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Verticillium dahliae secreted protein Vd424Y is required for full virulence, targets the nucleus of plant cells, and induces cell death

Lisen Liu[1,2] | Zhaohan Wang[1] | Jianing Li[1] | Ye Wang[1] | Jiachen Yuan[1,3] | Jingjing Zhan[1] | Peng Wang[1] | Yongjun Lin[2] | Fuguang Li[1] | Xiaoyang Ge[1,3]

[1]Institute of Cotton Research, Henan Normal University Research Base of State Key Laboratory of Cotton Biology, Henan, China

[2]National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China

[3]Zhengzhou Research Base, State Key Laboratory of Cotton Biology, School of Agricultural Sciences, Zhengzhou University, Zhengzhou, China

Correspondence
Xiaoyang Ge, Institute of Cotton Research, Henan Normal University Research Base of State Key Laboratory of Cotton Biology, Henan, China.
Emails: gexiaoyang@caas.cn (X.G.); aylifug@caas.cn (F.L.); yongjunlin@mail.hzau.edu.cn (Y.L.)

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Abstract

Fungal pathogens secrete effector proteins that regulate host immunity and can suppress basal defence mechanisms against colonization in plants. *Verticillium dahliae* is a widespread and destructive soilborne fungus that can cause vascular wilt disease and reduces plant yields. However, little is currently known about how the effectors secreted by *V. dahliae* function. In this study, we analysed and identified 34 candidate effectors in the *V. dahliae* secretome and found that *Vd424Y*, a glycoside hydrolase family 11 protein, was highly upregulated during the early stages of *V. dahliae* infection in cotton plants. This protein was located in the nucleus and its deletion compromised the virulence of the fungus. The transient expression of *Vd424Y* in *Nicotiana benthamiana* induced *BAK1*- and *SOBIR1*-dependent cell death and activated both salicylic acid and jasmonic acid signalling. This enhanced its resistance to the oomycetes *Phytophthora capsici* in a way that depended on its nuclear localization signal and signal peptides. Our results demonstrate that Vd424Y is an important effector protein targeting the host nucleus to regulate and activate effector-triggered immunity in plants.

KEYWORDS

cell death, effector, nuclear localization signal, *Verticillium dahliae*, virulence

1 | INTRODUCTION

Pathogenic microorganisms are responsible for a variety of diseases affecting plants. Despite the wide range of defence strategies that plants have developed to trigger immune responses, pathogenic microorganisms still represent a serious threat to agriculture. These defence strategies include pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI), a process through which PAMPs are recognized by plasma membrane-bound receptors located at the cell surface, inducing primary defence response (Akira et al., 2006). This process can be suppressed by pathogen-delivered effector proteins, which can also cause infections (Houterman et al., 2008;

Lisen Liu and Zhaohan Wang contributed equally to this work.

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de Jonge et al., 2010; Stergiopoulos & de Wit, 2009; Stergiopoulos et al., 2012). Plants have acquired a second layer of immune response known as effector-triggered immunity (ETI), a process through which host cells detect the presence of a pathogen's effectors via R proteins. These two kinds of immunity are involved in the accumulation of reactive oxygen species (ROS), callose deposition, and the regulation of hormone signalling genes (Dodds & Rathjen, 2010; Tsuda & Katagiri, 2010). Immune signalling contains PTI and ETI and is initiated from either the extracellular space or the cytoplasm of the cell through the interaction of the pathogen molecule and plant receptor, though this signalling typically must be transduced into the plant nucleus to activate the expression of defence genes (Eulgen & Somssich, 2007). The barley MLA10 protein can recognize the effector AVR_{A10} and induce nuclear association between the receptor and WRKY transcription factors, which is needed to activate ETI (Shen et al., 2007). In *Fusarium oxysporum*, the effector AVR2 triggers ETI via recognition by the resistance protein I-2 and subsequent translocation into the plant nucleus (Ma et al., 2013). In *Arabidopsis*, the PTI signalling pathway also relies on the function of transcription factors (Eulgen & Somssich, 2007).

Previous studies have demonstrated that pathogen effectors can attenuate ETI (Chisholm et al., 2006; Petre & Kamoun, 2014), making it important to study effector functions to better understand pathogenic mechanisms. Biotrophic and necrotrophic pathogens are responsible for delivering apoplastic and intracellular effector proteins that regulate innate host immunity. This includes RxLR effectors secreted from oomycetes (Qiao et al., 2013; Wu et al., 2019; Yang et al., 2017) that can directly hijack plant resistance pathways (Du, Mpina, et al., 2015; King et al., 2014) and interfere with endoplasmic reticulum stress (Jing et al., 2016) and epigenetic regulation (Hou et al., 2019; Kong et al., 2017; Qiao et al., 2013).

The fungus *Verticillium dahliae* is a hemibiotrophic pathogen that progresses through biotrophic and necrotrophic stages during infection. *V. dahliae* is a soilborne vascular pathogen that infects plants through the roots and colonizes the xylem vessels of different plants species, such as cotton, potato, pepper, *Arabidopsis*, tomato, and *Nicotiana benthamiana* (Lo Presti et al., 2015; Pantelides et al., 2010; Wang, Cai, et al., 2004). *V. dahliae* is similar to other pathogens in that it uses apoplastic and intracellular effector proteins to combat plant immunity and successfully colonize the host (Lo Presti et al., 2015). Examples include the following: *Ave 1* is the first cloned *V. dahliae* effector and is recognized by a membrane-bound receptor-like protein in tomatoes (de Jonge et al., 2012); *Vdlcs1* is an isochorismate synthase able to suppress salicylic acid (SA) signalling during host colonization (Liu et al., 2014); *VdSCP41* is located in the nucleus and can suppress plant immunity by disrupting the transcriptional activity of host genes (Qin et al., 2018); *VdCUT11* is an apoplastic effector that induces cell death and triggers defence responses in *N. benthamiana*, cotton, and tomato (Gui et al., 2018). These studies demonstrated that *V. dahliae* effectors have evolved to target host regulatory proteins and disrupt the host defence signal network during the infection process.

The secretome of *V. dahliae* is composed of nearly 800 proteins (Klosterman et al., 2011). However, relatively few of these secreted proteins have been functionally characterized, and a better understanding of how *V. dahliae* facilitates plant immunity could provide insights into ways to improve host plant resistance. In this study, we assessed the variation of the Vd991 strain and compared the published Vd991 genome with our de novo assembly of the *V. dahliae* 991 genome (unpublished data), finding that they were highly conserved. We also analysed 34 candidate effectors from secreted proteins and identified a potential secreted protein, Vd424Y, that induces cell death in tobacco, *Arabidopsis*, and pepper leaves. Vd424Y is a member of the glycoside hydrolase family 11 (GH11) and targeted the nucleus of the host cells. Its presence was necessary for *V. dahliae* to be fully virulent in cotton. Moreover, we observed that whether or not Vd424Y triggered cell death depended on its nuclear localization signal (NLS) and signal peptide (SP), and that its ability to induce cell death was dependent on *BAK1* and *SOBIR1*. Finally, we demonstrated that a Vd424Y-induced immune response in *N. benthamiana* leaves could depend on its translocation from the plant cell apoplastic space to the nucleus.

2 | RESULTS

2.1 | Identification of a *V. dahliae* secreted protein that elicits plant cell death

Few proteins secreted by *V. dahliae* capable of inducing cell death have been reported. We analysed the genomes of *V. dahliae* strain 991 (Vd991) and 34 effectors with a signal peptide (SP), which were individually cloned into potato virus X (PVX) vector pGR107 (Table S1). We then performed transient expression in *N. benthamiana* to screen potential candidate *V. dahliae* effectors capable of inducing cell death. Green fluoresecnt protein (GFP) and Bcl-2-associated X protein (BAX) were used as negative and positive controls, respectively. *Agrobacterium* carrying each of the effectors was injected into the leaves of 4-week-old *N. benthamiana* plants. Seven days after infiltration, we identified a candidate effector EVM0004916 (hereafter designated Vd424Y) that induced intense cell death (Figure 1a). Western blot analysis demonstrated that both GFP and Vd424Y were expressed (Figure 1c), with a significant increase in the electrolyte leakage around the region of Vd424Y expression compared to regions of GFP expression. This is consistent with the cell death phenotype (Figure 1b). Additionally, the *Agrobacterium*-mediated transient expression of *Vd424Y* in pepper and *Arabidopsis thaliana* induced cell death, which did not occur in the GFP control (Figure S1). These findings highlighted the ability of Vd424Y to induce cell death in various plant species.

Vd424Y encodes a 223 amino acid protein with a conserved glycoside hydrolase domain (Figure S2), and has a signal peptide (SP) encoded by the first 60 nucleotides (Figure S2) along with a predicted nuclear localization signal (NLS) (Dingwall & Laskey, 1991) encoded

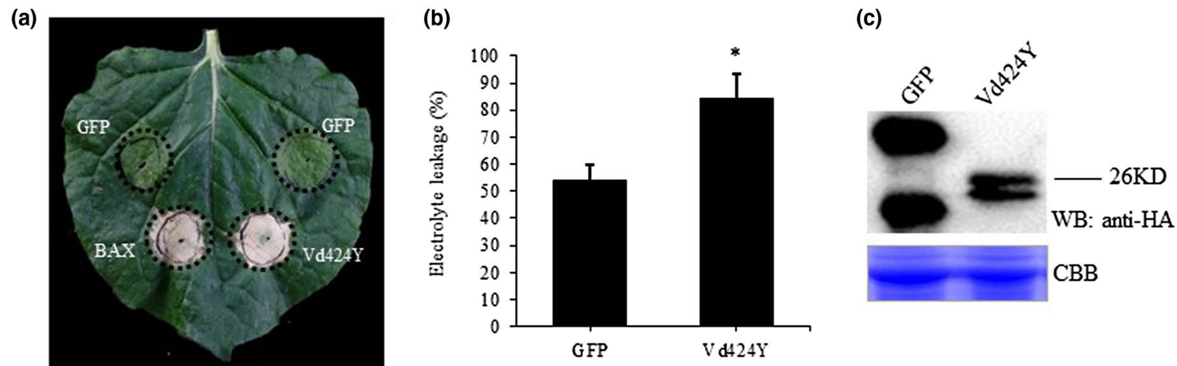

FIGURE 1 Vd424Y is an elicitor of cell death. (a) Vd424Y induces cell death in *Nicotiana benthamiana*. Four-week-old plants were used to express PVX-GFP-HA, PVX-Vd424Y-HA, and PVX-BAX. Photographs were taken 7 days after *Agrobacterium* infiltration. (b) Quantification analysis of cell death via electrolyte leakage measurement. The data shown represent the mean ± *SE* estimated from three biological replicates. Significant *p* values ($p < .05$) for a Student's *t* test are represented by *. (c) Western blotting (WB) analysis of protein levels in *N. benthamiana* transiently expressing green fluorescent protein (GFP) control (left) and Vd424Y fused with HA tag (right). Proteins were stained with Coomassie brilliant blue R-250 (CBB) to confirm equal loading

by 33 nucleotides (Figure S3). This indicates that Vd424Y could have secretory functioning and target the host protein in the nucleus. As shown in Figure S3, only Avr1b^{SP} and Vd424Y^{SP} constructs grew well on the YPRAA medium. However, the empty vector pSUC2 and the YTK12 strain did not grow well. These results indicate that the SP of Vd424Y is essential for secretory functioning.

2.2 | The NLS and SP are required for Vd424Y-induced cell death

Subcellular localization demonstrated that Vd424Y is predominantly located in the plant nuclei (Figures 2c and S4a). This suggests it could be closely related to the presence of a putative NLS (Figure S2). To investigate whether the NLS had a significant effect on the Vd424Y-induced cell death due to its location in the nucleus, we performed NLS mutation analysis and fused a nuclear export signal (NES) to the effector (Vd424Y-nls). Three vectors were individually expressed in *N. benthamiana* via *Agrobacterium tumefaciens*-mediated infiltration, and yellow fluorescent protein (YFP) fluorescence was detected in the infiltrated leaves using a confocal microscope at 48 hr postinfiltration (hpi). These vectors included Vd424Y-nls-YFP, in which the NLS residues were mutated to alanines (Figures 2a and S4), Vd424Y-nls-NES-YFP, in which Vd424Y-nls was fused with a NES (Du, Berg, et al., 2015) at the C-terminus (Figure 2a), and Vd424Y-NES-YFP, in which Vd424Y was fused with an NES at the C-terminus (Figure 2a). As shown in Figure 2c, the YFP signal intensity in the nuclei expressing Vd424Y-nls was weaker than the signalling intensity in the nuclei expressing Vd424Y-YFP. Vd424Y-YFP also displayed a significantly stronger YFP signal intensity in the nucleus than both Vd424Y-nls-NES and Vd424Y-NES. These results indicate that the predicted NLS in Vd424Y affects nuclear localization, and that the NES is capable of guiding Vd424Y out of the nucleus. Additionally, we performed a transient expression assay demonstrating that Vd424Y-nls, Vd424Y-nls-NES, and Vd424Y-NES were unable to induce intense cell death (Figure 2b). This suggests that NLS-mediated nuclear localization is required for Vd424Y to induce cell death.

To determine whether or not Vd424Y-induced cell death depends on Vd424Y secretion, we constructed an SP-deleted (amino acids 1–20; Figure S2) mutant (Vd424Y$^{21\text{-}223}$, hereafter designated Vd424N; Figure 2a) and fused the SP of *N. benthamiana* pathogenesis-related protein (NbPR1) to produce Vd424N-SP$_{NbPR1}$ (Figure 2a). Both of these *Vd424Y* variants were expressed in *N. benthamiana*. Immunoblot analysis confirmed the presence of Vd424N and Vd424N-SP$_{NbPR1}$ (Figure S5). We found that Vd424N did not induce cell death in *N. benthamiana*, while the expression of Vd424N-SP$_{NbPR1}$ did (Figure 2b). This suggests that SP is required for Vd424Y-induced cell death and that Vd424Y must target the extracellular space for cell death to occur. These results demonstrate that NLS and SP are necessary for Vd424Y to induce intense cell death, and that a Vd424Y-induced immune response in *N. benthamiana* leaves depends on its translocation from the plant's apoplastic space to the nucleus.

2.3 | Vd424Y triggers plant immunity responses

To determine whether Vd424Y-activated cell death was associated with plant immune response, we examined a hypersensitive response (HR)-specific marker gene (*NbHIN1*) (Takahashi et al., 2004) in *N. benthamiana* leaves following *Vd424Y* infiltration via *Agrobacterium*. Analysis of expression levels demonstrated that 3 days after infiltration *NbHIN1* was significantly activated by Vd424Y but not by Vd424N or Vd424Y-nls (Figure 3). To confirm whether Vd424Y-trigged immunity involved hormone signalling pathways, we used quantitative reverse transcription PCR (RT-qPCR) analysis to detect the SA-dependent immunity marker genes *NbPR1a* and *NbPR2* (Dean et al., 2005), and the jasmonic acid (JA)-dependent immunity gene *NbPR4* (Asai & Yoshioka, 2009; Rodriguez et al., 2014). These marker genes were significantly induced in *N. benthamiana*

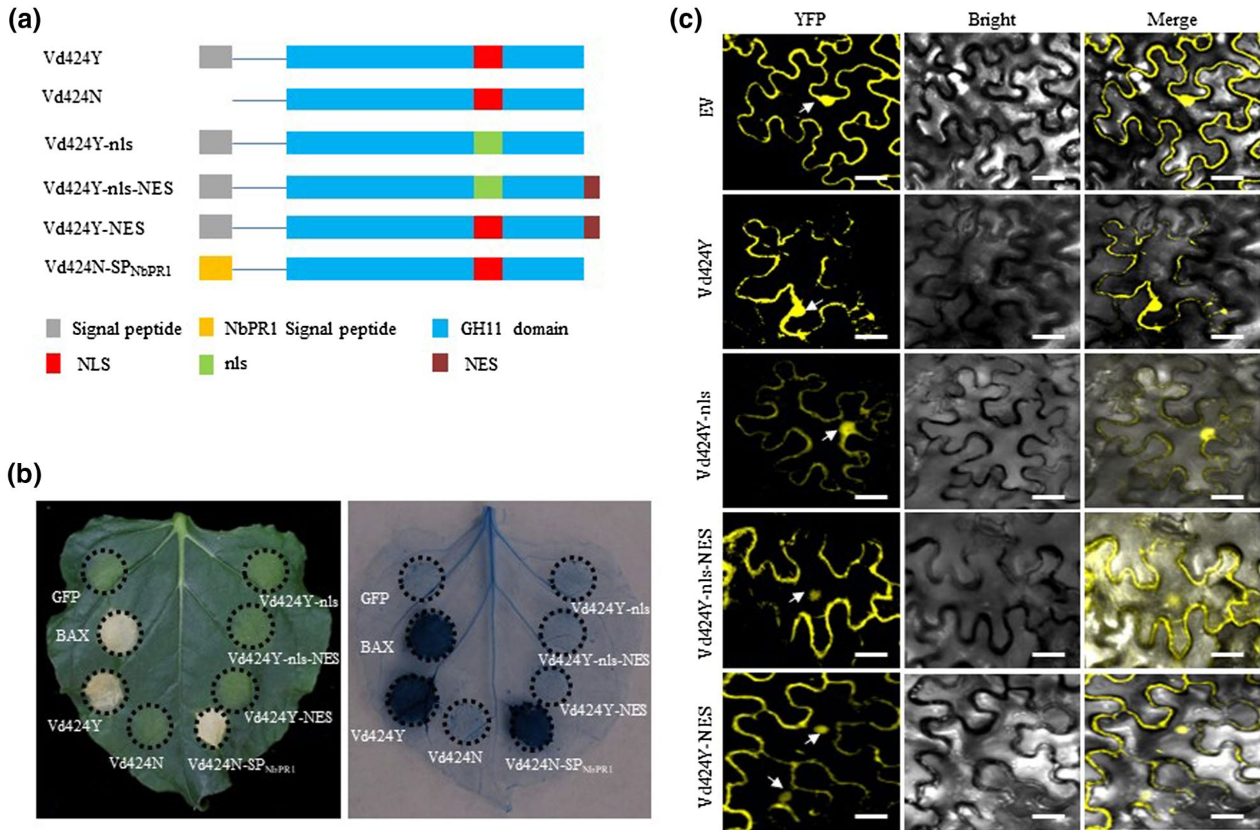

FIGURE 2 Signal peptide and nuclear localization signal are required for Vd424Y-induced cell death. (a) Schematic illustration of the Vd424Y deletion mutants: Vd424Y, the full-length sequence of the candidate effector; Vd424N, effector variant lacking the signal peptide (SP) sequence; Vd424Y-nls, effector variant where the predicted nuclear localization signal (NLS) was mutated to alanine residues; Vd424Y-nls-NES, the variant Vd424Y-nls was fused with a NES at C-terminus; Vd424Y-NES, effector variant where the NES at C-terminus was tagged to the wildtype Vd424Y allele; Vd424N-SPNbPR1, effector variant with a fused SP of *Nicotiana benthamiana* pathogenesis-related protein (NbPR1). (b) *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* carrying *Vd424Y*, *Vd424N*, *Vd424Y-nls*, *Vd424Y-nls-NES*, *Vd424Y-NES*, *Vd424N-SP$_{NbPR1}$*, positive control BAX, and control green fluorescent protein (GFP). Representative photographs of *N. benthamiana* leaves were taken after 7 days. (c) Subcellular localizations of Vd424Y-YFP, Vd424Y-nls-YFP, Vd424Y-nls-NES-YFP, and Vd424Y-NES-YFP in *N. benthamiana* on *A. tumefaciens*-mediated transient expression. Fluorescence was detected by confocal microscopy 48 hr postinfiltration. Bars, 40 μm

leaves expressing Vd424Y, and their expression levels were higher than in leaves expressing the variants Vd424N and Vd424Y-nls (Figure 3). Furthermore, we demonstrate that Vd424Y (but not its variants Vd424N and Vd424Y-nls) was able to induce the expression of PTI-related marker genes, including *NbCYP71D20* (Heese et al., 2007), *NbPti5*, *NbWRKY7*, and *NbWRKY8* (Ishihama et al., 2011; Nguyen et al., 2010) (Figure 4). This confirms that Vd424Y is a microbial PAMP. These results suggest that Vd424Y can be recognized by *N. benthamiana* and activate plant immunity in a manner dependent on its NLS and SP.

2.4 | *NbBAK1* and *NbSOBIR1* are required for Vd424Y-induced cell death in *N. benthamiana*

BAK1 and *SOBIR1* are receptor-associated kinases that participate in various PAMP-triggered immune responses and are activated by cell death-inducing proteins secreted by fungi or oomycetes (Heese et al., 2007; Ma et al., 2015; Nie et al., 2019). Vd424Y properties that induce cell death in the extracellular space are dependent on the SP, so we tested whether *BAK1* and *SOBIR1* mediate Vd424Y-induced cell death in *N. benthamiana*. We performed virus-induced gene silencing (VIGS) to silence *NbBAK1* and *NbSOBIR1* in *N. benthamiana* leaves, and used RT-qPCR to confirm the silencing of *NbBAK1* and *NbSOBIR1* 21 days after VIGS-mediated gene silencing (Figure 5b). These leaves were then agroinfiltrated with Vd424Y, Vd424Y variants (Vd424N, Vd424Y-nls, and Vd424N-SP$_{NbPR1}$), and BAX. Immunoblotting analysis confirmed that Vd424Y and its variants were expressed in *N. benthamiana* leaves inoculated with *pTRV2:BAK1*, *pTRV2:SOBIR1*, and *pTRV2:GFP* (Figure 5c) but did not induce cell death in either *NbBAK1*- or *NbSOBIR1*-silenced plants. However, the positive control BAX retained its ability to induce cell death (Figure 5a). In contrast, Vd424Y and Vd424N-SP$_{NbPR1}$ were able to activate cell death in control plants (Figure 5a). These results

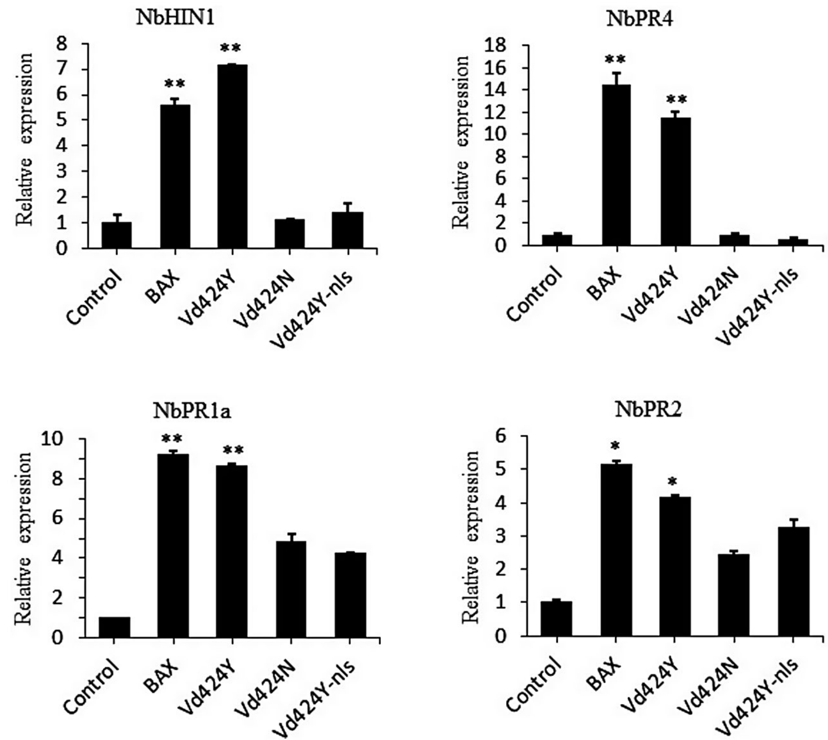

FIGURE 3 Vd424Y induces plant defence responses in *Nicotiana benthamiana*. Relative expression of hypersensitive-response-specific and defence-related marker genes in *N. benthamiana* infiltrated with *Agrobacterium tumefaciens* carrying *Vd424Y*, *Vd424N*, and *Vd424Y-nls*. At 3 days postinfiltration (dpi), total RNA was extracted and transcript levels were detected by quantitative reverse transcription PCR. *NbActin* was used as the internal reference gene. The data shown represents the mean across three independent experiments. Bars indicate ± *SE*. Significance levels $p < 0.05$ and $p < 0.01$ are represented by * and **, respectively

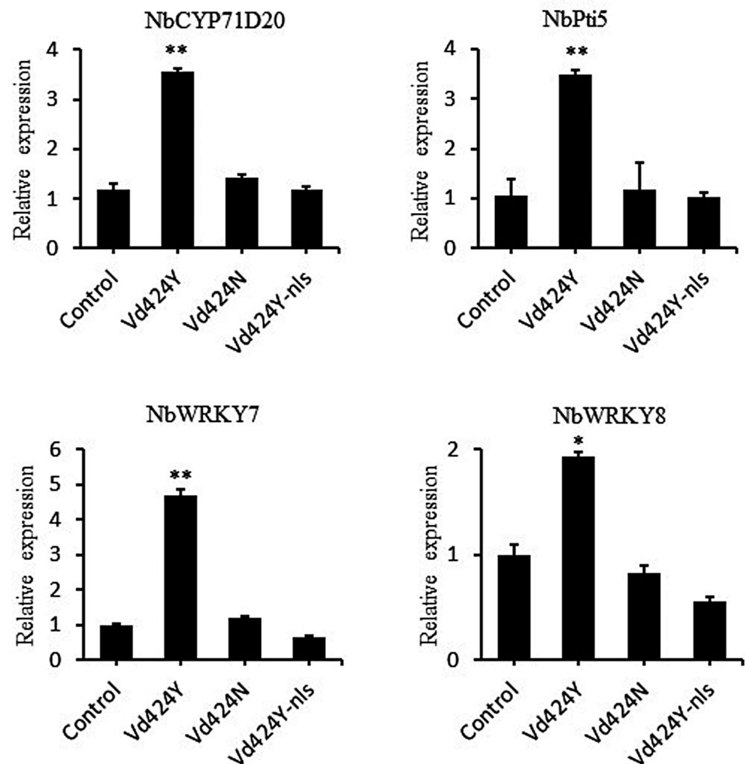

FIGURE 4 Vd424Y promotes transcription of PAMP-PTI marker genes in *Nicotiana benthamiana*. Relative transcript levels of *NbCYP71D20*, *NbPti5*, *NbWRKY7*, and *NbWRKY8* were analysed in *N. benthamiana* infiltrated with *Agrobacterium tumefaciens* carrying *Vd424Y*, *Vd424N*, and *Vd424Y-nls*. At 3 days postinfiltration, total RNA was extracted and transcript levels were detected by quantitative reverse transcription PCR. *NbActin* was used as the internal reference gene. The data shown represent the mean across three independent experiments. Significance levels $p < 0.05$ and $p < 0.01$ are represented by * and **, respectively

indicate that Vd424Y-mediated plant cell death depends on *NbBAK1* and *NbSOBIR1*. We further investigated whether Vd424Y-triggered cell death involved other signalling components by silencing *NbNDR1* and *NbEDS1* in *N. benthamiana* leaves (Figure S6). Although immunoblotting analysis confirmed that Vd424Y and its variants were expressed in leaves inoculated with *pTRV2:NDR1*, *pTRV2:EDS1*, and *pTRV2:GFP*, the silencing of either *NbNDR1* or *NbEDS1* did not affect Vd424Y-induced cell death (Figure S6).

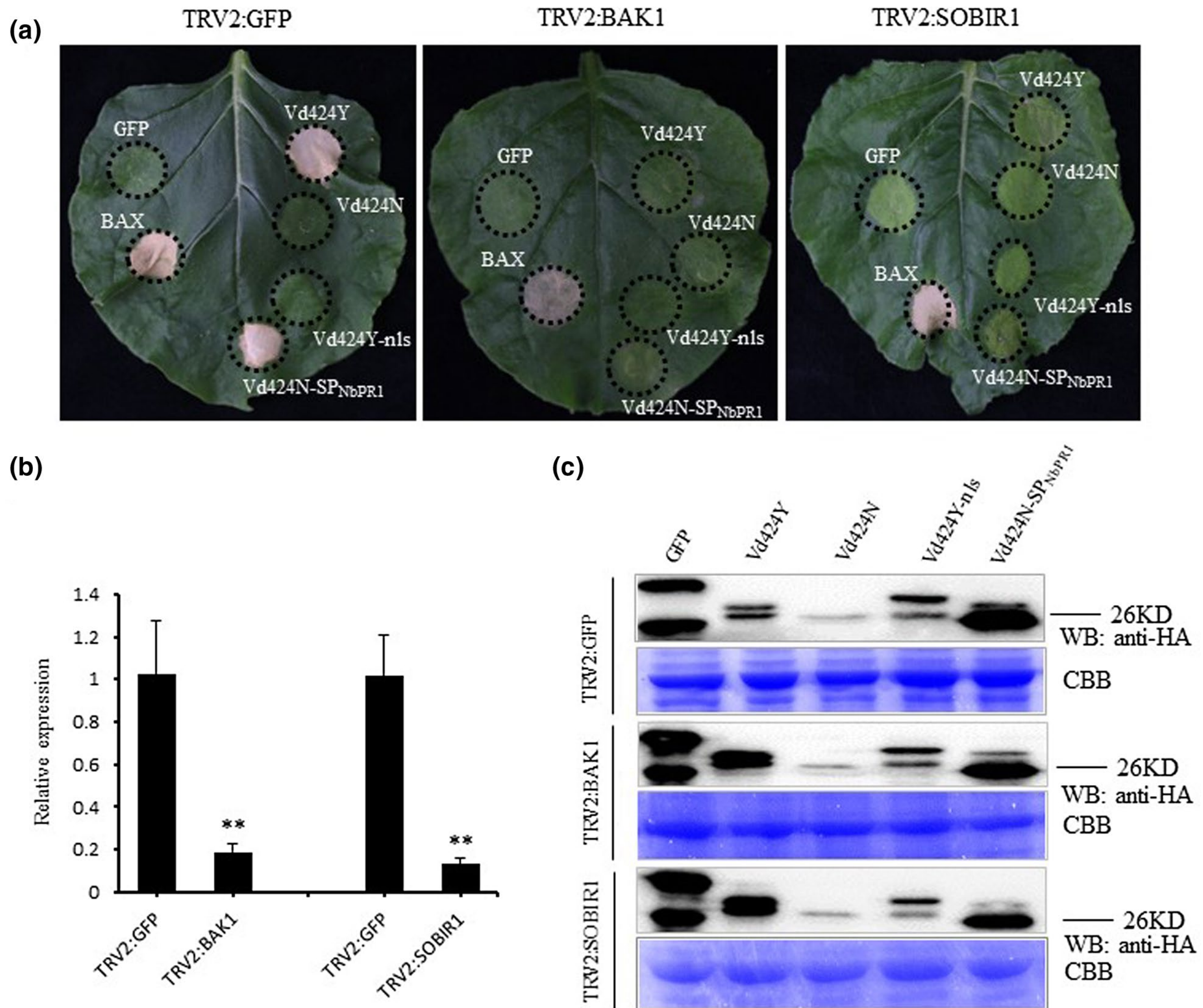

FIGURE 5 *NbBAK1* and *NbSOBIR1* are required for Vd424Y-induced cell death. (a) Virus-induced gene silnecing (VIGS) technology was used to silence *NbBAK1* and *NbSOBIR1* by inoculation with TRV constructs (pTRV2:GFP, pTRV2:*NbBAK1*, and *NbSOBIR1*) in *Nicotiana benthamiana* plants. Three weeks after inoculation, GFP, BAX, Vd424Y, and Vd424Y mutations were transiently expressed in *NbBAK1*- and *NbSOBIR1*-silenced *N. benthamiana* plant leaves. Photographs were taken 7 days after agroinfiltration. The experiment was carried out three times with five plants for each TRV construct. (b) The expression levels of *NbBAK1* and *NbSOBIR1* after VIGS treatment as evaluated by quantitative reverse transcription PCR. *NbActin* was used as the internal reference gene. Mean and *SE* were calculated from three independent experiments. Bars indicate ± *SE*. Significance level $p < 0.01$ is represented by *. (c) Western blot (WB) analysis of green fluorescent protein (GFP), Vd424Y, and Vd424Y mutations protein fused with HA tags after transient expression in *N. benthamiana* leaves. Proteins were stained with Coomassie brilliant blue (CBB) to confirm equal loading

2.5 | Vd424Y enhances *N. benthamiana* resistance to oomycete pathogens

To investigate whether Vd424Y modulates plant resistance to the oomycete pathogen *Phytophthora capsici*, we transiently expressed Vd424Y, Vd424N, and Vd424Y-nls in *N. benthamiana* leaves. *P. capsici* zoospores were inoculated onto the infiltrated area 24 hr after *Agrobacterium* infiltration (Figure 6a), and the size of the resulting lesions was recorded at 48 hr postinoculation (hpi). As shown in Figure 6b,c, the biomass and lesion size of *Phytophthora* in leaves expressing Vd424Y were both significantly lower than in leaves expressing Vd424N, Vd424Y-nls, and an empty vector control. These results strongly demonstrate that Vd424Y triggers plant defence responses and alters plant immunity, enhancing *N. benthamiana* resistance to oomycete pathogens. We also found that both SP and NLS are essential for enhancing Vd424Y's resistance in *N. benthamiana*.

2.6 | Vd424Y contributes to *V. dahliae* virulence

To evaluate the expression profile of *Vd424Y* during the early stages of *V. dahliae* infections in cotton, we inoculated cotton roots with fresh *V. dahliae* spores and collected the infected cotton root samples at different time points. RT-qPCR analysis indicated that

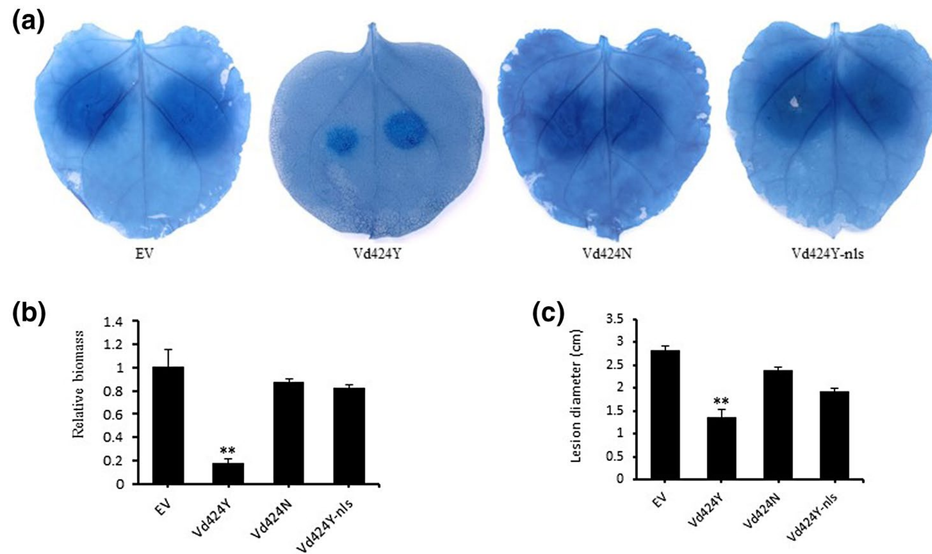

FIGURE 6 Expression of Vd424Y in *Nicotiana benthamiana* enhanced resistance to an oomycete pathogen. Leaf regions transiently expressing Vd424Y, Vd424N, and Vd424Y-nls were inoculated with zoospores of *Phytophthora capsici* strain 35. (a) Resulting lesions visualized using trypan blue staining. (b) Quantitative PCR analysis of relative *Phytophthora* biomass following *P. capsici* infection. Infected leaves ($n = 10$) were collected 48 hr after infection, after which DNA was isolated and qPCR analysis was performed. (c) Size of the lesions caused by *P. capsici* infection on plant leaves expressing Vd424Y and Vd424Y mutations

Vd424Y's expression was strongly upregulated during the early stages of cotton infection by *V. dahliae* (Figure S7), reaching its peak at 3 hpi. This strong induction indicated that *Vd424Y* could promote *V. dahliae* infection.

To determine whether Vd424Y is involved in fungal virulence, we generated *V. dahliae* mutants by replacing the Vd424Y gene with a hygromycin resistance gene (Figure S8) according to methods previously described (Gao et al., 2010; Gui et al., 2017). There were similarities in the growth rate and colony morphology of Vd424Y knockout mutants (ΔVd424Y) and the wild type Vd991 (Figure 7a,b). However, compared to the wild type, ΔVd424Y mutants caused fewer disease symptoms and had a lower disease index 26 days after pathogen inoculation in cotton plants (Figure 7c,d). We found that complementing ΔVd424Y mutants resulted in disease symptoms and colony morphology similar to the wild type Vd991 (Figure 7a,d), highlighting the important role that Vd424Y plays in *V. dahliae* virulence during cotton infection.

3 | DISCUSSION

V. dahliae is a soilborne fungal pathogen that destroys the xylem of host cells and causes injury to plants (Fradin & Thomma, 2006; Klosterman et al., 2009). Relatively few secreted effector proteins have been found to play a role in virulence. Therefore, it is necessary to identify virulence-related effectors and determine their potential roles and pathogenic mechanisms. In this study, we analysed transient expression in *N. benthamiana* leaves to identify the toxic effectors that induced intense cell death. One of the effectors secreted by *V. dahliae*, Vd424Y, can trigger strong cell death in *N. benthamiana*, pepper, and *Arabidopsis* (Figures 1 and S1). This indicates that Vd424Y could act as a PAMP during host plant infection.

After assessing the yeast signal trap assay system, we found that the fusion of the signal peptide of Vd424Y into the invertase gene resulted in the secretion of invertase in yeast (Figure S3). This indicates that Vd424Y was most probably secreted into the extracellular space during infection of the host plant. The transient expression of Vd424Y lacking the signal peptide, Vd424N, did not induce cell death (Figure 2b), suggesting that the extracellular secretion of Vd424Y was needed to induce cell death in *N. benthamiana*. These effector-mediated cell death effects have been previously reported in numerous pathogens. PsXEG1, a glycoside hydrolase family 12 (GH12) protein, acts as a PAMP in soybean and *N. benthamiana*, in which SP was required for PsXEG1-induced cell death by targeting the apoplast (Ma et al., 2015). VmE02 exhibited cell-death-inducing activity in *N. benthamiana*, while an SP-mediated apoplastic location is required to fully induce death of the cell (Nie et al., 2019).

Pathogenic effectors located in the cell nuclei are recognized by their host and have been identified in pathogenic bacteria, fungi, oomycetes, and mutualistic fungi (Kloppholz et al., 2011; Szurek et al., 2001). *PSR1* was identified in oomycetes and targeted the RNAi component PINP1 to disturb the defence mechanism in plants (Qiao et al., 2013, 2015). A VdSCP7-induced immune response was dependent on its nuclear localization (Zhang et al., 2017). Similar research found that *Vd424Y*-induced immunity depends on its nuclear localization, while defence signalling is probably initiated in the host nucleus. Our research demonstrated that certain *V. dahliae* effectors with SP and NLS are secreted into extracellular space and subsequently enter the plant cell nucleus, altering plant immunity. This study assessed Vd424Y-activated innate immune responses

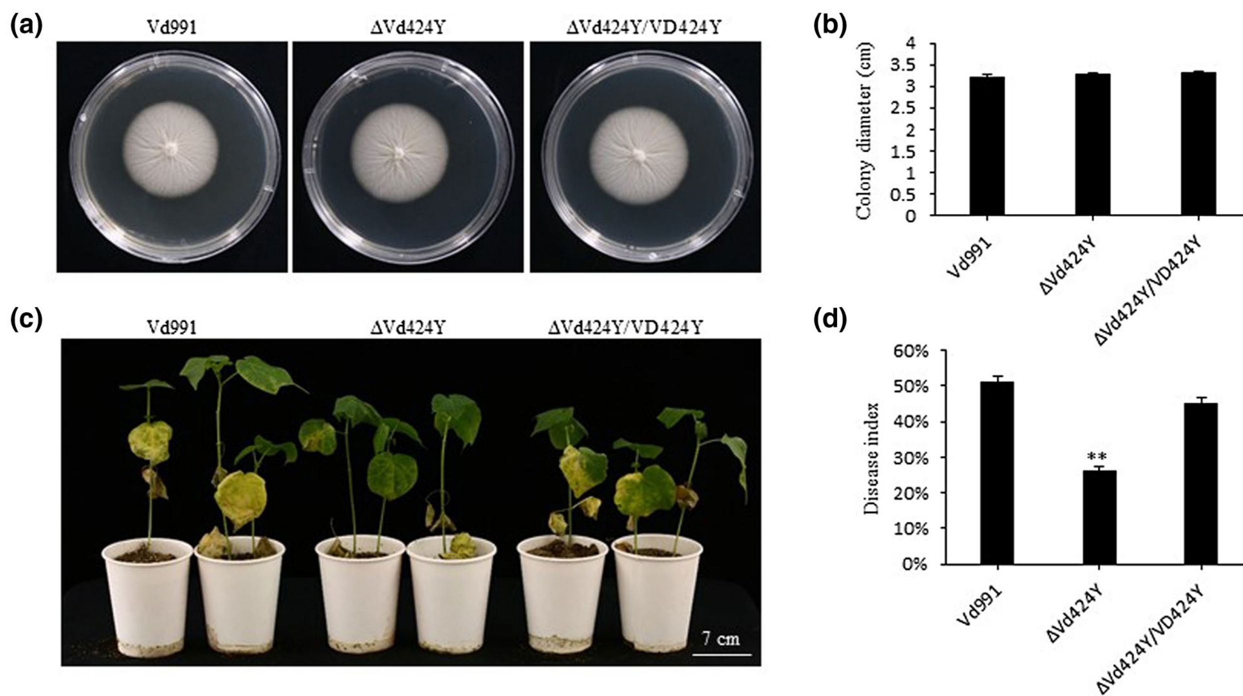

FIGURE 7 Vd424Y contributes to the virulence of *Verticillium dahliae*. (a) Images of Vd991 (wild type), ΔVd424Y mutant, and ΔVd424Y/VD424Y-complementation transformants cultured on potato dextrose agar plates at 25 °C for 7 days in the dark. (b) Colony diameter of Vd991, ΔVd424Y mutant, and ΔVd424Y/VD424Y transformants. The data shows the mean across three independent replicates. (c) Images of disease symptoms on cotton 26 days postinoculation (dpi). The cotton cultivar Jimian 11 was inoculated with Vd991, ΔVd424Y mutant, and ΔVd424Y/VD424Y transformants. Images are representative of three independent experiments. (d) Disease index of the cotton plants at 26 dpi. The disease index (DI) was calculated as the following formula: DI = [(Σdisease grades × number of infected plants)/(total checked plants × 4)] × 100%. Seedlings were classified into five grades (0, 1, 2, 3, and 4). The data represent the mean across three independent experiments

in *N. benthamiana*, pepper, and *Arabidopsis*, including the upregulation of HR-specific marker genes and the activation of SA- and JA-mediated resistance pathways (Figure 3). Therefore, we speculated that Vd424Y is a potential PAMP. Consistent with our hypothesis, Vd424Y dramatically activated the expression of PTI marker genes (Figure 4). Two PRR-RLKs, *BAK1* and *SOBIR1*, were shown to be necessary for Vd424Y-triggered cell death in *N. benthamiana* (Figure 5), further confirming Vd424Y to be a PAMP. This is similar to the case of VdCUT11 and VmE02: VdCUT11-induced plant defence responses in *N. benthamiana* were dependent on both *BAK1* and *SOBIR1* (Gui et al., 2018) and VmE02 triggered cell death in *N. benthamiana* depending on the presence of *BAK1*, *SOBIR1*, *HSP90*, and *SGT1* (Nie et al., 2019). We observed that SP and NLS are essential for Vd424Y-trigged immune responses, because they activate the expression of marker genes involved in SA and JA signalling and PTI, while mutations in the SP and the NLS weaken expression of the above marker genes (Figures 3 and 4). These results demonstrated that immune responses in *N. benthamiana* leaves that are induced by the Vd424Y effector protein depend on its translocation from the plant's apoplatic space to the nucleus. Phylogenetic analysis demonstrated that Vd424Y is conserved in different *Verticillium* species and that its homologs are present in several fungi species (Figure S9), suggesting that Vd424Y may have a conserved function in triggering plant immunity.

We also performed *V. dahliae* infection assays in cotton plants and found that Vd424Y was highly expressed at the early stages of cotton infection by *V. dahliae* (Figure S7). This indicates that Vd424Y is closely related to the virulence of *V. dahliae* when it has infected cotton. Vd424Y knockout mutants did not affect the growth rate and colony morphology of Vd991 but weakened its virulence. Similar findings were reported in *Fusarium graminearum* (Yang et al., 2021), where the secreted ribonuclease Fg12 was significantly induced at the early stages of *F. graminearum* infection in soybean. Deleting the secreted ribonuclease Fg12 reduced the virulence of *F. graminearum*. In conclusion, our results demonstrate that Vd424Y can efficiently activate of plant immunity.

4 | EXPERIMENTAL PROCEDURES

4.1 | Strains and plant growth conditions

The wildtype *V. dahliae* strain 991 (Vd991) was grown in the dark on potato dextrose agar (PDA) at 25 ℃. The *P. capsici* strain 35 (Table S2) was also grown in the dark in 10% V8 juice agar at 25 ℃. *Escherichia coli* DH5α, which was cultured in a Luria-Bertani (LB) medium at 37 ℃, was used for plasmid construction, whereas *A. tumefaciens*

GV3101 (pSoup), which was grown in LB medium at 28 °C, was used for the agroinfiltration of plants. *N. benthamiana*, *Capsicum annuum*, and *A. thaliana* were grown in a greenhouse under a 16 hr light/8 hr dark cycle at 22 ± 1 °C. Cotton plants were also grown in a greenhouse under a 16 hr light/8 hr dark cycle at 26 ± 1 °C.

4.2 | Plasmids construction

The *V. dahliae* candidate effector EVM0004916 (Vd424) with SP (Vd424Y), was amplified from a Vd991 cDNA library with gene-specific primers (Table S3) using TKS Gflex DNA polymerase (Takara). The fragment was then cloned into the PVX vector pGR107-HA (Yang et al., 2021) using the ClonExpress II One Step Cloning Kit (Vazyme Biotech Co. Ltd). The variants Vd424N (without SP), Vd424Y-nls (with the mutated NLS), and Vd424N-NbPR1-SP (with the SP of NbPR1 fused into Vd424N) were cloned into pGR107. For subcellular localization, the Vd424Y, Vd424Y-nls, Vd424Y-nls-NES (Vd424Y-nls fused with the NES), and Vd424Y-NES (Vd424Y fused with the NES) variants were cloned into the Gateway entry vector QBV3 and then into the destination expression vector pEG101 (Earley et al., 2006). For *Phytophthora* infection assays, the QBV3-Vd424Y, QBV3-Vd424N, and QBV3-Vd424Y-nls were ligated into the pEG100 vector. For VIGS assays, NbBAK1, NbSOBIR1, NbEDS1, NbNDR1, GFP, and phytoene desaturase (PDS) were cloned into pTRV2 (Dong et al., 2007).

4.3 | Yeast signal sequence trap

A yeast secretion system was performed to validate the function of the predicted SP (Yin et al., 2018). Specifically, Vd424Y's predicted SP was cloned into pSUC2T7M13ORI (pSUC2) using specific primers (Table S2), and the resulting plasmid was then transformed into the YTK12, an invertase mutant yeast strain (Oh et al., 2009). The positive colonies were screened on a CMD−W medium (0.67% yeast N base without amino acids, 0.075% W dropout supplement, 2% sucrose, 0.1% glucose, 2% agar) and incubated on YPRAA medium (1% yeast extract, 2% peptone, 2% raffinose, 2 mg/ml antimycin A) to assay invertase secretion. The empty pSUC2 and pSUC2-Avr1b^{SP} vectors were used as negative and positive controls, respectively.

4.4 | VIGS in *N. benthamiana*

In TRV-mediated gene silencing assays, the plasmid constructs pTRV1, pTRV2-NbBAK1, pTRV2-NbSOBIR1, pTRV2-NbEDS1, pTRV2-NbNDR1, pTRV2-GFP, and pTRV2-PDS were introduced into *A. tumefaciens* GV3101. The two primary leaves of four-leaf-stage *N. benthamiana* seedlings were injected with a mixture (1:1 ratio) of *A. tumefaciens* culture (OD_{600} = 1.8) containing pTRV1 and pTRV2-genes. The pTRV2-PDS was used to evaluate VIGS efficiency, while the pTRV2-GFP served as control. The RNA extracted from leaves was used to validate the efficiency of gene silencing by RT-qPCR.

4.5 | Infection assays

To infect cotton plants with *V. dahliae*, 4-week-old Jimian 11 plants were inoculated with 10 ml conidial suspensions following previously published methods (Gong et al., 2017). The disease index (DI) was calculated according to previously described protocols (Gong et al., 2017). The infected plants were classified into five grades (grade 0, 1, 2, 3, and 4) according to the symptoms on the cotyledons and new leaves (Wang, Chen, et al., 2004; Zhang et al., 2012).

To infect *N. benthamiana* with *P. capsici*, 4-week-old *N. benthamiana* plants expressing the empty vector pEG100 (used as control), Vd424Y, Vd424N, and Vd424Y-nls were inoculated with *P. capsici* strain 35 using a previously published protocol (Zhang et al., 2019). Disease development was evaluated by staining lesions with trypan blue (Xiong et al., 2014) and assessing the biomass of *P. capsici* (Zhang et al., 2019). This procedure was repeated twice for validation and provided similar results.

4.6 | RT-qPCR analysis

Total RNA was isolated using Tiangen RNAprep Pure Plant Plus Kit (DP441), quantified and used as a template for reverse transcription with the PrimeScript RT reagent Kit (Takara). The RT-qPCR assays were performed using the 2× Wiz Universal SYBR Green qPCR Master Mix (BCS, No. SPE00005). The *NbActin* gene was used as internal control. The sequences of each of the primers used in the different RT-qPCR assays are listed in Table S2.

4.7 | Subcellular localization assays

Recombinant plasmids were transformed into *A. tumefaciens* GV3101. The resulting strains were then expressed in 4-week-old *N. benthamiana* leaves using a previously described protocol (Qiao et al., 2015). Forty-eight hours after *Agrobacterium*-infiltration, yellow fluorescence from YFP was detected using a LSM780 confocal microscope.

4.8 | Protein extraction and western blotting

For transient expression analysis, the protein was extracted from the leaves of 4-week-old *N. benthamiana* plants after *Agrobacterium*-infiltration. The infiltrated leaves were subsequently collected after 48 hr, ground in liquid nitrogen, and mixed with an equal volume of cold protein isolation buffer (1 mM EDTA pH 8.0, 20 mM Tris-HCl pH 7.5, 5 mM dithiothreitol, 150 mM NaCl, 0.1% sodium dodecyl sulphate [SDS], 10% glycerol, and 1 × protease inhibitor cocktail

[Sigma-Aldrich]). The mixture was centrifuged at 4 °C for 10 min at 13,000 × g, transferred to a new tube, and boiled in protein sample buffer for 5 min. Proteins were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare).

4.9 | Bioinformatics analysis

SP prediction was implemented using the SignalP-5.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The putative bipartite NLS (Ding et al., 1991) was predicted using the cNLS Mapper (nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi).

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AUTHOR CONTRIBUTIONS

X.Y.G., L.S.L., F.G.L., and Y.J.L. planned and designed the research. L.S.L., Z.H.W., and J.N.L. performed the research. L.S.L., Z.H.W., J.N.L., Y.W., J.C.Y., J.J.Z., and P.W. analysed the data. L.S.L., X.Y.G. F.G.L., and Y.J.L. wrote the paper. All authors commented on the article before submission.

DATA AVAILABILITY STATEMENT

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

ORCID

Xiaoyang Ge https://orcid.org/0000-0003-3428-2942

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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