VdNPS, a Nonribosomal Peptide Synthetase, Is Involved in Regulating Virulence in *Verticillium dahliae*

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ABSTRACT

Nonribosomal peptide synthetases (NPS) are known for the biosynthesis of antibiotics, toxins, and siderophore production. They are also a virulence determinant in different phytopathogens. However, until now, the functional characterization of NPS in *Verticillium dahliae* has not been reported. Deletion of the *NPS* gene in *V. dahliae* led to the decrease of conidia, microsclerotia, and pathogenicity. Δ *VdNPS* strains were tolerant to H₂O₂, and the genes involved in H₂O₂ detoxification, iron/copper transport, and cytoskeleton were differentially expressed in Δ *VdNPS*. Interestingly, Δ *VdNPS* strains exhibited hypersensitivity to salicylic acid (SA), and the genes involved in SA hydroxylation were up-regulated in Δ *VdNPS* compared with wild-type

V. dahliae under SA stress. Additionally, during infection, Δ *VdNPS* induced more pathogenesis-related gene expression, higher reactive oxygen species production, and stronger SA-mediated signaling transduction in host to overcome pathogen. Uncovering the function of VdNPS in pathogenicity could provide a reliable theoretical basis for the development of cultivars with durable resistance against *V. dahliae*-associated diseases.

Keywords: analytical and theoretical plant pathology, host response, nonribosomal peptide synthetase, reactive oxygen species, salicylic acid, vegetative growth, *Verticillium dahliae*, virulence

Nonribosomal peptides (NRPs) are an important group of natural products that exhibit a wide range of biological and pharmacological activities in medicine and agriculture, such as daptomycin, penicillin, and vancomycin (Sieber and Marahiel 2005). δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine is the firstly identified NRP in biosynthetic pathways of β -lactam antibiotics 40 years ago, of which penicillin and cephalosporin are the most well-known (Byford et al. 1997). Fungal toxins are a category of NRPs, including the HC-toxin produced by race 1 of the maize pathogen *Cochliobolus carbonum* (Panaccione et al. 1992), AM-toxin produced by the apple pathotype *Alternaria alternata* (Johnson et al. 2000), and ennatin produced by *Fusarium* spp. (Haese et al. 1993). These products are assembled by NRP synthetases (NPSs) that consist of a series of catalytic domains without the involvement of ribosomes (Fischbach and Walsh 2010). NPSs not only catalyze the synthesis of secondary metabolites but also determine virulence and iron metabolism. For example, AaNPS6 is involved in maintaining iron homeostasis via siderophore iron uptake and is a conserved virulence determinant through resisting to toxic reactive oxygen species (ROS) in the citrus fungal pathogen, *Alternaria alternata* (Chen et al. 2013), as well as in plant pathogenic ascomycetes, such as *C. heterostrophus*, *C. miyabeanus*, *F. graminearum*, and *Alternaria brassicicola* (Oide et al. 2006).

Deletion of NPS6 orthologs results in reduced virulence and hypersensitivity to H₂O₂ (Oide et al. 2006). As the homolog of NPS6 in *Aspergillus nidulans* and *Alternaria alternata*, VdNPS was first mentioned by Wang et al. (2018), who reported that nonribosomal peptide synthetases (VDAG_05314) was highly up-regulated under iron sufficiency compared with that under conditions of iron starvation in *V. dahliae* XS11 strain and Δ *VdHapX* strains. However, no further functional characterization of VdNPS was reported later.

Verticillium dahliae Kleb. is a soilborne, hemibiotrophic, phytopathogenic fungus that causes wilt disease in over 200 species in economically important crops (Pegg and Brady 2002), such as cotton, potato, tomato, and sunflower. Every year, the global economic losses caused by *Verticillium* wilt exceeds \$1.8 billion. Due to the long viability of rest structures (microsclerotia), broad host range, and the limited activity of fungicide against the fungus once it enters the (root) vascular system, the disease is difficult to control (Fradin and Thomma 2006; Luo et al. 2014). During its initial biotrophic stage, the synthesis of cell-wall-degrading enzymes (CWDEs), the release of effectors, and the ability to suppress plant basal resistance contribute to the successful infection and tissue colonization of *V. dahliae* in hosts. To overcome pathogen attack, plants establish a variety of defense responses, including callose deposition (Gómez et al. 1999), oxidative burst (Apel and Hirt 2004), synthesis of pathogenesis-related (PR) proteins induced by salicylic acid (SA), jasmonic acid (JA), or ethylene (ET) (van Loon et al. 2006), and localized cell death via the hypersensitive response (Hwang and Hwang 2011; Kim and Hwang 2011).

Upon recognition of pathogen's infection, as a core component of the early plant immune response (Daudi et al. 2012), ROS burst in the apoplast (Torres 2010; Torres et al. 2006) and then diffuse into the cytoplasm and serve as signals, interacting with other signaling processes such as phosphorylation cascades, calcium signaling, and hormone-mediated pathways (Kovtun et al. 2000; Mou et al. 2003). ROS, primarily superoxide, and H₂O₂, produced by plasma membrane-localized NADPH oxidases and class III peroxidases (Doke et al. 1996; Guo et al. 2011), are regarded as one of the fastest

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defense reactions against pathogen attack (Apostol et al. 1989). However, excessively high levels of ROS can disturb the immune responses of plants and ultimately result in cellular injury and tissue damage (Krishnamurthy and Rathinasabapathi 2013; Velloso et al. 2010), thus weakening the resistance of plants to pathogen infection (Puertollano et al. 2011; Lorrain et al. 2003). In plant defense, ROS always occur in conjunction with other plant signaling molecules, especially SA (Torres et al. 2006). The accumulation of ROS drives the production of SA, which subsequently enhances ROS production (Shirasu et al. 1997). These two immune molecules drive the hypersensitive reaction (HR) and mediate the establishment of systemic acquired resistance (SAR) (Durrant and Dong 2004). In most plants, free SA represents a key compound in the activation of plant defense against biotrophic and hemibiotrophic pathogens (Grant and Lamb 2006), and its accumulation is necessary for the induction of HR and the expression of marker *PR* genes (Alvarez 2000; Dong 2004). JA is another key hormone involved in plant immune responses to necrotrophic pathogens and herbivorous insects (Bari and Jones 2009; Ton et al. 2002). Upon hypersensitive necrosis against *V. dahliae* infection in cotton, not only does the level of SA increase, but also JA synthesis is strongly enhanced during the early stage (Grant and Jones 2009; Seo et al. 2001). Thus, during biotrophic stage, suppressing the immune system mainly the SA-dependent defenses is a strategy of *V. dahliae* to promote disease (Gkizi et al. 2016). ROS/hormone-mediated signaling pathways, resistant proteins (Gao et al. 2013b; Gao et al. 2011; Li et al. 2014, 2016; Qin et al. 2004; Sun et al. 2014; Yang et al. 2015), and intrinsic metabolites (Gao et al. 2013a; Luo et al. 2001; Tan et al. 2000; Xu et al. 2011) improve our understanding of the complicated innate defense systems of host plants against *V. dahliae*. However, little is known about the mechanism of NRPS in virulence and the relationship with host immunity. The research was aimed to illustrate the functional mechanism of *VdNPS* in *V. dahliae* by (i) demonstrating the influence of *VdNPS* deletion on vegetative growth and virulence of *V. dahliae*; (ii) clarifying the role of *VdNPS* in response to H₂O₂ stress; and (iii) exploring the function of *VdNPS* involved in SA tolerance and repressing host immunity.

MATERIALS AND METHODS

Strains, culture conditions, and plant lines. The defoliating type *V. dahliae* strain V991 was first isolated from cotton that originated in Xinjiang, China and used as a wild-type (WT) strain for transformation in this study. The strain and its transformants were stored at -80°C in the form of a conidial suspension in 30% glycerol and were reactivated on potato-dextrose agar (PDA) medium at 25°C. Conidia for infection assays were cultured in potato dextrose broth (PDB) medium. Basal media modified (BMM) was used for inducing microsclerotial development (Luo et al. 2019). *Agrobacterium tumefaciens* AGL-1 was used for genetic transformation, which was grown in YEB (0.5% sucrose, 0.1% yeast extract, 1% tryptone, 0.05% MgSO₄ · 7H₂O, pH 7.0) agar/liquid medium. Cotton (*Gossypium hirsutum* L.) and tobacco (*Nicotiana benthamiana*), which were used for pathogenicity assays, were grown in growth chambers at 25°C with 16-h photoperiods and 55% humidity.

Identification of positive randomly insertional transformants. The construction and identification of T-DNA randomly insertional mutants was performed as Luo et al. (2016). The randomly insertional mutants were constructed using pPK2 vector by *Agrobacterium tumefaciens* mediated transformation (ATMT). To detect the T-DNA insertion sites, hiTAIL-PCR (Liu and Chen 2007) was used for amplifying the flanking sequences of the positive transformants. The insertional sites were finally identified through sequencing and blasting with the genome of *V. dahliae* VdLs.17 strain (http://fungi.ensembl.org/Verticillium_dahliae/Info/Index) (Klosterman et al. 2011). The primers of hiTAIL-PCR were listed in Supplementary Table S1.

Vector construction and genetic transformation. To generate the knockout plasmids pPK2-*VdNPS*, 892 bp of upstream and 925 bp of downstream fragments were amplified from the genomic DNA with the primer pairs of F-U-*VdNPS*/R-U-*VdNPS* and F-D-*VdNPS*/R-D-*VdNPS*, respectively. A 3,032-bp hygromycin resistant cassette was amplified from the vector *pSilent-1* using primers F-P-*VdNPS*/R-T-*VdNPS*. These three fragments were fused to U-*hph*-D using primers F-U-*VdNPS*/R-D-*VdNPS* as described previously. The U-*hph*-D fragment was inserted into pPK2 binary vector by double enzyme digestion with *EcoRV*/*Xba*I. The complementation fragment, which contained the open reading fragment (ORF) of the *VdNPS* and its native promoter region, was amplified by PCR with primers F/R-in-*VdNPS*, and linked into pPNK2 complementation vector by *Hind*III/*Spe*I. pPNK2 was modified from pPK2 by replacing the *HygB*-resistance cassette with the *Neo*-resistance cassette. The knockout and complementation plasmids were transformed into *Agrobacterium tumefaciens* AGL-1, and the classical ATMT was performed as specified in Luo et al. (2016). Deletion mutants (Δ *VdNPS*) were screened on PDA supplemented with hygromycin at 50 mg/liter, and complementation transformants (Δ *VdNPS*:*VdNP*) were screened with G418 at 100 mg/liter. All the deletion transformants were identified by PCR screening using F-*hph*/R-*hph* to confirm whether the *hph* resistant cassette was inserted into the genome, using F-*VdNPS*/R-*VdNPS* to confirm whether the ORF of *VdNPS* was deleted successfully, and using F/R-in-*VdNPS* to confirm whether the *HygB* resistant cassette was randomly inserted into the genome or replaced the target ORF of *VdNPS* through homologous recombination. The fragment amplified using F/R-in-*VdNPS* was sequenced further to ensure the target gene was deleted successfully. The complementation mutants were also identified as above to check if the deletion mutant was complemented or not.

The deletion and complementation mutants of *VdSAH* were generated as above. The fragment size of each PCR product was listed in Supplementary Table S1. Furthermore, the overexpression vector of *VdSAH* was constructed using fusion PCR. Enhanced PtpC promoter and TtpC terminator of *Aspergillus nidulans* *gpdA* gene were amplified from *pSilent-1* vector by primer pairs of F/R-P2-*VdSAH* and F/R-T2-*VdSAH*, respectively. F/R-GFP-*VdSAH* was used to amplify GFP from plasmid pPHGK, and F/R-*VdSAH* was used to amplify the ORF of *VdSAH* from genomic DNA, and the two fragments were fused as *VdSAH-GFP* using F-*VdSAH*/R-GFP-*VdSAH*. Following, PtpC promoter, *VdSAH-GFP*, and TtpC terminator were fused to PtpC-*VdSAH-GFP*-TtpC using F-P2-*VdSAH*/R-T2-*VdSAH*. The overexpression fragment was linked into pPK2 by *Xba*I/*Hind*III digestion. All primers are listed in Supplementary Table S1.

Measuring the response of *V. dahliae* to abiotic stresses. In order to test the sensitivity of the *V. dahliae* to osmotic stress, oxygen radicals, and plant hormones, WT and transformants were cultured in PDB for 5 days and filtered through two layers of lens paper to prepare conidia suspension at 10⁷ spores/ml. Three microliters of conidia suspension of WT and transformants were individually pipetted onto PDA containing 1 M NaCl, 1.0 M sorbitol, 2 mM H₂O₂, 3.5 mM JA, and 3.5 mM SA. Transformants and WT strains were cultured at pH = 4.0 and 12.0 to measure the effects of pH on their growth. Strains were also exposed to ultraviolet rays for 1 h to test their viability. Colony diameters were measured 7 days postinoculation (dpi). All the experiments were repeated three times. To determine whether both iron and Cu²⁺ were involved in the response to H₂O₂, 0, 1, 5, 10, 20, 30, and 50 μ M Fe²⁺ and Cu²⁺ were added to PDA medium, which contained 0 and 5 mM H₂O₂. The growth of each colony was observed at 25°C. Each experiment was repeated three times.

Pathogenicity assays. WT *V. dahliae* and transformants were cultured in PDB for 5 days to prepare inoculum, which was adjusted to approximately 1 × 10⁷ conidia/ml. Virulence of WT, deletion mutants, overexpression mutants, and complementation mutants

were performed on 6-week-old intact *G. hirsutum* L. and *N. benthamiana* using unimpaired root-dip inoculation method as previously described (Fradin et al. 2009; Luo et al. 2016). The fungal biomass in the infected cotton roots were quantified by semiquantitative RT-PCR as described by Luo et al. (2016). Further, in order to test the colonization of WT and transformants in soil, the tested soil was reused to measure the germination of *G. hirsutum* L. seeds. The plants were incubated at 25°C with 16-h/8-h light/dark cycle and 55% humidity to enable disease symptoms to develop. The disease severity was evaluated by the percentage of plants that showed wilting symptom at 20 dpi after inoculation. The disease grade was classified as follows: 0 (no symptoms), 1 (>0 to 25% wilted leaves), 2 (25 to 50% wilted leaves), 3 (50 to 75% wilted leaves), and 4 (75 to 100% wilted leaves). The disease index was counted as $\Sigma = (\text{number of necrosis leaves} \times \text{disease grade}) / (\text{total number of leaves} \times 4) \times 100$. The virulence assays were repeated three times, and each time 12 plants were analyzed.

RNA extraction, RNA-seq, and quantitative real-time PCR (qRT-PCR). Total RNA was extracted using RNAiso Plus reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions, which was free of genomic DNA. For qRT-PCR, cDNA was reverse transcribed using PrimeScript RTase (Takara, Dalian, China), and qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, U.S.A.) in a CFX96 thermocycler (Bio-Rad). The expression levels of target genes of *V. dahliae* were quantified relative to the constitutively expressed β -tubulin (*Vdtubulin*), and the transcription level of *PR* genes (*NPRI*, *PR1*, *PR5*, *PDF1.2*) of *G. hirsutum* was normalized using constitutively expressed histone 3 (*Vdhist3*). All the experiments had three independent biological replicates. Gene-specific primers designed for qRT-PCR are listed in Supplementary Table S2. To study the resistance mechanism of *VdNPS* against ROS in *V. dahliae*, we compared the gene expression profiles between the WT and Δ *VdNPS* under the treatment of 0, 2, and 6 mM H_2O_2 for 12 h. RNA sequencing and analysis were conducted as specified in Luo et al. (2019). The raw data were mapped to the *V. dahliae* VdLs.17 genome database (http://fungi.ensembl.org/Verticillium_dahliae/Info/Index) (Klosterman et al. 2011). The RNA-seq analysis of the defense response of *G. hirsutum* against *V. dahliae* was performed similarly. After inoculation for 0, 3, 5, 7, and 9 days, the *G. hirsutum* roots and leaves were collected and prepared for RNA-seq. The cotton genome database (<https://www.cottongen.org/organism/Gossypium/hirsutum>) (Yu et al. 2014) was used for data mapping. Data analysis and annotation were performed according to Luo et al. (2019).

ROS measurement. For detecting ROS accumulation, the roots of cotton were stained with diaminobenzidine (DAB) at 1 mg/ml as previously described (Yoshioka et al. 2003). The content of hydrogen peroxide in plant roots and leaves was measured by titanium sulfate method (Eisenberg 1943; Satterfield and Bonnell 1955). The combination of H_2O_2 and titanium sulfate (or titanium chloride) forms peroxide-titanium complex, which is yellow precipitation. The precipitation can be dissolved by H_2SO_4 and determined by colorimetry at 415 nm wavelength. Within a certain range, the color is linearly related to the concentration of H_2O_2 .

Hoechst staining. Hoechst staining is used to visualize the nucleus integrity. Before staining the nuclei of WT and Δ *VdNPS*, conidia were harvested from 5-day-old cultures and filtered through two layers of lens paper. Harvested conidia were treated with 0, 2, and 6 mM H_2O_2 for 0, 12, 24, 48, and 96 h, respectively, and was recollected by centrifuging at 1,000 rpm, 5 min. The conidia were washed twice using 1× PBS buffer and were resuspended to 1×10^7 spores/ml in 10 $\mu\text{g/ml}$ Hoechst 33342 (Invitrogen, Shanghai, China). The suspension was incubated for 30 min in the dark at 25°C according to the kit instructions. For fluorescence images, cells were analyzed with UV/488 nm dual excitation, and emission was measured with standard DAPI filters on Leica confocal fluorescence microscope (Leica TCS SP8, German).

LC-MS/MS analysis. The fermentation broths of WT and Δ *VdNPS* were collected after 3, 5, 7, and 9 days and were filtered to eliminate fungi. The total metabolites were extracted with ethyl acetate and dissolved in methanol. Internal standard substances (2-chlorophenylalanine) were added followed by vortexing for 30 s, and then underwent ultrasonic disruption for 10 min on ice and incubation for 1 h at −20°C to precipitate metabolites. Centrifugation was performed at 13,000 rpm for 15 min at 4°C, and the supernatant was prepared for ultra high performance liquid chromatography-quadrupole time of flight-mass spectrometry (UHPLC-QTOF-MS) analysis. Metabolome data were processed using R package XCMS (version 3.2) (Fraga et al. 2010).

Bioinformatic and statistical analysis. Conserved domains of proteins were analyzed on online Pfam (<http://pfam.xfam.org/>) (El-Gebali et al. 2018). Phylogenetic tree was constructed using MEGA6.0 by the neighbor-joining method. The bootstrap replicate is 1,000. The heatmap was drawn by HemI software (Heatmap Illustrator, version 1.0) (Deng et al. 2014). Statistics were performed using IBM SPSS software. The differences among independent groups were determined by one-way analysis of variance and Student's *t* test. The significance level was chosen to be **P* < 0.05 and ***P* < 0.01.

RESULTS

Identification and characterization of *VdNPS* in *V. dahliae*. *VdNPS* (EGY14150) is annotated as N-(5-amino-5-carboxypentanoyl)-L-cysteinyl-D-valine synthase in *V. dahliae* VdLs.17 genome database. Functional domain analysis of the protein indicated that *VdNPS* contains three kinds of conserved domains (Fig. 1A): one is a typical AMP binding domain for adenylation (Pfam00501; A); the two other domains are a PP binding domain for phosphopantetheine attachment (Pfam00550; PP), and a condensation domain catalyzing amide bond formation (Pfam00668; C), which appear in conjunction as PP-C. The A domain occurs in triplicate, and the PP-C domain repeats six times in *VdNPS*. Based on the published architecture of NRPSs (Marahiel 2016; Tarry and Schmeing 2015), the conserved domains were organized to form three modules to incorporate each amino acid into the end product (δ -(L- α -aminoadipyl)-L-cysteine-D-valine, $\text{C}_{14}\text{H}_{25}\text{N}_3\text{O}_6\text{S}$, molecular mass: 363.65) (Fig. 1A). Phylogenetic analysis indicated that *VdNPS* is an ortholog of *NPS6* in *F. graminearum* (identity: 76.4%) and *Alternaria brassicicola* (identity: 70.4%) (Fig. 1B). *VdNPS* was firstly isolated through T-DNA random insertion. The inserted mutant (*IMΔVdNPS*) showed decreased virulence and microsclerotial formation (Supplementary Fig. S1A and B). Through sequencing the right-border flanking sequence, the mutant was identified to disrupt the encoding region of the fourth condensation domain of *VdNPS* (Supplementary Fig. S1C). Because the left-border flanking sequence was amplified unsuccessfully, we constructed knockout and complementation mutants through homologous recombination for further research.

Deletion and complementation mutants of *VdNPS*. Owing to the large ORF of *VdNPS* (15,337 bp), it was difficult to replace the entire ORF with *HygB* resistance cassette. Thus, we replaced only the 2,500 bp of the ORF from the initiation codon, which contained an integral set of domain (A-PP-C). Subsequent molecular verification of transformants showed that three colonies (No. 2, No. 3, and No. 10) were deleted successfully (Supplementary Fig. S2A and C), which were named Δ *VdNPS*-2, Δ *VdNPS*-3, and Δ *VdNPS*-10, respectively. The deletion mutant Δ *VdNPS*-2 was further used for constructing complementation mutant, and Δ *VdNPS*-2 and Δ *VdNPS*-3 were used for further measurement of pathogenicity and abiotic stress response. Through PCR amplification and sequencing, the No. 2 transformant was confirmed as the complementation mutant, annotated as Δ *VdNPS*:*VdNPS* (Com) (Supplementary Fig. S2B and C).

Decreased conidiation and microsclerotial formation in Δ *VdNPS* and its response to abiotic stress. Although there was no difference among Δ *VdNPS*, Com, and WT with respect to

colony diameter and growth rate, the formation of melanized microsclerotia in $\Delta VdNPS$ was much weaker than that in Com and WT (Fig. 2A and B). In addition, the $\Delta VdNPS$ mutants produced significantly fewer conidia than WT and Com ($P < 0.05$) (Fig. 2C).

Further abiotic stress response showed that under 1.0 M NaCl, 1.0 M sorbitol, ultraviolet radiation, pH 4.0, and pH 12.0 conditions, the vegetative growth of the $\Delta VdNPS$ mutant was not significantly different to that of Com and WT strains. However, the $\Delta VdNPS$

Fig. 1. The characterization of nonribosomal peptide synthetases (NPS) in *Verticillium dahliae*. **A**, Overall architecture of VdNPS shows three different modules, which incorporate amino acids into the end product (δ -(L- α -aminoadipyl)-L-cysteine-D-valine). Module boundaries are defined based on Pfam analysis and previous reports (Marahiel 2016; Tarry and Schmeing 2015). Each extension module comprises three core domains: one for adenylation (A, shown in blue), one for catalyzing peptide bond formation (C, shown in orange), and one for phosphopantetheine attachment (PP, shown in red). **B**, Phylogenetic relationship among nonribosomal peptides (NRPs) from different fungi. The amino acid sequences of 18 NRPs from public databases (<https://www.ncbi.nlm.nih.gov>) distributed into two clades showed high level of similarity. The percentage of trees in which the associated proteins clustered together (at least 50% in 1,000 bootstrap replicates) is shown next to the branches. The phylogenetic tree was constructed using MEGA6.0 by the neighbor-joining method.

Fig. 2. Phenotype of $\Delta VdNPS$. **A**, The colony phenotype of $\Delta VdNPS$. Reduced microsclerotia formed in $\Delta VdNPS$. **B**, No significant difference existed in the growth rate of $\Delta VdNPS$ mutants and wild type (WT). The x-axis represents growth days. **C**, The decreased conidiation in $\Delta VdNPS$. The statistical analysis was performed using *t* test ($*P < 0.05$). The experiment was repeated three times ($n = 3$).

showed significant resistance to H₂O₂ and hypersensitivity to SA and JA (Supplementary Fig. S3).

The analysis of metabolites in Δ VdNPS mutants. After fermenting for 7 days, total metabolites of WT and Δ VdNPS were analyzed by HPLC-MS. The results showed the mass-spectrum peak of 363.7 (8-(L- α -aminoadipyl)-L-cysteine-D-valine) did not exist in the Δ VdNPS mutant (Fig. 3A). Further metabolic profiling indicated that compared with WT, 213, 179, 142, and 103 metabolites were differentially regulated in Δ VdNPS at 3, 5, 7, and 9 days, respectively (Fig. 3B). The differentially regulated metabolites were enriched in the biosynthesis of secondary metabolites (ko01110), and biosynthesis of antibiotics (ko01130) (Fig. 3C to F); and over 66.7% differentially regulated metabolites in these pathways were down-regulated in Δ VdNPS (Supplementary Table S3). It is worth noting that no metabolite associated with siderophores was detected in both WT and Δ VdNPS, while the biosynthesis of β -lactam antibiotics carbapenem (ko00332) and staurosporine (ko00404) were enriched in Δ VdNPS (Fig. 3C to F). Carbapenems are used to treat serious infections caused by extended-spectrum β -lactamase carrying pathogens (Gupta et al. 2011). L-Proline and L-glutamate were differentially synthesized metabolites involved in the carbapenem biosynthesis, which were down-regulated in Δ VdNPS ($P < 0.05$, $\text{Log}_2\text{FC} < 0$) (Supplementary Table S3). The antibiotics staurosporine is an inhibitor of phospholipid/Ca⁺⁺-dependent protein kinase, and it could induce programmed cell death (Jacobsen et al. 1996; Tamaoki et al. 1986). L-Proline is a key protein in staurosporine biosynthesis, which was down-regulated in Δ VdNPS at 3, 5, and 7 days (Supplementary Table S3). The results indicated that VdNPS is more likely an enzyme for metabolite biosynthesis than for siderophore production as NPS6 in *Alternaria alternata*, *C. heterostrophus*, *F. graminearum*, and *Alternaria brassicicola* (Chen et al. 2013; Oide et al. 2006). However, the biosynthesis of specific metabolites and

antibiotics mediated by VdNPS need further isolation and identification.

VdNPS deletion decreased H₂O₂ sensitivity. In vitro tests indicated that Δ VdNPS strains exhibited decreased sensitivity to different concentrations of H₂O₂ than WT. The IC₅₀ and lethal concentration of H₂O₂ was 2 and 4 mM in WT, respectively; while those in Δ VdNPS was 4 and 6 mM, individually (Fig. 4A and Supplementary Fig. S4A). To elucidate the molecular mechanism of H₂O₂ tolerance in the Δ VdNPS strain, we examined the gene expression by transcriptional profiling analysis under the stress of 0, 2, and 6 mM H₂O₂. Results showed that the genes encoding cell wall protein and cytoskeleton complex protein were significantly up-regulated in Δ VdNPS, while the genes encoding membrane intrinsic protein were significantly down-regulated (Supplementary Table S4). Additionally, genes encoding DNA repair protein were significantly up-regulated in Δ VdNPS (Supplementary Table S4), and Hoechst staining analysis indicated that high concentration of H₂O₂ led to nuclear degradation in Δ VdNPS and WT (Supplementary Fig. S5). NADPH oxidases (Nox) is the major enzymatic producer of ROS (Egan et al. 2007), which is important for the appressoria formation, while the VDAC_00930 encoding NoxB was down-regulated under H₂O₂ stress both in WT and Δ VdNPS; and compared with WT, the expression of NoxB was down-regulated in Δ VdNPS. Importantly, the expression levels of three categories of genes associated with H₂O₂ detoxification were differentially expressed under H₂O₂ stress in Δ VdNPS, including *cat* (VDAG_09115), *sod* (VDAG_00019, VDAG_07230, VDAG_08724, VDAG_09837), and *pod* (VDAG_03116). Without H₂O₂, compared with WT, VDAG_00019, VDAG_07230, and VDAG_09115 were up-regulated by 9-fold, 3.9-fold, and 3.82-fold in Δ VdNPS, respectively. Under the stress of 2 mM H₂O₂, VDAG_00019 and VDAG_07230 were up-regulated by 2.86-fold and 3.12-fold in Δ VdNPS, respectively (Fig. 4B). These data suggested that

Fig. 3. The influence of VdNPS on the biosynthesis of metabolites in *Verticillium dahliae*. **A**, High performance liquid chromatography-mass spectrometry analysis of the total metabolites between Δ VdNPS and wild type (WT) after 7 days fermentation. The y-axis indicates intensity, the x-axis represents mass-to-charge ratio (m/z). Arrow indicated the target peak of VdNPS, which only exists in WT. **B**, Number of differentially expressed metabolites (DEMs) in Δ VdNPS versus WT groups. The y-axis represents the number of DEMs. **C, D, E, and F**, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially regulated metabolites between WT and Δ VdNPS, which were fermented for 3, 5, 7, and 9 days individually. The biosynthesis of antibiotics (ko01130), biosynthesis of secondary metabolites (ko01110), staurosporine biosynthesis (ko00404), and carbapenem biosynthesis (ko00332) were enriched in DEMs.

$\Delta VdNPS$ alleviated oxidative stress by regulating the expression of genes associated with strengthening cell structure, detoxifying H_2O_2 , and inhibiting ROS production in vivo.

VdNPS involved in the response to H_2O_2 stress via iron/copper. H_2O_2 function in the form of high reactive hydroxyl radical ($OH^\bullet$). During the process of transforming H_2O_2 to $OH^\bullet$, Fe^{2+} release and reduce to Fe^{3+} , and stimulates O_2^- transformation to $OH^\bullet$ through the Fenton reaction (Kohanski et al. 2010, 2007). Unexpectedly, during the response to H_2O_2 , transcriptome sequencing revealed that besides iron, copper was also involved in the response to H_2O_2 . Under the treatment of 0, 2, and 6 mM H_2O_2 , genes encoding siderophore iron transporter (VDAG_03807 and VDAG_08042) were down-regulated in $\Delta VdNPS$, but genes encoding ferric reductase (VDAG_03652 and VDAG_06705) were up-regulated in the presence of 2 mM H_2O_2 , and VDAG_02046 was down-regulated in the presence of 6 mM H_2O_2 (Fig. 4D). As the cofactor of Cu/Zn SOD, Cu could induce cellular antioxidants scavenged ROS under optimal level (Baker 1987), and excessive Cu could catalyze the production of ROS (Aust et al. 1985). Contrary to iron related transporter and enzymes, genes encoding copper transporter (VDAG_06704, VDAG_06151) and copper amine oxidase (VDAG_07314) were up-regulated under the stress of 2 and 6 mM H_2O_2 (Fig. 4D).

Due to an important role of iron/copper in detoxification of oxidative stress, we assessed whether deletion of VdNPS affected H_2O_2 sensitivity through iron/copper uptake. Results showed that under normal condition, exogenous application of lower concentration ($<30 \mu M$) of Fe^{2+} and Cu^{2+} did not affect the growth of $\Delta VdNPS$ and WT. In the presence of 5 mM H_2O_2 , exogenous application of Fe^{2+} ($<10 \mu M$) and Cu^{2+} ($<30 \mu M$) relieved the H_2O_2 damage to WT; while the iron/copper-excess did not affect the growth of $\Delta VdNPS$ under H_2O_2 stress (Fig. 4C and Supplementary Fig. S4B to E). The results implied that VdNPS is essential for *V. dahliae* to confront oxidative stress, and iron/copper regulate H_2O_2 response coordinately.

$\Delta VdNPS$ strains exhibited hypersensitivity to SA via up-regulation of SA hydroxylation-associated genes. SA is a multitasking and pleiotropic molecule, which could induce plant immune responses and has direct antifungal activity. In vitro inhibition tests showed that $\Delta VdNPS$ strains were hypersensitive to SA (Fig. 5A and B). To further explore the function of VdNPS in response to SA stress, we monitored the expression of key genes involved in SA hydroxylation. The hydroxylation of SA leads to loss of function, which regulates the excessive accumulation of SA and maintains SA homeostasis (Zhang et al. 2017). Four genes associated with SA hydroxylation exist in *V. dahliae*, including

Fig. 4. The response mechanism of $\Delta VdNPS$ to H_2O_2 stress. **A**, Growth inhibition of $\Delta VdNPS$, wild type (WT) and complementation mutant (Com) under different concentrations of H_2O_2 . The deletion of VdNPS decreased the sensitivity to H_2O_2 stress in *V. dahliae*. **B**, Normalized expression of genes encoding catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) in WT and $\Delta VdNPS$ under the stress of 0, 2, and 6 mM H_2O_2 . CAT: VDAG_09115; SOD: VDAG_00019, VDAG_07230, VDAG_08724, and VDAG_09837; and POD: VDAG_03116. **C**, Colony phenotype of tested strains under exogenous application of Fe^{2+} and Cu^{2+} . Exogenous application of lower concentration ($<30 \mu M$) of Fe^{2+} and Cu^{2+} did not affect the growth of $\Delta VdNPS$ and WT, while in the presence of 5 mM H_2O_2 , exogenous application of Fe^{2+} ($<10 \mu M$) and Cu^{2+} ($<30 \mu M$) relieved the H_2O_2 damage to WT. **D**, Normalized expression of genes encoding copper/siderophore iron transporter, ferric reductase transmembrane component, and metalloprotease in WT and $\Delta VdNPS$ under the treatment of 0, 2, and 6 mM H_2O_2 . The y-axis indicates the relative transcripts compared with the housekeeping gene *Vdtubulin*. Bars represent the standard error from three independent replicates. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; t test) between $\Delta VdNPS$ and WT.

salicylate hydroxylase *VdSAH1* (VDAG_04960), salicylate hydroxylase *VdSAH2* (VDAG_07329), salicylate hydroxylase *VdSAH3* (VDAG_09318), and salicylate 1-monooxygenase *VdSAH4* (VDAG_01968). The four proteins are nonsecretory. The quantitative RT-PCR analysis showed that the transcript levels of the *VdSAH1*, *VdSAH2*, *VdSAH3*, and *VdSAH4* were significantly up-regulated in $\Delta VdNPS$ under SA stress compared with those in WT on the same conditions (Fig. 5C). Further functional characterizations of *VdSAHs* showed that the knockout mutants ($\Delta VdSAH1-4$) were hypersensitive to SA, while the overexpression mutants (*PttrpC::SAH1-4*) were tolerant to SA (Fig. 5D and Supplementary Fig. S6). The same phenomenon was observed under JA and H₂O₂ stress (Supplementary Figs. S6 and S7A). Additionally, the deletion of *VdSAH1-4* significantly weakened the virulence of *V. dahliae*, and the disease indexes were lower than 12.5%, while those of overexpression strains, Com, and WT exceeded 85% (Fig. 5E to F). During infection, $\Delta VdSAH1-4$ strains induced higher expression of *NPR1*, *PR1*, *PR5*, and *PDF1.2* in cotton than that induced by WT, overexpression strains, and Com (Supplementary Fig. S7B). The results indicated that $\Delta VdNPS$ was hypersensitive to SA through mediating SA hydroxylation via

general down-regulation of salicylate hydroxylase and salicylate 1-monooxygenase, thus, to weaken the virulence of *V. dahliae*.

Reduced virulence of $\Delta VdNPS$ mutants. *G. hirsutum* and *N. benthamiana* were used to test the pathogenicity of *V. dahliae*. Twenty days after inoculation, the plants infected by Com and WT showed severe wilting symptoms (disease index, DI > 95%) (Fig. 6A); while the intact plants infected by $\Delta VdNPS$ grew normally and the DI (17.5%) was significantly lower ($P < 0.01$) (Fig. 6B). Semiquantitative RT-PCR analysis showed that the biomass of WT, Com, and $\Delta VdNPS$ in the cotton shoots was not significantly different (Fig. 6C). Compared with the expression in cotton inoculated with WT, the expressions of *NPR1*, *PR1*, *PR5*, and *PDF1.2* at 9 dpi were significantly up-regulated in cotton inoculated with $\Delta VdNPS$ (Fig. 6D). Diaminobenzidine (DAB) staining was carried out on cotton roots to detect the production of H₂O₂ after inoculating for 9 days. The results showed that the roots inoculated with $\Delta VdNPS$ were stained dark brown, which was a deeper color than those inoculated with WT and Com (Fig. 6E). The H₂O₂ content reached 72.248 mol/g (fresh weight) in the roots inoculated with $\Delta VdNPS$, which was significantly higher than that observed in response to WT ($P < 0.01$) (Fig. 6E).

Fig. 5. *VdNPS* strains exhibited hypersensitivity to salicylic acid (SA) via up-regulation of SA hydroxylation-associated genes. **A**, $\Delta VdNPS$ was hypersensitive to SA. **B**, The inhibited colony growth of $\Delta VdNPS$ mutants under the SA stress. **C**, Normalized expression of *VdSAH1*, *VdSAH2*, *VdSAH3*, and *VdSAH4* under 3.5 mM SA treatment for 0, 2, 4, 8, and 24 h in *Verticillium dahliae*. Expression was normalized against housekeeping gene *Vdtubulin*. **D**, Phenotype of *VdSAH* mutants under the treatment of SA. Compared with wild type (WT) and complementation mutant (Com), the knockout mutants $\Delta VdSAH$ were unable to grow in the presence of SA. **E**, *VdSAH* is required for full *V. dahliae* virulence. The disease symptoms on intact cotton plants were scored at 20 days postinoculation with indicated strains. **F**, The disease indexes of $\Delta VdSAH$ mutants were lower than 12.5%. $\Delta VdSAH1-4$ represent knockout mutants; *PttrpC::SAH1-4* represent overexpression mutants under the *Aspergillus nidulans* trpC promoter; and Com1-4 represent complementation mutants of *VdSAH1-4* using its own promoter, respectively. Three biological replicates ($n = 3$) were used for this study. The statistical analysis was performed using a *t* test (* $P < 0.05$; ** $P < 0.01$).

The host response against $\Delta VdNPS$ strain and WT *V. dahliae*. In order to illustrate the phenomenon of reduced virulence in $\Delta VdNPS$, we monitored the changes of transcriptional level in host infected by $\Delta VdNPS$ and WT. At 9 dpi, the cotton plants infected by WT *V. dahliae* started to wilt, while those infected by $\Delta VdNPS$ were still healthy. The transcriptional profile and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that during the infection, plant hormone signal transduction (ko04075) and plant–pathogen interaction (ko04626) enriched after 5-day-inoculation (Supplementary Fig. S8, Supplementary Table S5). With respect to plant hormone signal transduction (ko04075), JA- and SA-mediated signaling pathways were significantly enriched

for defense response. Compared with WT, the differentially expressed genes (DEGs) involved in JA- and SA-mediated signaling pathways were all up-regulated in $\Delta VdNPS$ at 5 dpi (Fig. 7A), including jasmonate-zim domain family proteins (JAZ) (evm.TU.Gh_A03G1341 and evm.TU.Gh_D02G1776) and basic-helix-loop-helix transcription factors MYC2 (evm.TU.Gh_A08G1412, evm.TU.Gh_A08G0962, and evm.TU.Gh_D08G1707) encoding genes in JA-mediated signaling pathway, basic/leucine zipper-type transcription factors TGA (evm.TU.Gh_A09G0903, evm.TU.Gh_A09G1184, evm.TU.Gh_D07G1078, and evm.TU.Gh_D09G1188), and PR1 (evm.TU.Gh_A12G0274) encoding genes in SA-mediated signaling pathway (Fig. 7F). At 7 dpi, DEGs encoding JAZ were up-regulated,

Fig. 6. VdNPS is a virulence factor in *Verticillium dahliae*. **A**, Necrosis and defoliation in intact cotton (*Gossypium hirsutum* L.) and tobacco (*Nicotiana benthamiana*) plants after 20 days postinoculation (dpi) with 4×10^7 conidia of *V. dahliae* mutants. The representative photos were taken at 20 dpi, when the plants infected with wild type (WT) were dead. The top plant is cotton, and the bottom plant is tobacco. **B**, Wilting symptoms on cotton plants were scored at 20 dpi with the indicated strains. The disease grade and disease index are shown above each column (** $P < 0.01$; t test). WT represents *V. dahliae* V991 isolate; $\Delta VdNPS$ represents the deletion mutant; CK represents plant roots inoculated with sterile water; and Com represents the complementation strain ($\Delta VdNPS$: *VdNPS*) under its own promoter. **C**, Semiquantitative RT-PCR analysis showed that there was no difference of fungal biomass in infected plant roots. **D**, Relative expression of *NPR1*, *PR1*, *PR5*, and *PDF1.2* in plants infected with the WT strain and mutants at 10 dpi, when the cotton inoculated with WT began to wilt. Expression was normalized against histone 3 encoding gene (*Ghhist3*). **E**, Diaminobenzidine (DAB) staining and H₂O₂ contents in cotton roots infected with the WT strain and *V. dahliae* mutants. FW, fresh weight. Scale bar: 50 μ m. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; t test) between each mutant and WT.

whereas DEGs encoding MYC2 were down-regulated (Fig. 7B and F); at 9 dpi, DEGs involved in JA-mediated signaling were down-regulated in $\Delta VdNPS$ (Fig. 7C and F). At 7 and 9 dpi, the gene expression in SA-mediated signaling pathway was not different between WT and $\Delta VdNPS$. Besides the classical JA- and SA-mediated defense response, ET, auxin, ABA, gibberellin, cytokinin, and brassinosteroid-mediated signaling pathways were involved in the response to pathogen invasion, which were summarized in Supplementary Figures S9 and S10. In the process of plant-pathogen interaction (ko04626), Ca^{2+} -mediated signaling pathways play an important role in $\Delta VdNPS$; however, fungal pathogen-associated molecular pattern (PAMP, Avr9) triggering immunity only occurred at 9 dpi (Fig. 7D and F). At 5 and 9 dpi, DEGs encoding calcium binding protein, calmodulin-like protein, and calcium-dependent protein kinase were all down-regulated in $\Delta VdNPS$; at 9 dpi, DEGs encoding the cyclic nucleotide-gated ion channel were up-regulated in $\Delta VdNPS$, whereas the plant receptor kinase BAK1 encoding gene was down-regulated (Supplementary Fig. S11). WRKY transcription factors 22 and 33 play an important role in disease resistance even though they are antagonistic (Supplementary Fig. S11). The above results indicated the enhanced plant hormone-mediated signal pathways, and the Ca^{2+} -WRKY- and Ca^{2+} -Rboh-mediated HR, cell wall

reinforcement and defense-related gene expressions induced by $\Delta VdNPS$ provided a likely explanation for the decreased virulence of $\Delta VdNPS$ mutants.

DISCUSSION

Since the discovery of penicillin, NPSs have been well characterized as important enzymes for synthesizing natural products in fungi and plant pathogens, including (i) β -lactam abiotic synthesis, such as penicillin and cephalosporin in *Penicillium chrysogenum* (Arnstein et al. 1959), *Acremonium chrysogenum*, and *Streptomyces clavuligerus* (Banko et al. 1986; Jensen et al. 1988); (ii) fungal toxins biosynthesis, such as HC-toxin in *C. carbonum* (Panaccione et al. 1992), AM-toxin in *Alternaria alternata* (Johnson et al. 2000), phytotoxin in *Dickeya zeae* (Cheng et al. 2013), and fusaoctatin A in *F. graminearum* (Jia et al. 2019); and (iii) siderophore production in *Alternaria alternata* (Chen et al. 2013), *C. heterostrophus*, *F. graminearum*, and *Alternaria brassicicola* (Oide et al. 2006). VdNPS was first mentioned by Wang et al. (2018), who reported that as the homolog of key siderophore biosynthetic genes in *Aspergillus nidulans* (Eisendle et al. 2003),

Fig. 7. Plant hormone signal transduction pathways and plant-pathogen interaction in cotton infected by wild type (WT) and $\Delta VdNPS$. **A to C**, After inoculating for 5, 7, and 9 days, differentially expressed genes (DEGs) in host were enriched in jasmonic acid (JA)- and salicylic acid (SA)-mediated signaling transduction pathways. At 5 dpi, DEGs involved in JA- and SA-mediated signaling pathways were all up-regulated. **D and E**, DEGs involved in plant-pathogen interaction. Ca^{2+} -mediated host defense and fungal pathogen-associated molecular pattern (PAMP) triggered immunity are two predominantly different pathways in response to WT and $\Delta VdNPS$ infection. The red rectangles represent up-regulated DEGs, and the green rectangles represent down-regulated DEGs. **F**, The normalized expression of DEGs involved in the JA- and SA-mediated signaling transduction pathways. The y-axis represents the normalized expression, the x-axis represents the infection time. Error bars represent standard deviation. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; t test).

NPS (VDAG_05314) was highly up-regulated under iron sufficiency compared with that under conditions of iron starvation in *V. dahliae* XS11 strain. However, no further functional characterization of *VdNPS* was reported later. In this research, through HPLC-MS analysis, we found that the end product of *VdNPS*, L- δ -(α -aminoadipoyl)-L-cysteinyl-D-valine (NRP), did not synthesize in the Δ *VdNPS* strain. Through phylogenetic analysis showed that the *VdNPS* was the ortholog of *AaNPS6*, which catalyzes the produce of coprogen siderophore, while the molecular weight and structure of *VdNRP* was different from that of siderophores. Further LC-MS profiling showed that no siderophore associated metabolites were significant differentially synthesized in Δ *VdNPS*, while the metabolic synthesis of carbapenem antibiotics and toxin staurosporine were down-regulated in Δ *VdNPS* strain. Additionally, the phenotypes of Δ *VdNPS* and WT *V. dahliae* were not different under iron stress. All the results implied that *VdNPS* is more likely a synthetase for metabolites biosynthesis than siderophore production.

Ignoring whether the NPSs are involved in abiotic synthesis, toxins biosynthesis, or siderophore production, they are important for fungal pathogenicity in different pathogens, such as *Magnaporthe oryzae* (Bohnert 2004; Song et al. 2015), *C. carbonum* (Panaccione et al. 1992), and *Aspergillus nidulans* (Eisendle et al. 2003). Loss of NPS in these fungi resulted in reduced virulence on hosts. The same decreased virulence was also observed in Δ *VdNPS* strain. The deletion of *VdNPS* led to reduced microsclerotial formation and lower conidiation in *V. dahliae*. However, contrary to *NPS6* in *Alternaria alternata*, *C. heterostrophus*, *F. graminearum*, and *Alternaria brassicicola* (Chen et al. 2013, Oide et al. 2006), the deletion mutants Δ *VdNPS* were less sensitive to oxidative stress. The tolerance to H_2O_2 was mediated by siderophore bypass Fenton reaction in *C. heterostrophus*, *F. graminearum*, *Alternaria alternata*, and *Alternaria brassicicola*, in which the iron application could restore the tolerance of *NPS6* deletion mutants to H_2O_2 (Chen et al. 2013; Oide et al. 2006). Intracellular ROS is important for the differentiation of appressoria-like structures, conidiation, and virulence in fungal pathogens (Oide et al. 2006; Chen et al. 2013); and ROS from pathogens are important for development and pathogenicity, which was confirmed by Nox family within filamentous fungi (Lara-Ortiz et al. 2003), such as in *B. cinerea* (Segmüller et al. 2008), *M. oryzae* (Egan et al. 2007), *C. purpurea* (Giesbert et al. 2008), *Alternaria alternata* (Hyon et al. 2010), and *V. dahliae* (Zhao et al. 2016). In *V. dahliae*, transcriptional profiling analysis indicated that compared with WT, the gene encoding NADPH oxidases (NoxB) was down-regulated under H_2O_2 stress in Δ *VdNPS*, which could inhibit the appressoria formation, thereby decreased the pathogenicity. Additionally, genes associated with cell structure strengthen DNA repair, and H_2O_2 detoxification were up-regulated in Δ *VdNPS* under H_2O_2 stress. It maybe one factor for H_2O_2 tolerance in Δ *VdNPS*. In Fenton reaction, transformation from ferrous (Fe^{2+}) to ferric (Fe^{3+}) is the key step to change the ROS to the highly active $OH^\bullet$, which is the ROS damage form (Halliwell and Gutteridge 1992). Unexpectedly, as the genes encoding ferric associated transporters and reductase, and the genes encoding copper transporter and oxidase were differentially expressed, transcriptional profiling analysis indicated that besides iron, copper was involved in the response to H_2O_2 stress in *V. dahliae*. Under optimal level, copper induces cellular antioxidants to scavenge ROS thereby preventing the damage caused by the overproduction of ROS (Baker 1987). However, excessive copper will catalyze the ROS production via Haber Weiss and Fenton reactions (Aust et al. 1985). Without oxidative stress, iron and copper ($<30 \mu M$) had no influence on the growth of WT and Δ *VdNPS* strain; while under H_2O_2 stress, the exogenous application of iron ($<10 \mu M$) and copper ($<30 \mu M$) could restore the tolerance to H_2O_2 in WT but had no obvious influence on Δ *VdNPS*. The results indicated that the deletion of *VdNPS* caused insensitivity to the iron and copper change, but it regulated ROS response via Fenton reaction.

The induction of PR gene expression, ROS burst, and SA/JA production are common defense responses of plants against pathogen infection (Fonseca et al. 2018). Our molecular demonstration and RNA-sequencing results indicated that Δ *VdNPS* induced most PR gene expression, higher ROS production, and stronger SA/JA-mediated signaling transduction. During the process of plant-pathogen interactions, fungi have evolved three strategies to interfere the host SA homeostasis and SA-mediated plant defenses (Chanclud and Morel 2016) as follows: (i) producing and secreting chorismate mutase as a virulence factor, such as *Cmu1* in *Ustilago maydis* (Djamei et al. 2011); (ii) synthesizing secretory enzymes to degrade SA precursors to decrease SA levels in the plant host, such as isochorismatase in *V. dahliae* (Liu et al. 2014); and (iii) synthesizing salicylate hydroxylases to convert bioactive SA into an inactive compound, such as *U. maydis* (Rabe et al. 2013) and *Epichloë festucae* (Ambrose et al. 2015). In this study, we found that Δ *VdNPS* was hypersensitive to SA stress. Further molecular characterization showed that the genes encoding salicylate hydroxylase was up-regulated in Δ *VdNPS* compared with WT, and the loss of these genes caused decreased virulence and inability to grow under SA stress. Researches have reported that SA could diffuse from cell to cell through membrane, thereby reducing the SA accumulation and maintain SA homeostasis (Shulaev et al. 1997). Base on the results, we speculated that during pathogen infection, the host SA synthesis was induced, the intracellular SA mediated the establishment of SAR (Durrant and Dong 2004), and the extracellular SA have direct antifungal activity. The salicylate hydroxylases in pathogens detoxify SA through SA hydroxylation and thereby promotes disease. It is the first report that Δ *VdNPS* induced stronger SA-mediated defense response and hypersensitivity to SA via up-regulation of salicylate hydroxylases. The relationship between *VdNPS* and *VdSAH* needs further demonstration.

In summary, *VdNPS* is a key virulent determinant in *V. dahliae* (Fig. 8). Uncovering the mechanism of actions of *VdNPS* should facilitate the development of cultivars with durable resistance against *V. dahliae*-associated diseases.

Data availability statement. The transcriptome data for this study were deposited into the NCBI database as a BioProject (accession numbers PRJNA587035 and PRJNA587036).

Fig. 8. A proposed working model of *VdNPS* functions in oxidative and salicylic acid (SA) stress response. Under H_2O_2 stress, Δ *VdNPS* inhibited the transformation of H_2O_2 to $OH^\bullet$ through up-regulating the expression of *VdCAT*, *VdSOD*, and *VdPOD*, and Fe^{2+}/Cu^{2+} -mediated Fenton reaction. Under SA stress, the genes associated SA hydroxylation were induced in wild-type *Verticillium dahliae*, thus stimulating the SA detoxification. However, the expression of genes encoding salicylate hydroxylases were down-regulated in Δ *VdNPS*, and the Δ *VdNPS* mutants were hypersensitive to SA stress. Black lines indicate the response of Δ *VdNPS* to H_2O_2 stress. Gray/blue lines represent the response mechanism of Δ *VdNPS* to SA stress. Dotted lines represent that it's unsure whether the functional mechanism is direct or indirect.

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