A secreted ribonuclease effector from *Verticillium dahliae* localizes in the plant nucleus to modulate host immunity

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Abstract
The arms race between fungal pathogens and plant hosts involves recognition of fungal effectors to induce host immunity. Although various fungal effectors have been identified, the effector functions of ribonucleases are largely unknown. Herein, we identified a ribonuclease secreted by *Verticillium dahliae* (VdRTX1) that translocates into the plant nucleus to modulate immunity. The activity of VdRTX1 causes hypersensitive response (HR)-related cell death in *Nicotiana benthamiana* and cotton. VdRTX1 possesses a signal peptide but is unlikely to be an apoplastic effector because its nuclear localization in the plant is necessary for cell death induction. Knockout of VdRTX1 significantly enhanced *V. dahliae* virulence on tobacco while *V. dahliae* employs the known suppressor VdCBM1 to escape the immunity induced by VdRTX1. VdRTX1 homologs are widely distributed in fungi but transient expression of 24 homologs from other fungi did not yield cell death induction, suggesting that this function is specific to the VdRTX1 in *V. dahliae*. Expression of site-directed mutants of VdRTX1 in *N. benthamiana* leaves revealed conserved ligand-binding sites that are important for VdRTX1 function in inducing cell death. Thus, VdRTX1 functions as a unique HR-inducing effector in *V. dahliae* that contributes to the activation of plant immunity.

Keywords
host immunity, secreted ribonuclease, VdRTX1, *Verticillium dahliae*
1 | INTRODUCTION

Plant diseases caused by fungal pathogens threaten worldwide agricultural production and food security (Fisher et al., 2012). To combat fungal infection, plants have evolved innate immune responses (Zipfel, 2014). The first layer of defence in response to fungi is activated by plant recognition of pathogen-associated molecular patterns (PAMPs) (Jones & Dangl, 2006; Zipfel, 2008). PAMP-triggered immunity (PTI) is a conserved mechanism in plants, marked by similar sets of physiological responses that contribute to defence, including accumulation of reactive oxygen species (ROS), callose deposition, and induction of defence-related genes (Boller & Felix, 2009; Zipfel, 2008). Typically, plant perception of microbial PAMPs is mediated through pattern recognition receptors (PRRs), which are membrane-localized leucine-rich repeat proteins or kinases, including brassinosteroid insensitive 1 (BRI1)-associated kinase (BAK1) and suppressor of BAK1-interacting receptor kinase 1 (SOBIR1) (Liebrand et al., 2013; Roux et al., 2011). For successful plant colonization and systemic infection, fungal pathogens must overcome host immunity. This is usually achieved by secreting a variety of small proteins called effectors into plant cells (Rovenich et al., 2014). In turn, effectors can also be recognized by the plant surveillance system to trigger a second layer of defence responses termed effector-triggered immunity (ETI) (Lo Presti et al., 2015). ETI often results in a localized cell death, or hypersensitive response (HR), at the zone of invasion, an action that restricts fungal growth into neighbouring plant cells (Dodd & Rathjen, 2010). These flexible mechanisms in the activation or suppression of plant immunity shape the complex arms race between pathogenic fungi and host plants. The repertoire of fungal effectors plays important roles in host infection and subsequent disease development (Bialas et al., 2018). The evidence now accumulated suggests that fungal effectors function in a pathogen lifestyle-dependent manner and their expression may be temporally and spatially regulated (Giraldo & Valent, 2013). Fungal effectors can be divided into two classes based on their sites of action in the plant cell: apoplastic and cytoplasmic. Intracellular effectors can target subcellular organelles, including the nuclei, chloroplasts, and mitochondria (Doehlemann & Hemetsberger, 2013). One prominent example is the Ecp6 effector in the tomato leaf mould fungus Cladosporium fulvum, which sequesters chitin oligosaccharides that are released from the cell walls of invading hyphae during host infection and suppresses chitin-induced plant PTI (de Jonge et al., 2010). Fungal pathogens also secrete many effectors into the cytoplasm to target the phytohormone biosynthetic pathways, which are key signalling molecules in plants that elicit immune responses against a variety of pathogens (Han & Kahmann, 2019); the effectors interfere with the hormone signalling pathways usually by binding to some host enzymes. For instance, the biotrophic maize smut pathogen Ustilago maydis secretes high amounts of the Cmu1 effector encoding a chorismate mutase during plant colonization to channel chorismate into the phenylpropanoid pathway, thereby preventing the biosynthesis of salicylic acid (SA), a central defence signal in plants against biotrophic pathogens (Djamei et al., 2011; Rabe et al., 2013). Fungal effectors targeting the nucleus have also been reported in recent years (Liu et al., 2014; Zhang et al., 2017). The host nuclear-located effector Isc1 from both Phytophthora sojae and Verticillium dahliae is delivered into the host cells to inhibit salicylate metabolism by hydrolysing an SA precursor (Liu et al., 2014). The V. dahliae small cysteine-containing protein VdSCP7 is transported to plant nuclei to inhibit or induce plant defence (Zhang et al., 2017). Functional analysis of nuclear effectors from other pathogens during their interactions with hosts have been reported. The Meloidogyne in-cognita nuclear effector MIEFF1 interacts with Arabidopsis thaliana cytoplasmic glyceraldehyde-3-phosphate dehydrogenase and promotes parasitism in A. thaliana (Truong et al., 2021). The effector MiSSP7 from the ectomycorrhizal fungus Laccaria bicolor localizes to the plant nucleus and interacts with PJAZ6, a negative regulator of jasmonic acid (JA)-induced gene regulation in Populus trichocarpa. This interaction prevents JA-dependent degradation of PJAZ6, resulting in the suppression of JA-induced host defences (Plett et al., 2014).

V. dahliae is a soilborne fungus, and the causal agent of a vascular wilt disease in over 200 plant species, including many economically important crops (Inderbitzin & Subbarao, 2014; Klosterman et al., 2009). The fungus survives in soil for more than 14 years in the form of resting structures called microsclerotia (Wilhelm, 1955). On induction by root exudates, microsclerotia are triggered to germinate and the hyphae that emerge penetrate the root surface (Zhang et al., 2019). Invasive hyphae of V. dahliae colonize the vascular tissues, resulting in leaf wilting and ultimately, in some instances, plant death. Like other pathogens, the secreted proteins, including many toxins and effectors, play important roles during V. dahliae infection (Chen et al., 2016; Gui et al., 2017, 2018; Liu et al., 2013; Qin et al., 2018; Santanam et al., 2013; Zhang et al., 2017; Zhou et al., 2012). These proteins can function both in the apoplastic and cytoplasmic spaces, and some are involved in the modulation of plant immune responses. For example, two glycoside hydrolase 12 (GH12) proteins, VdEG1 and VdEG3, target the apoplast in Nicotiana benthamiana to trigger plant cell death and the PTI response by recognition of VdEG1 or VdEG3 via the plant immune receptors BAK1 and SOBIR1 and can be suppressed by another V. dahliae-secreted protein containing a carbohydrate-binding module family 1 (VdCBM1) (Gui et al., 2017). In addition, the plant nuclear-localized effectors from V. dahliae are also important modulators of host immunity. The nuclear-localized effector VdSCP41 targets the master immune regulators CBFp60g and SARD1 in Arabidopsis and GhCBP60b in cotton to inhibit the transcription of defence genes, thereby promoting virulence of V. dahliae (Qin et al., 2018).

Although multiple roles are ascribed to fungal ribonucleases, secreted ribonucleases may mediate complex interactions between organisms in their environmental niches (Hill et al., 1983; Olombrada et al., 2017a). For instance, anisoplpin from Metarhizium anisopliae shows ribonucleolytic activity and cytotoxicity against
between nontoxic RNases and ribotoxins is that the latter also secrete nontoxic RNases. The main structural difference extends to prokaryotes and yeasts as well (Kettles et al., 2008). Functional roles of secreted ribonucleases in phytopathogens have also been reported. Secreted Zymoseptoria tritici toxic ribonuclease Zt6 is a functional ribonuclease that selectively cleaves both plant and animal rRNA species. In addition, Zt6 functions as an effector and induces plant cell death in a SOBIR1/BAK1-independent way.

Besides these toxic extracellular fungal RNases, fungal species also produce them (Herrero-Galán et al., 2008). Secreted RNases in fungal pathogens block host immune responses through secretion of CBM1-type effectors that can disrupt host immune responses activated by VdRTX1 recognition.

2 | RESULTS

2.1 | V. dahliae VdRTX1 encodes a ribonuclease that induces cell death in N. benthamiana

The genome of V. dahliae strain Vd991 encodes 739 predicted secreted proteins (Chen et al., 2018). Pfam domain analyses among these revealed three ribonuclease domain-containing proteins encoded by the loci VEDA_03750, VEDA_05283, and VEDA_02049, named VdRTX1 (Verticillium dahliae Ribotoxin 1), VdRTX2, and VdRTX3, respectively. Each of these RTX proteins contains an N-terminal signal peptide and a typical ribonuclease domain (Figure 1a). Phylogenetic analysis of these three proteins with representative ribotoxin and nontoxic RNase protein sequences, including the recently identified RNase-like effector (CSEPO0064; Pennington et al., 2019) from the biotrophic fungal pathogen Blumeria graminis, revealed the convergence of nontoxic RNases because VdRTX1 and VdRTX3 shared closer evolutionary ties to the nontoxic RNase F1 (PDB ID: 1FUS; Noguchi, 2010) from Fusarium moniliforme (Figure 1b). In addition, VdRTX1 and VdRTX3 displayed higher sequence identities to the nontoxic RNase than the ribotoxin, and VdRTX2, a possible paralog of VdRTX1 or VdRTX3 (Figure 1b), showed high identities to the nontoxic RNase but not to the ribotoxin (Figure S1). Thus, these results suggested that VdRTX genes may function as the nontoxic RNases in V. dahliae.

Agrobacterium tumefaciens-mediated transformation was used to transiently express each of the three VdRTX genes in N. benthamiana leaves. The sequences encoding VdRTX1, VdRTX2, and VdRTX3, including their secretion signals, were cloned individually into the binary PVX vector pGR107, resulting in a fused C-terminal FLAG tag to each of the proteins (Jones et al., 1999). The pGR107 vectors encoding the cell-death-inducing Bcl-2-associated protein X (BAX) and the green fluorescent protein (GFP) gene were used as positive and negative controls, respectively. Interestingly, transient expression of these genes in N. benthamiana demonstrated that only VdRTX1 triggered typical cell death in N. benthamiana leaves 5 days after infiltration (Figure 1c). Immunoblot analysis of the total protein extract from the agroinfiltrated area of the leaves confirmed effective protein production by all three VdRTX genes (Figure 1d). Therefore, these results suggest that VdRTX1, containing a ribonuclease encoding sequence, can trigger cell death in N. benthamiana and potentially is involved in immunity manipulation in host-V. dahliae interactions.

2.2 | VdRTX1 functions as a ribonuclease to induce host immunity in N. benthamiana

We hypothesized that VdRTX1-induced cell death is probably caused by RNA degradation as VdRTX1 encodes a ribonuclease
To test this hypothesis, the dependence of VdRTX1-induced cell death on RNA degradation was determined in cell lysis experiments. As with RNase A, the integrity of rRNA was degraded when incubated with the cell-free protein extracted from \( N. \) benthamiana leaves expressing VdRTX1 at 48 h, resulting in the loss of the main peaks of 18S and 28S rRNA (Figure 2a). However, the positive control of cell-free protein from leaves transiently expressing VdSCP126 (an apoplastic effector that induces cell death on \( N. \) benthamiana leaves) did not result in degradation of the RNA (Figure 2a). These results strongly suggest that VdRTX1 possesses ribonuclease activity and can be used as a functional ribonuclease for RNA degradation.

To test whether cell death induction by VdRTX1 is caused by the immune responses during host–V. dahliae interactions, the characteristics of cell death were determined in VdRTX1-expressing \( N. \) benthamiana leaves, including ROS accumulation and callose deposition. As expected, the \( N. \) benthamiana leaves expressing GFP exhibited no visible ROS or callose deposition at 2 days postinoculation (dpi). By contrast, VdRTX1-expressing leaves showed a strong ROS accumulation and callose deposition at 2 dpi, suggesting that the transient expression of VdRTX1 activates the plant immune responses (Figure 2b–d). To further confirm whether VdRTX1 triggers immune responses in \( N. \) benthamiana, we measured the relative expression of several genes associated with plant defence responses after transient expression. Indeed, the plant defence-related genes, especially the SA signalling genes NbPR1, NbPR2, NbPR5, and GLNb (Seo et al., 2010), were significantly up-regulated in \( N. \) benthamiana leaves 24 h postinfiltration (hpi) (Figure 2e), indicating a strong immune response elicited by VdRTX1 in plants. Notably, the HSR203 gene, a hallmark of HR in plant immunity, was also significantly up-regulated, suggesting that VdRTX1 triggers a plant immune response. Following the determination of the cell-death-inducing function of VdRTX1 in \( N. \) benthamiana, we wanted to examine if it is also common in other plant species, especially cotton. We expressed VdRTX1 protein and analysed its cell-death-inducing function in cotton cotyledons, with \( N. \) benthamiana leaves serving as a control. Twenty-four hours after infiltration, the protein triggered cell death in both \( N. \) benthamiana leaves and cotton cotyledons (Figure S2). These results strongly suggest that VdRTX1 functions as an effector, and the ribonuclease activity is required for VdRTX1-induced host immunity in \( N. \) benthamiana.

2.3 | VdRTX1 is probably not an apoplastic effector

VdRTX1 is predicted to encode a ribonuclease with a signal peptide (Figure 1a). To test the secretion activity of the N-terminal signal peptide of VdRTX1, we performed yeast signal trap assays based on the requirement for secretion of invertases for yeast growth on medium with raffinose as the sole carbon source (Jacobs et al., 1997). The coding sequence of the predicted signal peptide of VdRTX1 (1–17 amino acids) was fused in-frame to the yeast invertase sequence within the vector pSUC2, and transformed into the invertase mutant yeast strain YTK12. YTK12 was independently transformed with a construct encoding the
functional signal peptide of Avr1b or an empty pSUC2 vector as positive and negative controls, respectively (Oh et al., 2009). Like the Avr1b signal peptide positive control, the signal peptide from VdRTX1 also conferred the ability of YTK12 to grow on YPRAA medium (Figure 3a), indicating that the signal peptide of VdRTX1 mediates extracellular secretion of VdRTX1.
As defined previously, fungal effectors can induce plant cell death both in the apoplastic space and in the cytoplasm (Ma et al., 2015). Apoplastic fungal effectors usually rely on the plant receptor-like protein kinase SERK3/BAK1 and/or the suppressor of BAK1-interacting receptor kinase 1 (SOBIR1) to participate in host–pathogen interaction (Albert et al., 2015). Because the VdRTX1 protein induced cell death in plant cells, we hypothesized that this induction might be BAK1/SOBIR1-dependent. To

**FIGURE 3** VdRTX1 functions intracellularly and independently from immune receptors of *Nicotiana benthamiana*. (a) Secretory activity assays of the predicted signal peptide of VdRTX1 from *Verticillium dahliae* using the yeast signal trap system. The predicted signal peptide sequence with two additional amino acids (1–19) of VdRTX1 protein were fused in-frame to the mature yeast invertase, enabling secretion of invertase and resulting in yeast growth on YPRAA medium. CMD–W (minus tryptophan, Trp) plates were used to select yeast strain YTK12 transformed with the pSUC2 vector. Growth on YPRAA was used to indicate invertase secretion. The functional signal peptide of Avr1b was used as a positive control. (b) The silencing efficiency of the immunity receptor genes *NbBAK1* and *NbSOBIR1* on *N. benthamiana*. *N. benthamiana* leaves from 3-week-old plants were subjected to virus-induced gene silencing (VIGS) by inoculation with tobacco rattle virus (TRV) constructs TRV:BAK1, TRV:SOBIR1, and TRV:GFP (green fluorescent protein) as a negative control. The silencing efficiency of *NbBAK1* or *NbSOBIR1* was determined by TRV:BAK1 or TRV:SOBIR1 compared with TRV:GFP using reverse transcription-quantitative PCR (RT-qPCR) analysis with *N. benthamiana EF-1α* as a reference gene. Means and standard errors from three biological replicates are shown. Asterisks ** indicate significant differences (*p* < 0.01). (c–e) Analysis of the associations of BAK1 and SOBIR1 with VdRTX1. VdRTX1 was transiently expressed in gene-silenced plants that were subjected to VIGS by inoculation with TRV constructs for 3 weeks: TRV:BAK1 (c), TRV:SOBIR1 (d), and TRV:GFP (e). Transiently expression of BAX and GFP in the gene-silenced leaves were used as positive and negative controls, respectively. The phenotypes of induced cell death were photographed 5 days later. (f) Immunoblot analysis of VdRTX1 proteins transiently expressed in gene-silenced (*NbBAK1* or *NbSOBIR1*) *N. benthamiana* leaves 5 days following agroinfiltration. Ponceau S-stained RuBisCO protein was used as a total protein loading control. (g) Cell-death-inducing activity analyses using a construct expressing the VdRTX1 protein without the signal peptide (VdRTX1SP). Close to the right of the schematic is a photograph of the *N. benthamiana* leaves transiently expressing VdRTX1SP protein. The right-most line indicates immunoblotting results of expressed proteins, in which the top asterisk (*) stands for immunoblotted protein and the bottom hash (#) indicates Ponceau S-stained RuBisCO protein used as a total protein loading control.
test this hypothesis, we constructed virus-induced gene silencing (VIGS) vectors based on the recombinant tobacco rattle virus (TRV) for targeted silencing of BAK1 or SOBIR1 gene expression in tobacco. Three weeks after virus inoculation, when plant transcripts of BAK1 and SOBIR1 were significantly decreased by VIGS, as determined by reverse transcription-quantitative PCR (RT-qPCR; Figure 3b), plants were agroinfiltrated with either pGR107-VdRTX1 or pGR107-BAX vectors for transient expression. The results showed that the BAX protein induced normal cell death lesions in BAK1, SOBIR1, and GFP-silenced N. benthamiana leaves at 3 dpi. Similarly, VdRTX1 protein also triggered obvious cell death symptoms on the same leaves (Figure 3c-e). Immunoblotting confirmed that VdRTX1 was successfully expressed, at the expected size, in N. benthamiana silenced with TRV:BAK1, TRV:SOBIR1 or TRV-GFP (Figure 3f). Moreover, transient expression of VdRTX1 lacking the signal peptide sequence (VdRTX1SP) retained the ability to induce cell death in N. benthamiana leaves (Figure 3g), which indicated that VdRTX1 can function in the cytoplasm but not independent of secretion to the extracellular environment by its signal peptide. Thus, our data suggest that the plant cell death induced by the secretion of VdRTX1 from V. dahliae is not dependent on the membrane-localized BAK1/SOBIR1 proteins, supporting the hypothesis that VdRTX1 is probably not an interactor with BAK1 or SOBIR1 in the plant apoplastic space.

2.4 VdRTX1-induced cell death is plant nucleus-dependent

As predicted by cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi), VdRTX1 contains a nuclear localization signal (NLS) sequence (26–57 amino acids) in the mid-region of the protein. Expression of a truncated sequence of VdRTX1 lacking the NLS sequence resulted in the loss of its ability to induce cell death in N. benthamiana leaves 5 dpi (Figure 4a). These results indicate that nuclear localization is essential for the cell-death-inducing function of VdRTX1. To test whether the NLS facilitates nuclear localization, the full-length VdRTX1 was C-terminally fused with the GFP gene in the pBinGFP4 vector to generate pBin-VdRTX1-GFP, which was agroinfiltrated and transiently expressed in N. benthamiana leaves. The truncated sequence of VdRTX1 without the NLS sequence (VdRTX1SNLS) but with a GFP gene fusion was also prepared and inserted into the pBinGFP4 vector for comparative analyses. As expected, infiltration results showed that VdRTX1-GFP protein predominantly accumulated in the plant nucleus; however, the subcellular location of VdRTX1SNLS was disrupted and distributed into the cytoplasm and to the inner sides of the plasma membrane (Figure 4b). These observations suggest that VdRTX1 localizes in the plant nucleus.

To further examine whether nuclear localization of VdRTX1 is required for cell death induction in N. benthamiana, two variants, VdRTX1NES (N-terminally tagged in its coding region with a nuclear export signal [NES]; Du et al., 2015) and VdRTX1mNES (N-terminally fused with a nonfunctional mutated NES sequence; Du et al., 2015), were separately expressed in N. benthamiana. Like the VdRTX1SNLS, VdRTX1NES was exported and VdRTX1mNES was relocated into the nucleus owing to the mutation in the NES sequence (Figure 4b). VdRTX1NES failed to induce cell death because of its failure to locate into the plant nucleus, whereas the VdRTX1mNES protein caused normal cell death owing to its ability for nuclear localization (Figure 4c). Western blots confirmed the successful expression of the two variants in N. benthamiana leaves (Figure 4d). Taken together, these results demonstrate that nuclear localization mediated by the NLS sequence in VdRTX1 is essential to induce plant cell death.

2.5 Putative ligand-binding sites are critical for VdRTX1 function

Fungal RNases are assumed to directly target host RNA molecules for cleavage. To confirm the binding of VdRTX1 to RNA, putative ligand-binding sites were predicted by the I-TASSER server (Yang & Zhang, 2015). The results showed that VdRTX1 contains 13 putative ligand-binding sites (Y68, positions 70–76: HRYNNYE, E88, R106, H121, N127, and F129 (Figure 5a). Sequence alignment of VdRTX1 with the known RNases showed that most of the putative ligand-binding sites display divergence among RNases, except three (E88, R106, and H121) that are absolutely conserved in the ribonuclease family (Figure 5b). To further determine if the function of VdRTX1 depends on the predicted ligand-binding sites, six amino acid residues (Y68, 75Y, R106, H121, N127, and F129) were individually mutated to alanine residues (Figure 5c), and the induction of cell death was examined by transient expression in N. benthamiana leaves (Figure 5d).

Interestingly, although the mutation of putative ligand-binding sites Y75A, R106A, or N127A had no effect on the cell-death-inducing function of VdRTX1, the necrosis phenotype showed that the transient expression of the protein harbouring mutations in Y68A, H121A, or F129A each abolished the cell death activity of VdRTX1 in N. benthamiana leaves (Figure 5e). Based on the importance of ligand-binding sites to ribonuclease activity, we postulated that it may be the mutation of the ligand-binding site in the ribonuclease domain in VdRTX1 that led to the loss of cell death activity. To test our hypothesis, we evaluated the capacity of rRNA degradation of these VdRTX1 mutants using a cell lysis experiment. As shown in Figure S3, similar to the wild-type VdRTX1 and the positive RNase A control, mutants that caused necrosis phenotypes (Y75A, R106A, or N127A) still maintained their ability to degrade rRNA, while mutations that interfered with the cell-death-inducing activity (Y68A, H121A, or F129A) significantly affected their ribonuclease activity. Thus, the ribonuclease activity of VdRTX1 is essential for its cell-death-inducing activity. Any mutation affecting VdRTX1 ribonuclease activity can weaken its necrosis-inducing activity.
FIGURE 4  Cell death in *Nicotiana benthamiana* depends on the nuclear localization of VdRTX1. (a) Identification the cell-death-inducing activity of VdRTX1 protein from *Verticillium dahliae* without the nuclear localization sequence (VdRTX1ΔNLS). Close to the right of the schematic is a photograph of the *N. benthamiana* leaves transiently expressing VdRTX1ΔNLS. The right-most line indicates immunoblotting results of expressed proteins, in which the top asterisk (*) stands for immunoblotted protein and the bottom hash (#) indicates Ponceau S-stained RuBisCO protein used as a total protein loading control. (b) Subcellular localization of green fluorescent protein (GFP)-tagged VdRTX1 variants in *N. benthamiana*. VdRTX1 and VdRTX1ΔNLS, N-terminally tagged in its coding region with a nuclear export signal (VdRTX1NES) and with a nonfunctional mutated NES sequence (VdRTX1mNES) fused with the GFP, were transiently expressed in *N. benthamiana* leaves. The fluorescent signal of GFP was observed using confocal microscopy. GFP served as a negative control. 4′,6-diamidino-2-phenylindole (DAPI) staining was used to visualize the nuclei. Arrowheads point to the nuclei. Bars, 5 µm. (c) Identification of the cell-death-inducing activity of transient expression of VdRTX1NES and VdRTX1mNES in *N. benthamiana* leaves. The wild-type VdRTX1 and GFP were used as positive and negative controls, respectively. The phenotype of cell death was photographed at 5 days following agroinfiltration. (d) Immunoblot analyses to confirm protein expression in *N. benthamiana* leaves transiently expressing the indicated proteins using anti-HA antibodies. RuBisCO stained by Ponceau S was used as a total protein loading control.
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2.6 | VdRTX1-induced plant immune responses can be suppressed by VdCBM1

The activation of plant immune responses by VdRTX1 could limit plant infection by V. dahliae. We hypothesized that V. dahliae might employ effectors for inhibition of VdRTX1-triggered plant immunity to facilitate successful infection. Previously, we had identified a carbohydrate-binding module 1 (VdCBM1) functioning as a suppressor of plant immunity by blocking the immune responses triggered by a broad-spectrum effector from V. dahliae (Gui et al., 2017, 2018; Wang et al., 2020). We therefore tested whether VdCBM1 could suppress VdRTX1-induced plant defences. Strikingly, co-expression of VdRTX1 and VdCBM1 in N. benthamiana leaves did not result in cell death, even 5 days after coagroinfiltration, whereas coexpression of VdRTX1 with the negative control, GFP, resulted in normal cell death (Figure 6a). Immunoblot assays performed on total protein extracted from the coinfiltration areas confirmed the presence of VdRTX1 and coexpression of proteins in the plant tissue (Figure 6b). Conductivity assays from the coinfiltrated areas of N. benthamiana leaves revealed that electrolyte leakage occurred 3 days after coagroinfiltration of VdRTX1 and GFP constructs, but that the electrolyte leakage was significantly reduced following the coagroinfiltration of VdRTX1 and VdCBM1 constructs (Figure 6c). Furthermore, VdRTX1-induced callose deposition and ROS accumulation in N. benthamiana were effectively suppressed by VdRTX1 and VdCBM1 coexpression (Figure 6d,e). As shown above, VdRTX1 triggered immunity by significantly up-regulating SA signalling-related genes (Figure 2e); however, another coinfiltration test with VdISC1, an SA signalling suppressor (Liu et al., 2014), did not suppress the cell death triggered by VdRTX1 (Figure 6a). Therefore, unlike other common effectors, VdRTX1 appears to employ a specific mechanism to activate SA signalling for induction of immunity in N. benthamiana. Together, these results revealed that plant defence responses induced by VdRTX1 can be inhibited by the V. dahliae immunity suppressor VdCBM1.
2.7 Knockout of VdRTX1 enhances the virulence of V. dahliae on tobacco

To gain insight into the possible functions of VdRTX1 in the pathogenesis of V. dahliae, we first determined expression patterns of three VdRTXs during infection of the roots of the natural host cotton plants in a time-course using RT-qPCR. RNA from the axenic culture of V. dahliae was used for comparison. Analyses of the results revealed that although the transcripts of three VdRTXs were up-regulated during V. dahliae infection of cotton, the expression of VdRTX1 was especially high within 5 days after plant inoculation and reached high levels at 2 dpi, suggesting that VdRTX1 responds to plant inoculation and is highly expressed during plant infection (Figure S4). Thus, these data implied that VdRTX1 may be involved in V. dahliae pathogenesis during infection of plant hosts.

Having established that VdRTX1 triggers host immune responses, which could inhibit fungal growth during plant infection, we reasoned that the deletion of VdRTX1 might promote plant infection by V. dahliae. To test this hypothesis, we generated a V. dahliae mutant strain with a VdRTX1 deletion through homologous recombination-based replacement of the region encoding VdRTX1 with a hygromycin resistance cassette in the genome of V. dahliae strain VD8. The resulting gene deletion strain ΔVdRTX1 was confirmed by PCR sequencing and subsequently used for growth and plant infection assays. Deletion of VdRTX1 had no effect on fungal growth on potato dextrose agar (PDA) and the ΔVdRTX1 strain was also capable of producing melanin, similar to the wild-type strain VD8, indicating that VdRTX1 does not play a major role, if any, in fungal growth and development (Figures S5 and S6). However, when the ΔVdRTX1 strain was inoculated to N. benthamiana plants, it caused significantly more disease symptoms than the wild-type strain (Figure 7a). Fungal biomass assays by quantitative PCR confirmed that N. benthamiana plants inoculated with the ΔVdRTX1 strain contained significantly more fungal biomass than those inoculated with the wild-type strain (Figure 7c). Complementation of the...
VdRTX1 deletion mutant with the wild-type VdRTX1 gene restored virulence in complemented strains to levels comparable to those observed for the wild-type strain (Figure 7a,c). These results indicate that VdRTX1 negatively regulates the virulence of *V. dahliae* on *N. benthamiana* plants during pathogenesis. Similarly, we also tested the virulence of the ΔVdRTX1 mutant on cotton plants but the deletion of VdRTX1 did not significantly reduce the virulence of *V. dahliae* (Figure 7b,d), probably because of different interaction mechanisms of VdRTX1 between *N. benthamiana*–*V. dahliae* and cotton–*V. dahliae* pathogenesis. Thus, VdRTX1 is involved in the induction of plant immunity during *V. dahliae* infection of host plants, and differentially contributes to virulence dependent on plant host species.

2.8 | Cell-death-induction function of VdRTX1 homologs specifically confined to *Verticillium* spp.

Secreted ribonucleases are produced by several fungal species to mediate complex interactions between organisms (Olombrada et al., 2017). To examine whether the pathogenicity function of VdRTX1 is widely present in other fungi, the taxonomy of VdRTX1 homologs in fungi was systematically investigated. In total, 1162 homologs that encode predicted secretory ribonucleases (PFAM: PF00545) were identified, including 756 species from Ascomycota and Basidiomycota (Table S1). An identity matrix constructed by the reciprocal BLAST analysis of all VdRTX1 homologs showed that the VdRTX1 homologs are significantly

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**FIGURE 7** Virulence assays of VdRTX1 deletion mutants on *Nicotiana benthamiana* and cotton plants. (a) Disease symptoms of *N. benthamiana* plants inoculated with the various *Verticillium dahliae* strains. Plants inoculated with sterile water (Mock) and wild-type (WT) *V. dahliae* VD8 served as negative and positive controls, respectively. Development of Verticillium wilt symptoms on plants inoculated with the VdRTX1 deletion mutant (ΔVdRTX1) and the corresponding complemented transformant strains (ECVdRTX1-1 and ECVdRTX1-2) was observed and photographs were taken 3 weeks following inoculation. There were three replicates of nine plants. (b) Susceptible cotton (*Gossypium hirsutum* 'Junmian 1') seedlings were root-dipped with sterile water (Mock), wild-type (WT) *V. dahliae* VD8, and the VdRTX1 gene deletion mutant (ΔVdRTX1), along with the corresponding complemented transformant strains (ECVdRTX1-1 and ECVdRTX1-2). Photographs of disease symptoms were taken 3 weeks following inoculation (top). *V. dahliae* colonization-induced plant stem discoloration in longitudinal sections is shown (bottom). There were three replicates of 25 plants each. In planta fungal biomass was assessed by quantitative PCR in *N. benthamiana* (c) and cotton (d) inoculated with the indicated *V. dahliae* strains. Error bars represent standard errors. Asterisks ** indicate significant differences (*p* < 0.01), calculated by unpaired Student’s *t* tests.
divergent with identities at <65% (Figure 8a). Interestingly, one block in the identity matrix that displayed high identity values belonged to the VdRTX1 homologs from *Verticillium* spp. (Figure 8a). This suggested that the VdRTX1 homologs are more highly conserved *Verticillium* spp. than in other genera. To further see if the highly conserved VdRTX1 homologs among *Verticillium* spp. are unique, the identities of reciprocal BLAST within VdRTX1 homologs from 10 selected genera were compared. The results showed that the distribution of identity values from *Verticillium* spp. (median value 96.38%) are significantly higher than in the other nine genera (all median values less than 90%) (Figure 8b). Sequence alignment showed that VdRTX1 homologs from *Verticillium* spp. showed divergence in only 11 residues (Figure 8c), unlike the case from *Fusarium* spp. in which nearly half of the residues of VdRTX1 homologs showed sequence divergence (Figure S7). Finally,}

![Figure 8](image)

**FIGURE 8** Identification and functional analysis of VdRTX1 homologs in *Verticillium* spp. and other fungi. (a) Identity matrix of VdRTX1 homologs from fungi. Reciprocal BLAST analysis of the VdRTX1 homologs was performed using the BLASTP program (e-value < 1e−5) to find all pairwise matches among 1162 VdRTX1 homologs. The identity matrix was constructed with all pairwise combinations. The number in parentheses by each fungal grouping represents the total number of VdRTX1 homologs. The blue blocks at the bottom of the identity matrix represent the selected genus for identity comparison with VdRTX1 homologs from *Verticillium* spp. in (b). Numbers on the left side of the identity matrix represent the 24 selected VdRTX1 homologs examined for cell death detection by transient expression in (d). (b) Identity comparison of VdRTX1 homologs from *Verticillium* spp. with the selected genus. The left and right numbers in the square brackets represent the total number of VdRTX1 homologs and the number of species in the selected genus, respectively. (c) Sequence alignment of the VdRTX1 homologs from *Verticillium* spp. (d) Detection of cell death induction by selected VdRTX1 homologs, as indicated on the left side of the identity matrix in (a). *Nicotiana benthamiana* leaves from 4-week-old plants were examined at 5 days following transient expression of the selected homologs. VdRTX1 and green fluorescent protein (GFP) were used as positive and negative controls, respectively.
VdRTX1 homologs from 24 species were randomly selected from fungi from Ascomycota and Basidiomycota to test the cell-death-induction function on N. benthamiana (Figure 8a and Table S2). Interestingly, none of the candidates induced cell death on N. benthamiana leaves similar to VdRTX1 (Figure 8d), which strongly suggested that VdRTX1 homologs in other fungi are not involved in plant immunity. Thus, the function of VdRTX1 in virulence appears specific to Verticillium spp. among these fungi.

3 | DISCUSSION

Fungal pathogens have evolved sophisticated strategies to combat host defences by delivering an array of secreted effector proteins to modulate plant immune responses (Biafas et al., 2018). These effectors are typically small, highly divergent proteins between fungal species, and even between strains within a single species, making it difficult at best to predict their biological functions through bioinformatic approaches (Francescetti et al., 2017). Here, we identified and characterized VdRTX1, a 138 amino acid, secreted protein from V. dahliae whose expression was induced during early stages of plant infection. Interestingly, VdRTX1 contains a ribonuclease domain very similar to many well-known fungal ribotoxins (Olobrada et al., 2017), suggesting VdRTX1 is an RNase-like protein. VdRTX1 triggered plant immune responses leading to cell death in both N. benthamiana and cotton (Figure 52), but VdRTX1 homologs from other fungi failed to cause cell death in N. benthamiana leaves. Our studies thus demonstrated that VdRTX1 functions as a unique cell-death-inducing effector with RNase activity in Verticillium spp. and contributes to pathogenesis.

The V. dahliae ribonuclease proteins have functional signal peptides (Figure 3a), raising intriguing questions on the purpose of fungal secreted RNases. Our initial hypothesis was that the secreted RNases function primarily in protecting fungi from hostile environmental conditions. For instance, secreted ribonucleases produced by some fungal species, such as sarcin from Aspergillus giganteus, are primarily involved in self-defence and fungal development (Brandhorst & Kenealy, 1992; Yang & Kenealy, 1992). Previous studies have isolated double-stranded RNA viruses from V. dahliae (Cao et al., 2011; Feng et al., 2013), suggesting that V. dahliae can be infected by RNA viruses and, therefore, might have evolved secreted RNases as a protective mechanism. Deletion of VdRTX1 from V. dahliae yielded no observable morphological or developmental phenotype (Figures S5 and S6), suggesting that VdRTX1 in V. dahliae might not be directly involved in regulating development. The plant cell-death-inducing ability of Z. tritici Zt6 and its broad toxicity is conserved in other prokaryotes and yeasts (Kettles et al., 2018); this finding suggests that Zt6 may have important antimicrobial and niche protection roles. Whether VdRTX1 is also antimicrobial remains to be analysed.

When VdRTX1 was transiently expressed in N. benthamiana leaves, it triggered cell death similar to that caused by the HR elicitor BAX (Lacomme & Santa Cruz, 1999). Although we cannot exclude the possibility of the toxicity of VdRTX1 causing cell death as a result of the direct cleavage of N. benthamiana rRNA by VdRTX1 RNase activity, there is an alternative possibility that VdRTX1 might be recognized by the host plant to induce host immunity and thus act as an Avr protein. Indeed, the results indicated that transiently expressed VdRTX1 in N. benthamiana leaves induced typical plant defence responses (Figure 2c.d), besides its direct toxicity. This mechanism is similar to the well-known Nep1-like proteins (NLPs) in many phytopathogens, which play dual roles as both toxin-like virulence factors and elicitors of plant innate immune responses (Lenarčič et al., 2019; Oome et al., 2014; Ottmann et al., 2009; Quot et al., 2006). The NLP family of proteins is also present in V. dahliae genomes and has been shown to induce necrotic lesions and trigger defence responses in N. benthamiana, Arabidopsis, and cotton. This suggests that NLPs that play dual roles in plant cells might be involved in the transition from biotrophic to necrotrophic phases during plant infection (Santhanam et al., 2013; Zhou et al., 2012). Similarly, VdRTX1 might play dual roles during the interactions between V. dahliae and its host plants.

Z. tritici Zt6 works as a functional ribonuclease and can cleave both plant and animal rRNA species (Kettles et al., 2018). An open question is whether VdRTX1 has RNase activity. This hypothesis originated from our observation that VdRTX1 harbours a typical ribonuclease domain (Figure 1a). Protein structure prediction in this study revealed that the mature VdRTX1 protein exhibited striking structural similarity to the F1 RNase from F. moniliforme, a small, secreted ribonuclease that specifically hydrolyses a phosphodiester bond at the 3’ end of guanosine in single-stranded RNA (Omori et al., 1972; Vassylyev et al., 1993), indicating that VdRTX1 might have guanosine-specific RNase activity. This prompted us to examine whether VdRTX1 would exhibit ribonucleolytic activity on RNA molecules. Indeed, analysis of the total proteins extracted from N. benthamiana leaves transiently expressing VdRTX1 revealed strong RNase activity that degraded rRNA (Figure 2a), and this RNase activity was not observed following expression of the cell-death-induction effector VdSCP126 (Wang et al., 2020). The mutations of the putative ligand-binding sites Y68A, H121A, or F129A each abolished both the cell-death-inducing activity of VdRTX1 in N. benthamiana leaves and its RNase activity (Figures 5d and S3). These results demonstrated that VdRTX1 is a bona fide RNase in V. dahliae that participates in the host immune response.

Even though many fungal ribonucleases have been characterized, evidence of their subcellular locations has been lacking (Lacadena et al., 2007; Olobrada et al., 2017). An unusual aspect of VdRTX1 is that its function is dependent on the nuclear localization in the host cells (Figure 4). It is well established that fungal apoplastic effectors commonly trigger plant defence responses through their perception by the plant membrane-localized immune receptors BAK1 and SOBIR1 (Doelemann & Hemetsberger, 2013; Liebrand et al., 2013). For example, the cell-death-inducing activity of three V. dahliae secreted proteins in N. benthamiana, VdEG1, VdEG3, and VdCut11 (Güi et al., 2017, 2018), occurs through the BAK1/SOBIR1-mediated signalling pathway. Silencing of BAK1 or SOBIR1 genes in N. benthamiana significantly attenuated the cell-death-induction activity of these proteins. As expected, VdRTX1-induced plant cell death was BAK1- and SOBIR1-independent (Figure 3c,d). Thus, VdRTX1 is not recognized...
by these plant immune receptors in the apoplast. These lines of evidence indicate that VdRTX1 is unlikely to be an apoplastic effector.

An intriguing question remains as to why V. dahliae secretes VdRTX1 that enters plant cells. One possible explanation could be that VdRTX1 belongs to a group of ancient RNase effectors widespread in microorganisms, whose function is primarily in fungal self-defense and development, which might help ward off certain biotic threats within their environment, such as RNA viruses (Martínez-Ruiz et al., 1999). Indeed, VdRTX1 homologs are widely distributed among fungi (Figure 8a), which indicates that fungi employ the ribonuclease function to tackle the challenges from biotic stresses. Singularly, it seems that the cell-death-induction function is specifically confined to Verticillium spp., as all other VdRTX1 homologs selected from other fungi were incapable of inducing cell death on N. benthamiana (Figure 8d). This does not mean that ribonucleases from other fungi are nonfunctional, as the fungal ribonuclease-like effector protein CSEP0064 represses plant immunity and interferes with degradation of host ribosomal RNA (Pennington et al., 2019).

It is possible that plant hosts have evolved to recognize VdRTX1 resulting in cell death induction for activation of the immune response. Interestingly, VdRTX1 homologs from Verticillium spp. displayed significantly lower sequence diversity than other fungal genera (Figures 8b,c and S5). This is probably a result of the strictly asexual reproduction in Verticillium spp., which limits the accumulation of beneficial mutations during evolution (de Jonge et al., 2013). These highly conserved VdRTX1 homologs in Verticillium spp. might each contribute to host plant recognition and activation of host immune responses. However, it remains an intriguing question as to why VdRTX1 secreted by V. dahliae has become important in determining the outcomes of certain plant-pathogen interactions.

In conclusion, we identified a novel RNase effector, VdRTX1, in the soilborne pathogen V. dahliae. The dual roles of VdRTX1 both in direct cytotoxicity and in the induction of immunity were demonstrated to contribute to disease establishment. Future work will aim at understanding how fungal RNase effectors are recognized by plants and the biological significance of these RNases in virulence, and also in the relatively lengthy biotrophic phase of host–V. dahliae interactions. This study extends our understanding of fungal effectors, illustrating that diverse secreted proteins in fungi bearing conserved functional domains are yet to be fully appreciated and explored in plant-pathogen interactions.

4 | EXPERIMENTAL PROCEDURES

4.1 | Fungal culture, plant material, and growth conditions

The V. dahliae strain VD8 was cultured on potato dextrose agar (PDA) in the dark for 4 days at 25°C. Cotton and N. benthamiana were grown at 25°C in a greenhouse at 70% relative humidity with a 14 h/10 h, day/night regime. A. tumefaciens strains AGL-1 and GV3101 were cultured on lysogeny broth (LB) medium with the appropriate antibiotics at 28°C.

4.2 | Yeast signal sequence trap system

Functional validation of the predicted signal peptide was performed as described previously (Jacobs et al., 1997). The VdRTX1-encoded signal peptide was fused in frame in the pSUC2 vector to the truncated invertase gene (SUC2) lacking both the initiation methionine and signal peptide. The resulting construct, pSUC2 (VdRTX1), was transformed into the yeast strain YTK12 and screened on CMD-W (lacking tryptophan) medium. The positive control included the strain YTK12 transformed with the functional signal peptide of Avr1b (pSUC2 [Avr1b]) and the negative control was transformed with pSUC2 only (pSUC2 [noSP]). The transformants were incubated on YPRAA (2% raffinose) medium to assay invertase secretion.

4.3 | Transient expression analysis in N. benthamiana

A. tumefaciens strains were grown in LB medium with appropriate antibiotics at 28°C to an optical density (OD600) of 0.8. Cultures were harvested and resuspended in 10 mM MgCl2, 10 mM MES, and 200 µM acetosyringone, pH 5.6, to an OD600 of 0.8–1.0. For coinfiltration, the cultures carrying the constructs of interest, including the control constructs, were mixed in a 1:1 ratio to OD600 = 0.5 for each. The resuspended cultures were incubated in the dark at 28°C for 3 h. All gene sequences and variants were separately cloned into the PVX vector pGR107 and A. tumefaciens GV3101 containing the appropriate vector was infiltrated into 4-week-old N. benthamiana leaves with a 1-ml syringe. Agroinfiltration assays were performed on N. benthamiana plants using the Bcl-2-associated X protein (BAX) and green fluorescent protein (GFP) as positive and negative controls, respectively. Leaf phenotypes were photographed at 5 dpi. Each assay was performed on six leaves from three individual plants, and performed at least three times for each analysis.

4.4 | VIGS in N. benthamiana

VIGS was performed using recombinant TRV assays as described previously (Liu et al., 2002). The constructs pTRV1, pTRV2:BAK1, or pTRV2:SOBIR1 were introduced into A. tumefaciens GV3101. The A. tumefaciens strain harbouring pTRV1 was mixed with the strain carrying pTRV2:BAK1 or pTRV2:SOBIR1 in a 1:1 ratio to achieve a final OD600 for each strain of 0.5. The cocultures were infiltrated into the lowest three noncotyledonous leaves of 3-week-old plants. The plasmid pTRV2:GFP was used as the control. The silencing efficiency of NbBAK1 or NbSOBIR1 was validated by RT-qPCR. The experiment was performed three times, using five plants for each TRV construct.

4.5 | Confocal microscopy

The full-length VdRTX1 coding region and its variants, including VdRTX1 protein without the nuclear localization sequence
fluorescence microscopy, epidermal cell layers of N. benthamiana leaves were collected and examined at 2 dpi. Plant cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). The fluorescence was visualized using an LSM T-PMT confocal microscopy system (Leica). The GFP and DAPI fluorescence were excited using specific excitation and emission wavelengths for the microscopy system (Leica). The GFP and DAPI fluorescence were stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen).

4.6 RNA extraction and RT-qPCR

RNA was extracted using an EASY spin kit. Reverse transcription was performed on 1 μg of total RNA using Transcript II One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgene). Quantitative real-time PCR was performed using the following conditions: 10 min at an initial 95°C denaturation step followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. All reactions were conducted in triplicate. The V. dahliae elongation factor 1-α gene (EF-1α) was used as a reference to quantify fungal colonization. The cotton 18S rRNA gene and NbEF-1-α in N. benthamiana were used as internal references. Relative transcript levels of these genes were calculated using the 2−ΔΔCt method (Livak & Schmittgen, 2001). Unpaired Student’s t tests were performed to determine statistical significance, and p < 0.05 between three treatments groups was considered statistically significant. Primers are listed in Table S3.

4.7 Detection of ROS, callose, and electrolyte leakage

ROS accumulation was detected using 3,3′-diaminobenzidine (DAB) staining. Infiltrated N. benthamiana leaves were treated with DAB, incubated on a shaker for 8 h in the dark, and destained with 95% ethanol. Samples were subsequently observed under light microscopy. Callose deposition was observed as described previously (Clay et al., 2009; Schenk et al., 2014). Agroinfiltrated N. benthamiana leaves were transferred to 95% ethanol for destaining and washed two or three times with water, stained with 0.1% aniline blue in 150 mM K2HPO4, pH 9.5, incubated for 2 h in the dark, and imaged under fluorescence microscopy. Electrolyte leakage assays for N. benthamiana were performed as described previously (Oh et al., 2010). Briefly, nine leaf discs (1 cm diameter) were harvested from the Agrobacterium-infiltrated regions of nine leaves of three plants 2 days after coinfiltration of indicated constructs and incubated in 9 ml of deionized water with shaking at 165 rpm at 25°C for 2 h. Ion conductivity was then measured using a conductivity meter with Probe LE703 (Mettler-Toledo). The assays were repeated three times.

4.8 Pathogenicity assays

Pathogenicity assays with V. dahliae were performed on susceptible and resistant cotton and N. benthamiana, including VdRTX1 targeted deletion strains, complementary transformants, and the wild-type strain VDB. Two-week-old cotton seedlings or 4-week-old N. benthamiana plants were inoculated with 5 × 10⁶ conidia/ml by a root-dip method (Zhou et al., 2013). Disease symptoms associated with inoculated plants were observed at 21 dpi for cotton or at 12 dpi for N. benthamiana. Wilt-associated vascular discoloration of cotton was observed in longitudinal sections of the shoots 3 weeks following inoculation. Fungal biomass of V. dahliae strains in cotton and N. benthamiana was determined using quantitative PCR as previously described (Santhanam et al., 2013). The primers used are listed in Table S3.

4.9 Cell-free protein for ribonuclease activity test

To test the ribonuclease activity of VdRTX1, a cell-free protein (i.e., total proteins) extract from leaves transiently expressing VdRTX1 was used for the RNA degradation assays. In brief, 4-week-old N. benthamiana leaves were infiltrated with VdRTX1 and nine leaf discs (1 cm diameter) were harvested from the Agrobacterium-infiltrated regions of nine leaves in three plants after 2 days. The samples were ground with lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 900 g × 10 min. Subsequently, 0.5 volume of phenol was added to the supernatant, then it was shaken well on ice for 10 min, followed by centrifugation at 900 g × 10 min. Four volumes of methanol containing 100 mM ammonium acetate were added to precipitate the phenol layer at −20°C for 2 h. The precipitated protein was spun down at 900 g × 10 min. The protein was washed twice with a methanol/ammonium acetate mixture and stored in 80% acetone at −20°C before using. The leaves infiltrated with VsSCP126, which can induce cell death (Wang et al., 2020) or sterile water were set as positive and negative controls, respectively. The concentration of total protein was assessed using the Bradford method. Subsequently, the equivalent cell-free protein of each sample was incubated with 2 μg of N. benthamiana RNA for 15 min. The integrity of the RNA was determined using a LabChip GX Touch HT Nucleic Acid Analyzer (Perkin Elmer) to measure the fluorescence signals of the 28S, 18S, and 5S rRNA corresponding to the samples.
to the primary peaks in RNA of high integrity. RNase A (10 mg/ml) was set as the control for RNA degradation.

4.10 | Western blots

Plant tissue was harvested at 2 dpi from the *A. tumefaciens*-infiltrated leaf sites and frozen in liquid nitrogen. Protein extraction from plant materials used RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA). After lysis, the sample was centrifuged at 10,000–14,000 × g for 5 min and the supernatant was removed for additional analysis. The samples were fractionated using 12% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (Sangon Biotech) using standard procedures. Blots were blocked using phosphate-buffered saline (PBS) with Tween 20 (PBST) with milk (5% wt/vol) for 1.5 h, washed four times in PBST for 5 min, followed by addition of the primary monoclonal antibody (TransGen Biotech) in PBST with milk at a 1:5000 dilution for 1.5 h. The membranes were washed five times in PBST for 5 min in each wash before being probed with a mouse horseradish peroxidase-conjugated secondary antibody (Invitrogen) diluted to 1:8000. Blots were washed 10 times in PBST before imaging.

4.11 | Bioinformatics analysis

The conserved domain of VdRTXs was predicted by the online tool SMART (Letunic et al., 2021). Clustal X v. 1.83 software was used for the multiple sequence alignment (Thompson et al., 1997). Signal peptides and signal peptide cleavage sites were predicted using SignalP v. 4.0 software (D-score cut-off set to 0.500) (Petersen et al., 2011). The nuclear localization signal sequence of VdRTX1 was deduced using the web-based program of cNLS Mapper (Kosugi et al., 2009). The known fungal ribotoxin and nontoxic RNase protein sequences (gene accession numbers indicated in Figure 1b) were selected for phylogenetic analysis, and the unrooted tree of the VdRTX1 homologs was generated using the MEGA 6 program (Tamura et al., 2013). To identify putative ligand-binding sites in VdRTX1, the protein sequence without the signal peptide was submitted to the I-TASSER software tool (http://zhanglab.ccmb.med.umich.edu/ITASSER/). To analyse the VdRTX1 homologs in fungi, a preliminary BLASTP analysis using the VdRTX1 protein sequence against the NCBI nr protein database and MycoCosm database (DOE Joint Genome Institute) identified 1520 and 1335 homologs, respectively. Redundant sequences were removed and results filtered to not exceed a length of 276 amino acids, twice the length of VdRTX1. The filtered VdRTX1 homolog set was subjected to ribonuclease domain prediction by HMMER against the HMM profile of PF000545 and the returned hits with E values <1e−7 and scores >25 were selected for further secretory prediction by combination SignalP v. 4.0 to define with signal peptide, WoLF Psort software (Horton et al., 2007) to identify the extracellular location, and Phobius (Kall et al., 2007) and TMHMM v. 2.0 (Krogh et al., 2001) to filter the proteins with transmembrane domains. This filtering resulted in 1162 VdRTX1 homologs with ribonuclease domains and secretory characteristics. Subsequently, reciprocal BLAST analysis of 1163 VdRTX1 homologs (including VdRTX1) was performed using the BLASTP program (e-value <1e−5) to find all pairwise matches. The matrix was constructed by the identity value of all pairwise combinations among the VdRTX1 homologs.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Albert, I., Böhm, H., Albert, M., Feiler, C.E., Imkampe, J., Wallmeroth, N. et al. (2015) An RLP23-SOBIR1-BAK1 complex mediates NLP-triggered immunity. *Nature Plants*, 1, 15140.
Biafas, A., Zess, E.K., De la Concepcion, J.C., Franceschetti, M., Pennington, H.G., Yoshida, K. et al. (2018) Lessons in effector and NLR biology of plant–microbe systems. *Molecular Plant-Microbe Interactions*, 31, 34–45.
Boller, T. & Felix, G. (2009) A Renaissance of elicitors. Perception of microbe-associated molecular patterns and danger signals by pattern recognition receptors. *Annual Review of Plant Biology*, 60, 379–406.
Brandhorst, T.T. & Kenealy, W.R. (1992) Production and localization of restrictocinin *Aspergillus restrictus*. *Journal of General Microbiology*, 138, 1429–1435.
Cao, Y.- F., Zhu, X.- W., Xiang, Y.-U., Li, D.- Q., Yang, J.- R., Mao, Q.- Z. et al. (2011) Genomic characterization of a novel dsRNA virus detected in the phytopathogenic fungus *Verticillium dahliae* Kleb. *Virus Research*, 159, 73–78.
Chen, J.Y., Liu, C., Gui, Y.J., Si, K.W., Zhang, D.D., Wang, J. 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 Phytologist*, 217, 756–770.
Chen, J.Y., Xiao, H.L., Gui, Y.J., Zhang, D.D., Li, L., Bao, Y.M. et al. (2016) Characterization of the Verticillium dahliae exporoteome involved in pathogenicity from cotton-containing medium. *Frontiers in Microbiology*, 7, 1709.

Clay, N.K., Adio, A.M., Denoux, C., Jander, G. & Ausubel, F.M. (2009) Glucosinolate metabolites required for an Arabidopsis innate immune response. *Science*, 323, 95–101.

Djamei, A., Schipper, K., Rabe, F., Ghosh, A., Vincon, V., Kahn, J. et al. (2011) Metabolic priming by a secreted fungal effector. *Nature*, 478, 395–398.

Dodds, P.N. & Rathjen, J.P. (2010) Plant immunity, towards an integrated view of plant-pathogen interactions. *Nature Reviews Genetics*, 11, 539–548.

Doehlemann, G. & Hemetsberger, C. (2013) Apoplastic immunity and its suppression by filamentous plant pathogens. *New Phytologist*, 198, 1001–1016.

Du, Y., Berg, J., Govers, F. & Bouwmeester, K. (2015) Immune activation mediated by the late blight resistance protein R1 requires nuclear localization of R1 and the effector AVR1. *New Phytologist*, 207, 735–747.

Feng, Z., Zhu, H., Li, Z., Shi, Y., Zhao, L., Liu, L. et al. (2013) Complete genome sequence of a novel dsRNA mycovirus isolated from the phytopathogenic fungus *Verticillium dahliae* Kleb. *Archives of Virology*, 158, 2621–2623.

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L. et al. (2012) Emerging fungal threats to animal, plant and ecosystem health. *Nature*, 484, 186–194.

Francescetti, M., Maqbool, A., Jiménez-Dalmaroni, M.J., Pennington, H.G., Kamoun, S. & Banfield, M.J. (2017) Effectors of filamentous plant pathogens, commonalities amid diversity. *Microbiology and Molecular Biology Reviews*, 81, e00066-16.

Giraldo, M.C. & Valient, B. (2013) Filamentous plant pathogen effectors in action. *Nature Reviews Microbiology*, 11, 800–814.

Gui, Y.J., Chen, Y.J., Zhang, D.D., Li, N.Y., Li, T.G., Zhang, W.Q. et al. (2017) *Verticillium dahliae* manipulates plant immunity by glycosidohydrolase 12 proteins in conjunction with carbohydrate-binding module 1. *Environmental Microbiology*, 19, 1914–1923.

Gui, Y.J., Zhang, W.Q., Zhang, D.D., Zhou, L., Short, D.P.G., Wang, J. et al. (2018) A *Verticillium dahliae* extracellular cutinase modulates plant immune responses. *Molecular Plant-Microbe Interactions*, 31, 260–273.

Han, X. & Kahmann, R. (2019) Manipulation of phytohormone pathways by effectors of filamentous plant pathogens. *Frontiers in Plant Science*, 10, 822.

Herrero-Galán, E., Lacadena, J., Martínez-del-Pozo, A., Boucas, D.G., Olmo, N., Oñederra, M. et al. (2008) The insecticidal protein hirustin A from the mite fungal pathogen *Hirsutella thompsonii* is a ribotoxin. *Proteins*, 72, 217–228.

Hill, C., Dodson, G., Heinemann, U., Sutcliffe, W., Mitsuji, S., Nakamura, K. et al. (1983) The structural and sequence homology of a family of microbial ribonucleases. *Proceedings of the National Academy of Sciences of the United States of America*, 34, 7022–7026.

Hirsutella thompsonii -saturated secreted protein from highly virulent defoliating cotton witches’ broom. *New Phytologist*, 207, 735–747.

Jacobs, K.A., Collinson, C.J., Adams-Collier, C.J. et al. (2007) WoLF PSORT: protein localization predictor. *Nucleic Acids Research*, 35(Web server issue), W585–W587.

Inderbitzin, P. & Subbarao, K.V. (2014) *Verticillium dahliae* systematics and evolution: how confusion impedes *Verticillium* wilt management and how to resolve it. *Phytopathology*, 104, 564–574.

Jacobs, K.A., Collins-Racie, L.A., Colbert, M., Duckett, M., Golden-Fleet, M., Kelleher, K. et al. (1997) A genetic selection for isolating cDNAs encoding secreted proteins. *Gene*, 198, 289–296.

Jones, J.D. & Dangl, J.L. (2006) The plant immune system. *Nature*, 444, 323–329.

Jones, L., Hamilton, A.J., Voinnet, O., Thomas, C.L., Maule, A.J. & Baulcombe, D.C. (1999) RNA–DNA interactions and DNA methylation in post-transcriptional gene silencing. *The Plant Cell*, 11, 2291–2301.

de Jonge, R., Bolt, M.D., Kombrink, A., van den Berg, G.C., Yadeta, K.A. & Thomma, B.P.H.J. (2013) Extensive chromosomal reshuffling drives evolution of virulence in an asexual pathogen. *Genome Research*, 23, 1271–1282.

de Jonge, R., van Esse, H.P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R. et al. (2010) Conserved fungal LysM effector EcP6 prevents chitin-triggered immunity in plants. *Science*, 329, 953–955.

Kall, L., Krog, A. & Sonnhammer, E.L.L. (2007) Advantages of combined transmembrane topology and signal peptide prediction – the Phobius web server. *Nucleic Acids Research*, 35, W429–W432.

Kettles, G.J., Bayon, C., Sparks, A.A., Canning, G., Kanyuka, K. & Rudd, J.J. (2018) Characterization of an antimicrobial and phytotoxic ribonuclease secreted by the fungal wheat pathogen *Zymoseptoria tritici*. *New Phytologist*, 217, 320–331.

Klosterman, S.J., Atallah, Z.K., Vallad, G.E. & Subbarao, K.V. (2009) Diversity, pathogenicity, and management of *Verticillium* species. *Annual Review of Phytopathology*, 47, 39–62.

Klosterman, S.J., Subbarao, K.V., Kang, S., Veronese, P., Gold, S.E., Thomma, B.P.H.J. et al. (2011) Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. *PLoS Pathogens*, 7, e1002137.

Kosugi, S., Hasebe, M., Tomita, M. & Yanagawa, H. (2009) Systematic identification of yeast cell cycle-dependent nucleocyttoplasmic shuttling proteins by prediction of composite motifs. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 10171–10176.

Krog, A., Larsson, B., von Heijne, G. & Sonnhammer, E.L.L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of Molecular Biology*, 305, 567–580.

Kumar, S., Stecher, G. & Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33, 1870–1874.

Lacadena, J., Álvarez-García, E., Carreras-Sangrã, N., Herrero-Galán, E., Alegre-Coballada, J., García-Ortega, L. et al. (2007) Fungal ribotoxins, molecular dissection of a family of natural killers. *FEMS Microbiology Reviews*, 31, 212–237.

Lacomme, C. & Santa Cruz, S. (1999) Bax-induced cell death in tobacco is similar to the hypersensitive response. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 7956–7961.

Langhorst, U., Loris, R., Denisov, V.P., Doumen, J., Roose, P., Maes, D. et al. (1999) Dissection of the structural and functional role of a conserved hydration site in RNase T1. *Protein Science*, 8, 722–730.

Lenarčič, T., Pirc, K., Hodnik, V., Albert, I., Boršek, J., Magistrato, A. et al. (2019) Molecular basis for functional diversity among microbial Nep1-like proteins. *PLoS Pathogens*, 15, e1007951.

Letunic, I., Khedkar, S. & Bork, P. (2021) SMART: recent updates, new developments and status in 2020. *Nucleic Acids Research*, 49[D1], D458–D460.

Liebrand, T.W.H., van den Berg, G.C.M., Zhang, Z., Smit, P., Cordewener, J.H.G., America, A.H.P. et al. (2013) Receptor-like kinase SOBIR1/EVR interacts with receptor-like proteins in plant immunity against fungal infection. *Proceedings of the National Academy of Sciences of the United States of America*, 110, 10010–10015.

Liu, S.-Y., Chen, J.-Y., Wang, J.-L., Li, L., Xiao, H.-L., Adam, S.M. et al. (2013) Molecular characterization and functional analysis of a specific secreted protein from highly virulent defoliating *Verticillium dahliae*. *Gene*, 529, 307–316.

Liu, T., Song, T., Zhang, X., Yuan, H., Su, L., Li, W. et al. (2014) Unconventionally secreted effectors of two filamentous pathogens
target plant salicylate synthesis. Nature Communications, 5, 4686.

Liu, Y., Schiff, M. & Dinesh-Kumar, S.P. (2002) Virus-induced gene silencing in tomato. The Plant Journal, 31, 777–786.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods, 25, 402–408.

Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M. et al. (2015) Fungal effectors and plant susceptibility. Annual Review of Plant Biology, 66, 513–545.

Ma, Z., Song, T., Zhu, L., Ye, W., Wang, Y., Shao, Y. 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.

Martínez-Ruiz, A., Kao, R., Davies, J. & Martinez-del Pozo, A. (1999) Ribotoxins are a more widespread group of proteins within the filamentous fungi than previously believed. Toxicon, 37, 1549–1563.

Noguchi, S. (2010) Isomerization mechanism of aspartate to isoaspartate implied by structures of Ustilago spp. ribonuclease U2 complexed with adenosine 3′-monophosphate. Acta Crystallographica. Section D, Biological Crystallography, 66, 843–849.

Oh, C.S., Pedley, K.F. & Martin, G.B. (2010) Tomato 14-3-3 protein 7 positively regulates immunity-associated programmed cell death by enhancing protein abundance and signaling ability of MAPKKKα. The Plant Cell, 22, 260–272.

Oh, S.K., Young, C., Lee, M., Oliva, R., Bozkurt, T.O., Cano, L.M. et al. (2009) In planta expression screens of Phytophthora infestans RXLR effectors reveal diverse phenotypes, including activation of the Solanum bulbocastanum disease resistance protein Rpi-bl2. The Plant Cell, 21, 2928–2947.

Olobrada, M., Lázaro-Gorines, R., López-Rodríguez, J.C., Martinez-Del-Pozo, A., Ónáderra, M., Maestro-López, M., et al. (2017a) Fungal ribotoxins: a review of potential biotechnological applications. Toxins, 9, pii, E71.

Olobrada, M., Medina, P., Budia, F., Gavilanes, J.G., Martinez-Del-Pozo, A. & García-Ortega, L. (2017b) Characterization of a new toxin from the entomopathogenic fungus Metarhizium anisopliae, the ribotoxin anisoplin. Biological Chemistry, 398, 135–142.

Omori, A., Sato, S. & Tamiya, N. (1972) Isolation and some properties of ribonuclease from Fusarium moniliforme. Biochimica et Biophysica Acta, 268, 125–131.

Oome, S., Raaymakers, T.M., Cabral, A., Samwel, S., Böhmer, H., Albert, I. et al. (2014) Nep1-like proteins from three kingdoms of life act as a microbe-associated molecular pattern in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America, 111, 16955–16960.

Ottmann, C., Luberacki, B., Küfner, I., Koch, W., Brunner, F., Weyand, M. et al. (2009) A common toxin fold mediates microbial attack and plant defense. Proceedings of the National Academy of Sciences of the United States of America, 106, 10359–10364.

Pennington, H.G., Jones, R., Kwon, S., Bonciani, G., Thieron, H., Chandler, T. et al. (2019) The fungal ribonuclease-like effector protein CSEP0064/BECl054 represses plant immunity and interferes with degradation of host ribosomal RNA. PLoS Pathogens, 15, e1007620.

Petersen, T.N., Brunak, S., von Heijne, G. & Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nature Methods, 8, 785–786.

Plett, J.M., Daguere, Y., Wittulsky, S., Vayssière, A., Deveau, A., Melton, S.J. et al. (2014) Effector MiSSP7 of the multisituistic fungus Laccaria bicolor stabilizes the Populus JAZ6 protein and represses jasmonic acid (JA) responsive genes. Proceedings of the National Academy of Sciences of the United States of America, 111, 8299–8304.

Qin, J., Wang, K., Sun, L., Xing, H., Wang, S., Li, L. et al. (2018) The plant-specific transcription factors CBP60g and SARD1 are targeted by a Verticillium secretory protein VdSCP4I to modulate immunity. eLife, 7, pii, e34902.

Qutob, D., Kemmerling, B., Brunner, Frédéric, Küfner, I., Engelhardt, S., Gust, A.A. et al. (2006) Phytotoxicity and innate immune responses induced by Nep1-like proteins. The Plant Cell, 18, 3721–3744.

Rabe, F., Ajami-Rashidi, Z., Doehlemann, G., Kahmann, R. & Djamei, A. (2013) Degradation of the plant defense hormone salicylic acid by the biotrophic fungus Ustilago maydis. Molecular Microbiology, 89, 179–188.

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N. et al. (2011) The Arabidopsis leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. The Plant Cell, 23, 2440–2455.

Rovenich, H., Boshoven, J.C. & Thomma, B.P. (2014) Filamentous pathogen effector functions of pathogens, hosts and microbiomes. Current Opinion in Plant Biology, 20, 96–103.

Santhanam, P., van Esse, H.P., Albert, I., Faino, L., Nürnberg, T. & Thomma, B.P. (2013) Evidence for functional diversification within a fungal NEP1-like protein family. Molecular Plant-Microbe Interactions, 26, 278–286.

Schenk, S.T., Hernandez-Reyes, C., Samans, B., Stein, E., Neumann, C., Schikora, M. et al. (2014) N-acyl-homoserine lactone primes plants for cell wall reinforcement and induces resistance to bacterial pathogens via the salicylic acid/oxylipin pathway. The Plant Cell, 26, 2708–2723.

Seo, P.J., Kim, M.J., Park, J.-Y., Kim, S.-Y., Jeon, J., Lee, Y.-H. et al. (2010) Cold activation of a plasma membrane-tethered NAC transcription factor induces a pathogen resistance response in Arabidopsis. The Plant Journal, 61, 661–671.

Steyaert, J. (1997) A decade of protein engineering on ribonuclease T1. Atomic dissection of the enzyme–substrate interactions. European Journal of Biochemistry, 247, 1–11.

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.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research, 25, 4876–4882.

Truong, N.M., Chen, Y., Mejías, J., Soulé, S., Mulet, K., Jauannot, M. et al. (2021) The Meliodogyne incognita nuclear effector MIEFF1 interacts with Arabidopsis cystosolic glyceraldheyde-3-phosphate dehydrogenases to promote parasitism. Frontiers in Plant Science, 12, 641480.

Vassylev, D.G., Katayanagi, K., Ishikawa, K., Tsujimoto-Hirano, M., Danno, M., Pähler, A. et al. (1993) Crystal structures of ribonuclease F1 of Fusarium moniliforme in its free form and in complex with 2′GMP. Journal of Molecular Biology, 230, 979–996.

Wang, D., Tian, L., Zhang, D.D., Song, J., Song, S.S., Yin, C.M. et al. (2020) Functional analyses of small secreted cysteine-rich proteins identified candidate effectors in Verticillium dahliae. Molecular Plant Pathology, 21, 667–685.

Wilhelm, S. (1955) Longevity of the Verticillium wilt fungus in the laboratory and field. Phytopathology, 45, 180–181.

Yang, J.Y. & Zhang, Y. (2015) iTASSER server: new development for protein structure and function predictions. Nucleic Acids Research, 43(W1), W174–W181.

Yang, R. & Kenealy, W.R. (1992) Effects of amino-terminal extensions of ribonuclease A on its catalytic and regulatory activities. The Journal of Biological Chemistry, 267, 16801–16805.

Zhang, D.D., Wang, J., Wang, D., Kong, Z.Q., Zhou, L., Zhang, G.Y. et al. (2019) Population genomics demystifies the defoliation phenotype in the plant pathogen Verticillium dahliae. New Phytopathology, 222, 1012–1029.

Zhang, L., Ni, H., Du, X., Wang, S., Ma, X.W., Nürnberg, T. et al. (2017) The Verticillium-specific protein VdSCP7 localizes to the
plant nucleus and modulates immunity to fungal infections. *New Phytologist*, 215, 368–381.
Zhou, B.J., Jia, P.S., Gao, F. & Guo, H.S. (2012) Molecular characterization and functional analysis of a necrosis- and ethylene-inducing, protein-encoding gene family from *Verticillium dahliae*. *Molecular Plant-Microbe Interactions*, 25, 964–975.
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*. *Journal of Genetics and Genomics*, 40, 421–431.
Zipfel, C. (2008) Pattern-recognition receptors in plant innate immunity. *Current Opinion in Immunology*, 20, 10–16.
Zipfel, C. (2014) Plant pattern-recognition receptors. *Trends in Immunology*, 35, 345–351.

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