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Permalink

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Journal

Molecular Plant Pathology, 23(8)

ISSN

1464-6722

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Publication Date

2022-08-01

DOI

10.1111/mpp.13213

Peer reviewed

ORIGINAL ARTICLE

Molecular Plant Pathology WILEY

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*

Chun-Mei Yin and Jun-Jiao Li contributed equally to this work.

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 (Dodds & 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 (Białas 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 incognita* 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 PtJAZ6, a negative regulator of jasmonic acid (JA)-induced gene regulation in *Populus trichocarpa*. This interaction prevents JA-dependent degradation of PtJAZ6, 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; Santhanam 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 CBP60g 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, anisoplin from *Metarhizium anisopliae* shows ribonucleolytic activity and cytotoxicity against

insect cells (Olombrada et al., 2017b). Standing out among examples of these types of interactions are the fungal ribotoxins, which exert their toxicity by entering cells and cleaving a single phosphodiester bond located within a universally conserved sequence of the larger RNA molecule (rRNA) of the ribosome, known as the sarcin-ricin loop (SRL) (Lacadena et al., 2007). This cleavage leads to inhibition of protein biosynthesis followed by cellular death by apoptosis. Ribotoxins are produced by several fungal species, mostly from the genus *Aspergillus*, although other entomopathogenic fungi such as *Hirsutella thompsonii* or *M. anisopliae* also produce them (Herrero-Galán 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. Furthermore, the toxicity of Zt6 is not limited to plant cells, but extends to prokaryotes and yeasts as well (Kettles et al., 2018). Besides these toxic extracellular fungal RNases, fungal species also secrete nontoxic RNases. The main structural difference between nontoxic RNases and ribotoxins is that the latter contain longer and positively charged loops, suggested to be the structural basis of their toxicity (Olombrada et al., 2017). A well-known representative of a large family of nontoxic ribonucleolyitc proteins is the RNase T1, which is secreted by species in the genera of *Aspergillus* and *Penicillium* (Langhorst et al., 1999). The catalytic activity of RNase T1 has obvious specificity for guanine and cleaves the P–O 5′ ester bond of GpN in single-stranded RNA sequences by a *trans*-phosphorylation reaction to produce 2′,3′-cyclophosphamide. RNase T1 acts as a cyclizing RNase, in which each of the active site residues His_{40}, Glu_{58}, and His_{92} is required for its catalytic activity (Steyaert, 1997). In *V. dahliae*, the genome also encodes ribonucleases with signal peptides (Klosterman et al., 2011), which suggests that these proteins are secreted into the extracellular space and hence may participate in host–pathogen interactions. However, a potential effector function of *V. dahliae* secreted ribonucleases in modulating host plant immunity is unknown.

In this study, we demonstrate that the *V. dahliae* genome encodes three secreted ribonucleases of which only one, VdRTX1,

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 (CSEP0064; 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 a 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

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In this study, we demonstrate that the *V. dahliae* genome encodes three secreted ribonucleases of which only one, VdRTX1, can induce cell death in *N. benthamiana* when transiently expressed in leaves. *VdRTX1* expression was induced after inoculation of plants and was abundantly up-regulated during early stages of plant infection by *V. dahliae*. VdRTX1 was secreted and translocated into the plant nucleus, resulting in the initiation of host immune responses. To counteract host defences, a secreted VdCBM1 protein of *V. dahliae* blocked the induction of plant immunity elicited by VdRTX1. The cell death-induction function of VdRTX1 homologs is specifically confined to *Verticillium* spp. This study identified a novel fungal effector containing a ribonuclease domain, and similar effector activity remains to be explored in related proteins with ribonuclease domains. We propose that some fungal pathogens block host immune responses through secretion

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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
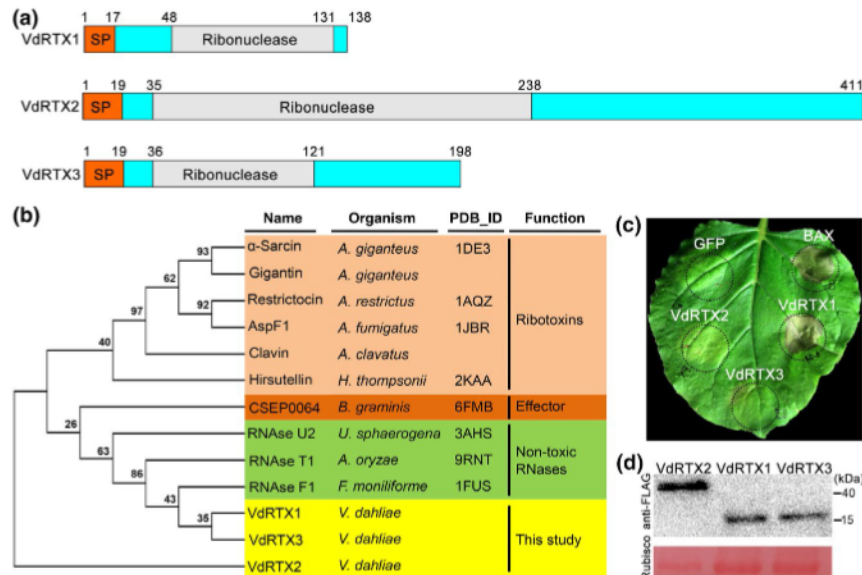
FIGURE 1 *VdRTX1* from *Verticillium dahliae* encodes a ribonuclease that induces cell death. (a) Schematics of the three secreted ribonucleases in *V. dahliae* and their domain structures. SP, signal peptide. (b) Phylogenetic relationship of fungal ribotoxin and nontoxic RNase protein sequences, as well as three secreted ribonuclease proteins identified in this study. The phylogenetic tree was prepared using MEGA 7.0 using maximum-likelihood algorithm (Kumar et al., 2016). (c) Cell-death-induction assays using VdRTX1, VdRTX2, and VdRTX3 in *Nicotiana benthamiana* 4-week-old plants were conducted at 5 days following agroinfiltration. Bcl-2-associated X protein (BAX) and green fluorescent protein (GFP) were used as positive and negative controls, respectively. (d) Immunoblotting analysis in *N. benthamiana* leaves transiently expressing the proteins VdRTX1, VdRTX2, and VdRTX3. Ponceau S-stained RuBisCO protein was used as a total protein loading control

domain (Figure 1a,c). 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. benthamina* 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. benthamina* 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 rRNA 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. benthamina*.

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
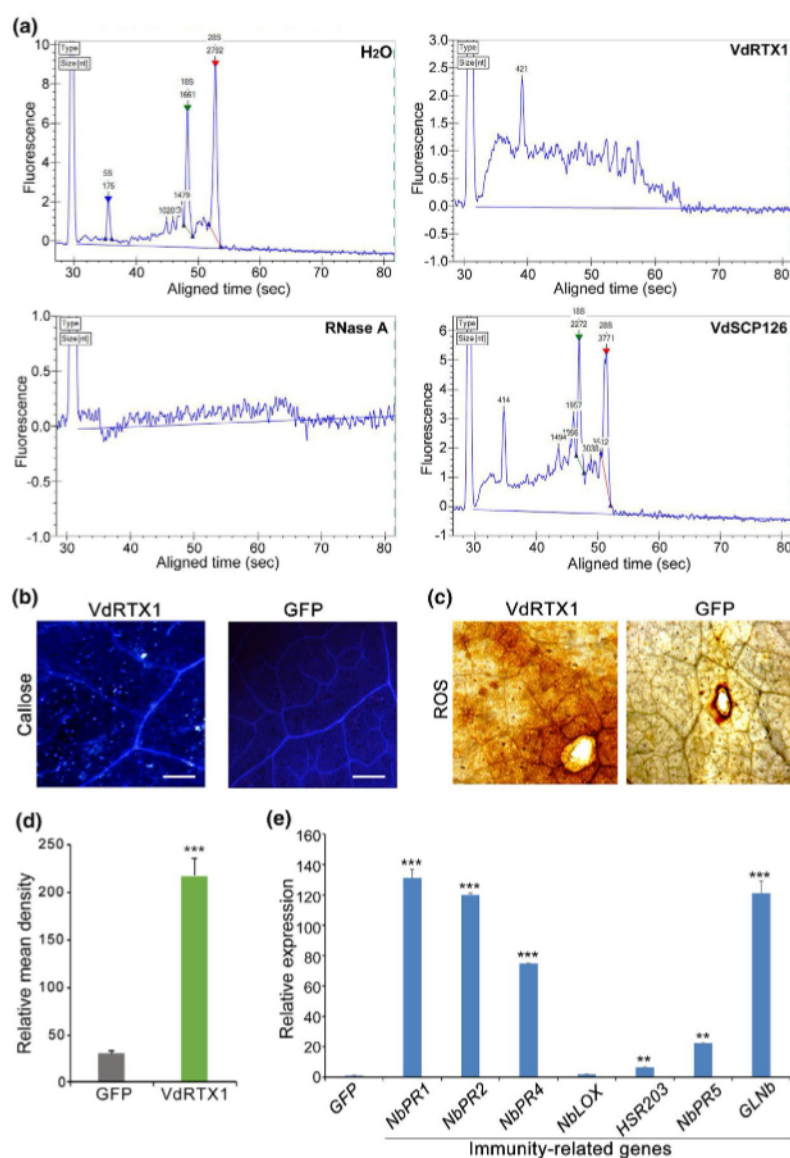
FIGURE 2 VdRTX1 triggers immunity in *Nicotiana benthamiana* that is dependent on its ribonuclease activity. (a) Analysis of RNA degradation. Degradation of the plant ribosomal RNA was determined following incubation with the total proteins extracted from *N. benthamiana* leaves after transient expression of *VdRTX1* for 48 h. *N. benthamiana* leaves injected with the cell-death-inducer VdSCP126 or sterile water were used as positive and negative controls, respectively. RNase A was used as a control to demonstrate degradation of rRNA. The integrity of RNA of the main peaks of 18S rRNA (green triangle box) and 28S rRNA (red triangle box) was detected by the LabChip GX Touch HT Nucleic Acid Analyzer (Perkin Elmer). (b) Identification of callose deposition in *N. benthamiana* leaves transiently expressing *VdRTX1*. Callose deposition in *N. benthamiana* leaves from 4-week-old plants was detected 2 days after transient expression of
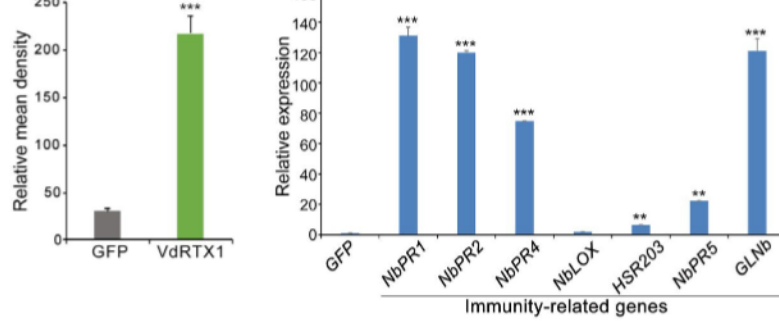
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functional signal peptide of Avr1b or an empty pSUC2 vector as positive and negative controls, respectively (Oh et al., 2009). Like the Avr1b signal peptide positive control, the signal peptide from VdRTX1 also conferred the ability of YTK12 to grow 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
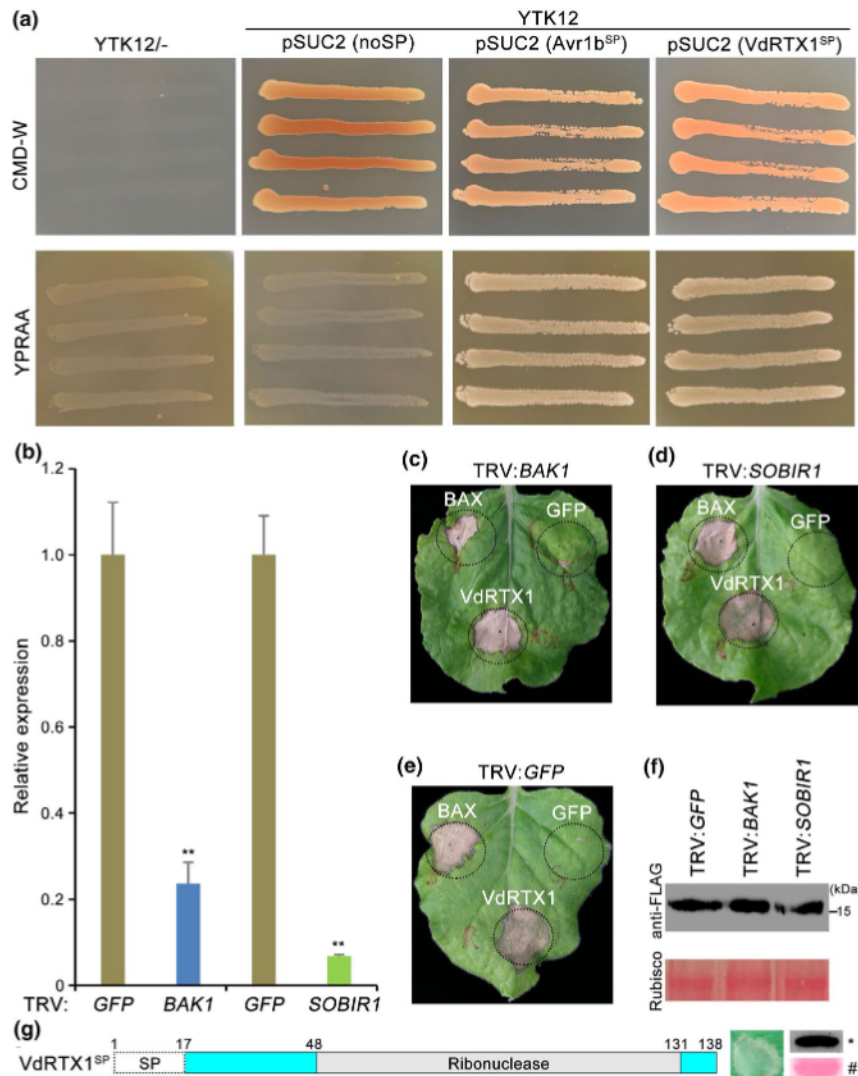

FIGURE 3 VdRTX1 functions intracellularly and independently from immune receptors of *Nicotiana benthamiana*. (a) Secretory activity assays of the predicted signal peptide of VdRTX1 from *Verticillium dahliae* using the yeast signal trap system. The predicted signal peptide sequence with two additional 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 (VdRTX1^SP). Close to the right of the schematic is a photograph of the *N. benthamiana* leaves transiently expressing VdRTX1^SP 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 (VdRTX1^{SP}) 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. benthami-*

export signal [NES]; Du et al., 2015) and VdRTX1^{mNES} (N-terminally fused with a nonfunctional mutated NES sequence; Du et al., 2015), were separately expressed in *N. benthamiana*. Like the VdRTX1$^{\Delta NLS}$, VdRTX1^{NES} was exported and VdRTX1^{mNES} was relocated into the nucleus owing to the mutation in the NES sequence (Figure 4b). VdRTX1^{NES} failed to induce cell death because of its failure to locate into the plant nucleus, whereas the VdRTX1^{mNES} 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. Western blots confirmed all the site-directed mutants were successfully expressed 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

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 (*VdRTX1*$^{\Delta NLS}$) 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 *VdRTX1*$^{\Delta NLS}$ 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, VdRTX1^{NES} (N-terminally tagged in its coding region with a nuclear

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. Western blots confirmed all the site-directed mutants were successfully expressed 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.
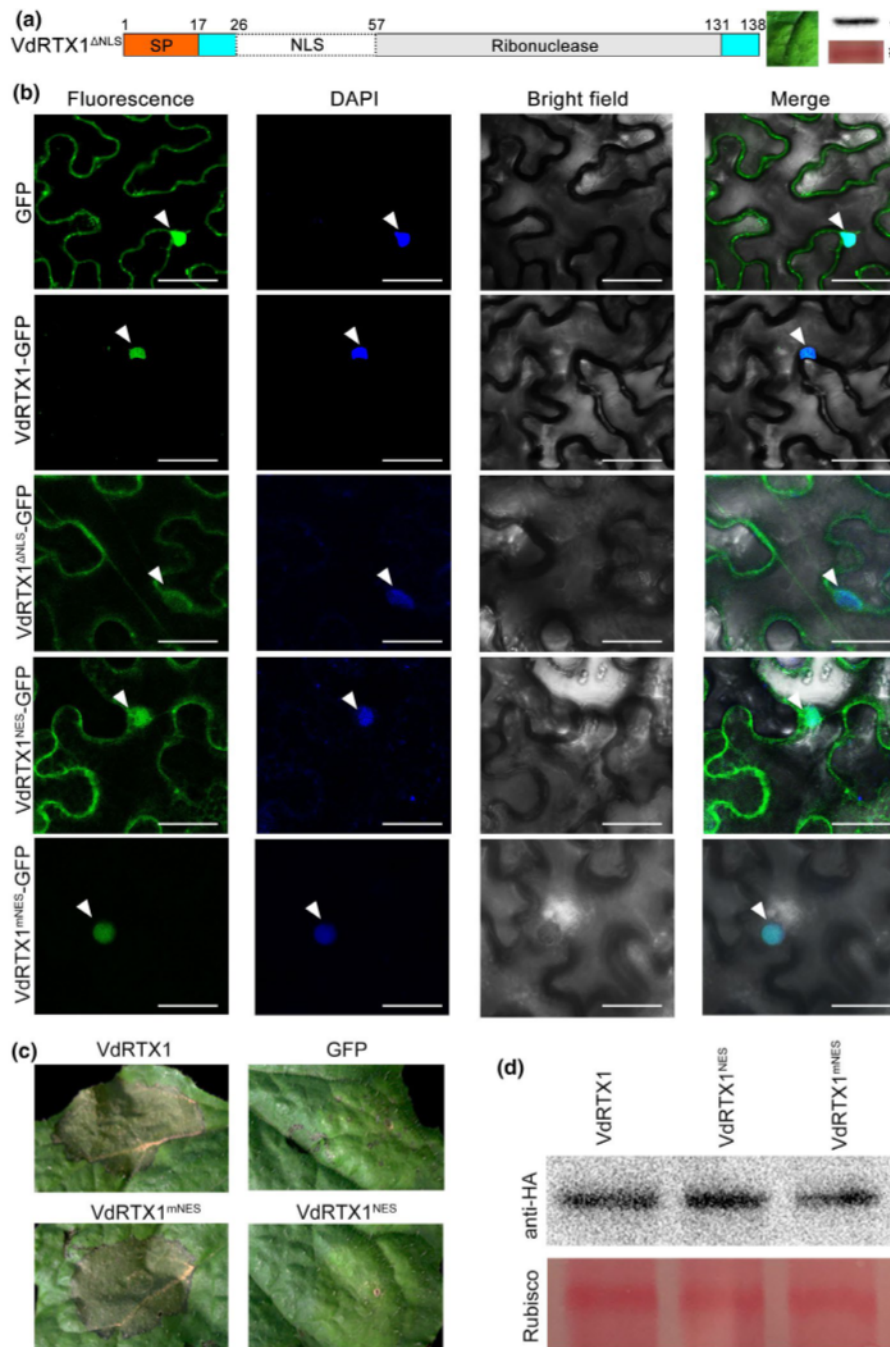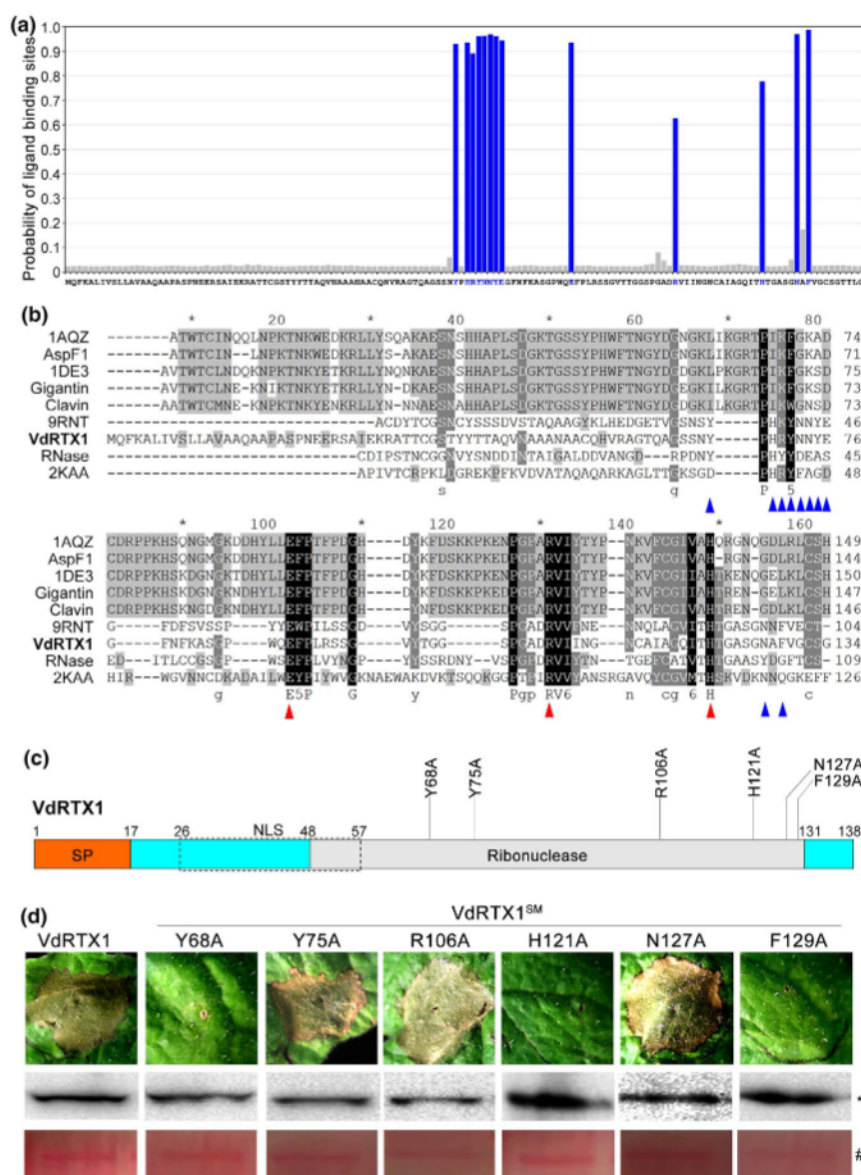
FIGURE 4 Cell death in *Nicotiana benthamiana* depends on the nuclear localization of VdRTX1. (a) Identification the cell-death-inducing activity of VdRTX1 protein from *Verticillium dahliae* without the nuclear localization sequence (VdRTX1$^{\Delta NLS}$). Close to the right of the schematic is a photograph of the *N. benthamiana* leaves transiently expressing VdRTX1$^{\Delta NLS}$. 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 VdRTX1$^{\Delta NLS}$, N-terminally tagged in its coding region with a nuclear export signal (VdRTX1^{NES}) and with a nonfunctional mutated NES sequence (VdRTX1^{mNES}) 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 VdRTX1^{NES} and VdRTX1^{mNES} 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

FIGURE 5 The putative ligand-binding sites of VdRTX1 are required for cell death induction in *Nicotiana benthamiana* leaves. (a) Prediction of the putative ligand-binding sites in VdRTX1 from *Verticillium dahliae*. The putative ligand-binding sites were predicted by the online software of I-TASSER tool (http://zhanglab.ccmb.med.umich.edu/I-TASSER) and the probability of ligand-binding sites was filtered with C-score >0.5. (b) Multiple sequence alignment of typical fungal ribotoxins and nontoxic RNases with the sequence of the VdRTX1 protein. Moderately and highly conserved amino acid residues are shaded grey and black, respectively. The triangles represent the putative ligand-binding sites, with blue and red representing the divergent and absolutely conserved putative ligand-binding sites of VdRTX1 compared to known RNases, respectively. (c) Mutation schematic of putative ligand-binding sites in VdRTX1 for transient expression in *N. benthamiana*. The putative ligand-binding sites were individually mutated to an alanine residue. (d) Cell-death-inducing activity analyses of the putative ligand-binding site mutations in VdRTX1. *N. benthamiana* leaves from 4-week-old plants were examined 5 days after transient expression of *VdRTX1* mutation constructs. The construct expressing the full-length VdRTX1 protein-coding region was used as the positive control. Immunoblotting results of expressed proteins are shown under the leaves, in which the asterisk (*) stands for respective immunoblotted proteins and the hash (#) indicates Ponceau S-stained RuBisCO protein used as a total protein loading control
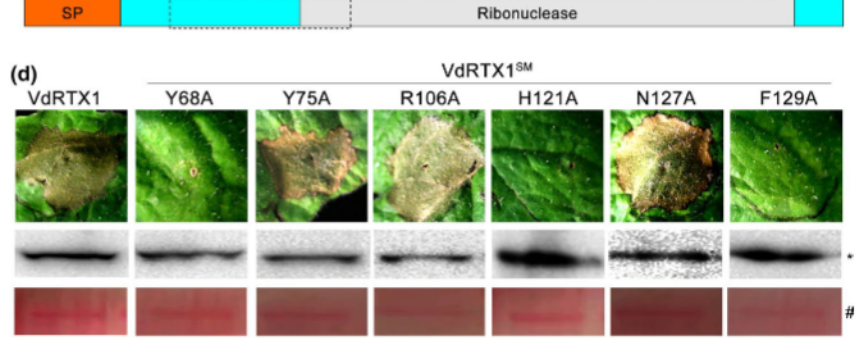

2.6 | VdRTX1-induced plant immune responses can be suppressed by VdCBM1

The activation of plant immune responses by VdRTX1 could limit

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 coag-roinfiltration of *VdRTX1* and *GFP* constructs, but that the electrolyte

in VdRTX1. *N. benthamiana* leaves from 4-week-old plants were examined 5 days after transient expression of *VdRTX1* mutation constructs. The construct expressing the full-length VdRTX1 protein-coding region was used as the positive control. Immunoblotting results of expressed proteins are shown under the leaves, in which the asterisk (*) stands for respective immunoblotted proteins and the hash (#) indicates Ponceau S-stained RuBisCO protein used as a total protein loading control

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 agroinfiltration, 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.
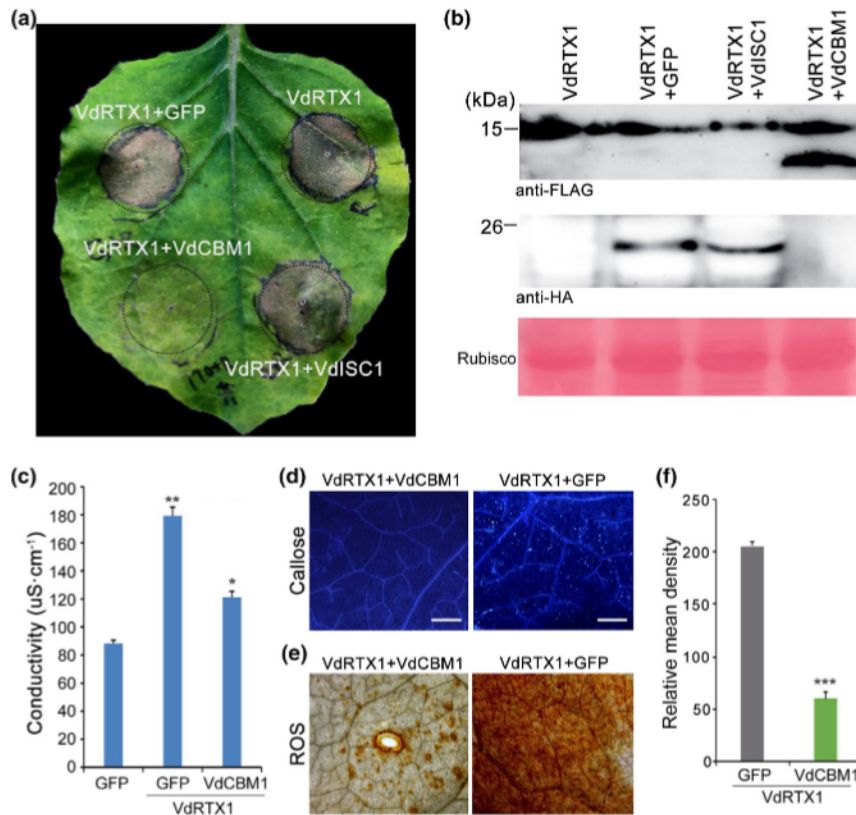
FIGURE 6 The effector VdCBM1 from *Verticillium dahliae* suppresses the VdRTX1-activated plant cell death and defence responses. (a) *VdRTX1* from *V. dahliae* was transiently expressed in 4-week-old *Nicotiana benthamiana* leaves together with *VdCBM1* or with *VdISC1* by coagroinfiltration of the respective expression constructs. Expression of green fluorescent protein (*GFP*) with *VdRTX1* or *VdRTX1* alone were used as cell death references. Photographs depicting cell death lesions were taken at 48 h postinfiltration. (b) Immunoblotting analysis of VdRTX1 and coagroinfiltrated proteins expressed in *N. benthamiana* leaves. VdRTX1 and VdCBM1 were fused with a FLAG-tag while VdISC1 and GFP were fused with an HA-tag. Ponceau S-stained RuBisCO is shown as a total protein loading control. (c) Electrolyte leakage in *N. benthamiana* leaves expressing *VdRTX1* together with *VdCBM1* or the negative control *GFP* was assayed at 48 h after coagroinfiltration. (d) Callose deposition in *N. benthamiana* leaves transiently expressing *VdRTX1* together with *VdCBM1* and the *GFP* negative control was measured by staining with aniline blue. Bar = 200 μm. (e) Accumulation of reactive oxygen species (ROS) in *N. benthamiana* leaves was determined at 12 h after coinfiltration of constructs expressing VdRTX1 with VdCBM1 proteins. Leaves were stained with 3,3′-diaminobenzidine (DAB). (f) Quantitative analysis of DAB staining results of tobacco leaves by ImageJ. Data represent the mean density of DAB-stained area from three leaves. Statistical significance was calculated using Student's t test. Asterisks *** indicate significant difference ($p < 0.001$)

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 *VdRTX*s 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 *VdRTX*s 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 Δ*VdRTX1* 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 Δ*VdRTX1* 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 Δ*VdRTX1* 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 Δ*VdRTX1* 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. bethamiana*–*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
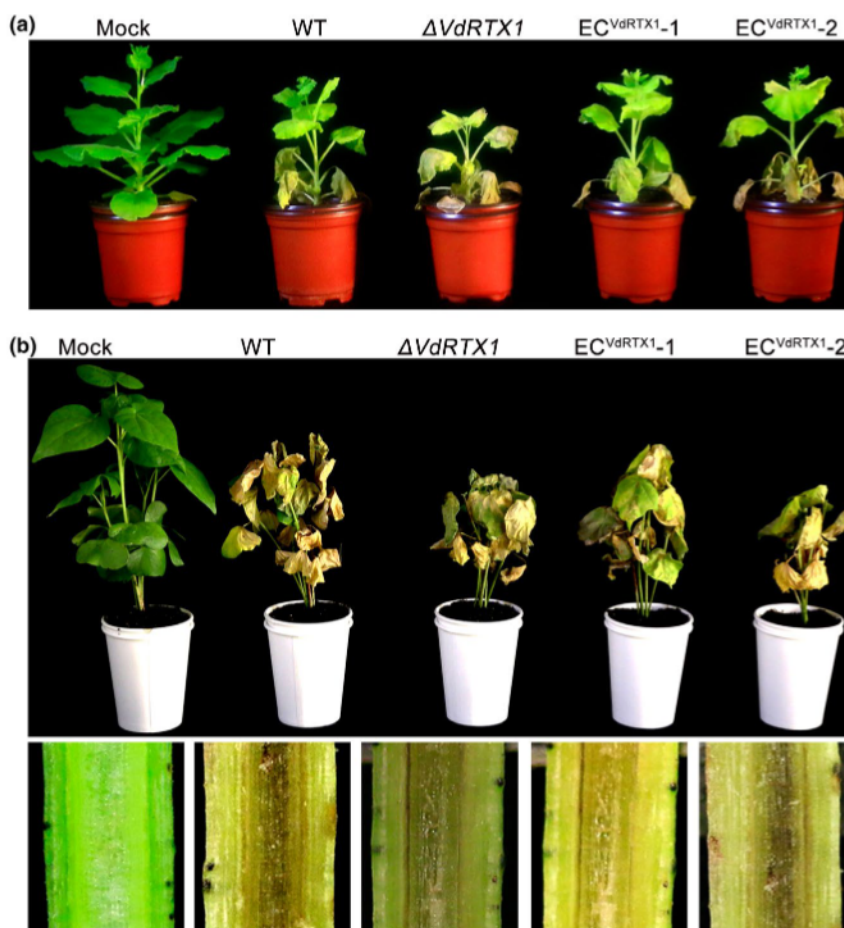

FIGURE 7 Virulence assays of VdRTX1 deletion mutants on *Nicotiana benthamiana* and cotton plants. (a) Disease symptoms of *N. benthamiana* plants inoculated with the various *Verticillium dahliae* strains. Plants inoculated with sterile water (Mock) and wild-type (WT) *V. dahliae* VD8 served as negative and positive controls, respectively. Development of Verticillium wilt symptoms on plants inoculated with the VdRTX1 deletion mutant (ΔVdRTX1) and the corresponding complemented transformant strains (EC^VdRTX1-1 and EC^VdRTX1-2) 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

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 (Δ*VdRTX1*) and the corresponding complemented transformant strains (EC^VdRTX1-1 and EC^VdRTX1-2) 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 (Δ*VdRTX1*), along with the corresponding complemented transformant strains (EC^VdRTX1-1 and EC^VdRTX1-2). Photographs of disease symptoms were taken 3 weeks following inoculation (top). *V. dahliae* colonization-induced plant stem discolouration 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
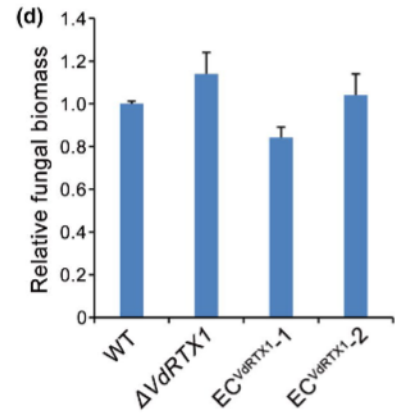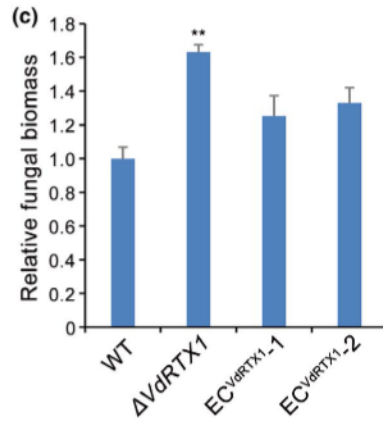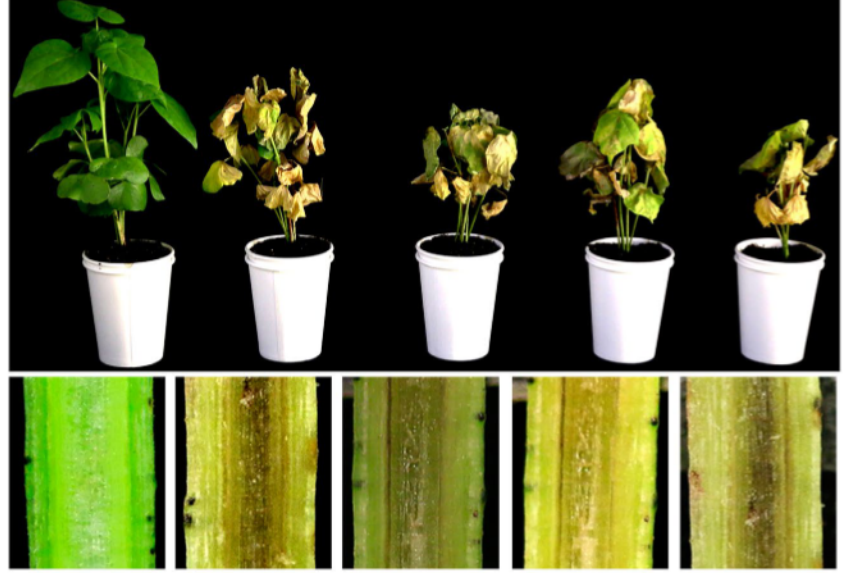
divergent with identities at <65% (Figure 8a). Interestingly, one block in the identity matrix that displayed high identity values belonged to the VdRTX1 homologs from *Verticillium* spp. (Figure 8a). This suggested that the VdRTX1 homologs are more highly conserved *Verticillium* spp. than in other 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,
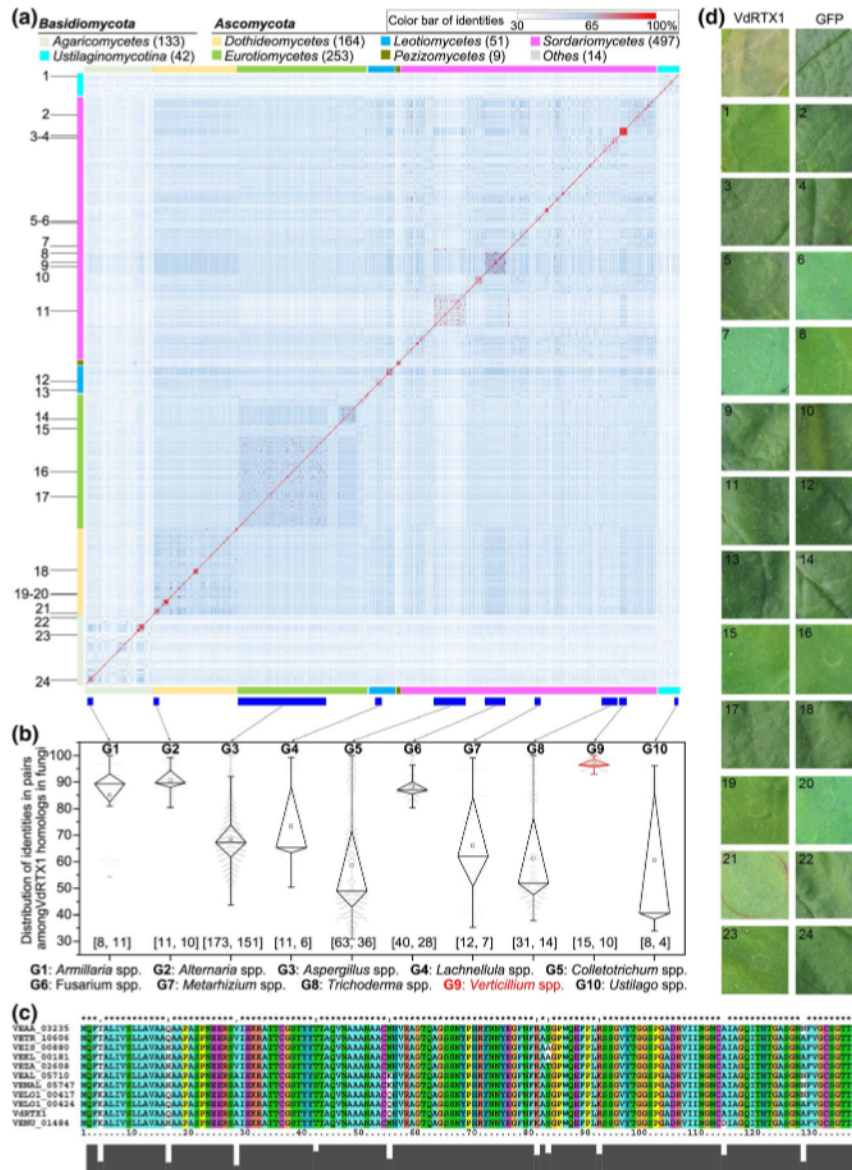

FIGURE 8 Identification and functional analysis of VdRTX1 homologs in *Verticillium* spp. and other fungi. (a) Identity matrix of VdRTX1 homologs from fungi. Reciprocal BLAST analysis of the VdRTX1 homologs was performed using the BLASTP program (e-value < 1e−5) to find all pairwise matches among 1162 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 (Białas 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 (Franceschetti 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 (Olombrada 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 S2), 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 pro-

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; Qutob 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

ing VdRTX1 is an RNase-like protein. VdRTX1 triggered plant immune responses leading to cell death in both *N. benthamiana* and cotton (Figure S2), 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

creted 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 (Figure2a), 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; Olombrada 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 (Doehlemann & 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 (Gui 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-defence 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^{SP}), 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^{SP}]) 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 (OD$_{600}$) of 0.8. Cultures were harvested and resuspended in 10 mM MgCl$_2$,10 mM MES, and 200 μM acetosyringone, pH 5.6, to an OD$_{600}$ 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 OD$_{600}$ = 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 OD$_{600}$ 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

($VdRTX1^{\Delta NLS}$), N-terminally tagged in its coding region with a nuclear export signal ($VdRTX1^{NES}$) and with a nonfunctional mutated NES sequence ($VdRTX1^{mNES}$), were inserted into the pBGFP4 vector to generate a C-terminal fusion with GFP under the control of cauliflower mosaic virus (CaMV) 35S promoter. Plasmids harbouring GFP alone (empty vector, p35S::GFP) were used as controls. All these vectors were introduced into A. tumefaciens GV3101 for transient expression in N. benthamiana. For observations via 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 GFP (488 and 495–530 nm) and DAPI (405 and 440–470 nm). Each photograph was scanned for an exposure time of 1 s. Confocal microscopy was performed at least twice with independent N. benthamiana leaf samples.

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 TransScript 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-1α in N. benthamiana were used as internal references. Relative transcript levels of these genes were calculated using the $2^{-\Delta\Delta Ct}$ 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

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 VD8. Two-week-old cotton seedlings or 4-week-old N. benthamiana plants were inoculated with 5×10^6 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 discolouration 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 $MgCl_2$, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 900 $g \times$ for 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 $\times$ g for 10 min. Four volumes of metha-

was performed on 1 μg of total RNA using TranscriptⅡ 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-1α* in *N. benthamiana* were used as internal references. Relative transcript levels of these genes were calculated using the $2^{-\Delta\Delta Ct}$ 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 K_2HPO_4, 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.,

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 $MgCl_2$, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 900 g × for 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 for 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 for 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 *VdSCP126*, 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 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 to 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.

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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