acknowledgements

We gratefully acknowledge X.F. Dai of the Chinese Academy of Agricultural Sciences for providing the vectors for gene knockout and complementation. We thank L. Yao of the Beijing Academy of Agriculture and Forestry for providing the pYBA1132 plasmid. This work was supported by the National Natural Science Foundation of China (31772151 and 31272086).

References

Abad Lr, D’Urzo Mp, Liu D, Narasimhan Ml, Reuveni M, Zhu Jk, Niü X, Singh Nk, Hasegawa Pm, Bressan Ra. 1996. Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. Plant Science 118, 11–23.

Anil Kumar S, Hima Kumar P, Shravan Kumar G, Mohanalatha C, Kavi Kishor Pb, 2015. Osmotin: a plant sentinel and a possible agonist of mammalian adiponectin. Frontiers in Plant Science 6, 163.

Bowling Sa, Guo A, Cao H, Gordon As, Klessig Df, Dong X. 1994. A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. The Plant Cell 6, 1845–1857.

Buchner V, Nachmias A, Burstein Y. 1982. Isolation and partial characterization of a phytotoxic glycopeptide from a protein–lipopolysaccharide complex produced by a potato isolate of Verticillium dahliae. FEBS Letters 138, 261–264.

Choquer M, Fournier E, Kunz C, Levier C, Pradier JM, Simon A, Viaud M. 2007. Botrytis cinerea virulence factors: new insights into a necrotrophic and polypathogenic fungus. FEBS Microbiology Letters 277, 1–10.

Chruszcz M, Chapman Md, Osinski T, Solberg R, Demas M, Porebski Pj, Majorek Ka, Pomés A, Minor W. 2012. Alternaria alternata allergen Alt a 1: a unique β-barrel protein dimer found exclusively in fungi. The Journal of Allergy and Clinical Immunology 130, 241–247.e9.

Cooper Rm, Wood Rks. 1980. Cell wall degrading enzymes of vascular wilt fungi. III. Possible involvement of endo-pectin lyase in Verticillium wilt of tomato. Physiological Plant Pathology 16, 285–300.

Cramer Ra, Lawrence Cb. 2003. Cloning of a gene encoding an Alt a 1 isoallergen differentially expressed by the necrotrophic fungus Alternaria brassicicola during Arabidopsis infection. Applied and Environmental Microbiology 69, 2361–2364.
Cramer RA, Lawrence CB. 2004. Identification of Alternaria brassicicola genes expressed in planta during pathogenesis of Arabidopsis thaliana. fungal Genetics and Biology 41, 115–128.

Dobinson KF, Grant SJ, Kang S. 2004. Cloning and targeted disruption, via Agrobacterium tumefaciens-mediated transformation, of a trypsin protease gene from the vascular wilt fungus Verticillium dahliae. Current Genetics 45, 104–110.

Enkerli J, Felix G, Boller T. 1999. The enzymatic activity of fungal xylanase is not necessary for its elicitor activity. Plant Physiology 121, 391–397.

Fesel PH, Zuccaro A. 2016. β-glucan: crucial component of the fungal cell wall and elavate MAMP in plants. fungal Genetics and Biology 90, 53–60.

Fradin EF, Thomma BP. 1999. The enzymatic activity of fungal xylanase modulates plant immune responses. Molecular Plant-Microbe Interactions 12, 71–86.

Frías M, González C, Brito N. 2011. BcSp1, a cerato-platanin family protein, contributes to Botrytis cinerea virulence and elicits the hypersensitive response in the host. New Phytopathol 192, 483–495.

Frías M, González M, González C, Brito N. 2016. BcIEB1, a Botrytis cinerea secreted protein, elicits a defense response in plants. Plant Science 250, 115–124.

González M, Brito N, González C. 2017. The Botrytis cinerea elicitor protein BcIEB1 interacts with the tobacco PR5-family protein osmotin and protects the fungus against its antifungal activity. New Phytopathol 118, 11–14.

Govrin EM, Levine A. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen Botrytis cinerea. Current Biology 10, 751–757.

Grenier J, Potvin C, Trudel J, Asselin A. 1999. Some thaumatin-like proteins hydrolyse polymeric beta-1,3-glucans. The Plant Journal 19, 473–480.

Gui YJ, Zhang WQ, Zhang DD, et al. 2018. A Verticillium dahliae extracellular cutinase modulates plant immune responses. Molecular Plant-Microbe Interactions 31, 260–273.

Hu X, Reddy AS. 1997. Cloning and expression of a PR5-like protein from Arabidopsis: inhibition of fungal growth by bacterially expressed protein. Plant Molecular Biology 34, 949–950.

Jones JD, Dangl JL. 2006. The plant immune system. Nature 444, 323–329.

Kettles GJ, Bayon C, Canning G, Rudd JJ, Kanyuka K. 2017. Apoplastic recognition of multiple candidate effectors from the wheat pathogen Zymoseptoria tritici in the nonhost plant Nicotiana benthamiana. New Phytopathol 213, 338–350.

Klaričzynski O, Plesse JM, Juy VC, Kopp M, Klaore AB, Fritig B. 2000. Linear beta-1,3-glucans are elicitors of defense responses in tobacco. Plant Physiology 124, 1027–1038.

Liang Y, Cui S, Tang X, Zhang Y, Qiu D, Zeng H, Guo L, Yuan J, Yang Y. 2018. An asparagine-rich protein NbHrip1 modulate virulence and elicits the hypersensitive response in the host. New Phytologist 211, 1201–1207.

Liu SY, Chen JY, Wang JL, Li L, Xiao HL, Adam SM, Dai XF. 2013. Molecular characterization and functional analysis of a specific secreted protein, elicits a defense response in plants. Plant Science 303.

Lv S, Wang Z, Yang X, Guo L, Qiu D, Zeng H. 2016. Transcriptional profiling of rice treated with MoHrip1 reveal the function of protein elicitor in enhancement of disease resistance and plant growth. Frontiers in Plant Science 7.

Ma Z, Song T, Zhu L, et al. 2015. A Phytophthora sojae glycoside hydrolase 12 protein is a major virulence factor during soybean infection and is recognized as a PAMP. The Plant Cell 27, 2057–2072.

Menu-Bouaouiche L, Vriet C, Peumans WJ, Barre A, Van Damme P. 2003. A molecular basis for the endo-beta 1,3-glucanase activity of the thaumatin-like proteins from edible fruits. Biochimie 85, 123–131.

Min K, Ha SC, Hasegawa PM, Bressan RA, Yun DJ, Kim KK. 2004. Crystal structure of osmotin, a plant antifungal protein. Proteins 54, 170–173.

Mitakakis TZ, Barnes C, Tovey ER. 2001. Spore germination increases allergen release from Alternaria. The Journal of Allergy and Clinical Immunology 107, 388–390.

Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kakuh S, Shibuya N. 2007. CERK1, a LysM receptor kinase essential for antifungal immunity in Arabidopsis. Proceedings of the National Academy of Sciences, USA 104, 19613–19618.

Pegg GF. 1965. Phytoxotoxin production by Verticillium albo-atrum Reinke et Berthold. Nature 206, 1228–1229.

Pei J, Grishin NV. 2001. AL2CO: calculation of positional conservation in a protein sequence alignment. Bioinformatics 17, 700–712.

Pennington HG, Gheorghe DM, Damerum A, Pliego C, Sapan PD, Cramer R, Bindschedler LV. 2016. Interactions between the powdery mildew effector BEC1054 and barley proteins identify candidate host targets. Journal of Proteome Research 15, 826–839.

Pressey R. 1997. Two isoforms of NP24: a thaumatin-like protein in tomato fruit. Phytochemistry 44, 1241–1245.

Quarantin A, Glasenapp A, Schäfer W, Favaron F, Sella L. Involvement of the Fusarium graminearum cerato-platanin proteins in fungal growth and plant infection. Plant Physiology and Biochemistry 109, 220–229.

Schmittgen TD, Livak KJ. 2006. Analyzing real-time PCR data by the comparative Ct method. Nature Protocols 3, 1101–1108.

Sella L, Gazzetti K, Castiglioni C, Schäfer W, Favaron F. 2014. Fusarium graminearum possesses virulence factors common to fusarium head blight of wheat and seedling rot of soybean but differing in their impact on disease severity. Phytopathology 104, 1201–1207.

Sharma P, Gaur SN, Arora N. 2013. In silico identification of IgE-binding epitopes of osmotin protein. PLoS ONE 8, e54755.

Singh NK, Hanaka AK, Hasegawa PM, Bressan RA. 1985. Proteins associated with adaptation of cultured tobacco cells to NaCl. Plant Physiology 79, 126–137.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30, 2725–2729.

Tjamos SE, Fletematikas E, Paplomatases EJ, Katinakis P. 2005. Induction of resistance to Verticillium dahliae in Arabidopsis thaliana by the biocontrol agent K-165 and pathogen-related proteins gene expression. Molecular Plant-Microbe Interactions 18, 555–561.

Twaroch TE, Arcalis E, Sterflinger K, Stöger E, Swoboda I, Valenta R. 2012. Predominant localization of the major Alternaria allergen Alt a 1 in the cell wall of airborne spores. The Journal of Allergy and Clinical Immunology 129, 1148–1149.

van Loon LC, Rep M, Pieterse CM. 2006. Significance of inducible defense-related proteins in infected plants. Annual Review of Phytopathology 44, 135–162.

Velazhahan R, Muthukrishnan S. 2004. Transgenic tobacco plants constitutively overexpressing a rice thaumatin-like protein (PR-5) show enhanced resistance to Alternaria alternata. Biologia Plantarum 47, 347–354.

Wang B, Yang X, Zeng H, Liu H, Zhou T, Tan B, Yuan J, Guo L, Qiu D. 2012. The purification and characterization of a novel hyperensitive-like response-inducing elicitor from Verticillium dahliae that induces resistance responses in tobacco. Applied Microbiology and Biotechnology 93, 191–201.

Wang YJ, Chai CJ, Gou JY, Mao YB, Xu YH, Jiang W, Chen XY. 2004. VdNEP, an elicitor from Verticillium dahliae, induces cotton plant wilting. Applied and Environmental Microbiology 70, 4989–4995.

Yan X, Hui W, Ye Y, Zeng G, Ma R, Mi F, Yao L. 2012. pyBA100: an ease-of-use binary vector with LoxP-FRT recombinase site for plant transformation. Molecular Plant Breeding 10, 371–379.

Yang G, Tang L, Gong Y, Xie J, Fu Y, Jiang D, Li G, Collinge DB, Chen W, Cheng J. 2017. A cerato-platanin protein SsCP1 targets plant PR1 and contributes to virulence of Sclerotinia sclerotiorum. New Phytopathol 120, 1031–1037.

Yang Y, Zhang Y, Li B, Yang X, Dong Y, Qiu D. 2018. A Verticillium dahliae peptidase lyase induces plant immune responses and contributes to virulence. Frontiers in Plant Science 9.

Zhang Y, Gao Y, Liang Y, Dong Y, Yang X, Yuan J, Qiu D. 2017a. The Verticillium dahliae SnodProt1-like protein VdCDP1 contributes to virulence and triggers the plant immune system. Frontiers in Plant Science 8, 1880.

Zhang Y, Liang Y, Dong Y, Gao Y, Yang X, Yuan J, Qiu D. 2017b. The Magnaporthe oryzae Alt A-1 like protein MoHrip1 binds to the plant plasma membrane. Biochemical and Biophysical Research Communications 492, 55–60.
Zhang Y, Liang Y, Qiu D, Yuan J, Yang X. 2017c. Comparison of cerato-platanin family protein BcSpl1 produced in Pichia pastoris and Escherichia coli. Protein Expression and Purification 136, 20–26.

Zhang Y, Zhang Y, Qiu D, Zeng H, Guo L, Yang X. 2015. BcGs1, a glycoprotein from Botrytis cinerea, elicits defence response and improves disease resistance in host plants. Biochemical and Biophysical Research Communications 457, 627–634.

Zhou R, Zhu T, Han L, Liu M, Xu M, Liu Y, Han D, Qiu D, Gong Q, Liu X. 2017. The asparagine-rich protein NRP interacts with the Verticillium effector PevD1 and regulates the subcellular localization of cryptochrome 2. Journal of Experimental Botany 68, 3427–3440.

Zhu W, Ronen M, Gur Y, et al. 2017. BcXYG1, a secreted xyloglucanase from Botrytis cinerea, triggers both cell death and plant immune responses. Plant Physiology 175, 438–456.

Zipfel C. 2008. Pattern-recognition receptors in plant innate immunity. Current Opinion in Immunology 20, 10–16.

Zipfel C. 2009. Early molecular events in PAMP-triggered immunity. Current Opinion in Plant Biology 12, 414–420.