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Research Article

Cytotoxic function of xylanase VdXyn4 in the plant vascular wilt pathogen *Verticillium dahliae*

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D.D.Z., J.Y.C., K.V.S., and X.F.D. designed the research. D.W. contributed to the functional analysis of VdXyn4 and data analysis. J.S. identified the six xylanases and analyzed the enzymic activity of VdXyn4. J.J.L., R.L., and Z.Q.K. assisted with individual experiments. D.D.Z., J.Y.C., and D.W. prepared the manuscript that was edited by S.J.K. and K.V.S., D.W., and J.Y.C. contributed equally. None of the authors have conflicts of interest with this manuscript.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/general-instructions) is: Dan-Dan Zhang (zhangdandan@caas.cn).

Abstract

Phytopathogen xylanases play critical roles in pathogenesis, likely due to their ability to degrade plant structural barriers and manipulate host immunity. As an invader of plant xylem vessels, the fungus *Verticillium dahliae* is thought to deploy complex cell wall degrading enzymes. Comparative genomics analyses revealed that the *V. dahliae* genome encodes a family of six xylanases, each possessing a glycosyl hydrolase 11 domain, but the functions of these enzymes are undetermined. Characterizing gene deletion mutants revealed that only *V. dahliae* xylanase 4 (VdXyn4) degraded the plant cell wall and contributed to the virulence of *V. dahliae*. VdXyn4 displayed cytotoxic activity and induced a necrosis phenotype during the late stages of infection, leading to vein and petiole collapse that depended on the enzyme simultaneously localizing to nuclei and chloroplasts. The internalization of VdXyn4 was in conjunction with that of the plasma membrane complexLeucine-rich repeat (LRR)-receptor-like kinase suppressor of BIR1-1 (SOBIR1)/LRR-RLK BRI1-associated kinase-1 (BAK1), but we could not rule out the possibility that VdXyn4 may also act as an apoplastic effector. Immune signaling (in the SA–JA pathways) induced by VdXyn4 relative to that induced by known immunity effectors was substantially delayed. While cytotoxic activity could be partially suppressed by known effectors, they failed to impede necrosis in *Nicotiana benthamiana*. Thus, unlike typical effectors, cytotoxicity of VdXyn4 plays a crucial intracellular role at the late stages of *V. dahliae* infection and colonization, especially following pathogen entry into the xylem; this cytotoxic activity is likely conserved in the corresponding enzyme families in plant vascular pathogens.

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Introduction

Xylan, the major constituent of hemicellulose in plant cell walls that primarily contain xylose and arabinose, is the second most abundant polysaccharide on earth (Bastawade, 1992). Xylanase is believed to play critical roles in the degradation of the complex hemicellulose although several other hydrolytic enzymes are needed for complete degradation (Shallom and Shoham, 2003; Collins et al., 2005). Based on their primary structures, xylanases mainly belong to the glycosyl hydrolase families 10 (GH10) and 11 (GH11) (Jeffries, 1996; Collins et al., 2005; Zhou et al., 2008). GH10 endoxylanases typically have a high molecular weight ($\geqslant$30 kDa) and a low isoelectric point (pI), but GH11 endoxylanases are often characterized by a low molecular mass (typically around 22 kDa) and a high pI (Törrönen and Rouvinen, 1997; Subramaniyan and Prema, 2002). For both families, two glutamate moieties act as catalytic residues in the enzymatic reaction (Henrissat and Davies, 1997; Rye and Withers, 2000).

In phytopathogens, the enzymes collectively known as xylanases are a part of the arsenal of carbohydrate-active enzymes (CAZymes) that breach the plant cell-wall barriers, and simultaneously can be recognized by the host plant to elicit defense responses. Because of the importance of xylanases in pathogen infection processes, many genes encoding xylanases in fungal pathogens have been identified and characterized. The expression of many genes encoding endoxylanases from microorganisms is induced during infection and plays important roles in pathogenicity (Wu et al., 1995, 2006a; Ruiz-Roldán et al., 1999; Gómez-Gómez et al., 2001, 2002; Brito et al., 2006; Noda et al., 2010; Yu et al., 2016). For instance, *SsXyl1*, encoding an endo-β-1,4-xylanase from *Sclerotinia sclerotiorum*, is expressed at very high levels and renders the pathogen more virulent (Yu et al., 2016). However, targeted disruptions of individual xylanase genes have revealed that these are not essential for pathogenicity (Apel et al., 1993; Gómez-Gómez et al., 2001; Wu et al., 2006b; Sella et al., 2013). The genetic and functional redundancy of multiple endoxylanases in each microorganism have been put forward as one of the main reasons for why the gene disruption mutants of endoxylanase remain virulent (Apel-Birkhold and Walton 1996; Gómez-Gómez et al., 2002). Moreover, the roles of xylanases in pathogenicity may not always be dependent on their enzymatic activity. In *Botrytis cinerea*, an endo-beta-1,4-xylanase belonging to family 11 of glycosyl hydrolases gene, *xyn11A*, contributes to virulence via its necrotizing activity and not by its catalytic activity (Brito et al., 2006; Noda et al., 2010).

In a classical model of plant–pathogen interactions, plants employ two defense systems in response to pathogen attacks. The first intimate contact between host plants and pathogen is established in the apoplast, in which plant pattern recognition receptors may recognize pathogen-associated molecular patterns (PAMPs). This recognition is dependent on the central regulator BAK1 (LRR- receptor-like kinase [RLK] BRI1-associated kinase-1) and/or suppressor of BIR1-1 (LRR-RLKSOBIR1) to induce PAMPs-triggered immunity (PTI). The second immunity system known as effector-triggered immunity (ETI) occurs through the intracellular nucleotide-binding/leucine-rich repeat receptors. To counter these two defense systems, pathogens secrete extracellular effectors to plant apoplast to suppress PTI, and transfer effectors to plant intracellular space to interfere with ETI, enabling the pathogens then to infect host plants and cause disease (Jones and Dangl, 2006; Zipfel, 2008). Xylanse is a well-known proteinaceous elicitor involved in PTI during host–pathogen interactions, since the ethylene-inducing xylanase (EIX) is conserved across genera (Enkerli et al., 1999). When these xylanases are expressed in *Nicotiana tabacum* or *Lycopersicon esculentum* leaves, they can induce defense responses to cause necrosis and hypersensitive cell death (Bailey et al., 1990; Avni et al., 1994; Enkerli et al., 1999). The EIX from *Trichoderma viride* has been extensively studied to elucidate the elicitor activity of fungal xylanases (Dean and Anderson, 1991; Yano et al., 1998, Ron and Avni, 2004). EIX induces ethylene production and rapid cell death, symptoms associated with the hypersensitive response in *N. tabacum* and *L. esculentum* plants (Avni et al., 1994). Similarly, the *Trichoderma reesei* xylanase II acts as an elicitor, inducing defense responses in tomato (Enkerli et al., 1999). Enzymatically inactive mutants from both xylanases retained elicitor activity, proving that endoxylanase activity is not necessary for elicitor activity (Avni et al., 1994; Enkerli et al., 1999). The EIX1 from *T. viride* is also an elicitor that interacts with the receptor-like protein (RLP) LeEix2 to induce defense responses (Bar et al., 2010). Similarly, the RLP NbEIX2 regulates the perception of *Verticillium dahliae* EIX-like protein VdEIX3 in *Nicotiana benthamiana* to induce immunity responses (Yin et al, 2020).

Verticillium dahliae is a soilborne plant pathogen with a broad range of host that invades the water-conducting xylem vessels of susceptible hosts to cause a vascular wilt disease in over 200 plant species (Fradin and Thomma, 2006; Klosterman et al., 2009; Inderbitzin and Subbarao, 2014). As specific colonizers of plant xylem vessels, *V. dahliae* encodes a complex array of cell wall degrading enzymes, presumably as adaptation to this unique ecological niche in a wide range of host species (Klosterman et al., 2011; Chen et al., 2018). A range of pectinolytic enzymes and their activities were previously hypothesized to accelerate plant cell wall degradation, especially polysaccharide lyase (*PL*) and a select glycoside hydrolase (*GH*) gene family (Klosterman et al., 2011). These cell wall degrading enzymes may degrade cell wall components and affect pathogen virulence, such as that shown for the PL (PL1 and PL3) and also the GH12 proteins (Chen et al., 2016; Gui et al., 2017). Alternatively, some of these cell wall degrading enzymes, or the plant cell wall fragments degraded by these enzymes, could be recognized by host plant to induce immunity, and some enzymes have previously been characterized in *V. dahliae* (Gui et al., 2017, 2018). In our previous study, comparative genomics showed that the *V. dahliae* genome encodes a family of six xylanases

(named VdXyn1–VdXyn6) with GH11 domains (Chen et al., 2018). With kaleidoscopic functions of xylanases in virulence, elicitor or necrosis activity, the mechanisms of xylanase involvement in pathogenicity during *V. dahliae* infection are unexplored and require further scrutiny.

In this study, the role of xylanases in *V. dahliae* pathogenicity was investigated systematically in plant systems. The main objectives of the current study were to: (1) identify the cell wall degrading activity and function of xylanases in virulence in *V. dahliae*; (2) investigate whether xylanases function as immunity effectors or necrosis factors; and (3) explore the mechanism of xylanase participation in pathogenicity during *V. dahliae*–host interactions.

Results

Identification of GH11 domain-containing xylanase in *V. dahliae*

From the bioinformatics analysis, six xylanases (VdXyn1–VdXyn6) with GH11 domain-containing proteins were identified in the Vd991 genome (Supplemental Table S1). Investigations of the basic structural characteristics of these xylanases showed that all VdXyns were predicted to be secreted proteins that possess the extracellular localization signal peptide (SP) and lack a transmembrane motif. Compared with the 94 xylanase members from 24 fungal species from *Sordariomycetes* (Supplemental Table S1), the xylanase family displayed a relative expansion in the nine *Verticillium* spp. (the majority of *Verticillium* spp. contained five members compared with the other genera that possessed three members; Supplemental Table S2), suggesting that the xylanase family could play important roles in *Verticillium* spp. The conserved GH11 domain of 31 xylanase members from other genera was also appended with the carbohydrate-binding module family (CBM1) and/or CBM60 domain, but not in *V. dahliae* xylanase (Figure 1A; Supplemental Table S1). Phylogenetic analysis showed that *V. dahliae* xylanase family members sorted into four distinct groups (G1–G4), with G1 (VdXyn3 and VdXyn4), G2 (VdXyn1), and G4 (VdXyn2 and VdXyn5) widely represented in other fungi, and the xylanase members displayed relatively higher sequence identities within groups (Figure 1A; Supplemental Table S1), suggesting that the *Sordariomycetes* have three types of conserved xylanases. However, G3 (VdXyn6) was only present in *Verticillium* spp (Figure 1A). Sequence alignment of the GH11 domain from VdXyns showed that this domain is highly conserved among the five xylanase members (VdXyn1–VdXyn5) and contains two conserved sites for enzymatic activity associated with a glutamic (Glu) acid residue (Figure 1B). However, VdXyn6 (166 aa) with the shortest sequence length displayed substantial sequence divergence at the C-terminus and lost a necessary active site associated with the Glu residue (Figure 1B), which was probably caused by gene prediction errors in the Vd991 genome (Chen et al., 2018). For these reasons, *VdXyn6* was not analyzed further. Instead, the five xylanase members *VdXyn1–VdXyn5* were examined further in this study.

VdXyn4 displays cell wall degradation activity

To investigate the role of the *V. dahliae* xylanases in cell wall degradation, xylanase gene deletion strains were generated to determine whether their defective function was associated with an ability to utilize plant cell wall components. Two random deletion strains of each of the five xylanase genes (Δ*Xyn1*–Δ*Xyn5*) were validated bypolymerase chain reaction (PCR) amplification and selected for further analysis (Supplemental Figure S1). Analyses of the growth phenotypes showed that the colony morphology of xylanase gene deletion mutants changed little when compared with the wild-type strain, retaining the white mycelia and similar colony size at 11 days after incubation on potato dextrose agar (PDA) medium (Supplemental Figure S2). This suggested that none of the *V. dahliae* xylanases are necessary for growth, similar to the wild-type strain, at least under the conditions tested, in which full nutrition was available. Furthermore, the ability of deletion strains to degrade cell wall components was assessed on C'zapek medium lacking the carbon source (sucrose), but supplemented with xylan, cellulose, and pectin, respectively. Relative to the wild-type strain, the colony sizes of the *VdXyn4* or *VdXyn5* mutants were significantly reduced when xylan was the carbon source (Figure 2, A and B), suggesting that *VdXyn4* and *VdXyn5* contribute to xylan degradation by *V. dahliae*. Unexpectedly, the colony sizes of *VdXyn4* or *VdXyn5* deletion strains also were significantly reduced when cultured on C'zapek medium (sucrose; Figure 2, A and C), which indicated that either *VdXyn4* or *VdXyn5* was also involved in basal metabolism involving other carbon sources. Their functions diverged, however, in the efficient utilization of cellulose and pectin as carbon sources. The colony diameters of Δ*VdXyn4* strains were significantly reduced compared to both wild-type and Δ*VdXyn5* strains (Figure 2, A, D, and E), suggesting that among the *V. dahliae* xylanase family, only *VdXyn4* participates in the metabolism of cellulose and pectin. These results strongly suggested that the two xylanase genes, *VdXyn4* and *VdXyn5*, are involved in utilizing xylan in addition to other carbon sources in *V. dahliae*, but only *VdXyn4* has a role in degrading other components of the cell wall, including cellulose and pectin.

VdXyn4 contributes to virulence of *V. dahliae* during infection of cotton

To explore whether the functional differences in cell wall degradation by *VdXyn1-VdXyn5* were also associated with virulence, expression levels of five xylanase genes and virulence of every gene deletion mutant were analyzed on cotton. Expression analysis showed that all xylanase genes were significantly upregulated during *V. dahliae* infection of cotton roots, especially the upregulation of *VdXyn4*, a 100-fold increase at 7 days post inoculation (Supplemental Figure S3A). Virulence assays showed that only two

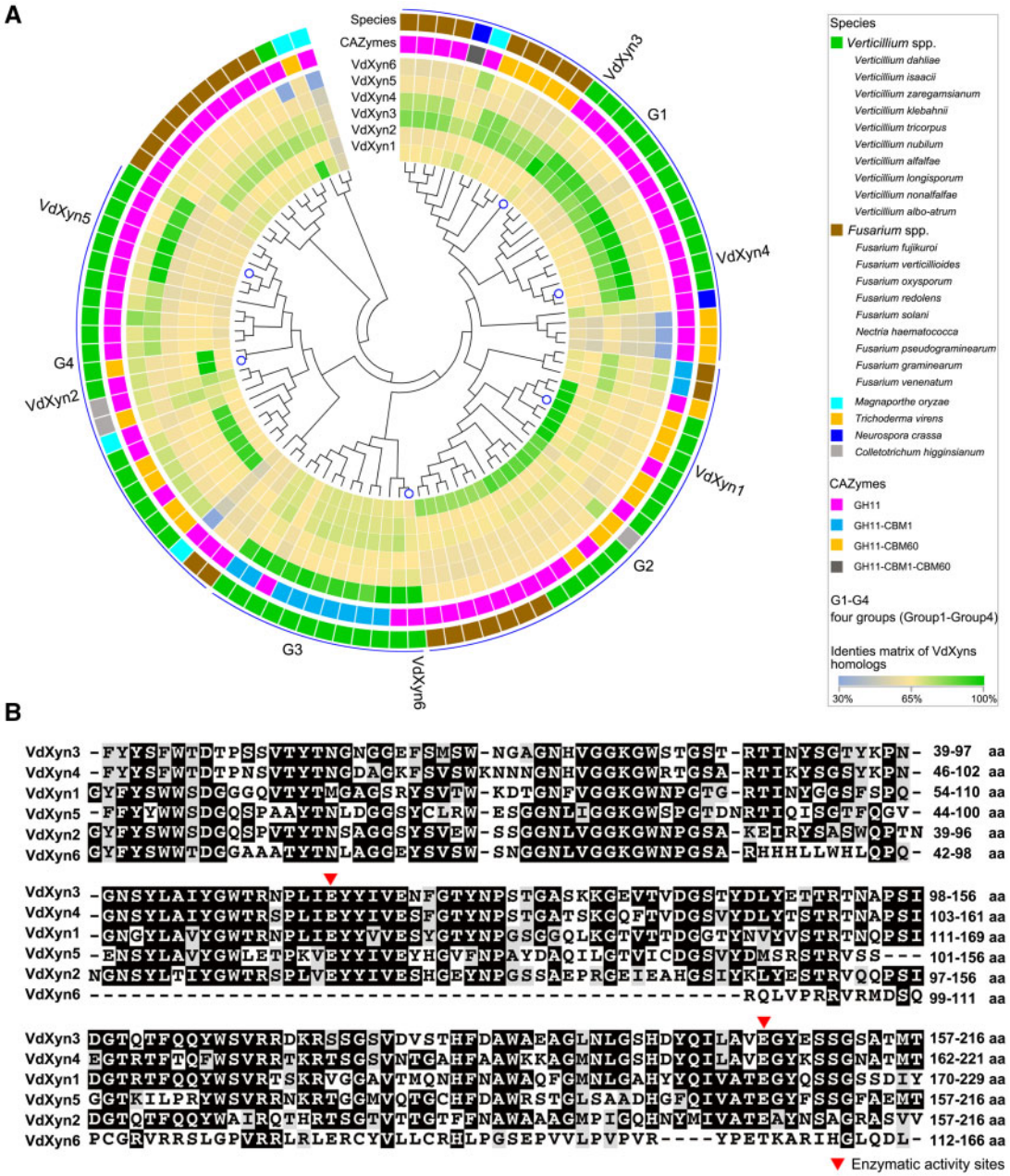

Figure 1 Bioinformatics of the glycoside hydrolase family 11 xylanase in *V. dahliae*. A, Phylogenetic analysis between *V. dahliae* glycoside hydrolase family 11 xylanase members and xylanases from other 23 species of *Sordariomycetes*. B, Sequence alignment of the GH11 domain from six members of the GH11 protein family in *V. dahliae*. GH11 domain sequences were aligned using *Clustal X2*. Numbers represent the position of the amino acid (aa) residues. Conserved enzymatic activity sites are highlighted with red triangles. White/gray/black shaded letters: for the alignment sequence of each site, if $>50\%$ (including 50%) of the amino acid residues were identical, the identical amino acids at these sites were depicted in black, amino acids with same properties were marked in gray, and those with different properties were marked in white.

independent *VdXyn4* deletion transformants substantially reduced the Verticillium wilt symptoms on cotton, including leaf wilting, necrosis, and death, compared to the wild-type strain (Figure 3A; Supplemental Figure S3B). Symptom quantification and disease indices analyses revealed that *VdXyn4* knockout mutants were virulent, but at a reduced level compared with the wild-type strain in cotton plants at 3-weeks post inoculation (wpi; Figure 3B). Furthermore, quantification of the fungal biomass in cotton stems showed that colonization by Δ*VdXyn4* was significantly reduced compared with the wild-type strain (Figure 3C). However, strains that utilize xylan displayed virulence as high as that caused by the wild-type strain as measured by leaf wilt ratios, vascular discoloration, and fungal biomass (Figure 3, A–C; Supplemental Figure S3, B and C). Interestingly, stem sectioning of the inoculated plants showed that the

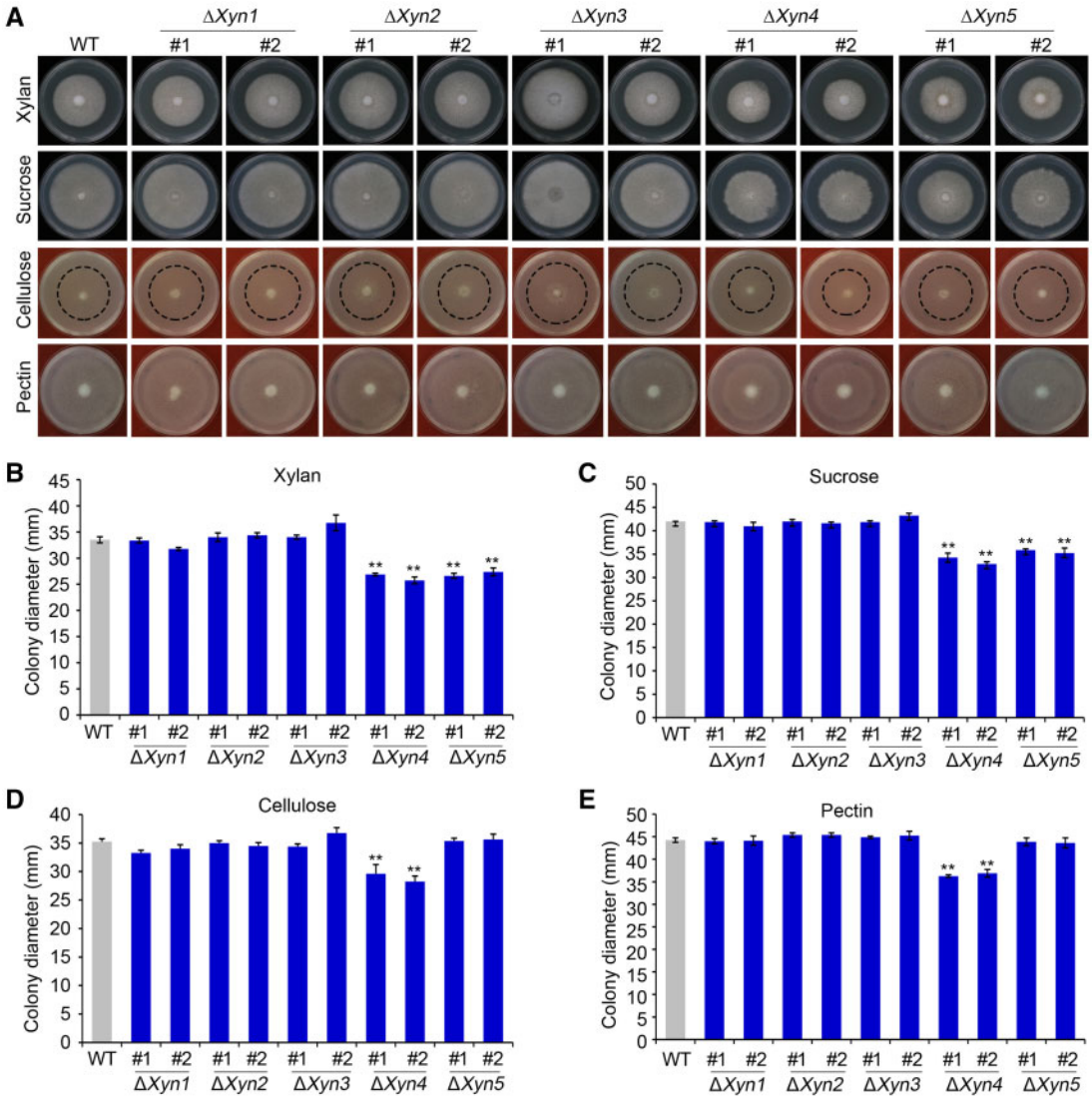

Figure 2 Growth phenotypes of five xylanase gene deletion mutants of *V. dahliae*. A, The growth phenotypes of five Δ*Xyn1*–Δ*Xyn5* deletion mutant strains of *V. dahliae* on basic C'zapek medium containing the xylan, sucrose, cellulose, and pectin as carbon sources, respectively. Images were captured after 11 days of incubation at 25°C. The dashed circle was used to mark the boundaries of colony growth on cellulose containing medium. Two independent transformations for each gene deletion mutant were investigated. B–E, The radial growth of the five gene deletion mutant strains on basic C'zapek medium agar plates that included xylan, sucrose, cellulose, and pectin as carbon sources. Error bars represent standard errors (SE) of the mean. Asterisks represent statistical significance at $P < 0.01$ based on unpaired Student's *t* tests.

Δ*VdXyn4* strain lost the ability to cause vascular discoloration compared with the wild-type strain (Figure 3D), indicating that the function of *VdXyn4* in virulence may be linked to its ability to degrade cell wall components in the xylem. Together, these results suggested that among the xylanase family members in *V. dahliae*, only *VdXyn4* plays a critical role in virulence on cotton.

Enzymatic activity of VdXyn4 is required for full virulence in *V. dahliae*

Previous studies revealed that two Glu residues within the active site are essential for xylanase activity (Henrissat and Davies, 1997). These sites correspond to Glu-119 and Glu-210 within the primary sequence of VdXyn4 (Figures 1B and 4A). To confirm whether the xylanase activity of VdXyn4 is dependent on these two Glu residues, the enzymatic activity was detected by the recombinant proteins with xylan as the substrate. The recombinant proteins from native VdXyn4 and site-directed mutant (VdXyn4$^{SM119-210}$, Glu replaced by Alanine) were purified using the small ubiquitin-related modifier (SUMO) tag. Assays of the enzymatic activity of the recombinant proteins revealed that VdXyn4 had the enzymatic activity necessary to degrade xylan into oligosaccharides and monosaccharides. The reducing oligosaccharides and monosaccharides can react with 3,5-dinitrosalicylic acid reagent in a boiling water bath to yield a

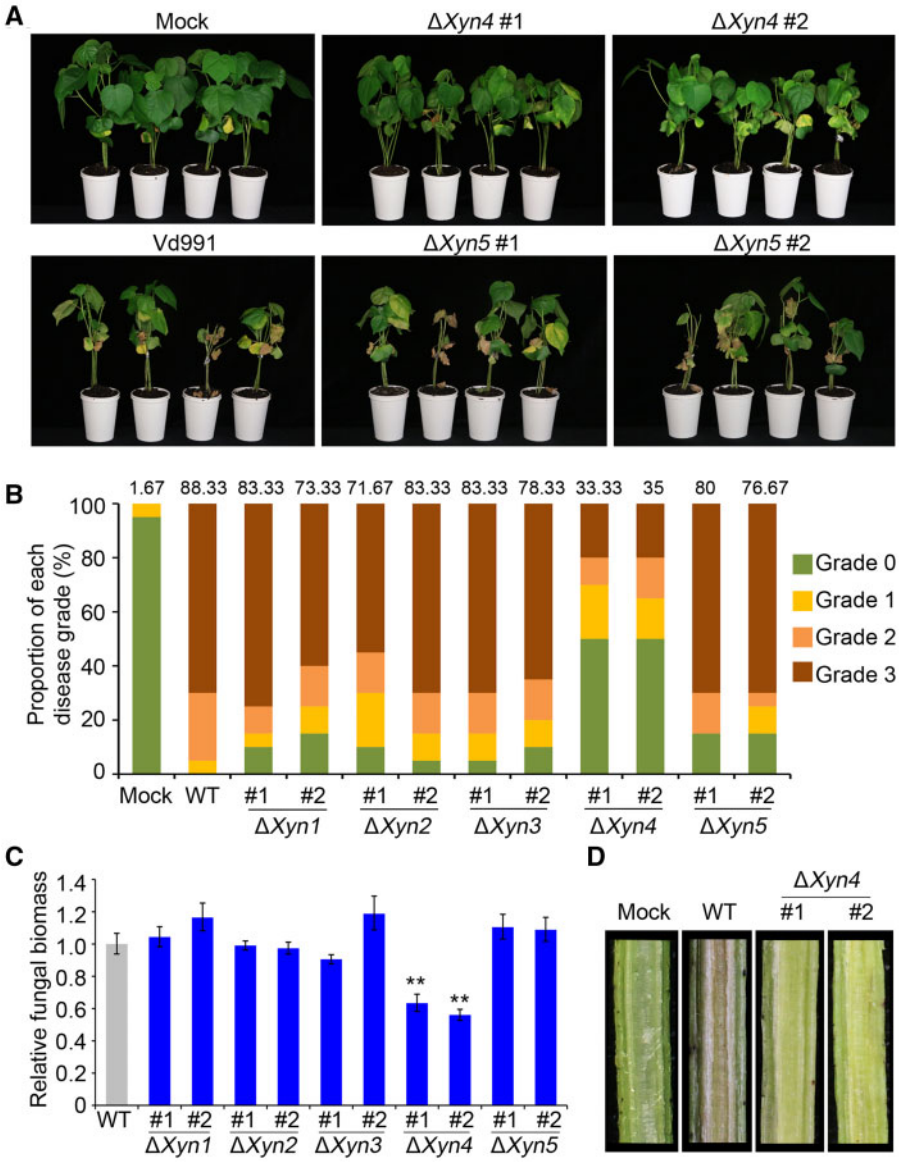

Figure 3 Virulence assays with five xylanase gene deletion mutant strains of *V. dahliae* on cotton. A, Assays to investigate the role of genes *VdXyn4* and *VdXyn5* in the virulence of *V. dahliae* on *Gossypium hirsutum* cv Junmian No. 1. Three-week-old seedlings of cotton plants were inoculated with the respective gene deletion strains with two independent transformations for each gene, in addition to inoculation with wild-type (WT) *V. dahliae* (Vd991), and sterile water (Mock). Verticillium wilt symptoms were photographed 3 wpi. B, The disease was evaluated on a 0–3 scale in which, Grade 0 was 0%–25% leaves wilted, Grade 1 where 25%–50% leaves wilted, Grade 2 where 50%–75% leaves wilted and Grade 3 where 75%–100% leaves wilted. The data were generated from two replicate experiments with a total of 20 cotton seedlings for each *V. dahliae* strain inoculated. The bar chart represents the proportion of each disease grade, and the numbers on top of the column represent the DI calculated from the disease grades. The ratings were conducted with 20 cotton seedlings at 3 wpi with each of the respective *V. dahliae* strains. C, Fungal biomass was quantified in cotton by quantitative PCR following inoculation of the deletion mutant strains at 3 wpi. The *V. dahliae* elongation factor 1-α (*EF-1α*) was used to quantify fungal biomass in the colonized tissue, and the cotton *18S* served as an endogenous plant control. Error bars represent SE of the mean. Asterisks represent statistical significance at $P < 0.01$ based on unpaired Student's *t* tests. D, Phenotypes of stem longitudinal sections of cotton plants inoculated with *VdXyn4* deletion strains, at 3 wpi.

color change indicative of xylan hydrolysis. The color of VdXyn4 reaction solution changed from yellow to dull red, whereas the mutagenized recombinant proteins ($Xyn4^{SM119–210}$) nearly lost the ability to hydrolyze xylan and there was no color change in the test solution (Figure 4B). Quantification of the enzymatic activity by absorbance levels in spectrophotometry showed that hydrolysis activity was significantly reduced in the recombinant proteins from $Xyn4^{SM119–210}$ (3.67 ± 0.19 U) compared to the native VdXyn4 (161.19 ± 7.50 U) protein (Figure 4C). These results

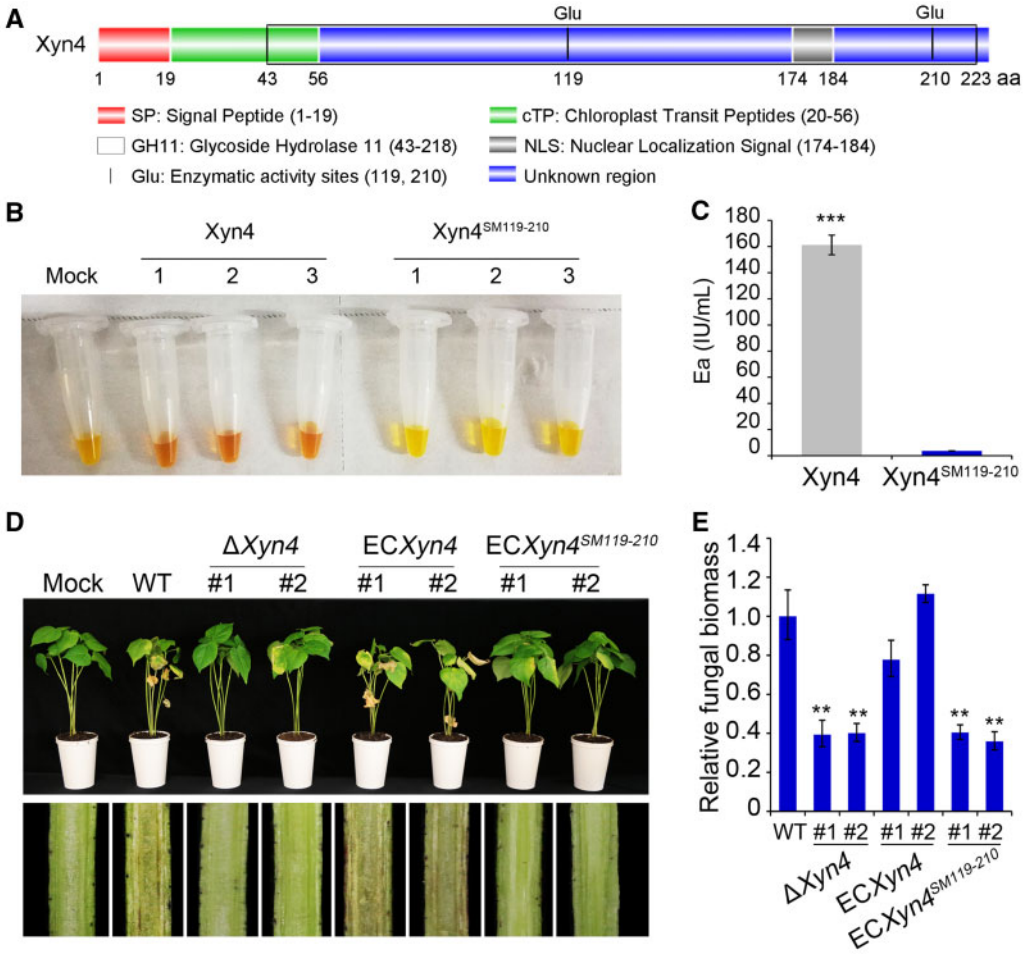

Figure 4 Function identification of enzyme activity sites of xylanase VdXyn4 from *V. dahliae*. A, Sequence characteristics of VdXyn4, including: SP, N-terminal cTP, Glu, two enzymatic activity sites, NLS sequence, Glycoside hydrolase 11 (GH11) domain. Numbers in schematic represent the position of the amino acid (aa) residues. B and C, Xylanase activity determination of WT and two site-directed mutants. Induction was carried out with beechwood xylan as the only carbon source in each case. Ea represents enzyme activity. Error bars represent SE of the mean. Asterisks represent statistical significance at $P < 0.001$ based on unpaired Student's *t* tests. D, Phenotypes of cotton seedlings inoculated with *VdXyn4* gene deletion strains and complementary transformants. The cotton seedlings were inoculated with sterile water (Mock), WT *V. dahliae*, *VdXyn4* gene deletion strains, and complementary transformants. The disease symptoms are shown at the top and the discoloration of the shoot longitudinal sections is shown at the bottom at 3 wpi. E, The fungal biomass of the gene deletion strains and corresponding ectopic transformants on cotton were determined by quantitative PCR. The *V. dahliae* elongation factor 1-α (*EF-1α*) was used to quantify fungal colonization, and the cotton *18S* served as an endogenous plant control. EC represents ectopic complement transformants. Error bars represent SE of the mean. Asterisks represent statistical significance at $P < 0.01$ based on unpaired Student's *t* tests.

further suggested that VdXyn4 belonged to the xylanase family and that the xylanase activity of VdXyn4 was indeed dependent on the two conserved Glu residues.

Furthermore, the strong correspondence between VdXyn4 xylanase activity and its virulence on cotton was further verified by the re-introduction of the native *VdXyn4* or $Xyn4^{SM119-210}$ gene into the *VdXyn4* deletion mutant. As expected, *VdXyn4* deletion mutants were restored to wild-type levels of virulence on cotton with the re-introduction of the native *VdXyn4*. Inoculated plants displayed typical leaf wilting, necrosis, and plant death. However, reintroduction of $Xyn4^{SM119-210}$ gene into the *VdXyn4* deletion mutant failed to restore virulence on cotton (Figure 4D). Quantification of fungal biomass provided further evidence that reintroduction of $Xyn4^{SM119-210}$ gene could not restore the fungal biomass in planta, compared with reintroduction of the native gene *VdXyn4* (Figure 4E). These results further support that *VdXyn4* is required for full virulence of *V. dahliae* and that this function is dependent on its xylanase activity.

By virtue of its cytotoxic activity, VdXyn4 induces necrosis phenotype along the vein tissue during the late stages of infection

Several xylanases have a proteinaceous elicitor activity that can induce defense responses in *N. tabacum* and *L. esculentum* (Beliën et al., 2006). To determine whether xylanase has

a similar elicitor activity in *V. dahliae*, the cell death inducing activity of xylanase genes was examined by transient expression of *VdXyn1–VdXyn5* in *N. benthamiana* leaves. Interestingly, agro-infiltration assays of five xylanase genes showed that only VdXyn4 caused leaf tissue collapse, not the cell death phenotype, at 10 d after agro-infiltration, but this was substantially later than the positive controls that used the programed cell death factor Bcl-2-associated X (BAX) or PAMPs immunity factor endoglucanase (VdEG1), which usually induced cell death around 3–4 d after infiltration (Figure 5A; Supplemental Figure S4A). Immunoblot analysis of the total protein extracted from the agro-infiltrated leaf area confirmed effective protein translation of all five xylanase family members (Supplemental Figure S4, B and C). These results suggested that unlike BAX and PAMPs, the xylanase VdXyn4 mainly induces leaf tissue collapse during later stages following infiltration into *N. benthamiana*. Interestingly, unlike the cell death induced by BAX or PAMPs, in which the cell collapse was restricted by the activation of defense response, the cell death induced by VdXyn4 gradually expanded through the leaf vein and petiole, and these tissues also showed the necrosis phenotype around 9 – 10 d after infiltration (Figure 5A; Supplemental Figure S4A). These results suggested that VdXyn4, mainly by its cytotoxicity, could induce cell necrosis in the vein tissue in *N. benthamiana*.

The correspondence between xylanase activity of VdXyn4 and its cytotoxic function was detected by the transient expression of the corresponding site-directed mutant genes ($Xyn4^{SM119}$, $Xyn4^{SM210}$, and $Xyn4^{SM119–210}$) and wild-type *VdXyn4* in *N. benthamiana* leaves. Unlike the native *VdXyn4*, $VdXyn4^{SM119}$, $Xyn4^{SM210}$, and $Xyn4^{SM119–210}$ could not induce leaf vein and petiole necrosis from 10 d after agro-infiltration in *N. benthamiana* leaves (Figure 5B). Immunoblot analysis showed that all *VdXyn4* site-directed mutant genes were unaffected in protein translation in *N. benthamiana* leaves (Supplemental Figure S4D), which further confirmed that the cytotoxic function of VdXyn4 was dependent on the xylanase activity in *V. dahliae*. Together, these results suggested that the VdXyn4 has the ability to

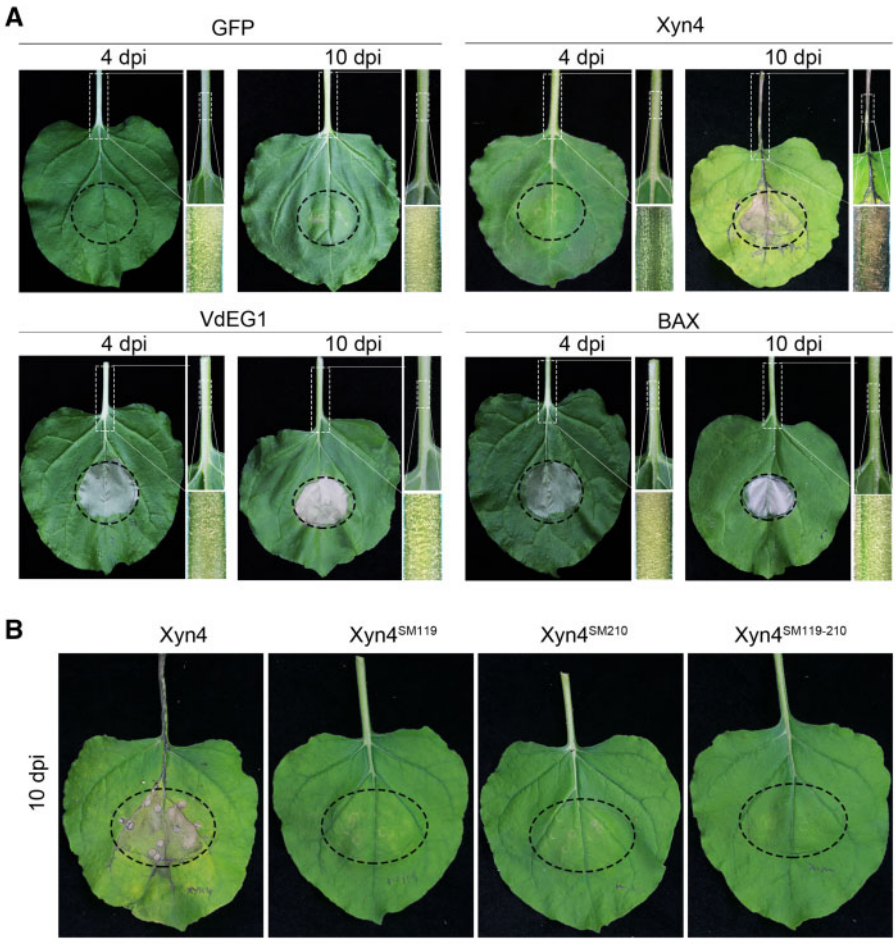

Figure 5 The cytotoxic activity analysis of VdXyn4 in *N. benthamiana*. A, Leaf cell-death induction and expansion through the leaf vein and petiole were observed for VdXyn4 expressed in *N. benthamiana* leaves from 4-week-old plants after 4 or 10 d. The enlarged picture on the right of each picture is the magnification of leaf petiole on the left, to better illustrate the cytotoxic activity in this tissue. PAMPs endoglucanase VdEG1 and BAX protein were used as positive controls; GFP was used as a negative control; dpi, days post inflitration. The dashed circles or ovals represent infiltrated area. B, Detection of cytotoxic activity of native gene (*Xyn4*) and site-directed mutants $Xyn4^{SM110}$, $Xyn4^{SM119}$, and $Xyn4^{SM110–SM119}$. Transient expression was assessed in *N. benthamiana* leaves from 4-week-old plants 10 d after agroinfiltration.

induce cell death in the *N. benthamiana* vein tissue by virtue of its cytotoxic activity that was dependent on the xylanase activity of VdXyn4.

Endocytic internalization into nuclei and chloroplasts is required for VdXyn4 to induce necrosis in *N. benthamiana*

Gene prediction showed that the protein encoded by *VdXyn4* contains a SP (19 aa at the *N*-terminus) indicative of extracellular localization (Supplemental Table S1). Yeast signal trap system showed that the SP of VdXyn4 had the ability to mediate the secretion of invertase after fusion with an invertase gene in yeast, and successfully conferred the ability of yeast strain YTK12 to utilize raffinose as the sole carbon source and grow normally (Supplemental Figure S4E). This suggested that VdXyn4 is an extracellular protein and that its secretion is mediated by the presence of the identified SP.

Prediction of the subcellular localization with the plant model suggested that VdXyn4 contains a chloroplast transit peptide (cTP) and nuclear localization signal (NLS), suggesting that VdXyn4 function may depend on its translocation into plant chloroplast or nucleus (Figure 4A; Supplemental Table S3). To further investigate the cellular process of necrosis induced by VdXyn4, its subcellular location and role in cell death were examined in *N. benthamiana* leaves. The VdXyn4– green fluorescent protein (GFP) fusion protein concentrated along the periphery of plasma membrane, partially overlapped with the fluorescence of cell membrane dye FM4-64, and was also abundant in the intracellular space, including on the nuclei, chloroplasts, and cytoplasmic vesicles. This fusion protein also overlapped with the fluorescence from the nuclear dye4',6-diamidino-2-phenylindole (DAPI) and chloroplast autofluorescence (Figure 6, A and B; Supplemental Figure S5). Similarly, when the SP of VdXyn4 was replaced with the plant SP of the pathogenesis-related 1 (PR1) protein (Van Loon and Ven Strien, 1999), there was a shift of protein secretion into the plant extracellular space. The recombinant protein was also localized at the *N. benthamiana* cell periphery of the plasma membrane, nuclei, chloroplasts, and cytoplasmic vesicles (Figure 6, A and B; Supplemental Figure S5). These results indicate that VdXyn4 functions intracellularly in *N. benthamiana*, especially on the cell periphery at the plasma membrane, and on nuclei and chloroplasts. To further verify if VdXyn4 can be secreted from *V. dahliae* and translocated into host cells, the *VdXyn4* sequence was fused with the *GFP* sequence and re-introduced into *V. dahliae VdXyn4* knockout mutant and screened. The positive transformants were named as EC_*Xyn4-GFP*. Subsequently, we co-incubated the conidial suspension of EC_*Xyn4-GFP* with onion epidermal cells for 4 d, when the Xyn4-GFP signals were observed in nuclei and at the periphery of plasma membrane (Supplemental Figure S6A). Furthermore, the conidial suspension of EC_*Xyn4-GFP* was injected into *N. benthamiana* leaves and examined at 2 d. EC_*Xyn4-GFP* signals clearly located on chloroplast overlapped with those of the chloroplast autofluorescence (Supplemental Figure S6B). In above two experiments, the wild-type *V. dahliae* strain with GFP alone was used as the negative control, in which case rare signals were detected around the host cells and most signals were concentrated in the mycelia and conidia of *V. dahliae* without secretion (Supplemental Figure S6). These results confirmed that Xyn4 could be secreted from *V. dahliae* and translocated into host cells and that it localizes at the peripheral plasma membrane, chloroplasts, and nuclei. Correspondingly, the recombinant proteins VdXyn4–GFP and VdXyn4^{PR1}–GFP all displayed similar cytotoxic activity to induce cell death on *N. benthamiana* leaves (Figure 6C).

Furthermore, the relationship between internalization and cytotoxic activity of VdXyn4 was determined by the absence of the SP (VdXyn4$^{\Delta SP}$), which failed to internalize since this construct was lacking in the extracellular localization signal. Interestingly, VdXyn4$^{\Delta SP}$ also concentrated peripherally on the cell membrane and in the nuclei, but it failed to locate on the chloroplasts (Figure 6, A and B). In addition, the recombinant protein VdXyn4$^{\Delta SP}$ could not induce necrosis in the *N. benthamiana* leaves (Figure 6C). Thus, the cytotoxic function of VdXyn4 occurred after intracellular localization, and this was dependent on the internalization process within the chloroplasts. To further confirm the function of VdXyn4 in the nuclei and chloroplasts, the NLS was deleted to generate *Xyn4*$^{\Delta NLS}$, and the cTP was deleted to generate *Xyn4*$^{\Delta cTP}$. As expected, NLS deletion disrupted localization on the nucleus and cTP deletion disrupted localization on the chloroplasts, and correspondingly both the recombinant protein Xyn4$^{\Delta NLS}$ and Xyn4$^{\Delta cTP}$ could not induce necrosis on *N. benthamiana* (Figure 6, A–C). Given the above observations, VdXyn4 is internalized into the *N. benthamiana* cells, where it concentrates at the nuclei and chloroplasts simultaneously, and its internalization is a necessary step for its cytotoxic and necrosis-inducing functions.

The membrane receptors BAK1 and SOBIR1 are probably required for the internalization of VdXyn4

Previous studies of the Eix1 xylanase from *T. viride* had revealed that this protein is an elicitor, that it interacted with the RLP LeEix2, and was internalized into *N. benthamiana* (Bar et al., 2010). Receptors BAK1 and SOBIR1 could displace Eix1 from the plasma membrane by endocytosis (Robatzek, 2006; Robatzek et al., 2006; Chinchilla et al., 2007a, 2007b; Liebrand et al., 2013). To test the involvement of BAK1 and SOBIR1 in the endocytic internalization of VdXyn4, virus-induced gene silencing (VIGS) constructs were prepared, based on the recombinant tobacco rattle virus (TRV), to target *NbBAK1* and *NbSOBIR1* expression in *N. benthamiana*, and RT-quantitative PCR (qPCR) analysis confirmed that the expression of *NbBAK1* or *NbSOBIR1* was markedly reduced 3 weeks after infiltration of TRV:*BAK1* (~20% expression level) or TRV:*SOBIR1* (~40% expression level) compared with infiltration with TRV:*GFP* (Figure 7A). As expected, unlike infiltration of the positive control BAX,

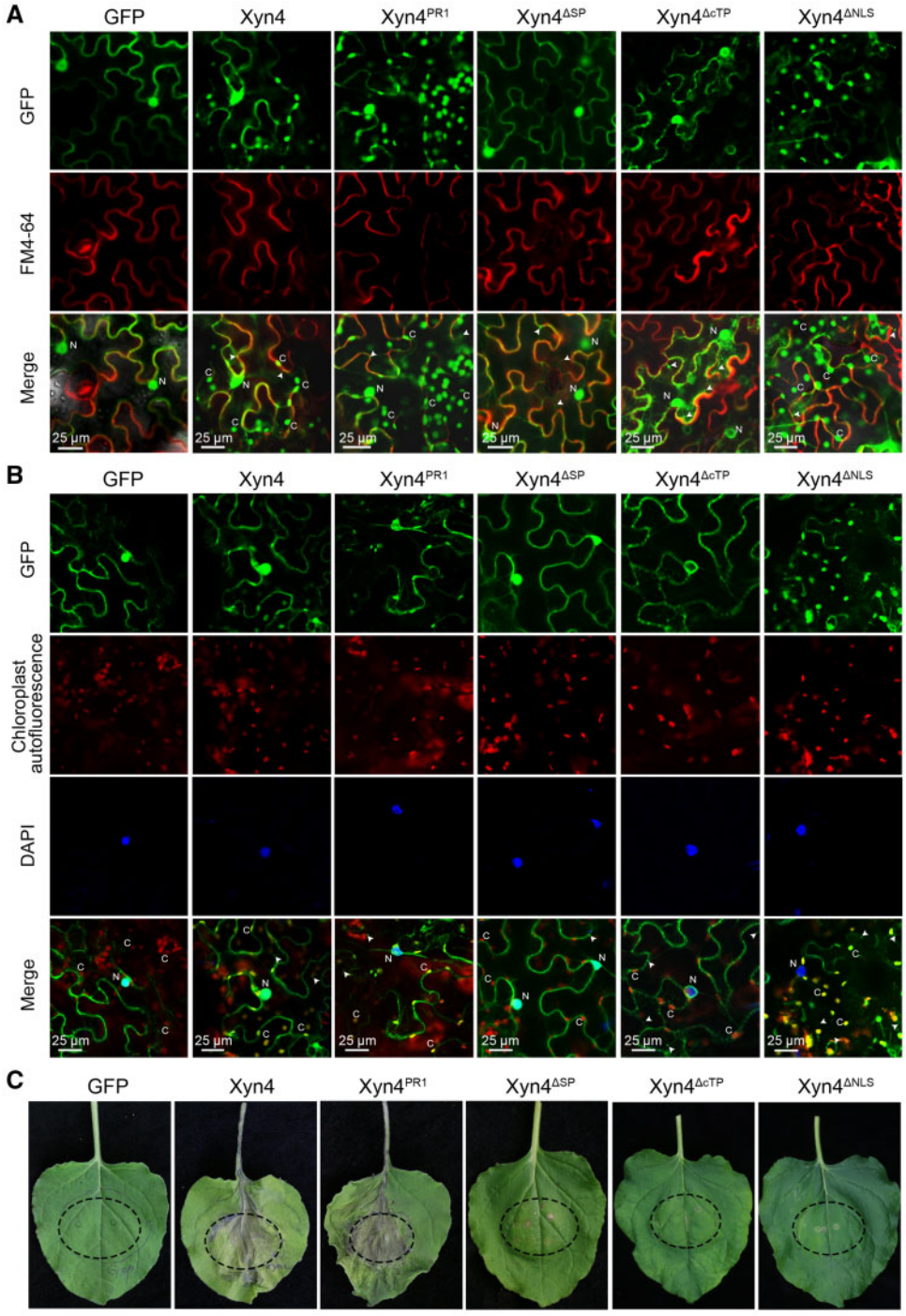

Figure 6 Endocytic internalization is required for the *V. dahliae* VdXyn4 to induce necrosis in *N. benthamiana*. A, B, Subcellular localization of the native gene (*Xyn4*) and mutant genes ($Xyn4^{PR1}$, $Xyn4^{\Delta SP}$, $Xyn4^{\Delta cTP}$, $Xyn4^{\Delta NLS}$) fused at the *C*-terminus to GFP; proteins were transiently expressed in 4-week-old *N. benthamiana* leaves and observed at 2 d post-agro-infiltration. pBin-GFP was used as control. Arrowheads, N, and C indicate cytoplasmic vesicle, nucleus, and chloroplast, respectively. The fluorochromes FM4–64 and DAPI, and chloroplast autofluorescence were used as fluorescence markers for the plasma membrane, nuclei, and chloroplasts, respectively. Bars = 25 μm. C, The cell death induction phenotypes of mutants in which the SP was replaced with PR1 ($Xyn4^{PR1}$), deletion of SP ($Xyn4^{\Delta SP}$), cTP deletion ($Xyn4^{\Delta cTP}$), and NLS deletion ($Xyn4^{\Delta NLS}$), were detected in *N. benthamiana* leaves from 4-week-old plants, 10 d after infiltration. The WT VdXyn4 and GFP were used as positive and negative controls, respectively. The dashed circles or ovals represent infiltrated area.

which caused cell death on *NbBAK1* or *NbSOBIR1*-silenced *N. benthamiana* leaves, transient expression of *VdXyn4* completely lost the ability to induce cell death on silenced *N. benthamiana* plants (Figure 7B). Immunoblotting analysis confirmed that *VdXyn4* was successfully expressed in the *N. benthamiana*-silenced plants infiltrated with TRV:*BAK1*,

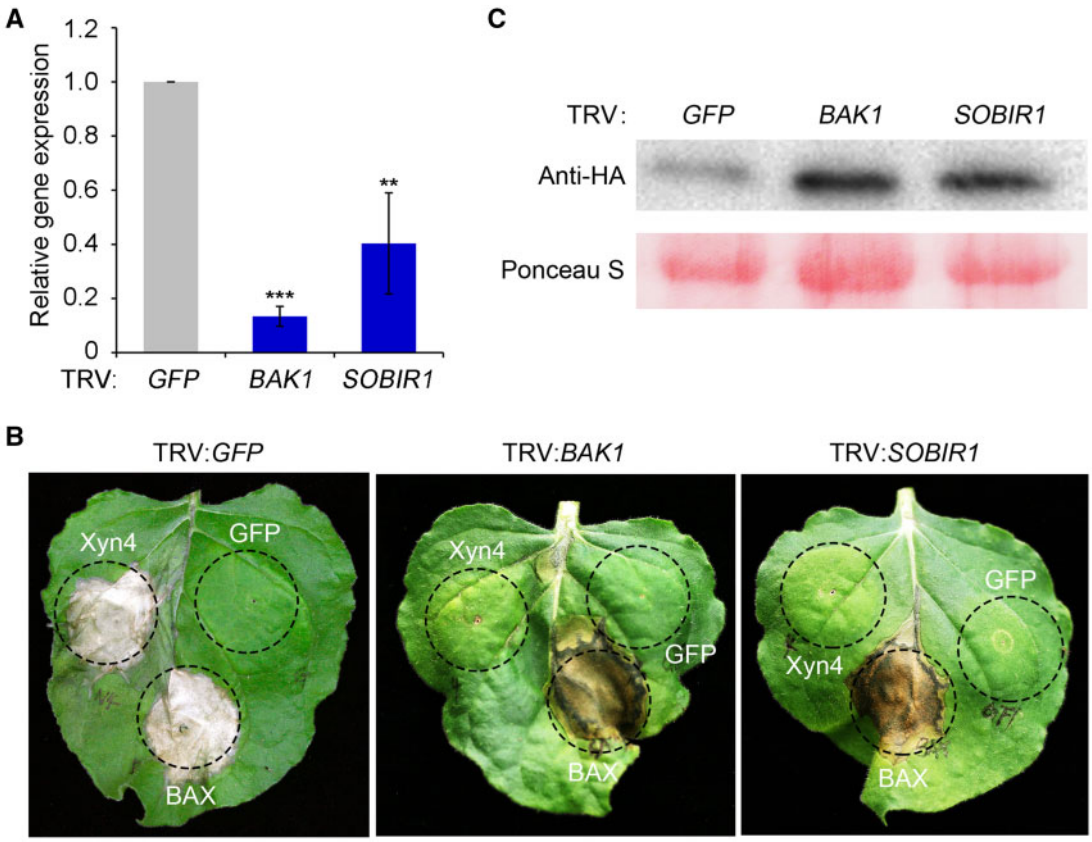

Figure 7 Analysis of the interactions of the *V. dahliae* VdXyn4 with the BAK1 and SOBIR1 in *N. benthamiana*. A, The silencing efficiency of *NbBAK1* and *NbSOBIR1* was determined by RT-qPCR analyses, *NbEF-1a* was used as a reference gene in relative expression analysis. Means and SE of the mean from three biological replicates are shown. *** and ** indicate statistical significance at $P < 0.001$ and $P < 0.01$, respectively, based on unpaired Student's *t* tests. B, BAK1 and SOBIR1 are required for VdXyn4-induced cell death in *N. benthamiana*. The cell death induction of VdXyn4 was not detected in *BAK1* or *SOBIR1* gene-silenced plants. The phenotypes of induced cell death were harvested at 10 dpi. The dashed circles or ovals represent infiltrated area. BAX protein and GFP were used as controls. C, Immunoblotting analysis of VdXyn4 fused to a HA-tag transiently expressed in *BAK1*-silenced and *SOBIR1*-silenced *N. benthamiana* leaves 60 h after agro-infiltration. Ponceau S-stained rubisco protein is shown as a total protein loading control.

TRV:*SOBIR1*, or TRV:*GFP* (Figure 7C). These results strongly suggest that VdXyn4 could be recognized by the BAK1/SOBIR1 complex to mediate the endocytic internalization, and thereby facilitating localization to the chloroplasts and nucleus to induce necrosis in *N. benthamiana*. Since BAK1/SOBIR1 are the core immunity receptors on the plasma membrane, and VdXyn4 showed extracellular interactions with BAK1/SOBIR1, VdXyn4 may also act as an apoplastic effector to generate DAMPs through its enzymatic activity, thereby inducing the immunity response.

Immunity induced by VdXyn4 is postponed while eliciting cytotoxic activity

In order to further detect how VdXyn4 compromises immunity to induce necrosis, the salicylic acid (SA) and jasmonic acid (JA) signaling defenses induced by VdXyn4 were compared to the known PAMPs VdEG1 (Gui et al., 2017), including *pathogenesis-related genes 1* and *2* (*PR1*, *PR2*) and *phenylalanine ammonialyase* (*PAL*) involved in SA signaling, and *PR4* and *lipoxygenase* (*LOX*) involved in JA signaling. For typical immunity induced by VdEG1, the gene markers of SA signaling (*PR1* and *PR2*) and JA signaling (*PR4*) were both significantly induced 2 d after agro-infiltration on *N. benthamiana* leaves, and retained high expression levels at 4 d (Figure 8, A and B). The JA signaling gene *LOX* was only highly upregulated at 4 d after agro-infiltration (Figure 8B). However, contrary to VdEG1, the transcript levels of all SA and JA signaling genes did not obviously change 2 d after agro-infiltration of *VdXyn4* on *N. benthamiana* leaves, and were upregulated until 4 d after agro-infilitration (Figure 8, C and D). These defense responses extended to 6 d after agro-infiltration, with the induction of SA signaling gene *PR1* and JA signaling genes (*PR4* and *LOX*; Figure 8, C and D). These results suggested that VdXyn4, unlike the classical PAMPs that quickly induce robust immunity (VdEG1 within 2–4 d in this study), significantly delayed induction of the defense response on *N. benthamiana*. Interestingly, JA signaling was continuously upregulated between 2 and 4 d following VdEG1 agro-infiltration, which coincided with the *N. benthamiana* leaf cell death processes (Figure 8B); however, unlike infiltration with VdXyn4, the strength in JA signaling was obviously reduced at 6 d compared to 4 d in *N.*

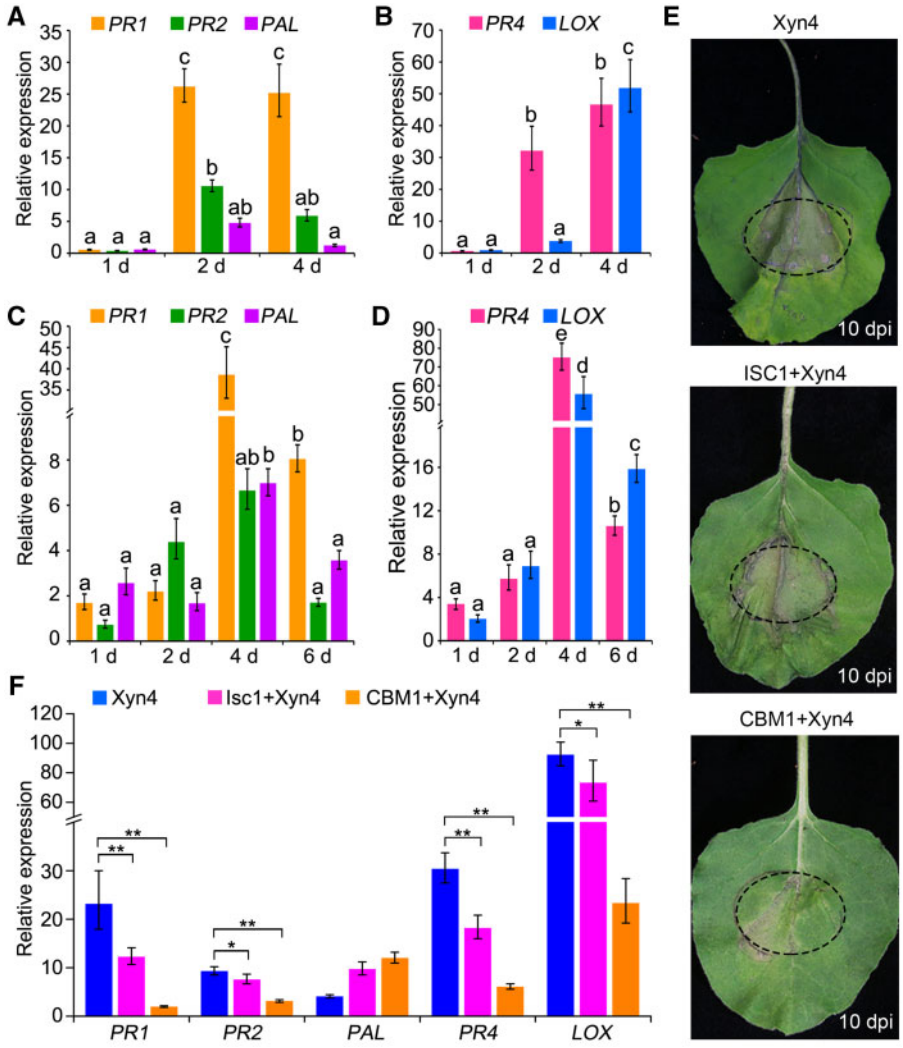

Figure 8 Expression of defense related genes in two different defense signaling pathways triggered by *V. dahliae* VdXyn4. A–D, Detection of transcripts of defense response genes related to SA signaling (*PR1*, *PR2*, and *PAL*) and JA signaling (*PR4* and *LOX*) pathways by RT-qPCR. The transcripts were detected in 4-week-old *N. benthamiana* leaves 1, 2, 4 d, respectively, after agro-infiltration with VdEG1, and 1, 2, 4, 6 d for VdXyn4. Error bars represent SE. Columns with different letters represent statistical significance $P < 0.05$, according to unpaired Student's *t* test. A, SA-signaling genes induced by VdEG1. B, JA-signaling genes induced by VdEG1. C, SA-signaling genes induced by VdXyn4. D, JA-signaling genes induced by VdXyn4. E, VdIsc1 and VdCBM1 cannot completely suppress induction of the cell-death like phenotype by VdXyn4 on *N. benthamiana* leaves. Assay for suppression of cell death-inducing activity of VdIsc1 and VdCBM1 by co-infiltration with VdXyn4 in 4-week-old *N. benthamiana* leaves, 10 d after agroinfiltration. BAX and GFP were used as a positive and negative control, respectively. The dashed circles or ovals represent infiltrated area. F, RT-qPCR analyses of defense-related gene expression at 4 d after agro-infiltration of constructs expressing VdXyn4 and in combination with expression of VdIsc1 or VdCBM1. Error bars represent SE. * and ** represent statistical significance at $P < 0.05$ and $P < 0.01$, respectively, according to unpaired Student's *t* tests.

benthamiana leaves (Figure 8D), resulting in reduced immunity that cannot block the cytotoxic activity of VdXyn4-induced leaf vein death. Therefore, VdXyn4 displayed cytotoxicity, though it may simultaneously induce delayed defense responses by manipulating JA signaling in the host.

Furthermore, as a phytopathogen, *V. dahliae* also employs effectors to surmount the typical immunity to facilitate host infection (Liu et al., 2014; Gui et al., 2017, 2018; Wang et al., 2020). To confirm cytotoxicity and immunity function of VdXyn4, the cell death-like phenotype and defense response were examined by using two known immunity suppressors (VdIsc1 and VdCBM1). As expected, unlike the co-infiltration of the suppressor with PAMPs that completely suppresses cell death (Gui et al., 2017; Wang et al., 2020), VdXyn4 partially retained the ability to induce cell death and vein necrosis in *N. benthamiana* leaves when co-infiltrated with VdIsc1 or VdCBM1, compared to a situation in which VdXyn4 was individually infiltrated (Figure 8E). Moreover, either VdIsc1 or VdCBM1 can also suppress defense responses (SA and JA signaling) that is involuntarily induced by the VdXyn4 cytotoxic function (Figure 8F), which likely facilitates necrosis of the vein tissue during *V. dahliae* infection. Taken together, VdXyn4 functions as a cytotoxicity factor, which compromises immunity responses

in *V. dahliae*—host interactions and ultimately causes vein tissue necrosis when *V. dahliae* colonizes the host plant vascular system.

Discussion

Xylanases catalyze the initial breakdown of the plant cell wall component xylan, and induce plant defense responses, thereby acting as one of the myriad weapons in the arsenal of a plant pathogen (Beliën et al., 2006). In this study, the structural characteristics of xylanase family members in *V. dahliae* were examined (Figure 1), and only one xylanase, VdXyn4, degraded cell wall components and contributed to virulence during *V. dahliae* infections on host plants (Figures 2 and 3). Although VdXyn4 could induce immune responses that were substantially delayed relative to the known PAMPs, it mainly displayed cytotoxic activity to induce necrosis that gradually spread to the foliar vein and petiole to overcome the deferred immunity during infection in *N. benthamiana*. The enzymatic activity of VdXyn4 is required for virulence and the necrosis-inducing function. The cytotoxicity function of VdXyn4 depended on its internalization into chloroplasts and nuclei, potentially through an unknown RLP receptor that co-opts BAK1 and SOBIR1 receptors, for its necrosis-inducing function. VdXyn4 may act as an apoplastic effector generating DAMPs to induce host immunity, which depends on BAK1/SOBIR1 receptors, but the induced immunity was not strong enough to inhibit later necrosis along the leaf vein and petiole (Figure 9).

Among the CAZymes, some xylanases are highly expressed during pathogen infections of the host and are viewed as secreted weapons in pathogen–host interactions (Ruiz-Roldán et al., 1999; Gómez-Gómez et al., 2001, 2002; Wu et al., 2006a, 2006b). In cereal pathogens, the offensive arsenal of xylanases plays crucial pathogenic function, since xylan represents a large proportion of the hemicellulose fraction of cereal cell wall matrices (Wanjiru et al., 2002). However, few xylanases have been implicated in virulence during pathogen–host interactions (Brito et al., 2006; Noda et al., 2010; Yu et al., 2016). In this study, the xylanase family member VdXyn4 from the hemi-biotrophic, soilborne pathogen, *V. dahliae* played a crucial role in virulence, and likely also degraded cell wall components during host infection (Figures 2–4). However, the potential pathogenic role of other xylanase members cannot be excluded because of the possibility of functional redundancy, which is a common phenomenon among CAZymes, such as the case of pectate lyase in *Nectria hematococca*, in which disruption of both pelA and pelD drastically reduced virulence, but not when either was disrupted individually (Rogers et al., 2000). Therefore, the deletion of other *V. dahliae* xylanase members alone probably caused no detectable decrease in virulence, unlike *VdXyn4*. This study systematically analyzes the virulence function of xylanase family members in *V. dahliae* and confirms that *VdXyn4* has a role in pathogenesis.

In necrotrophic pathogens, the necrosis-inducing xylanases may facilitate infection concurrent with the increased expression levels of many xylanases at later stages of infection (Yu et al., 2016). For hemi-biotrophic pathogens, the differential expression of endoxylanase genes may be associated with switching from a biotrophic to a necrotrophic phase on their respective hosts (Ruiz-Roldán et al., 1999; Gómez-Gómez et al., 2001, 2002). In general, *V. dahliae* is recognized as a hemi-biotrophic pathogen; the disease cycle begins as hyphae directly penetrating plant roots and colonizing the water-conducting xylem vessels, where they survive without causing obvious disease symptoms (Fradin and Thomma, 2006; Vallad and Subbarao, 2008; Klosterman et al., 2009) until plants transition from physiological growth to reproductive growth. Compared with other identified cell death inducing effectors, VdXyn4 exhibits a very strong cytotoxic activity, as the necrosis it induced gradually spreads around the leaf vein and petiole around 10 d after infiltration (Figure 5A; Supplemental Figure S4A). Furthermore, VdXyn4 could obviously stimulate the defense-related genes expression at 4 d after infiltration, especially of the JA pathway-related genes (*PR4* and *LOX*; Figure 8), and the JA pathway is generally believed to play a role in the host infection by hemi-biotrophic and necrotrophic pathogens. These findings indicate that VdXyn4 may play an important role in the transition from a biotrophic to a necrotrophic phase, or mainly function in necrotrophic phase during *V. dahliae* infection of the host plant. In addition, deletion of *VdXyn4* significantly reduced the ability to degrade cell wall components (cellulose, pectin, and xylan) and colonization of the xylem (decreased vascular discoloration; Figures 2 and 3, D), which further coincided with tissue collapse along the leaf veins and petiole (Figure 5A; Supplemental Figure S4A). These results suggest that the cytotoxic activity of VdXyn4 preferentially plays a role in the pathogenesis of *V. dahliae* in the unique niche of xylem vessels, and also, probably in the shift from biotrophic to necrotrophic phase.

Several xylanases act as elicitors to induce immune responses in the host, and the virulence and necrosis phenotypes are independent of their catalytic activity (Brito et al., 2006; Noda et al., 2010; Yu et al., 2016). In *B. cinerea*, a 25-residue peptide distinct from the xylanase activity site was responsible for its virulence (Brito et al., 2006; Noda et al., 2010; Frías et al., 2019). The activities of xylanase EIX and xylanase II in *T. viride* and *T. reesei*, respectively, were also similar, and the function of these elicitors was independent of the catalytic activity. (Enkerli et al., 1999; Furman-Matarasso et al., 1999; Rotblat et al., 2002). In just the submission of this research, one *V. dahliae* xylanase VdEIX3 was identified as a microbe-associated molecular pattern and recognized by NbEIX2 to induce *N. benthamiana* leaf death in one online paper finished by another group (Yin et al., 2020). Unfortunately, we could not functionally analyze VdEIX3 (equivalent to VdXyn6 in our research), because of the incomplete sequence of VdXyn6 caused by the sequencing error in the reference genome of Vd991 strain (Chen et al., 2018). In contrast to the typical immune responses caused by the above xylanase elicitors, there were five

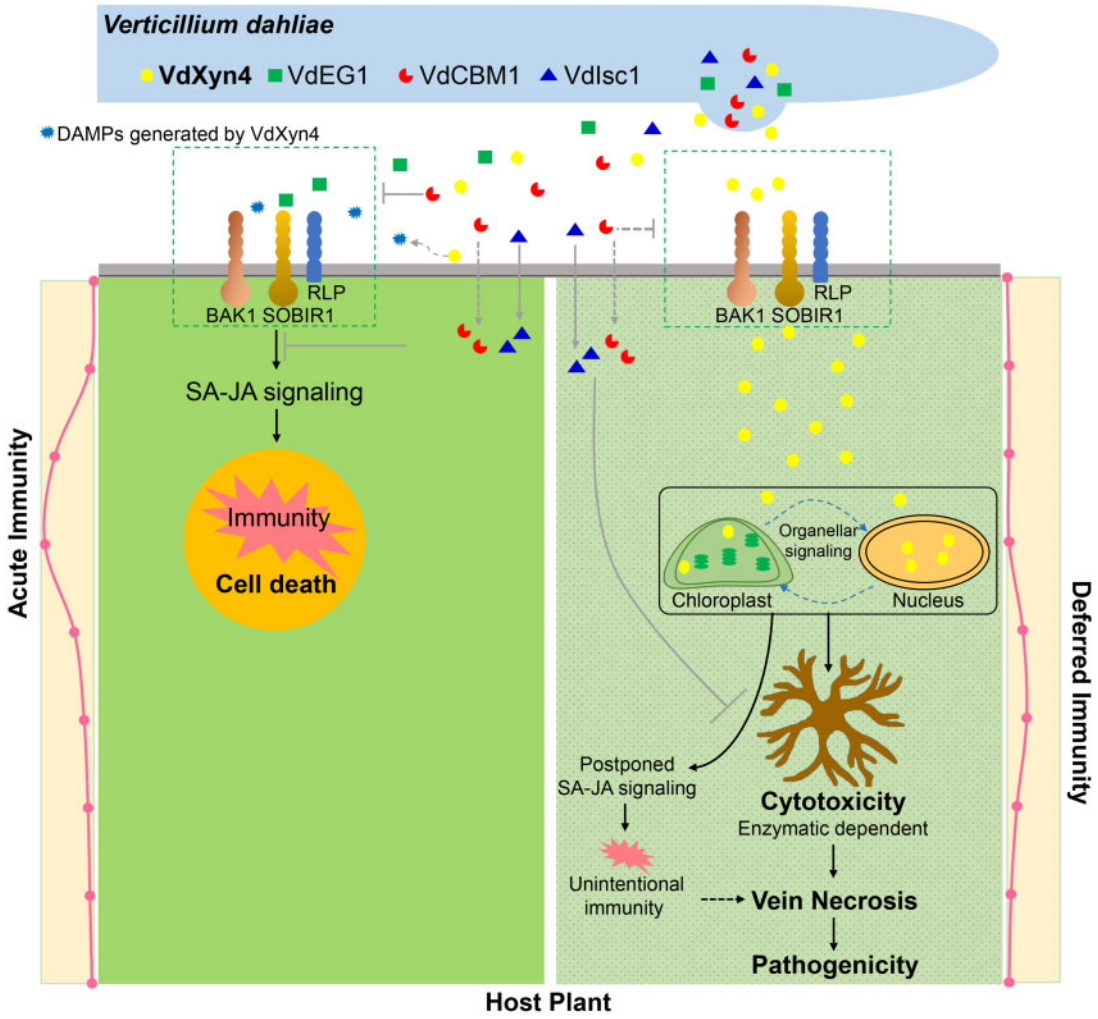

Figure 9 Model showing the role of the *V. dahliae* xylanase VdXyn4 in virulence through its cytotoxic function. *Verticillium dahliae* secretes the xylanase VdXyn4 into the apoplastic space and it internalizes into the plant intracellular space with the assistance of unknown plasma RLP that cooperates with SOBIR1/BAK1 receptors. Function of VdXyn4 in virulence depends on its enzymatic activity and the subcellular localization to the nuclei and chloroplasts and displays cytotoxic function to induce necrosis. During this necrotizing process, VdXyn4 induces immunity responses relating to SA–JA defense signaling. However, unlike the known PAMPs (as the VdEG1) that induce acute immunity, the immunity induced by VdXyn4 is substantially delayed and can be suppressed by known effectors (e.g. VdCBM1 and VdIsc1), which cannot impede necrosis. The end result is leaf vein and petiole cell collapse which facilitates the *V. dahliae* infection of the plant. But there is the possibility that VdXyn4 generates DAMPs to induce early immunity responses in the extracellular space. BAK1: LRR-RLK BR1-associated kinase-1; SOBIR1: LRR receptor-like kinase suppressor of BIR1-1; RLP: receptor-like protein; VdIsc1, a *V. dahliae* effector isochorismatases lacking a known SP, is thought to be delivered into host cells to hydrolyze a SA precursor and thereby inhibit salicylate metabolism (Liu et al., 2014). VdCBM1, carbohydrate-binding module family 1 protein domain containing effector, had the ability to suppress many PAMPs and DAMPs triggered immunity and cell death in *N. benthamiana* (Gui et al., 2017, 2018). Black lines indicate different pathways in the plant cell; gray lines indicate the effectors acting on the corresponding plant cell pathways; and the dashed and solid lines indicate the possible and the proven pathways, respectively; blue dashed lines indicate the organellar signaling between chloroplast and nucleus; green dashed lines indicate the RLP/SOBIR1/BAK1 complex; bold font indicates stronger reactions in plant cell versus normal reactions indicated by normal font; pink lines with circles indicate the time course from the effectors recognized by plant cell, and pink circles from top to bottom represent progress in days.

differences in this study: (1) the necrosis caused by VdXyn4 occurred typically around 10 d after inoculation (Figure 5A; Supplemental Figure S4A), substantially later than other *V. dahliae* effectors, such as the necrosis- and ethylene-inducing-like proteins (NLP1 and NLP2), VdEG1, and VdSCPs (Zhou et al., 2012; Santhanam et al., 2013; Gui et al., 2017; Wang et al., 2020); (2) the cell death induced by VdXyn4 could spread to the leaf vein and petiole, and these tissues also displayed tissue collapse around 10 d after infiltration (Figure 5A), unlike the cell death induced by PAMPs and xylanase elicitors, which was mainly restricted to the inoculated area by the initial immune responses (Gui et al., 2017; Frías et al., 2019); (3) the necrosis function of VdXyn4 was dependent on its enzymatic activity (Figure 5B), but most enzymes acting as PAMPs generally serve this function independent of their enzymatic ability (Gui et al., 2017; Frías

et al., 2019); (4) although VdXyn4 could induce an immune response and initiate the expression of defense-related genes, the upregulation was substantially delayed compared with the typical PAMPs or effectors (Brito et al., 2006; Noda et al., 2010; Yu et al., 2016; Gui et al., 2017; Zhang et al., 2017); and (5) the previously reported host immunity suppressors VdIsc1 and VdCBM1 (Liu et al., 2014; Gui et al., 2017) failed to suppress the necrosis phenotype induced by VdXyn4 completely, even if they could suppress the expression of defence-related genes (Figure 8), indicating that the necrosis phenotype caused by VdXyn4 is not a typical immune response. That is the most likely reason why the cell necrosis induced by VdXyn4 (same as VdEIX5) was not investigated or observed at 7 d after infiltration on *N. benthamiana* leaves (Yin et al., 2020). Thus, unlike the typical elicitors, VdXyn4 mainly functions as a cytotoxicity factor to facilitate the vascular niche adaptation and promote the switch from a biotrophic phase to the necrotrophic phase in this hemibiotrophic pathogen, but it also results in the unintentional induction of the deferred immunity (Figure 9).

Although most xylanases are secreted extracellularly by the pathogen during interactions with the host, only the xylanase EIX from *T. viride* functions in the cytoplasm by internalization after binding to the plant membrane receptor (Hanania et al., 1999). In this study, we confirmed that the xylanase VdXyn4 localized into periphery of plasma membrane, cytoplasmic vesicles, and on the nuclei and chloroplasts in *N. benthamiana* (Figure 6, A and B; Supplemental Figure S5). Its necrotic role depended on subcellular localization to nuclei and chloroplasts, and this localization was required for its cytotoxic activity (Figure 6, B and C). Interestingly, variants of VdXyn4$^{\Delta SP}$ were not localized on the chloroplasts, and unlike the native VdXyn4, could not induce cell death on *N. benthamiana* leaves (Figure 6, B and C). These results indicated that the initial extracellular location of VdXyn4 exported through the secretory pathway of fungus via the SP is vital for the intracellular localization of VdXyn4, especially on chloroplasts during the plant—*V. dahliae* interaction. Chloroplasts not only are photosynthetic organelles enabling photoautotrophy, but they are also hubs of integrating environmental stimuli that generate defense signals such as ROS, SA, JA, and nitrous oxide involved in immunity (de Torres et al., 2015; Serrano et al., 2016). Once organisms encounter biotic or abiotic stresses, chloroplasts serve as major sensors to communicate with other organelles, including the nucleus, mitochondria, and peroxisome, to establish plant immunity, and inhibit the expression of defense-related genes in the nucleus by a retrograde signal (Deslandes and Rivas, 2011; Shapiguzov et al., 2012). For instance, the plant defense regulator CPK16 can be imitated by proteins secreted from bacteria and viruses, and transferred from plasma membrane to chloroplasts to inhibit SA-induced defense through the plant nucleus (Medina-Puche et al., 2020). Therefore, this research further confirmed the importance of effectors located in plant chloroplasts and nucleus to manipulate host immunity. Chloroplast and nuclear localization are both indispensable for the cytotoxic activity of VdXyn4 that serves as a conduit for message delivery between chloroplasts and nuclei in VdXyn4-induced plant cell necrosis. While we also observed that VdXyn4 was located on the periphery of plasma membrane (Figure 6A), it is reasonable to question whether VdXyn4 functions as an apoplastic effector. However, based on the relationships between cell death inducing activity and subcellular localization of Xyn4 and its mutants Xyn4^{PR1}, Xyn4$^{\Delta SP}$, Xyn4$^{\Delta cTP}$, and Xyn4$^{\Delta NLS}$ (Figure 6), we conclude that Xyn4 functions mainly intracellularly. Its peripheral localization on the plasma membrane may be explained in the *in vivo* interaction of *V. dahliae*–host plants by temporary VdXyn4 localization at the plasma membrane during its transfer from the extracellular to intracellular space. Correspondingly, the VdXyn4 signal was also located on the cytoplasmic vesicles (Figure 6, A and B), which may facilitate the transport and internalization of VdXyn4 from the peripheral plasma membrane into nuclei and chloroplasts.

Based on the subcellular localization and cytotoxic activity of VdXyn4 and VdXyn4^{PR1} in the intercellular regions in *N. benthamiana* (Figure 6), endocytic internalization of VdXyn4 is highly plausible. The relationship between the effector internalization and plant cell death induction can be explained in two ways. First, for some pathogen-secreted proteins, the SPs can be deleted to induce plant cell death in transient expression systems, but with its SP intact, fails to induce cell death, suggesting that plant cells recognize SPs from fungi and direct the extracellular secretion of fungal proteins expressed in cells. The extracellular secretion peptides are unable to induce cell death outside of the plant cell (Xiao et al., 2014). Secreted proteins with the SP can act like proteins without SPs to induce cell death, provided they are internalized by plant cells in the absence of a pathogen (Lyu et al., 2016). Therefore, we can speculate that the SP can direct VdXyn4 secretion extracellularly, which then can be internalized into the *N. benthamiana* cells to cause necrosis.

The receptor-associated kinases SOBIR1 and BAK1, as plasma membrane-associated receptors, play crucial regulatory roles in cellular processes that cooperate with the plasma membrane receptors such as RLP, including immunity functions and mediation of stress responses (Liebrand et al., 2014). In this study, the VdXyn4 failed to cause necrosis in *NbBAK1-* or *NbSOBIR1*-silenced plants, suggesting that these two plasma receptors are required for the cytotoxic activity of VdXyn4 (Figure 7). VdXyn4 is primarily an intracellular protein based on its subcellular localization, while SOBIR1 and BAK1 are known to mainly interact with plasma membrane receptors (Hanania and Avni, 1997; Monaghan and Zipfel, 2012; Liebrand et al., 2013). The question that arises then is, how do we explain this paradox? Confocal laser-scanning observations revealed that green fluorescence signals of the VdXyn4–GFP fusion

were also observed in mobile cytoplasmic vesicles, and the many cytoplasmic vesicles with strong fluorescent signals were distributed along the cell periphery at the plasma membrane and also widely distributed into the nuclei and chloroplasts (Figure 6, A and B; Supplemental Figure S5), indicating that the cytoplasmic vesicles containing VdXyn4 were transported into both nuclei and chloroplasts. A similar mechanism has also been found in the immunity response induced by ethylene-induced xylanase EIX in *N. tabacum*. Confocal laser-scanning microscopy revealed that after binding to the *N. tabacum* plant membranes, EIX entered the cytoplasm by adhering to cytoplasmic vesicles (Hanania et al., 1999). Correspondingly, the receptor SlSOBIR1 of EIX mainly localizes to the plasma membrane and the fluorescence signals also were observed in mobile cytoplasmic vesicles, and thus SlSOBIR1 is speculated to be involved in the EIX transportation from the plasma membrane into the intracellular location by mobile cytoplasmic vesicles (Liebrand et al., 2013). Furthermore, the receptors BAK1 and SOBIR1 were required for receptor complexes displaced from the plasma membrane by endocytosis (Robatzek, 2006; Robatzek et al., 2006; Chinchilla et al., 2007a, 2007b). Therefore, the extracellular VdXyn4 is likely initially recognized by a plasma membrane receptor that cooperates with BAK1 and SOBIR1 to internalize the protein into the intracellular spaces through cytoplasmic vesicles, but the exact mechanism needs further clarification.

However, based on the results that VdXyn4 localized in the cell periphery at the plasma membrane, and induced a host necrotic phenotype that was dependent on its enzyme activity and mediated by BAK1 and SOBIR1 receptors, there remains the possibility that VdXyn4 also acts as an elicitor directly or generates DAMPs by degrading plant cell wall. In that way, VdXyn4 may activate the SA and JA pathway-related gene expression in the extracellular space. Yet, the early response was very weak (Figure 8, C and D) and not enough to inhibit the later necrosis along the *N. benthamiana* leaf vein and petiole (Figure 9).

In conclusion, the xylanase family member VdXyn4 from the plant pathogenic fungus *V. dahliae* plays a cytotoxic role during pathogenesis, a role that is dependent on the xylanase activity and its internalization into chloroplasts and nuclei. This internalization process was dependent on the plasma membrane receptors BAK1 and SOBIR1. Unlike the immunity caused by other typical PAMPs, VdXyn4 induction of immune response was postponed, while its cytotoxic function to cause necrosis was activated. This cytotoxic characteristic could not be blocked by the known immunity suppressors, VdIsc1 or VdCBM1 (Figure 9). These results further confirmed the crucial cytotoxic function of VdXyn4 during *V. dahliae* interactions with the host cells. Future efforts will be devoted to investigations on the mechanisms of VdXyn4 internalization, its cytotoxic function in plant vein tissues, and its potential interacting proteins in the host.

Materials and methods

Identification of xylanase genes family members in *V. dahliae* Vd991

Candidate xylanase gene family members were predicted using an HMMER package with the HMM profile of the GH11 domain (Glyco_hydro_11, Pfam ID: PF00457) queried against the *V. dahliae* Vd991 genome using the default parameters. The returned hits with *E*-values $<$ 1e-10 and scores $>$30 were manually selected. Secreted proteins were identified using four programs commonly used to identify protein localization, as described previously (Klosterman et al., 2011). Putative extracellular proteins containing a SP but lacking transmembrane domains were identified as secreted proteins. The orthologous genes in the *V. dahliae* VdLs.17 genome were determined by BLAST (Altschul et al., 1997). VdXyn1 corresponded to the predicted gene VEDA_07278 in Vd991 genome, encoding 716 amino acids. The length was somewhat unusual compared to other members of this family, and therefore was corrected using the reference genome of VdLs.17 genome. The final length of VdXyn1 is 237 amino acids; the corresponding gene ID in VdLs.17 genome is VDAG_03790, Accession Number: XM_009653874.1; Supplemental Table S1). Clustal X2 was used for multiple sequence alignment (Larkin et al., 2007). Conserved motifs and enzymatic activity sites were determined according to a PROSITE profile (PDOC00140). An unrooted tree was generated in MEGA6 program using the maximum-likelihood method with 1,000 bootstrap replicates (Tamura et al., 2013).

Generation of gene deletion mutants and mutant complementation

Targeted gene-deletion constructs were generated based on a previously described method (Liu et al., 2013). Briefly, ~1-kb flanking sequences of targeted genes (*VdXyn1–VdXyn5*) were amplified from *V. dahliae* Vd991 genomic DNA and fused to a 1.8-kb hygromycin-resistant cassette that consists of the *TrpC*-promoter, the hygromycin phosphotransferase gene and a *Nos*-terminator, were amplified with the Hyg-F and Hyg-R primers from the vector pCT-*Hyg*. The fusion fragment was introduced into the binary vector pGKO2-Gateway using a standard BP reaction. To generate complementation transformants, the wild-type gene *Xyn4* was cloned into the binary vector pCOM that carries geneticin resistance (Zhou et al., 2013), and reintroduced to the Δ*Xyn4* strains. The targeted gene deletion strains of *VdXyns* (Δ*Xyn1–*Δ*Xyn5*) and *VdXyn4*-complemented transformants were obtained using a previously described *Agrobacterium*-mediated transformation method (Liu et al., 2013). The positive targeted gene deletion of *VdXyn1–VdXyn5* was identified using PCR with three primer pairs: a *Hyg*-specific primer pair, the internal primer pairs of the gene itself and the flanking test primer pairs of *VdXyn1–VdXyn5* (Supplemental Table S4).

Phenotypic characterization

To determine the colony morphology of deletion mutants Δ*Xyn1*–Δ*Xyn5* on different carbon sources, conidial suspensions of *V. dahliae* strains were prepared at 2×10^6 conidia/mL and placed in the center of basic C'zapek medium agar plates that including either xylan (10.0 $g \cdot L^{-1}$), sucrose (30.0 $g \cdot L^{-1}$), pectin (10.0 $g \cdot L^{-1}$), or cellulose (10.0 $g \cdot L^{-1}$). The wild-type and gene deletion strains grown on PDA medium were used as controls. The growth phenotypes were investigated and colony diameters were measured after 11 d of incubation at 25°C. Each strain was cultured on five plates with three replicates. Unpaired Student's *t* tests were performed to determine statistical significance.

Fungal culture, inoculation, and virulence assays

To test the virulence functions of xylanase gene family members *VdXyn1*–*VdXyn5*, all the strains including the *VdXyn1*–*VdXyn5* targeted deletion strains, *VdXyn4* complemented transformants, and the wild-type strain Vd991, were shake-cultured in liquid C'zapek medium for 5 d at 25°C. Cotton plants (*Gossypium hirsutum* cv Junmian 1) were grown at 25°C in a greenhouse with alternating 14-h light and 10-h darkness. Three-week-old cotton seedlings were inoculated with 5×10^6 conidia/mL by a root-dip method for pathogenicity assays, as previously described (Gui et al., 2017). Disease symptoms were observed, and quantification was performed at 3 wpi following the method of Zhang et al. (2017). The disease index (DI) was calculated as = [∑(the seedling of every grade × relative grade)/(total seedlings × the most serious grade)] × 100 following Powell et al. (1971). Vascular discoloration of infected cotton was observed in longitudinal sections of the shoots 3 weeks after inoculation. Fungal biomass in cotton was determined as previously described (Santhanam et al., 2013). qPCR was performed using the SYBR premix Ex Taq II kit (TaKaRa, Kyoto, Japan) with the primer pairs listed in the supplementary information Supplemental Table S4. The *V. dahliae* elongation factor 1-α (*EF-1α*) gene was used to quantify fungal colonization, and the cotton *18S* gene served as an endogenous plant control. The qPCR conditions were as follows: an initial 95°C denaturation step for 5 min, followed by denaturation for 30 s at 95°C, annealing for 30 s at 60°C and extension for 30 s at 72°C for 40 cycles. Unpaired Student's *t* tests were performed to determine statistical significance at $P < 0.01$ between different treatments.

Transient gene expression assays and phenotypic observations

Transient gene expression assays were performed in 4-week-old *N. benthamiana* plant leaves using the wild-type and mutant genes including (1) the full-length coding sequences of xylanase genes (*Xyn1*–*Xyn5*); (2) $Xyn4^{\Delta SP}$ mutant, which lacks the sequence encoding the SP; (3) three site-directed catalytic residue mutants of *VdXyn4* ($Xyn4^{SM119}$, $Xyn4^{SM210}$, $Xyn4^{SM119-210}$); (4) $Xyn4^{PR1}$, in which the *N*-terminal SP-encoding region of VdXyn4 was replaced with that of the plant PR1 SP; (5) $Xyn4^{\Delta cTP}$, which lacks the cTP sequences; (6) $Xyn4^{\Delta NLS}$, which lacks NLS sequences; and (7) the positive control BAX and negative control GFP. All sequences contained a 3′-HA-tag and were cloned separately into the PVX vector pGR107 and transformed into the *Agrobacterium tumefaciens* strain GV3101. Agro-infiltration assays were performed on *N. benthamiana* plants using BAX and GFP as positive and negative controls, respectively (Gui et al., 2017). Leaf phenotypes were photographed at 4 or 10 d after infiltration. To examine the suppression of cell death induction, *A. tumefaciens* cells carrying pGR107:*VdXyn4* were co-infiltrated with pGR107:*VdCBM1* and pGR107:*VdIsc1*, respectively. Symptom development was monitored for 4 d in a time-course experiment until 10 d post-infiltration. Each assay was performed on six leaves from three individual plants and repeated at least three times. Total proteins were extracted using a P-PER Plant Protein Extraction Kit and Protease Inhibitor Cocktail Kit (Thermo Scientific, Waltham, MA, USA) from agroinfiltrated *N. benthamiana* leaves 60 h after infiltration. Transient protein expression in *N. benthamiana* was assessed using anti-HA antibodies (Sigma-Aldrich, St Louis, MO, USA). Total proteins of plant tissues on the polyvinylidene fluoride membrane were stained by Ponceau S solution (Sigma-Aldrich, St Louis, MO, USA).

Recombinant protein purification and enzyme activity assays

The wild-type *VdXyn4* and site-directed mutagenized gene $Xyn4^{SM119-210}$ were cloned and ligated into pSMART-I vector with a SUMO tag fused in-frame at the *N*-terminus. After transformation into *Escherichia coli* BL21(DE3), recombinant protein expression was induced by adding 1 mM IPTG for 8 h at 15°C. The cells were lysed by freezing and thawing, followed by sonication, and the recombinant protein was purified as previously described for other His_6-SUMO-tagged proteins (Lubin et al., 2013). The cleared lysate was incubated with nickel-NTA resin for 1–2 h at 4°C with mixing, and the tagged proteins were eluted batchwise with lysis buffer plus 300 mM imidazole. The *Xyn4* and $Xyn4^{SM119-210}$ were assayed for enzymatic activity using a modified version of the method of Bailey et al. (1992). Unless otherwise stated, reactions contained 1% (w/v) beechwood xylan in citrate–phosphate McIlvaine buffer, pH 5, plus the appropriate amount of enzyme in a final volume of 125 μL. Incubations were carried out at 35°C for 10 min and reactions were stopped by the addition of 187.5 μL of a dinitrosalicylic acid solution used to assay reducing sugars (Bailey and Pessa, 1990) and incubated 5 min in a boiling water bath. Finally, a DTX800 microplate reader (Beckman Coulter Inc., http://www.beckman.com) was used to read the absorbance of the samples at 540 nm. To determine optimal pH, assays were also made in McIlvaine buffer adjusted at pHs ranging from 3 to 7.

Yeast signal sequence trap system

Functional validation of the predicted SP was performed as described previously (Jacobs et al., 1997). The predicted SP

sequence of VdXyn4 was fused in-frame with the secretion-defective invertase gene in the vector pSUC2. The reconstructed plasmid pSUC2:*Xyn4*^{SP} was transformed into the yeast strain YTK12 and screened on CMD-W (lacking tryptophan) medium. Positive clones were confirmed by PCR using vector-specific primers (Supplemental Table S4). The positive transformants were incubated on YPRAA medium containing 2% raffinose. The recombinant YTK12 strain carrying the SP of Avr1b (pSUC2:*Avr1b*^{SP}) was used as a positive control, while the untransformed YTK12 strain and YTK12 strain transformed with an empty pSUC2 vector were used as negative controls.

Subcellular localization assays

To study the subcellular localization of VdXyn4 in planta, the wild-type gene *VdXyn4* and truncated genes (*Xyn4*$^{\Delta SP}$, *Xyn4*^{PR1}, *Xyn4*$^{\Delta cTP}$, and *Xyn4*$^{\Delta NLS}$) were introduced into the 5′-end of the gene encoding GFP driven by a cauliflower mosaic virus 35S promoter on the pBin:GFP4 vector to generate the recombinant expression vectors (pBin:*Xyn4-GFP*, pBin:*Xyn4*$^{\Delta SP}$*-GFP*, pBin:*Xyn4*^{PR1}*-GFP*, pBin:*Xyn4*$^{\Delta cTP}$*-GFP*, and pBin:*Xyn4*$^{\Delta NLS}$*-GFP*). The vectors were transformed into *A. tumefaciens* GV3101, and agro-infiltrated into 3-week-old *N. benthamiana* leaves. The empty vector pBin:GFP4 was used as a control. To observe fluorescence, the infiltrated *N. benthamiana* leaves were harvested 2 d post-agro-infiltration and directly imaged under a Leica TCS SP8 confocal microscope with an excitation wavelength of 488 nm, emission of 510 nm and 650–750 nm for GFP and chloroplast autofluorescence, respectively. Similarly, FM4-64 signals were observed at an excitation of 543 nm and emission at 562 nm, and for DAPI, excitation at 360 nm and emission at 460 nm.

VIGS in *N. benthamiana*

VIGS assays based on recombinant TRV were performed as described previously (Liu et al., 2002). The plasmid constructs pTRV1, pTRV2:*BAK1*, and pTRV2:*SOBIR1* were introduced into *A. tumefaciens*. *Agrobacterium* strains harboring either the pTRV2:*BAK1* or pTRV2:*SOBIR1* plasmid were combined with strains harboring the pTRV1 vector in a 1:1 ratio and co-infiltrated into two primary leaves of 3-week-old *N. benthamiana* plants. The plasmid pTRV2:*GFP* was used as the control. The effectiveness of the VIGS assay was evaluated using the phytoene desaturase (*PDS*) gene, as previously described (Liu et al., 2002). The silencing efficiency of *NbBAK1* or *NbSOBIR1* was validated by reverse transcription-quantitative PCR (RT-qPCR). The *N. benthamiana* elongation factor 1-α (*EF-1α*) was used as the reference gene, and relative transcript levels of *NbBAK1* and *NbSOBIR1* were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with each corresponding plant. The experiment was performed three times with six plants for each TRV construct per experiment.

Detection the transcripts of defense response genes

The samples from *N. benthamiana* were collected at 1, 2, 4, 6 d after infiltration of *VdXyn4*, and 1, 2, and 4 d for *VdEG1*. GFP control samples for each treatment time were also collected. The *N. benthamiana* leaf samples of co-expression of *VdXyn4* or *VdEG1* and *VdIsc1* or *VdCBM1* were obtained at 4 d after co-infiltration. GFP and the co-expression of Xyn4 or VdEG1 and GFP were used as controls. Total RNA was extracted using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen, USA), and the first-strand cDNA was synthesized using reverse transcriptase (Invitrogen, USA). RT-qPCR was carried out using the SYBR Premix ExTaq Kit (Takara, Kyoto, Japan) following the manufacturer's instructions. Gene expression levels of resistance-related genes (*PR1*, *PR2*, *PAL*, *PR4*, and *LOX*) were normalized to the *N. benthamiana EF-1α* gene. Relative transcript levels among various samples were determined using the $2^{-\Delta\Delta CT}$ method as described previously (Livak and Schmittgen, 2001).

Accession numbers

Sequence data from this article can be found in Supplemental Table S5. The prediction of encoding proteins by *VdXyn1* – *VdXyn6* projective on Vd991 genome can be found in the GenBank data libraries under accession numbers: VdXyn1, KAF3353319.1; VdXyn2, KAF3355309.1; VdXyn3, KAF3361332.1; VdXyn4, KAF3355673.1; VdXyn5, KAF3355674.1; VdXyn6, KAF3359032.1.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Polymerase chain reaction (PCR) analyses of single deletions of *VdXyn1-VdXyn5* in *Verticillium dahliae*.

Supplemental Figure S2. The growth phenotypes of five xylanase gene deletion mutants of *Verticillium dahliae*.

Supplemental Figure S3. Virulence analysis of *Verticillium dahliae* xylanase gene deletion mutants on cotton.

Supplemental Figure S4. Analyses of the *Verticillium dahliae* xylanase VdXyn4 cytotoxic activity in *Nicotiana benthamiana*.

Supplemental Figure S5. Subcellular localization of *Verticillium dahliae* xylanase VdXyn4 in *Nicotiana benthamiana*.

Supplemental Figure S6. The secretion and localization analysis of *Verticillium dahliae* xylanase VdXyn4.

Supplemental Table S1. Identities matrix of the xylanase genes between VdXyns in *Verticillium dahliae* and other fungi from *Sordariomycetes*.

Supplemental Table S2. CAZymes annotation of the xylanases in species from *Sordariomycetes*.

Supplemental Table S3. Prediction analysis of VdXyn4 localization.

Supplemental Table S4. Information on the primers used in this study.

Supplemental Table S5. Sequence information of VdXyn1–VdXyn6.

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