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# Thioredoxin VdTrx1, an unconventional secreted protein, is a virulence factor in *Verticillium dahliae*

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Understanding how plant pathogenic fungi adapt to their hosts is of critical importance to securing optimal crop productivity. In response to pathogenic attack, plants produce reactive oxygen species (ROS) as part of a multipronged defense response. Pathogens, in turn, have evolved ROS scavenging mechanisms to undermine host defense. Thioredoxins (Trx) are highly conserved oxidoreductase enzymes with a dithiol-disulfide active site, and function as antioxidants to protect cells against free radicals, such as ROS. However, the roles of thioredoxins in *Verticillium dahliae*, an important vascular pathogen, are not clear. Through proteomics analyses, we identified a putative thioredoxin (VdTrx1) lacking a signal peptide. VdTrx1 was present in the exoproteome of *V. dahliae* cultured in the presence of host tissues, a finding that suggested that it plays a role in host-pathogen interactions. We constructed a *VdTrx1* deletion mutant  $\Delta VdTrx1$  that exhibited significantly higher sensitivity to ROS stress, H<sub>2</sub>O<sub>2</sub>, and *tert*-butyl hydroperoxide (*t*-BOOH). *In vivo* assays by live-cell imaging and *in vitro* assays by western blotting revealed that while VdTrx1 lacking the signal peptide can be localized within *V. dahliae* cells, VdTrx1 can also be secreted unconventionally depending on VdVps36, a member of the ESCRT-II protein complex. The  $\Delta VdTrx1$  strain was unable to scavenge host-generated extracellular ROS fully during host invasion. Deletion of *VdTrx1* resulted in higher intracellular ROS levels of *V. dahliae* mycelium, displayed impaired conidial production, and showed significantly reduced virulence on *Gossypium hirsutum*, and model plants, *Arabidopsis thaliana* and *Nicotiana benthamiana*. Thus, we conclude that VdTrx1 acts as a virulence factor in *V. dahliae*.

## KEYWORDS

*Verticillium dahliae*, unconventional secreted protein, thioredoxin, ROS scavenging, virulence factor

## 1. Introduction

Reactive oxygen species (ROS), including superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical ( $OH^-$ ), are generated as natural by-products of oxygen metabolism in aerobes, and also function as part of host repertoire of defense-related activities (Broxton and Culotta, 2016). The critical roles of ROS in defense have been well established in numerous pathosystems (Heller and Tudzynski, 2011; Segal and Wilson, 2018). ROS can react rapidly and nonspecifically with macromolecules, causing molecular damage such as lipid peroxidation, protein oxidation, and mutations (Imlay, 2003). Host ROS accumulation can obstruct pathogen colonization through oxidative cross-linking of cell-walls (Camejo et al., 2016). The oxidative burst, a transient and rapid accumulation of ROS, is part of the initial defense response in higher plants to limit pathogen spread through induction of cell death near the penetration site to cause local necrosis (Lamb and Dixon, 1997).

Since ROS are highly toxic to microbes, pathogens must neutralize or scavenge excess ROS to survive and proliferate within the host. The first line of defense against ROS are the superoxide dismutases (SOD), which catalyze dismutation of  $O_2^-$  to  $O_2$  and  $H_2O_2$ , the latter then are removed by catalases, the glutathione system (composed of glutathione peroxidases, glutathione reductases, and glutaredoxins) or the thioredoxin system (composed of peroxiredoxin, thioredoxin, and thioredoxin reductase) (Lu and Holmgren, 2014; Staerck et al., 2017). It comes as no surprise that pathogen survival and virulence has been correlated with the activity of ROS scavenging enzymes in many pathogens. SODs of plant pathogenic fungi, such as *Botrytis cinerea* and *Fusarium graminearum*, play important roles in the detoxification of ROS and pathogenesis (Rolke et al., 2004; Yao et al., 2016). The thioredoxin systems employed by *Magnaporthe oryzae* (Wang et al., 2017) and *Sclerotinia sclerotiorum* to defend against host-generated oxidative stress also contribute significantly to virulence (Rana et al., 2021).

*Verticillium dahliae*, a destructive soilborne phytopathogenic fungus, causes Verticillium wilt disease in a wide range of hosts, including many economically important crops (Klosterman et al., 2009; Chen et al., 2021). As in other pathosystems, the interactions of *V. dahliae* with a variety of hosts are also accompanied by changes in the levels of ROS. For instance, *V. dahliae* triggered  $H_2O_2$  production in tomato early in the interaction (Gayoso et al., 2010); ROS production was also detected at infection sites during cotton and *V. dahliae* interactions (Zhang et al., 2017). Furthermore, many *V. dahliae* secreted proteins, such as the elicitor PevD1 (Wang et al., 2012), the PAMPs VdEG1/3 (Gui et al., 2017) and several small cysteine-rich effector proteins (Wang D. et al., 2020) can all induce host ROS accumulation. *V. dahliae* adopts multiple strategies to cope with oxidative stress. Transcription factors Vta2, Som1, and VdHapx relieve ROS stress by regulating expression of genes for oxidative stress response (Tran et al., 2014; Wang Y.L. et al., 2018; Bui et al., 2019). Chromatin remodeling, mediated by a histone-fold protein VdDpb4 and its interacting protein VdIsw2, facilitates the DNA damage repair of *V. dahliae* in response to plant ROS stress (Wang S. et al., 2020). Our previous studies have shown superoxide dismutase family mediating ROS detoxification by scavenging the primary superoxide anion  $O_2^-$  to  $H_2O_2$  under conditions of stress (Tian et al., 2021a,b). However, it

is unclear whether *V. dahliae* employs other ROS-detoxifying enzyme systems to remove excess  $H_2O_2$ .

To identify new pathogenicity factors facilitating *V. dahliae* infection, we analyzed the exoproteome of *V. dahliae* induced in cotton tissue-containing medium by iTRAQ (Chen et al., 2016; Wang J. et al., 2018). Using this approach, we identified a predicted thioredoxin (VdTrx1) lacking a signal peptide and containing five highly conserved sites (Chen et al., 2016). The above suggested that VdTrx1 may function in host-pathogen interactions and pathogenicity. Thioredoxins are small, ubiquitous proteins with a redox-active site (WCGPC) conserved throughout evolution (Holmgren, 1989). As hydrogen donors, thioredoxin cycle between their reduced dithiol [ $Trx-(SH)_2$ ] and oxidized disulfide ( $Trx-S_2$ ) forms to regulate many metabolic enzymes which form disulfide bonds during their catalytic cycle (Vignols et al., 2005). One of them is peroxiredoxin, an enzyme involved in the reduction of  $H_2O_2$  (Verdoucq et al., 1999).

Typically, secreted proteins in eukaryotic cells carry an N-terminal signal peptide, which direct their sorting to the lumen of the endoplasmic reticulum (ER). They are then transported to the extracellular space through the ER-Golgi route via vesicular carriers (Palade, 1975; Ferro-Novick and Brose, 2013). Although lacking an N-terminal signal sequence, the secreted human thioredoxin TXN1 was first identified as the adult T-cell leukemia-derived factor (ADF) (Wakasugi et al., 1990). Additionally, thioredoxin can be released from various types of mammalian cells to alter the extracellular redox state (Léveillard and Ait-Ali, 2017). In the plant pathogenic fungus, *Magnaporthe oryzae*, MoTrx2 also plays a critical role in scavenging extracellular ROS during host cell invasion and is likely a secreted protein, although there is no clear evidence for this currently (Wang et al., 2017).

The mechanism of secretion of thioredoxins is unconventional. Secretion of signal peptide-lacking antioxidant superoxide dismutase (SOD1) in *S. cerevisiae* requires a novel membrane compartment called CUPS (compartment for unconventional protein secretion), which marked by the presence of Golgi reassembly and stacking protein (GRASP; Cruz-Garcia et al., 2017). Secretion of *S. cerevisiae* SOD1 is accompanied by the export of thioredoxin such as thioredoxins Trx1/2 and peroxiredoxin Ahp1 in a GRASP-dependent manner (Cruz-Garcia et al., 2020). However, whether CUPS is involved in the secretion of similar antioxidant enzymes lacking a signal peptide in filamentous fungi is not clear.

The main objectives of this study were to: (1) confirm that VdTrx1 is secreted; (2) explore the molecular mechanism of VdTrx1 secretion; (3) determine the role of VdTrx1 in the clearance of intracellular ROS generated by *V. dahliae* itself as well as extracellular host-derived ROS during infection; and (4) determine the role of VdTrx1 in pathogenicity of *V. dahliae*.

## 2. Materials and methods

### 2.1. Fungal culture and plant growth

*V. dahliae* wild-type Vd991 (Chen et al., 2018) originally isolated from cotton was grown on potato dextrose agar solid medium (PDA, potato, 200 g/l; glucose, 20 g/l; agar 15 g/l) or complete medium broth (CM, yeast extract, 6 g/l; casein acids

hydrolyzate, 6 g/l; sucrose, 10 g/l) at 25°C. *Agrobacterium tumefaciens* AGL-1 for ATMT transformation of *V. dahliae* was cultured in LB medium at 28°C, as previously described (Wang S. et al., 2016). *Escherichia coli* competent DH5 $\alpha$  was used to amplify recombinant plasmids. Cotton (*G. hirsutum* 'Junmian No. 1'), *N. benthamiana* (LAB), and *Arabidopsis thaliana* (Columbia-0) were grown in a greenhouse of 25°C and 70% relative humidity with a 14 h/10 h, day/night cycle.

## 2.2. Bioinformatics analysis

The DNA and cDNA sequences of *VdTrx1* were retrieved from the genome database of *V. dahliae* (Chen et al., 2018). Homologs of *VdTrx1* in different species were searched by BLASTp using the National Center for Biological Information (NCBI) database.<sup>1</sup> The gene structure and multiple sequence alignments were determined by the Clustal-W of Bioedit v7.2.0.<sup>2</sup> Predictions of the domains and functional sites of *VdTrx1* were conducted using the CD-Search of NCBI. SignalP5.0 was used to predict signal peptide (Almagro Armenteros et al., 2019). SecretomeP v.2.0 (Bendtsen et al., 2004) and OutCyte 1.0 (Zhao et al., 2019) were used for predictions of unconventional secretion of *VdTrx1*.

## 2.3. Fungal transformation

To construct the *VdTrx1* knockout mutant, the upstream and downstream fragments of *VdTrx1* were amplified from the Vd991 genome, respectively. The hygromycin phosphotransferase gene cassette, which was amplified from the pUC-*Hyg*, the upstream and downstream fragments of *VdTrx1* were ligated together by fusion PCR and integrated into the *EcoRI/HindIII* sites of binary vector pGKO2 (Khang et al., 2005). Positive gene deletion strains were selected on PDA with 200  $\mu$ g/ml cefotaxime, 50  $\mu$ g/ml hygromycin, and 200  $\mu$ g/ml 5-fluoro-2'-deoxyuridine. To obtain the complemented strain, a fragment containing 1 kb 5'-upstream of the *VdTrx1* coding region, the *VdTrx1* coding sequence, and 0.5 kb of the *VdTrx1* 3'-flanking region was amplified from the genomic DNA of Vd991 and ligated into the pCOM vector (Zhou et al., 2013). Complementated strains were selected on PDA with 200  $\mu$ g/ml cefotaxime and 50  $\mu$ g/ml Geneticin (G418). To create *VdTrx1* overexpression strains, the CDS sequence of *VdTrx1* was cloned from the cDNA of Vd991. The fragment was integrated into the *KpnI* site of the pCOM-GFP vector to construct the *VdTrx1*-GFP fusion protein (Tian et al., 2021b). The CDS region of *VdTrx1* linked with the HA tag was integrated into the *SacI/XbaI* sites of the pCOM-TrpC vector to construct the *VdTrx1*-HA fusion protein (Zhou et al., 2013). The recombinant plasmids were transformed into the wild-type strain Vd991, and the mutant strains  $\Delta$ *VdGRASP*,  $\Delta$ *VdATG1*, and  $\Delta$ *VdVps36*. Primers used for expression profiling are listed in Supplementary Table S1.

## 2.4. Vegetative growth and conidiation assays

To observe the vegetative growth phenotype of fungi, 2  $\mu$ l of the conidial suspension with a concentration of  $5 \times 10^6$  conidia/ml were cultured on PDA, CM and MM (6 g/l NaNO<sub>3</sub>, 0.52 g/l KCl, 0.52 g/l, MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.52 g/l KH<sub>2</sub>PO<sub>4</sub>, 10 g/l glucose, 0.001% (w/v) thiamine and 0.1% (w/v) trace elements) medium at 25°C for 12 days. For sulfur utilization analysis, inorganic sulfur sulfite (SO<sub>3</sub><sup>2-</sup>) and organic sulfur L(+)-Cysteine (Coolaber) were added to MM medium at concentrations of 2 mM and 1.4 mM, respectively and growth was analyzed.

To analyze the sporulation of *V. dahliae*, the fungus was grown on PDA plates for 9 days. Three 5-mm-diameter blocks of the fungus were cut from the edge of the colony, placed in 1 ml of sterile water, and turbine oscillated for 1 min. The numbers of conidia were counted using a hemocytometer.

## 2.5. Stress and penetration analysis

The different concentrations of H<sub>2</sub>O<sub>2</sub> (1.0 mM, 1.5 mM, and 2.0 mM) and *tert*-Butyl hydroperoxide (*t*-BOOH) (0.2 mM, 0.3 mM and 0.4 mM) (Sigma-Aldrich) in the CM solid medium were used to detect the sensitivity of WT, *VdTrx1* mutant, and *VdTrx1* complemented strains to oxidative stress. Otherwise, 1 M sorbitol (Solarbio) and 200  $\mu$ g/ml Congo red (Sigma-Aldrich) were added to the CM medium to assay osmotic stress and cell-wall integrity.

To measure the penetration ability of the wild type, the *VdTrx1* mutant, and the *VdTrx1* complemented strains, 5  $\mu$ l of the spore suspensions of each, with concentrations of  $5 \times 10^6$  conidia/ml, was cultured on MM plate covered with sterilized cellophane membrane and incubated at 25°C. The cellophane membranes were removed after 4 days. The plates were further cultured for 3 days at 25°C to observe the hyphae that had passed through the membrane forming colonies.

## 2.6. Yeast signal sequence trap system

Functional validation of the *N*-terminal peptide of *VdTrx1* was performed as previously described (Jacobs et al., 1997). The nucleotide sequence of *VdTrx1* encoding the *N*-terminal 25 amino acids and CDS regions was cloned into the pSUC2 vector, and the resulting plasmids, pSUC2-*VdTrx1*<sup>N25</sup> and pSUC2-*VdTrx1*, were transformed into the yeast strain YTK12. Transformants were screened on CMD-W (lacking tryptophan) medium for 2 days at 30°C. The positive transformants, the negative controls (YTK12 and YTK12::pSUC2), and the positive control (pSUC2-SP<sup>Avr1b</sup>) were incubated with 10 mM acetic acid–sodium acetate solution (pH=4.7) and 10% sucrose medium at 37°C for 10 min. The supernatant was collected and incubated with 0.1% 2,3,5-triphenyl tetrazolium chloride (TTC) for 10 min. Invertase enzymatic activity was confirmed by an increase in insoluble red-colored triphenyl formazan due to the reduction of TTC.

## 2.7. Protein extraction and western blot

The mycelium of *V. dahliae* (WT::VdTrx1-HA,  $\Delta$ *VdGRASP*::VdTrx1-HA,  $\Delta$ *VdVps36*::VdTrx1-HA, and  $\Delta$ *VdATG1*::VdTrx1-HA)

1 <https://www.ncbi.nlm.nih.gov/>

2 <http://www.mbio.ncsu.edu/bioedit/>

in CM medium was ground into powder and suspended in the extraction buffer (RIPA lysis buffer: 500  $\mu$ l; phenylmethylsulfonyl fluoride (PMSF), 1 mM). Centrifugation at 12,000 rpm for 10 min at 4°C yielded total protein in the supernatant. For the extraction of secreted proteins, strains were cultured in CM medium for 5 days and transferred to MM medium for 3 days to collect the supernatant. The secreted proteins were fractionated from the supernatant by phenol extraction and stored in 80% acetone (Wang et al., 2011). The samples were separated using 12% SDS-PAGE and then transferred to Immobilon-P transfer membranes (Merck Millipore). The membranes were incubated with anti-HA (Abmart, M20003, 1:5000) and anti- $\beta$ -actin (Abclonal, AC006, 1:2000), then goat anti-mouse IgG-HRP (TransGen Biotech, HS201-01, 1:5000) and goat anti-rabbit IgG-HRP (TransGen Biotech, HS101-01, 1:5000) as secondary antibody, respectively. Chemiluminescence was detected with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore).

## 2.8. Confocal fluorescence microscopy

The onion intraepidermal cells were soaked in the spore suspensions of the *V. dahliae* strains WT::VdTrx1-GFP,  $\Delta$ VdGRASP::VdTrx1-GFP,  $\Delta$ VdATG1::VdTrx1-GFP, and  $\Delta$ VdVps36::VdTrx1-GFP for 30 min, and the onion intraepidermal cells were placed on 1% (w/v) water agar for 5 days at 25°C. The WT::VdEG1-GFP strain-infected onion epidermal cells were used as the positive control, while the WT::GFP strain-infected onion epidermal cells were used as the negative control. Fluorescence of GFP fusion protein was observed by laser confocal microscope (ZEISS, LSM 880) at emission and reception wavelengths of 488 and 510 nm.

## 2.9. Reactive oxygen species staining

The 3,3'-diaminobenzidine (DAB) was used as the stain to detect the reactive oxygen species (ROS) in cotton during the infection of *V. dahliae*. Briefly, sterile cotton seeds were placed on wet filter paper, and the germinated cotton sprouts were soaked in the sterile water (CK) or the conidial suspensions of Vd991,  $\Delta$ VdTrx1, and EC strains at a concentration of  $1 \times 10^7$  conidia/ml for 30 min after 4 days. The radicles soaked in water or conidial suspensions were then incubated on wet filter paper for 2 days and 5 days, respectively. The radicles were placed in the DAB staining solution (0.1% wt/vol DAB; Aladdin) in the dark and decolorized with 95% ethanol for 20 min. Samples were subsequently examined under light microscopy. For the detection of intracellular ROS levels, the strains were cultured in CM medium for 4 days and stained with 50  $\mu$ M 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma) for 20 min. The mycelium was rinsed three times with  $1 \times$  phosphate-buffered saline (PBS) and observed by confocal microscopy (Leica, TCS SP8).

## 2.10. RT-qPCR

To analyze the transcription levels of *VdTrx1* in response to H<sub>2</sub>O<sub>2</sub>, the Vd991 strain was cultured in CM liquid medium for 4 days, and

switched to incubate in CM liquid medium containing 1 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 3 h. Total RNA was isolated from fungal mycelium by using a total RNA Miniprep kit (Aidlab). The cDNA was reverse transcribed using TransScript II One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech). The transcription levels of *VdTrx1* relative to the constitutively expressed elongation factor 1- $\alpha$  of *V. dahliae* were quantified using the 2<sup>- $\Delta\Delta$ Ct</sup> method (Livak and Schmittgen, 2001).

To analyze the temporal pattern of *VdTrx1* expression,  $1 \times 10^8$  conidia/ml of wild-type strain was used to inoculate cotton. The roots of cotton were collected at 0, 0.5, 1, 2, 3, 4, 5, and 6 dpi, respectively. Total RNA of roots was extracted, and the qVdTrx1-F/R were used as the primers to detect the expression of *VdTrx1* during infection by using the 2<sup>- $\Delta\Delta$ Ct</sup> method (Livak and Schmittgen, 2001). The amplification reaction was carried out using 2 $\times$  Taq Pro Universal SYBR qPCR Master Mix (Vazyme). The experiment method included pre-denaturation at 95°C for 10 min, followed by 40 cycles of 95°C denaturation for 15 s, 60°C annealing for 30 s, and 72°C extension for 30 s. Primers used for expression profiling are listed in Supplementary Table S1.

## 2.11. Pathogenicity assays

Spore suspensions of the wild-type, mutant, and complemented strains at a concentration of  $1 \times 10^7$  conidia/ml each, were prepared for pathogenicity tests. Three-week-old susceptible cotton seedlings (*G. hirsutum* 'Junmian No. 1'), *N. benthamiana* and *Arabidopsis thaliana* (Col-0) were inoculated as previously described methods (Zhang et al., 2019; Sun et al., 2021). Twenty cotton plants, five *N. benthamiana* plants and five *Arabidopsis thaliana* plants were inoculated with each strain. The symptoms of Verticillium wilt were photographed 3 weeks later. Then cotton stems were cut longitudinally to observe vascular bundle browning. The vascular bundle browning in stems of at least ten cotton plants was observed.

For analyses of fungal biomass by qPCR, root-stem junctions of cotton and *N. benthamiana*, and the whole *Arabidopsis thaliana* plants were collected for DNA extraction. The quantification of *V. dahliae* DNA was carried out using the *V. dahliae* elongation factor 1 $\alpha$  (*VdEF-1 $\alpha$* ) with the cotton 18S rRNA (*Gh18S*), *N. benthamiana EF-1 $\alpha$*  (*NbEF-1 $\alpha$* ), or *A. thaliana* ubiquitin-specific protease 1 (*AtUBQ1*) genes used in normalization using the 2<sup>- $\Delta\Delta$ Ct</sup> method. The amplification reaction was carried out using 2 $\times$  Taq Pro Universal SYBR qPCR Master Mix (Vazyme). The experiment method included pre-denaturation at 95°C for 10 min, followed by 40 cycles of 95°C denaturation for 15 s, 60°C annealing for 30 s, and 72°C extension for 30 s. The primers used are listed in Supplementary Table S1.

## 3. Results

### 3.1. *VdTrx1* encodes a thioredoxin lacking a signal peptide

Exoproteome analysis of *V. dahliae* Vd991 induced by a cotton-containing medium revealed a putative thioredoxin VdTrx1 (VEDA\_00080, Gene-ID in VdLs.17 genome: VDAG\_09916) in the

extracellular proteins of *V. dahliae* (Chen et al., 2016). The 557 bp full-length *VdTrx1* gene contains three exons of 23, 145 and 189 bp and two introns of 121 and 79 bp (Figure 1A). The *VdTrx1* ORF encodes a polypeptide of 118 amino acids with a predicted molecular weight of 12.89 kDa and an isoelectric point of 4.77. Searches for conserved domains within *VdTrx1* by CD-Search of NCBI identified a typical thioredoxin motif (pfam00085, from N-26 to L-113) (Figure 1B). The amino acid alignment of the partial conserved domain of *VdTrx1* with other phytopathogen Trx1 orthologs showed they all contain the thioredoxin-specific redox-active site WCGPC (Figure 1C). SignalP analysis showed with a high degree of probability (0.9987, Figure 1D) that the protein sequence of *VdTrx1* lacks an *N*-terminal signal peptide. However, *VdTrx1* was predicted to be a potential non-conventional secreted protein, as predicted by OutCyte 1.0 (with UPS score of 0.598, above the threshold of 0.5) (Figure 1E) and SecretomeP 2.0 (with SecP scores of 0.599, above the threshold of 0.5) (Figure 1F). Together, these results suggested that *VdTrx1* is likely a thioredoxin lacking a signal peptide, which is exported into the extracellular space unconventionally.

### 3.2. *VdTrx1* has thioredoxin activity and contributes to the oxidative stress tolerance

To study the biological function of *VdTrx1*, gene deletion mutants were constructed by replacing the coding sequence of *VdTrx1* in the wild-type strain Vd991 genome with a hygromycin resistance cassette *via* homologous recombination. Deletion of the *VdTrx1* ORF was confirmed by PCR in five candidate transformants and two of them were arbitrarily selected for further characterization (Supplementary Figure S1A). Complemented strains (EC-1/2) were generated by reintroducing *VdTrx1* with its native promoter and a neomycin resistance cassette into  $\Delta VdTrx1$ -1/2 (Supplementary Figure S1B), respectively. The radial growth of the  $\Delta VdTrx1$  strains on complete medium (CM) was similar to that of the wild-type strain (Figure 2A).

Considering that a well-established cellular function of the thioredoxin is to neutralize ROS and therefore contribute to oxidative stress resistance (Meyer et al., 2009), the hyphal growth under H<sub>2</sub>O<sub>2</sub> stress between the wild-type and  $\Delta VdTrx1$  strains was then compared. As shown in Figures 1A,B, growth of  $\Delta VdTrx1$  on CM supplemented with 1.5 and 2 mM H<sub>2</sub>O<sub>2</sub> was significantly inhibited, with colony diameters reduced by 10 and 26%, respectively, relative to those of the wild-type strain. Hyphal growth was halted at 2.5 mM H<sub>2</sub>O<sub>2</sub>, for the  $\Delta VdTrx1$  strain but not the wild-type strain (Figures 2A,B). Reintroduction of *VdTrx1* into the  $\Delta VdTrx1$  strain restored the H<sub>2</sub>O<sub>2</sub> resistance to the wild-type level (Figures 2A,B).

The relative expression level of *VdTrx1* under H<sub>2</sub>O<sub>2</sub> stress was assessed *via* reverse transcription quantitative PCR (RT-qPCR). After treatment for 3 h with 1 mM H<sub>2</sub>O<sub>2</sub>, expression of *VdTrx1* was significantly upregulated 5-fold in the wild-type strain (Figure 2C). Moreover, upon exposure to another ROS stress, *t*-BOOH, the  $\Delta VdTrx1$  strain also displayed increased sensitivity, but this sensitivity was reduced by complementation (Supplementary Figures S2A,B). Together, these results indicate that *VdTrx1* has thioredoxin activity and contributes to oxidative stress tolerance in *V. dahliae*.

### 3.3. *VdTrx1* can be secreted by *Verticillium dahliae*

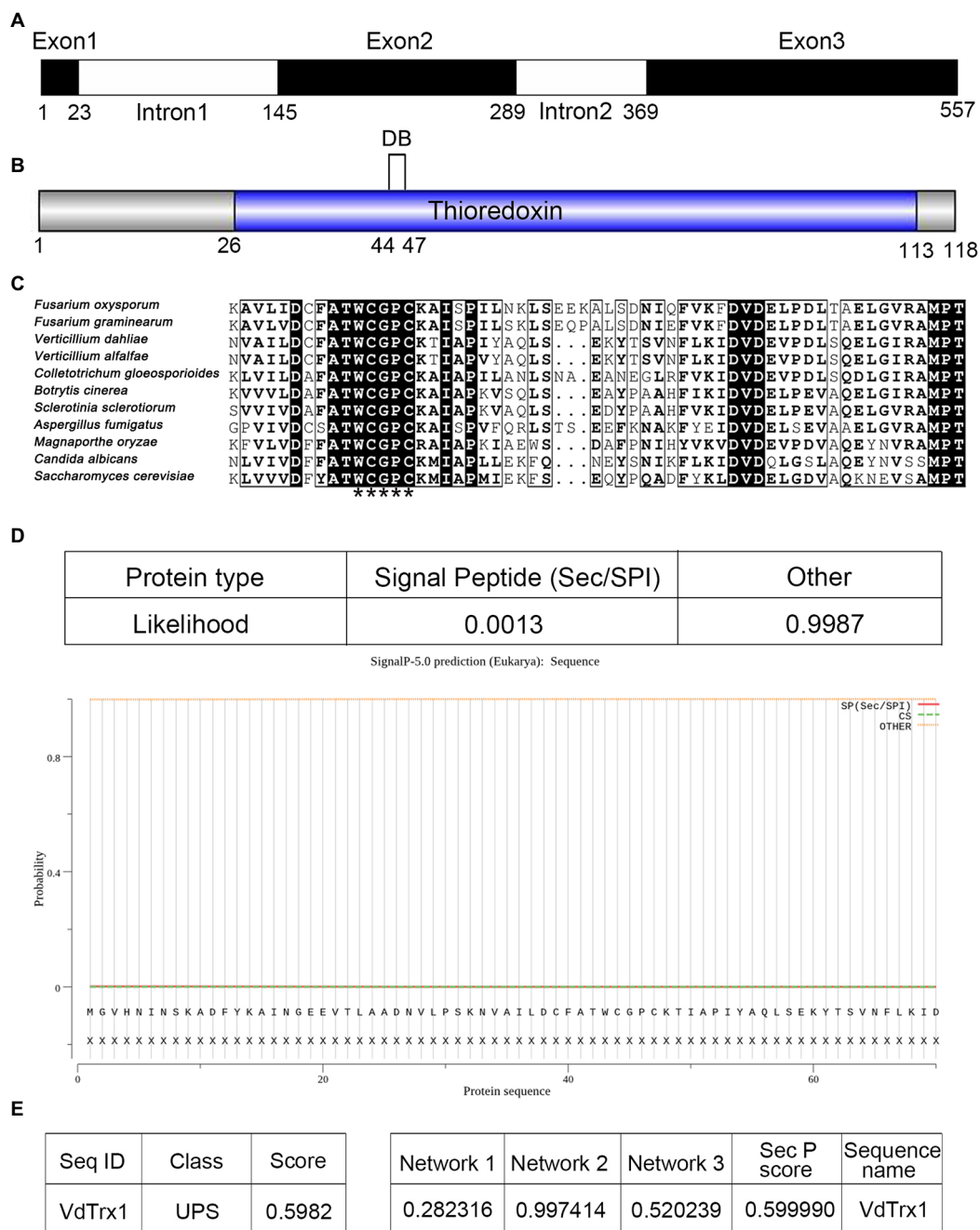
To verify the *N*-terminal of *VdTrx1* does not contain the functional signal peptide, as predicted by SignalP 5.0, we examined *VdTrx1* using a yeast signal sequence trap assay. The region encoding 25 amino acids of the *N*-terminus of *VdTrx1* was fused to the vector pSUC2, which carries an invertase gene lacking the region encoding a signal peptide, to generate pSUC2-*VdTrx1*<sup>N25</sup>. Invertase secretion was tested with a 2,3,5-triphenyl tetrazolium chloride (TTC) assay, in which secreted invertase catalyzes TTC in medium into the insoluble red compound. As expected, pSUC2-*VdTrx1*<sup>N25</sup> did not result in the catalysis of TTC in the medium (Figure 3A), indicating that the 25 aa *N*-terminal peptide of *VdTrx1* is not a functional signal peptide.

Since the bioinformatic analyses predicted *VdTrx1* to be an unconventionally secreted protein (Figure 1), we tested experimentally whether *VdTrx1* can be exported to the extracellular space. For this purpose, we first generated WT::*VdTrx1*-HA, a strain expressing a C-terminally HA-tagged *VdTrx1* in the wild-type background (Supplementary Figure S3). To examine whether *VdTrx1* is secreted, we performed western blotting of the culture supernatants of this *VdTrx1*-HA expressing strain using anti-HA antibody. Meanwhile, to eliminate the possibility that the *VdTrx1*-HA was detected in the culture supernatants due to cell lysis, intracellular cytoskeletal protein  $\beta$ -actin of *V. dahliae* was used as a control. As shown in Figure 3B, *VdTrx1*-HA was detected in both the mycelia and the culture supernatants of WT::*VdTrx1*-HA. In contrast, the  $\beta$ -actin protein was only clearly detected in mycelial protein extracts but not in culture supernatants under the same experimental conditions. These results therefore suggest that *VdTrx1* likely is secreted into culture medium *in vitro*.

To further confirm if *VdTrx1* is secreted *in vivo*, we examined the subcellular location of *VdTrx1* using an onion epidermal cell system. When the onion epidermal cells were inoculated with the WT::GFP strain expressing free GFP, green fluorescence was detected in the fungal hyphae or conidia, but not in the onion epidermal cells since GFP alone cannot be secreted into the extracellular space (Figure 3C). Signal-peptide containing VdEG1 is a known apoplastic elicitor secreted by *V. dahliae* during infection (Gui et al., 2017). In this experiment, VdEG1-GFP was observed around the plasma membrane in onion epidermal cells (Figure 3C). After incubation with the WT::*VdTrx1*-GFP strain expressing *VdTrx1*-GFP, the fluorescence was clearly detected both in the hyphal and the extracellular space around the onion epidermal cell plasma membrane (Figure 3C). Together, these results indicate that *VdTrx1* is not only an intracellular protein of *V. dahliae*, but also can be secreted extracellularly and translocated into the host apoplastic space.

### 3.4. Putative secretion mechanism of *VdTrx1*

We further investigated the molecular mechanism of *VdTrx1* secretion. In *S. cerevisiae*, secretion of superoxide dismutase (SOD1) lacking a signal peptide requires a novel membrane compartment called CUPS (compartment for unconventional protein secretion), which contains Golgi reassembly and stacking protein (GRASP), autophagy-related proteins, and proteins of the



**FIGURE 1** Gene cloning and bioinformatic analysis of VdTrx1 from *Verticillium dahliae*. **(A)** Structure of VdTrx1. Exons are black boxes and introns are white boxes. **(B)** Conserved domain of VdTrx1 predicted by CD-Search of NCBI. DB represent disulfide bond. **(C)** Alignment of conserved region of thioredoxins. Thioredoxin-specific redox-active sites are marked by asterisks. GenBank accession number of aligned sequences are *V. dahliae* (VEDA\_00080); *Verticillium alfalfae* (XP\_003005517.1); *Fusarium oxysporum* (EGU81922.1); *Fusarium graminearum* (ESU16261.1); *Magnaporthe oryzae* (EHA47211.1); *Botrytis cinerea* (EMR86794.1); *Aspergillus fumigatus* (XP\_753517.1); *Colletotrichum gloeosporioides* (EQB49288.1); *Candida albicans* (XP\_719372.1); *Saccharomyces cerevisiae* (YLR043C). **(D)** Signal peptide prediction of VdTrx1 using SignalP 5.0 program. OutCyte 1.0 **(E)** and SecretomeP 2.0 **(F)** were used to predict unconventional secretion characteristic of VdTrx1. UPS, unconventional protein secretions; Network 1, score of amino acid composition; Network 2, score of secondary structure prediction; Network 3, score of transmembrane helix prediction; SepP score, the average of three network scores.

endosomal sorting complex required for transport (ESCRT) machinery (Cruz-Garcia et al., 2017). Further, secretion of the *S. cerevisiae* thioredoxin (Trx1) lacking a signal-peptide also requires GRASP (Cruz-Garcia et al., 2020). Thus, we tested the involvement of VdGRASP in the secretion of VdTrx1. For this

purpose, we deleted the VdGRASP gene in *V. dahliae* (VdGRASP, VEDA\_09322, Gene-ID in VdLs.17 genome: VDAG\_03042) and introduced the construct VdTrx1-HA into the ΔVdGRASP strain, resulting in a strain overexpressing VdTrx1-HA in the VdGRASP deletion mutant. However, as in the wild-type strain, we observed

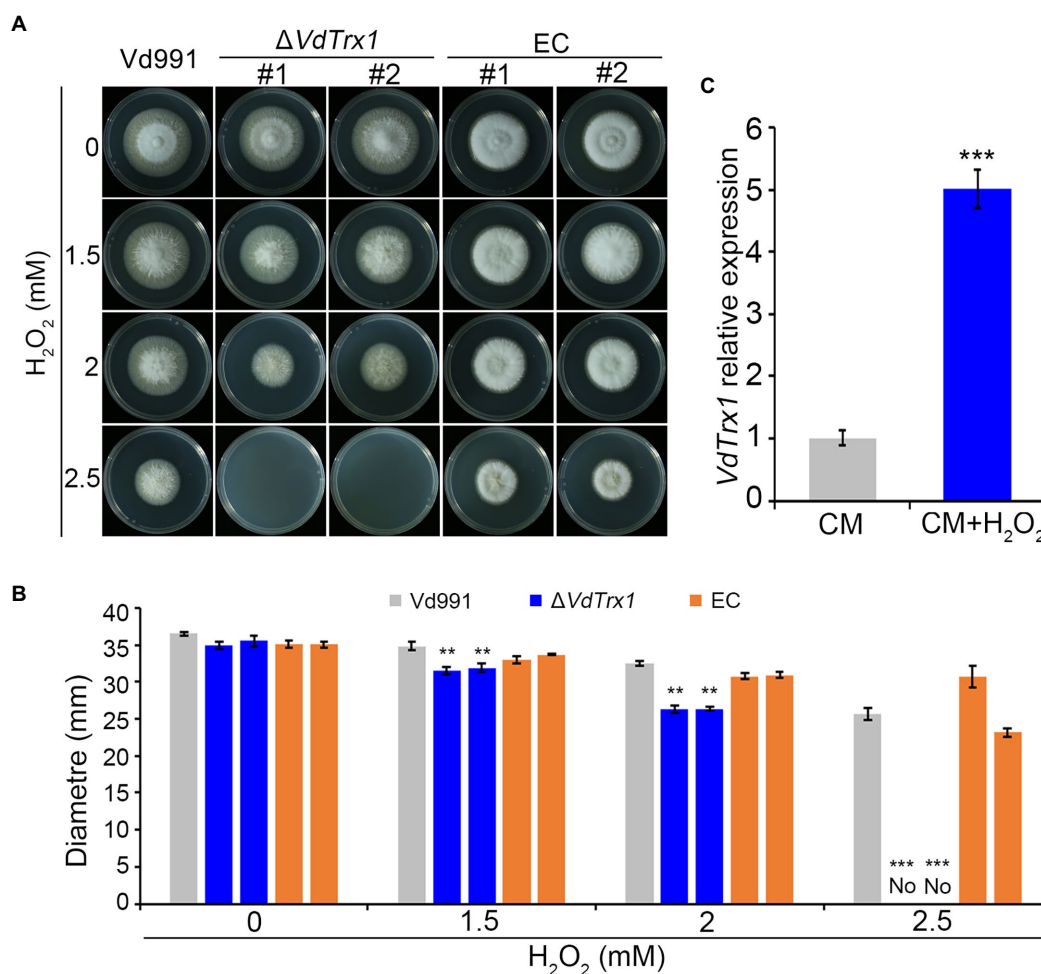


FIGURE 2

VdTrx1 of *V. dahliae* mediates the response to oxidative stress. (A) Radial growth of VdTrx1 deletion strains, complemented strains (EC#1 and #2) and wild-type strain (Vd991) on CM (complete medium) supplemented with H<sub>2</sub>O<sub>2</sub> at specified concentrations for 9 days. (B) Colony diameters of various *V. dahliae* strains on CM plates containing different concentration of H<sub>2</sub>O<sub>2</sub> following 9 days incubation. Means and standard deviations of the mean from three biological replicates are shown. Asterisks (\*\*) and (\*\*\*) denote significant differences  $p < 0.01$  and  $p < 0.001$ , respectively, according to Student's *t*-test. (C) Reverse transcription-quantitative PCR (RT-qPCR) of VdTrx1 transcripts in *V. dahliae* hyphae treated with 1.0 mM H<sub>2</sub>O<sub>2</sub> or incubated in CM only for 3 h. Total cDNA abundance in the samples was normalized to using VdEF-1 $\alpha$  gene as a control. In RT-qPCR, expression of VdTrx1 in the strain without H<sub>2</sub>O<sub>2</sub> treatment was set as 1. Bars indicate standard deviations from three biological replicates, \*\*\* denotes significant differences at  $p < 0.001$  (Student's *t*-test).

that VdTrx1 was also present in the supernatants derived from the  $\Delta$ VdGRASP::VdTrx1-HA strain (Figure 4A). Moreover, the onion cells inoculated with  $\Delta$ VdGRASP::VdTrx1-GFP, a strain expressing VdTrx1-GFP in  $\Delta$ VdGRASP background, also showed aggregation of fluorescence at the periphery of the onion epidermal cells, similar to that of WT::VdTrx1-GFP (Figure 4B). These results indicate that export of VdTrx1 is independent of VdGRASP.

We examined whether unconventional secretion of VdTrx1 is dependent on autophagy or ESCRT machinery. With a similar strategy, we deleted the VdATG1 (VdATG1, VEDA\_06789, Gene-ID in VdLs.17 genome: VDAG\_05745), which functions at the initial stage of autophagy, and VdVps36 (VdVps36, VEDA\_06789, Gene-ID in VdLs.17 genome: VDAG\_05745), the coding product of which is a member of ESCRT-II complex in *V. dahliae*. After expressing VdTrx1-HA in the VdATG1 and VdVps36 deletion background, we observed that the VdTrx1-HA

signal was absent in the supernatant of the VdVps36 mutant, but not in the supernatant of the VdATG1 mutant (Figure 4A). These results are consistent with our microscopic observations that after inoculating  $\Delta$ VdATG1::VdTrx1-GFP (a strain expressing VdTrx1-GFP in VdATG1 deletion background) and  $\Delta$ VdVps36::VdTrx1-GFP (a strain expressing VdTrx1-GFP in VdVps36 deletion background) on onion cells, GFP fluorescence around the onion epidermal cells disappeared in background of VdVps36 mutant rather than VdATG1 mutant (Figure 4B). Taking together, we conclude that the extracellular release of VdTrx1 is dependent on VdVps36 but not VdGRASP and VdATG1, and the secretion pathway of VdTrx1 in *V. dahliae* does not require the same set of components as Trx1 in *S. cerevisiae*. Moreover, the full-length coding sequence of VdTrx1 fused in-frame to invertase was not secreted from yeast, (Figure 3A), further confirming that the unconventional VdTrx1 secretion pathway in *V. dahliae* is not the same as in *S. cerevisiae*.



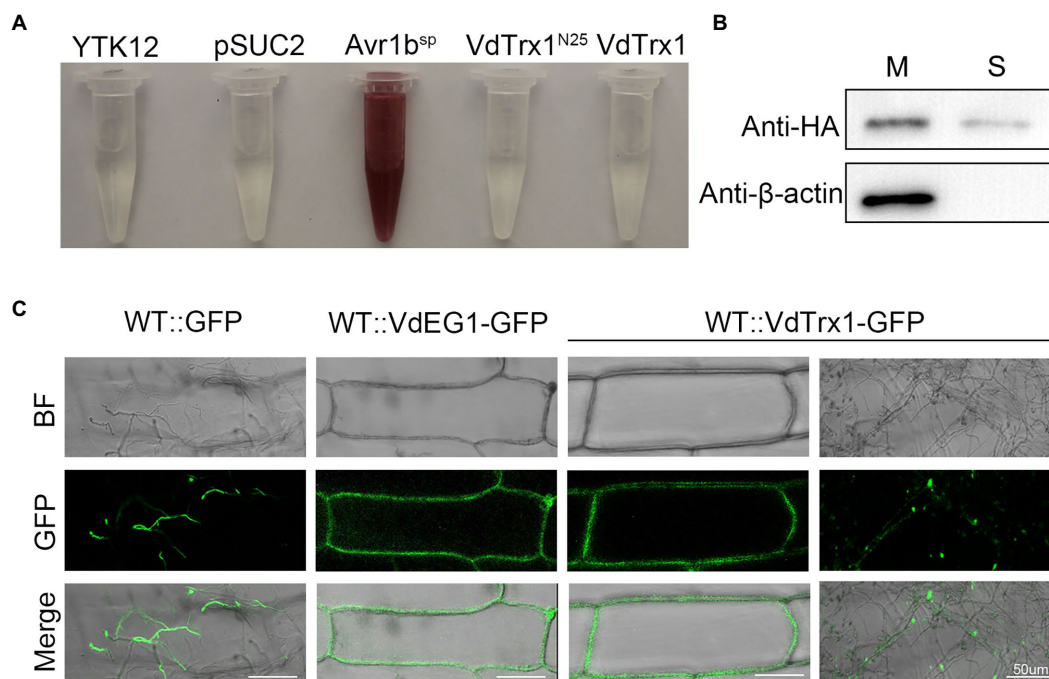


FIGURE 3

VdTrx1 can be secreted by *V. dahliae* to extracellular spaces. (A) Validation of the non-secretory function of the 25 aa *N*-terminal peptide and full-length sequence of VdTrx1 by a yeast signal trap assay. The region encoding the 25 aa *N*-terminal peptide and the full-length sequence of VdTrx1 were fused in-frame to the invertase sequence in the pSUC2 vector and transformed into yeast strain YTK12. The untransformed YTK12 strain and empty pSUC2 vector transformed into YTK12 were used as negative controls, while the pSUC2-Avr1b<sup>sp</sup> vector (integrating known functional signal peptide Avr1b<sup>sp</sup> into pSUC2) transformed YTK12 was used as a positive control. Only yeast strains that can secrete invertase converted 2,3,5- triphenyl tetrazolium chloride (TTC) to red triphenylformazan. (B) Western blotting assay demonstrates VdTrx1 secretion into culture filtrates. VdTrx1-HA was expressed in *V. dahliae* wild-type strain Vd991 to produce strain WT::VdTrx1-HA. Proteins extracted from mycelia (M) and culture supernatants (S) of strain WT::VdTrx1-HA were analyzed using western blotting with anti-HA or anti-β-actin antibodies, as indicated. (C) Live-cell imaging by confocal microscopy of VdTrx1. *V. dahliae* strains WT::VdTrx1-GFP, WT::GFP and WT::VdEG1-GFP were used to infect onion epidermal cells (the latter two strains were used as controls), respectively. Images were taken at 5 dpi using confocal laser scanning microscopy to perform a subcellular localization assay. Bar, 50μm.

### 3.5. VdTrx1 functions in neutralizing endogenous and exogenous ROS

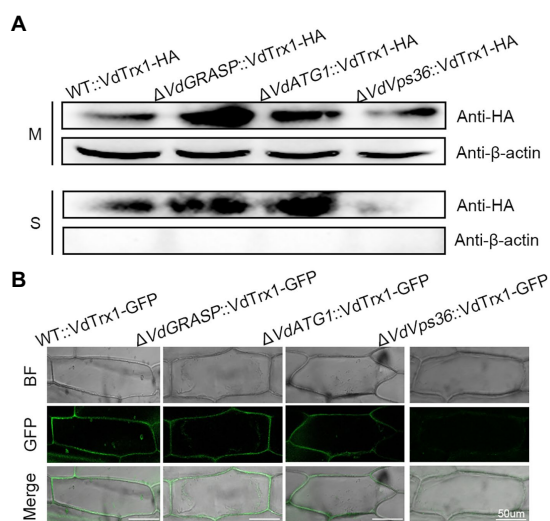
Since VdTrx1 has thioredoxin activity and is distributed both intracellularly and extracellularly, we investigated its role in scavenging endogenous and exogenous ROS. Intracellular levels of ROS were determined by DCFH-DA staining of *V. dahliae* mycelium after culturing in CM medium for 4 days. As shown in Figure 5A, the mycelia of the VdTrx1 deletion mutant displayed stronger bright green fluorescence compared to the mycelia of wild-type and complemented strains, indicating that the loss of VdTrx1 accompanied by increased accumulation of endogenous ROS and VdTrx1 functions in degrading intracellular ROS.

We further examined whether VdTrx1 could neutralize extracellular ROS generated by the host during infection. For this purpose, the change in ROS in cotton roots infected by *V. dahliae* strains was detected by DAB (3,3'-diaminobenzidine) staining, which produces a reddish-brown stain when oxidized by H<sub>2</sub>O<sub>2</sub>. In the relative early stages of infection (2 dpi), cotton roots inoculated by all strains displayed similar phenotypes, showing very few reddish-brown spots following DAB staining (Figure 5B). Potentially, few *V. dahliae* hyphae entered the root tissue at 2 dpi. In contrast, after 5 dpi, when the

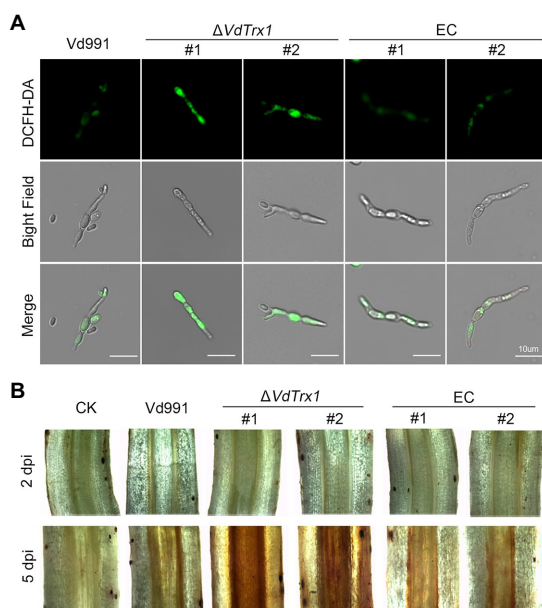
invading hyphae entered the xylem vessels and induced host-derived ROS generation (Tian et al., 2021b; Tian and Kong, 2022), cotton roots inoculated with the ΔVdTrx1 strain showed increased accumulation of H<sub>2</sub>O<sub>2</sub> in comparison with that of the wild-type strain, but decreased accumulation of H<sub>2</sub>O<sub>2</sub> was restored in the complemented strains (Figure 5B). Together, these results indicated that VdTrx1 functions in apoplast to neutralize host-derived ROS and plays roles in scavenging both endogenous and exogenous ROS produced in mycelia and in host defense, respectively.

### 3.6. VdTrx1 is essential for sulfite assimilation and conidiation in *Verticillium dahliae*

The VdTrx1 deletion mutants showed slightly reduced radial growth on potato dextrose agar (PDA) medium when compared with the wild-type strain and complemented transformants. However, the ΔVdTrx1 strain displayed no obvious mycelial growth defect when cultured in nutrient-rich CM medium (Figures 6A,B). To investigate the function of VdTrx1 in the response to cell-wall and hyperosmotic stress, the relevant *V. dahliae* strains were grown on CM supplemented with 200 μg/ml Congo red and 1 M sorbitol, respectively. As shown in



**FIGURE 4**  
Secretion mechanism of VdTrx1 from *V. dahliae*. **(A)** Proteins extracted from mycelia (M) and culture supernatants (S) of strain WT::VdTrx1-HA,  $\Delta$ VdGRASP::VdTrx1-HA,  $\Delta$ VdATG1::VdTrx1-HA and  $\Delta$ VdVps36::VdTrx1-HA were analyzed using western blotting with anti-HA or anti- $\beta$ -actin antibodies, as indicated. **(B)** The *V. dahliae* strains WT::VdTrx1-GFP,  $\Delta$ VdGRASP::VdTrx1-GFP,  $\Delta$ VdATG1::VdTrx1-GFP and  $\Delta$ VdVps36::VdTrx1-GFP were used to inoculate onion epidermal cells, respectively, at room temperature for 5 days followed by confocal microscopy imaging. Bar, 50  $\mu$ m.



**FIGURE 5**  
The role of VdTrx1 from *V. dahliae* in the degradation of ROS. **(A)** Hyphae from wild-type strain Vd991, VdTrx1 deletion strains, and the complemented strains were grown in liquid complete medium for 4 days. The fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFH-DA) was used to visualize the production of H<sub>2</sub>O<sub>2</sub>. Bar, 10  $\mu$ m. **(B)** Accumulation of H<sub>2</sub>O<sub>2</sub> in infected cotton roots. Detection of H<sub>2</sub>O<sub>2</sub> accumulation in cotton roots inoculated with sterile water, the wild-type strain Vd991,  $\Delta$ VdTrx1 and complemented strains at 2 days and 5 days post-inoculation, respectively, by 3,3'-diaminobenzidine staining.

**Figures 6A,B**, sensitivity of the  $\Delta$ VdTrx1 strain to the cell-wall and hyperosmotic stresses was comparable to that observed in wild-type strain Vd991, suggesting VdTrx1 is not essential for the response to cell-wall and hyperosmotic stress in *V. dahliae*.

Thioredoxin is an efficient reductant for 3'-phosphoadenosine 5'-phosphosulfate (PAPS) reductase, the enzyme involved in the reduction process of SO<sub>4</sub><sup>2-</sup> to SO<sub>3</sub><sup>2-</sup> (Berendt et al., 1995), which is a key step in sulfate assimilation. To investigate whether VdTrx1 participates in the sulfate assimilation process, we tested the growth rate of  $\Delta$ VdTrx1 in MM medium, which only contains SO<sub>4</sub><sup>2-</sup>. Radial growth of  $\Delta$ VdTrx1 was reduced by 20% relative to the wild-type strains and aerial hyphae production was severely impaired due to loss of VdTrx1 (**Figures 6A,B**). Further, supplementation of exogenous reduced sulfur (Na<sub>2</sub>SO<sub>3</sub>) and cysteine into MM medium could compensate for the defect seen in  $\Delta$ VdTrx1 (**Figure 6C**), suggesting that VdTrx1 is involved in sulfite assimilation in *V. dahliae*.

The effect of VdTrx1 disruption on conidia production was also studied, revealing a major role of VdTrx1 in conidiation. The  $\Delta$ VdTrx1 strain produced 81–85% fewer conidia than the wild-type strain. Although the complemented strain produced more conidia than  $\Delta$ VdTrx1, it did not reach the level of wild-type strain (**Figure 6D**). Together, these results indicated that VdTrx1 is not involved in cell-wall and hyperosmotic stress tolerance but is critical for sulfite assimilation and conidiation in *V. dahliae*.

### 3.7. VdTrx1 is required for virulence in *Verticillium dahliae*

To examine a possible contribution of VdTrx1 to the virulence of *V. dahliae*, VdTrx1 transcript levels were quantified by RT-qPCR *in planta* during infection of cotton roots. The results showed that the expression of VdTrx1 was upregulated after inoculation, reaching the maximum at 4 dpi, and suggesting that VdTrx1 responds to plant inoculation and may play an important role in pathogenicity (**Supplementary Figure S4A**). To investigate whether VdTrx1 plays a role during the initial colonization of *V. dahliae*, penetration ability was examined by incubation of all the transformants on cellophane membranes which were overlaid on the minimal medium (MM). Although  $\Delta$ VdTrx1 could not form normal aerial hyphae on this medium, they were capable of penetrating the cellophane membrane at 4 dpi (**Supplementary Figure S4B**), indicating that loss of VdTrx1 does not affect the penetration ability of *V. dahliae*.

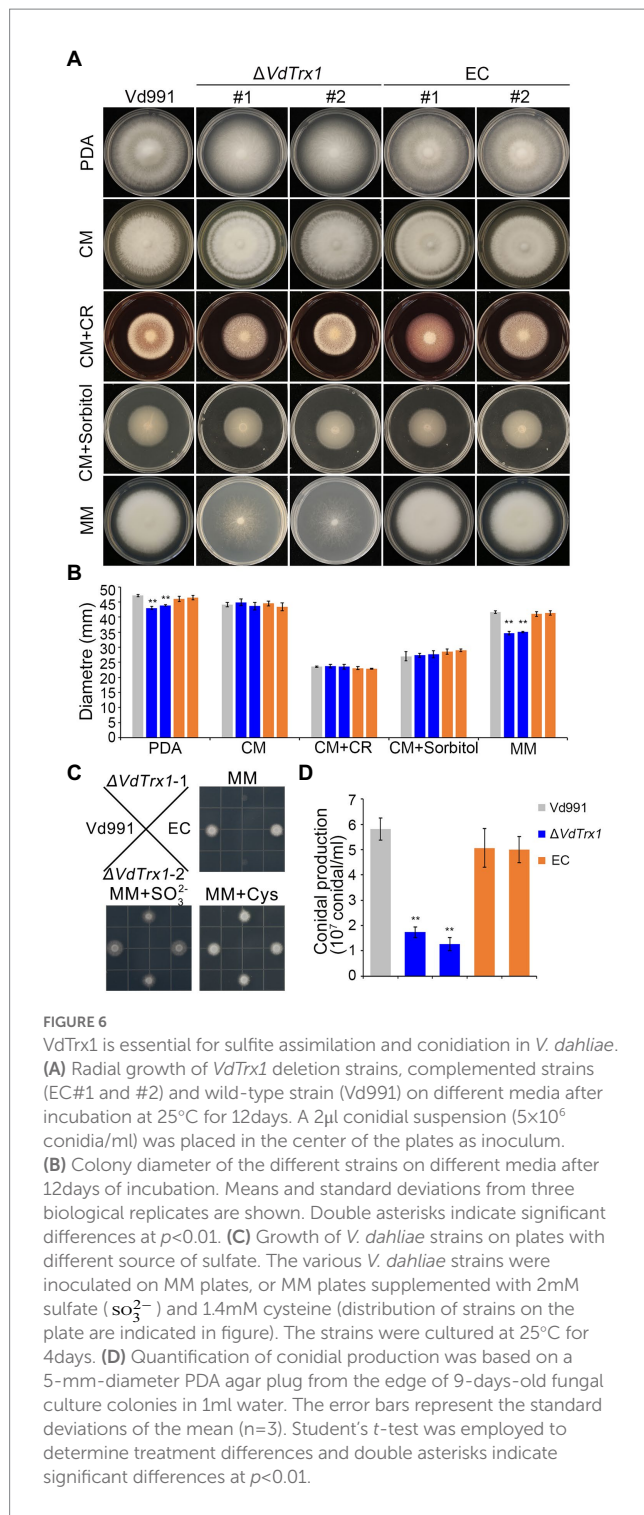
Further, the natural cotton host (*Gossypium hirsutum*) and the model plant species (*Arabidopsis thaliana* and *Nicotiana benthamiana*) were used to test the pathogenicity of the  $\Delta$ VdTrx1 strain and other *V. dahliae* strains. As expected, cotton inoculated with the wild-type strain showed typical disease symptoms, including wilting of leaves and vascular discoloration. In contrast, in cotton plants that were infected with the  $\Delta$ VdTrx1 strain, the disease severity was reduced, accompanied with a significant decrease in fungal biomass *in planta* of 61% compared with the biomass in the plants infected with the wild-type strain (**Figure 7A**). The virulence and fungal biomass of the complemented strains were comparable to those of the wild-type strain (**Figure 7B**). Like cotton, targeted deletion of VdTrx1 resulted in markedly compromised virulence on

## 4. Discussion

Although thioredoxins lacking a signal peptide have been considered as unconventional secreted proteins in mammalian cells, there has not been similar investigations on the secretory characteristics of thioredoxin in plant pathogenic fungi. In this study, we characterized the unconventionally secreted thioredoxin VdTrx1 and investigated its role in *V. dahliae*-plant interactions using a combination of approaches. The virulence function of VdTrx1 was demonstrated on three different hosts.

Secreted proteins typically carry an *N*-terminal signal peptide in eukaryotic cells and are transported through the conventional ER-Golgi secretion pathway. The majority of identified extracellular proteins in eukaryotes are secreted following this paradigm. However, proteomics analyses have revealed that a high percentage of proteins lacking signal peptides in the secretomes of fungi, including fungal phytopathogens (Rampitsch et al., 2013; González-Fernández et al., 2015). Recently, several studies have revealed the functions of unconventionally secreted proteins involved in plant-microbe interactions. For example, though the *Phytophthora sojae* isochorismatase PsIsc1 lacks a signal peptide, it can be secreted and translocated into soybean cells to suppress salicylate-mediated innate immunity *in planta* by hydrolyzing isochorismate, a precursor of salicylate (Liu et al., 2014). *Magnaporthe oryzae* oxysterol-binding protein-related proteins (MoORPs), normally described as intracellular proteins, are detected in intercellular fluids from barley plants infected with the fungus (Chen et al., 2022). MoORPs act as PAMP molecules to modulate plant innate immunity and promote virulence in *M. oryzae* (Chen et al., 2022). Two independent proteomics analysis also revealed that there are 56 and 99 potential proteins lacking signal peptides in the exoproteome of *V. dahliae* strains Vd07038 and Vd991, respectively, implying that unconventionally secreted proteins also play important roles during host-*V. dahliae* interactions (Chu et al., 2015; Chen et al., 2016). As an analogous analysis of *Phytophthora sojae* PsIsc1, the effector VdIsc1 in *V. dahliae* lacks a signal peptide and also plays a role in inhibiting salicylic acid-mediated plant immune response (Liu et al., 2014). Our previous study also found superoxide dismutase VdSOD1 lacking a signal peptide can be dispatched from *V. dahliae* cells and transferred into host apoplastic region to detoxify host-generated ROS in the form of  $O_2^-$  (Tian et al., 2021b). In this study, the presence of VdTrx1 in the culture filtrate was detected by western blot *in vitro*, and its apoplastic localization *in planta* was also observed by live-cell imaging, providing concrete evidence that VdTrx1 is an unconventionally secreted protein (Figure 3).

The removal of deleterious oxidants is a conserved function of thioredoxin (Koháryová and Kolárová, 2008). Not surprisingly, VdTrx1 functions in scavenging extracellular  $H_2O_2$ , further reducing the adverse effects of plant-derived ROS on pathogen (Figure 5B). Following the VdSOD1 scenario, the current research provided another example of an unconventionally secreted protein with a ROS-detoxifying function. The advantage of unconventional secretion of antioxidants may be that the oxidative environment in the lumen of the ER may result in the inactivation of sets of enzymes. In addition, cell starvation could lead to conventional protein secretion inhibition, while there is a net increase in the secretion of proteins lacking the signal peptide through an unconventional secretion pathway



both *Arabidopsis thaliana* plants and *N. benthamiana*, which were confirmed not only by observations of disease symptoms after plants were inoculated with the various fungal genotypes but also by measurements of fungal biomass *in planta* by quantitative PCR. All pathogenic defects of  $\Delta$ VdTrx1 were restored by complementation of VdTrx1 (Figures 7C–F). Overall, VdTrx1 is required for virulence not only on the natural host of cotton from which the original wild-type isolate used in this study was derived, but also on two other host plant species.

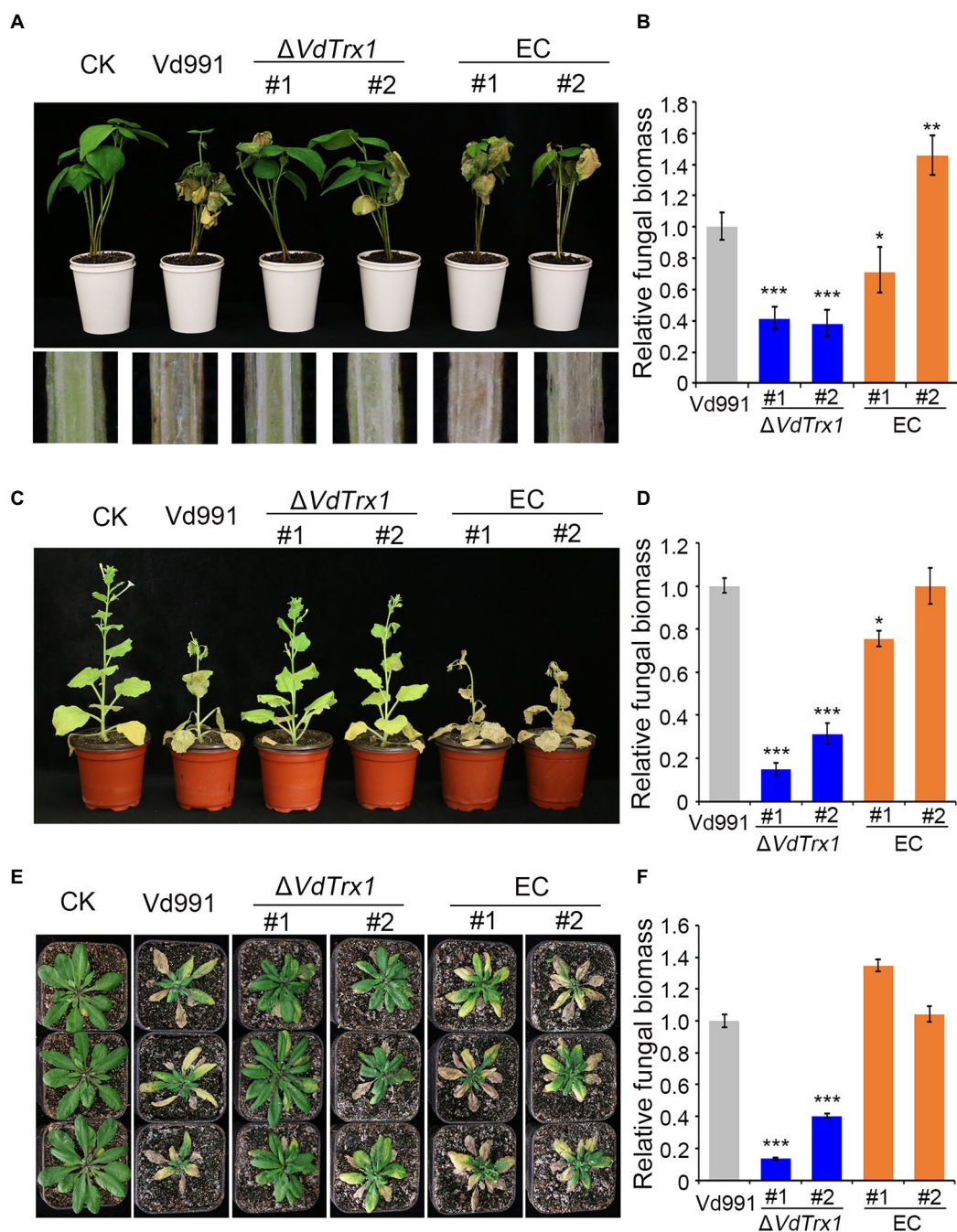


FIGURE 7

*VdTrx1* is required for the full virulence of *V. dahliae*. (A) The disease symptoms of cotton seedlings inoculated with sterile water (CK) or the *VdTrx1* deletion strains, complemented strains (EC#1 and #2) and wild-type strain (Vd991) at 21 days post-inoculation (Top). The discoloration of the inoculation shoot longitudinal sections is shown at the bottom. Phenotypes of *N. benthamiana* (C) and *A. thaliana* (E) plants inoculated with indicated strains. The fungal biomasses of each fungal strain in cotton (B), *N. benthamiana* (D) and *A. thaliana* (F) plants were determined by qPCR. Error bars represent standard deviations ( $n=3$ ). Asterisks \*\*\* indicate significant differences at ( $p<0.001$ ) based on the Student's *t*-test.

(Cruz-Garcia et al., 2020). *V. dahliae* colonizes the vascular bundles of plants, which is a nutrition-poor niche (Klosterman et al., 2011). Thus, conventional ER-Golgi secretion systems may be limited during infection and unconventional secretion of pathogens becomes a necessary complementary pathway to dispatch antioxidants for ROS-detoxification in vascular tissue. The findings indicate that searches for extracellular proteins in fungal plant pathogens, which

may be pathogenicity factors, should not be restricted to those bearing signal peptides.

At present, we are at the beginning stage of understanding the molecular mechanisms of secretion pathways for cargo proteins lacking signal peptides. The TMED10 of mice cells acts as a protein channel, which directly facilitates the translocation of proteins lacking a signal peptide into the ER-Golgi intermediate compartment

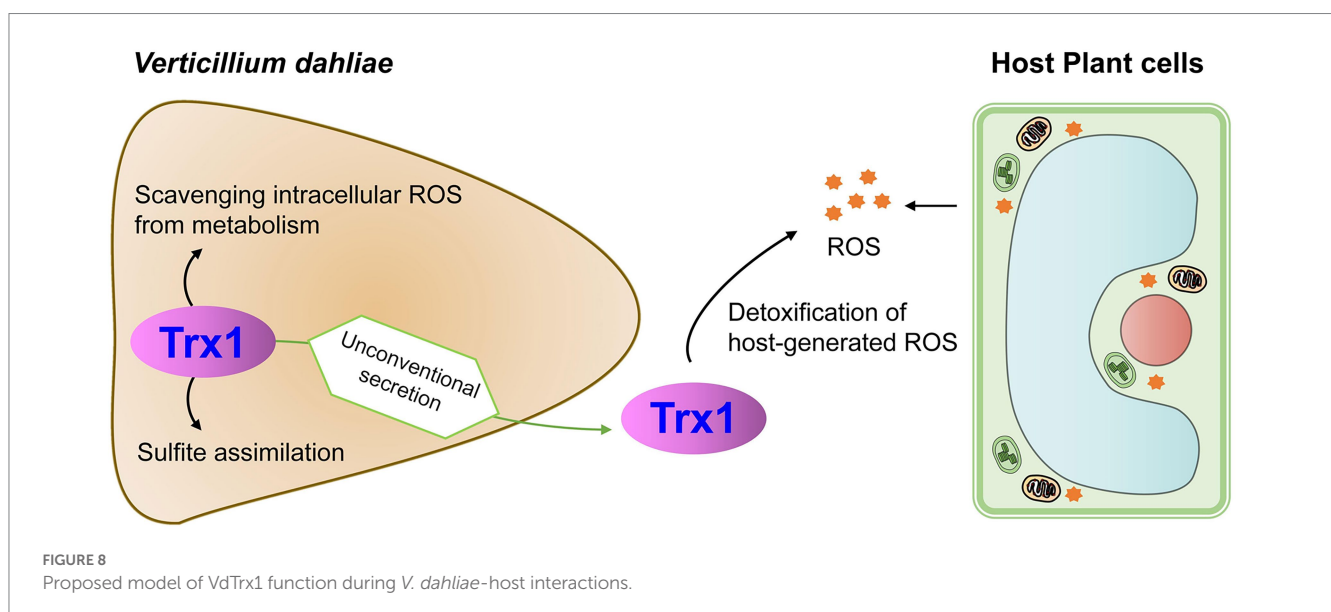
(ERGIC), and where the leaderless cargo enters other unknown vesicle carriers to be delivered out of the cell (Zhang et al., 2020). In *S. cerevisiae*, acyl-coenzyme A binding protein Acb1 and SOD1 lacking signal peptides are both captured into a cup-shape like membrane compartment called CUPS, which serves as a sorting station prior to their release into the extracellular space (Cruz-Garcia et al., 2017). Our previous research had shown that the secretion of VdSOD1 of *V. dahliae* requires the participation of VdGRASP, whose orthologous protein plays important roles in the formation and maturation of CUPS in *S. cerevisiae* (Tian et al., 2021b). However, the current study indicated the extracellular release of VdTrx1 is not in a VdGRASP dependent manner, but relies on VdVps36, which is a component of the ESCRT-II complex (Figures 4A,B). Thus, *V. dahliae* likely employs diverse unconventional secretory routes to translocate virulence-related proteins that lack signal peptides out of the cell.

Thioredoxins are ubiquitous small proteins with a redox-active site, which have been conserved throughout evolution in eukaryotes. As hydrogen donors, thioredoxin systems regulate many substrates including peroxiredoxin, an enzyme involved in the reduction of  $H_2O_2$ ; ribonucleotide reductase, required for dNTP synthesis; and PAPS reductase, which contributes to sulfate assimilation (Meyer et al., 2009). In this study, we found that  $\Delta VdTrx1$  showed a reduced radial growth rate accompanied by impaired aerial hyphae in the medium absence of  $SO_3^{2-}$ , indicating that VdTrx1 contributes to sulfite assimilation in *V. dahliae*, consistent with previous results obtained from analyses of other fungal phytopathogens such as *M. oryzae* (Wang et al., 2017) and *Podospora anserina* (Malagnac et al., 2007).

There is growing evidence that thioredoxin directly detoxifies ROS or acts as a regulator of ROS-induced signal transduction pathways. For instance, when the function of *S. sclerotiorum* SsTrx1 is inhibited by gene silencing, higher ROS accumulation was observed after inoculation of *A. thaliana*, and the direct role of *S. sclerotiorum* SsTrx1 in ROS detoxification was demonstrated, providing protection of the pathogen against oxidative stress (Rana et al., 2021). In the human fungal pathogen *C. albicans*, Trx1 regulates three distinct  $H_2O_2$  signaling proteins including protein kinases (Hog1 and Rad53) and the

transcription factor Cap1 to promote *C. albicans* survival in the host (da Silva Dantas et al., 2010). The thioredoxin protein of *M. oryzae* has two of the above-mentioned functions. On the one hand, MoTRX2 is important for the scavenging of ROS in the plant-fungus interaction (Wang et al., 2017); on the other hand, by directly interacting with kinase MoMst7, MoTRX2 regulates the activation of MAPK pathway in the pathogen that regulates its invasive growth and its ability to infect the plant (Zhang et al., 2016). In *V. dahliae*, members of MAPK pathway (VdHog1, VdPbs2 and VdSsk2) are involved in regulating the hyperosmotic stress response and cell-wall integrity (Tian et al., 2016; Wang Y.L. et al., 2016; Yu et al., 2019). In our current research, we showed that *V. dahliae* VdTrx1 is not essential for the response to cell-wall and hyperosmotic stress (Figure 6A). Instead, VdTrx1 functions in scavenging ROS both endogenously and exogenously (Figures 5A,B), suggesting the role of VdTrx1 in promoting pathogenesis may be dependent on its antioxidant functions rather than regulating MAPK signaling. Whether VdTrx1 is involved in the dNTP synthesis in *V. dahliae* requires further investigation.

Thioredoxins are important virulence factors in many pathogenic fungi, including the human pathogen *C. albicans* (da Silva Dantas et al., 2010) and phytopathogenic fungi, such as *M. oryzae* (Wang et al., 2017) and *S. sclerotiorum* (Rana et al., 2021). In our study, we found that *VdTrx1* was clearly induced during infection, suggesting VdTrx1 may play roles in host-pathogen interactions and in the pathogenicity of *V. dahliae* (Supplementary Figure S4A). Deletion of *VdTrx1* alleviated disease symptoms and reduced fungal biomass in three different hosts, suggesting that VdTrx1 was required for the virulence of *V. dahliae* (Figures 7A–C). Penetration assays showed that loss of *VdTrx1* did not damage the ability of the mutant strain to penetrate cellophane membrane, suggesting  $\Delta VdTrx1$  maintained the ability to infect and initially colonize host tissue (Supplementary Figure S4B). The reduced virulence of *VdTrx1* deletion mutant was likely due to its decreased viability in host vascular tissue (Rouached et al., 2009). First, loss-of-function of *VdTrx1* reduces the pathogen to counter the toxicity of host-derived ROS (Figure 5B). Secondly,  $SO_4^{2-}$ , absorbed



by plant root from the soil, is the main form of sulfur in plant vascular tissue. Loss of VdTrx1, which is involved in the reduction process of  $\text{SO}_4^{2-}$ , may render the  $\Delta\text{VdTrx1}$  strain unable to utilize sulfur effectively for normal growth (Figures 6A,B). Thirdly, according to studies of the invasion progress of *V. dahliae* on host plants, after successful epidermal invasion, conidia are formed in the xylem vessels to promote systemic propagation vertically (Tian and Kong, 2022). The decrease of sporulation ability of  $\Delta\text{VdTrx1}$  may slow down this invasive proliferation process (Figure 6D).

In conclusion, signal-peptide-lacking VdTrx1 is an unconventionally secreted thioredoxin with biological functions both inside and outside of the cells. Its presence in fungal tissue involves in scavenging intracellular ROS and sulfite assimilation. VdTrx1 can also be secreted unconventionally depending on VdVps36, detoxifying host-generated ROS during pathogen-host interactions (Figure 8). VdTrx1 is necessary for full virulence of *V. dahliae* on susceptible hosts.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repository and accession number(s) can be found in the article/Supplementary material.

## Author contributions

J-YC and LT conceived and designed the experiments. JZ, LT, J-JL, and HZ performed the experiments. D-DZ analyzed the data. LT, D-DZ, and JZ wrote the initial draft. J-YC, KS, SK, and X-FD wrote, reviewed, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2023.1130468/full#supplementary-material>

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