

Communication

Unconventionally Secreted Manganese Superoxide Dismutase VdSOD3 Is Required for the Virulence of *Verticillium dahliae*

Li Tian ¹ , Weixia Sun ¹, Junjiao Li ², Jieyin Chen ² , Xiaofeng Dai ², Nianwei Qiu ^{1,*} and Dandan Zhang ^{2,*}

¹ School of Life Science, Qufu Normal University, Qufu 273165, China; biotian@qfnu.edu.cn (L.T.); sunwxster@163.com (W.S.)

² Team of Crop Verticillium Wilt, c/o State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China; protectivecolor@yeah.net (J.L.); chenjieyin@caas.cn (J.C.); daixiaofeng@caas.cn (X.D.)

* Correspondence: nianweiqiu@qfnu.edu.cn (N.Q.); zhangdandan@caas.cn (D.Z.)

Abstract: Plant pathogens generally employ superoxide dismutase (SOD) to detoxify host defense reactive oxygen species (ROS), and to scavenge ROS derived from their own metabolism. However, the roles of SODs in an important vascular pathogen, *Verticillium dahliae*, are unclear. Our previous study has shown that a putative signal-peptide-lacking manganese superoxide dismutase (VdSOD3) is present in the exoproteome of *V. dahliae* cultured in tissues of host cotton, suggesting that VdSOD3 may be exported out of the fungal cells and contribute to the SOD activity extracellularly. Here, we confirm that the *N*-terminal of VdSOD3 is not a functional signal peptide by yeast signal trap assay. Despite lacking the signal peptide, the extracellular distribution of VdSOD3 was observed in planta by confocal microscopy during infection. Loss-of-function of *VdSOD3* decreased extracellular and intracellular SOD activities of *V. dahliae* by 58.2% and 17.4%, respectively. Deletion mutant of *VdSOD3* had normal growth and conidiation but showed significantly reduced virulence to susceptible hosts of cotton and *Nicotiana benthamiana*. Our data show that signal-peptide-lacking VdSOD3 is a dual function superoxide dismutase, localizing and functioning intracellularly and extracellularly. Whereas nonessential for viability, VdSOD3 plays a vital role in the virulence of *V. dahliae*.

Keywords: *Verticillium dahliae*; superoxide dismutase; unconventional secretion; virulence



Citation: Tian, L.; Sun, W.; Li, J.; Chen, J.; Dai, X.; Qiu, N.; Zhang, D. Unconventionally Secreted Manganese Superoxide Dismutase VdSOD3 Is Required for the Virulence of *Verticillium dahliae*. *Agronomy* **2021**, *11*, 13. <https://dx.doi.org/10.3390/agronomy11010013>

Received: 23 November 2020

Accepted: 21 December 2020

Published: 23 December 2020

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

In the course of infection, besides being challenged by reactive oxygen species (ROS) produced intracellularly as a natural byproduct of pathogen respiration, plant pathogens are additionally confronted with extracellular ROS generated by the host innate immune response [1]. These ROS have the oxidation capacity to damage virtually all organic and many inorganic components of the pathogen, leading to cellular dysfunction and eventually pathogen death [2]. Superoxide dismutases (SODs), the first-line enzymes against superoxide damage [3], are highly conserved metalloenzymes that use a redox-active cofactored metal to catalyze the conversion of superoxide anion to oxygen and hydrogen peroxide; the latter is further removed by catalase and peroxidase enzymes [4].

Manganese superoxide dismutase (Mn-SOD) is a SOD family member, which harbors Mn²⁺ as the metal cofactor [5]. Significant progress has been achieved in the functional analysis of Mn-SODs of specific pathogens. Mn-SODs contribute to pathogens' virulence such as *Cryptococcus neoformans*, *Metarhizium robertsii*, *Candida glabrata* and *Beauveria bassiana* [6]. However, other reports demonstrate a nonessential role of Mn-SOD in the virulence of pathogens, such as *Aspergillus fumigatus* and *Colletotrichum graminicola* [6]. These studies demonstrate that Mn-SODs differentially contribute to virulence on plant hosts.

The currently available Mn-SODs are generally considered intracellular enzymes lacking *N*-terminal signal peptides [7]. However, clear evidence shows that bacterial Mn-

SOD (termed SodA) is localized in the extracellular spaces. SodA has been reported to be exported to the periplasm or the culture supernatants of various bacteria [8–10]. In addition, the Mn-SOD activity can be detected from the supernatant of bacterial cultures [11]. Thus, Mn-SOD has been thought to be an unconventionally secreted protein in bacteria for a long time [12]. Fungi contain a Mn-SOD with a predicted mitochondrial targeting signal, typically called SOD2. In addition, a certain class of fungi also harbor a cytosolic Mn-SOD without this targeting signal, which is termed SOD3 [5]. Recently, increasing evidence suggests that fungal cytosolic SOD3 may also be exported extracellularly. For instance, SOD3 of certain fungi is recognized specifically by the host and is considered as a fungal allergen, most of which are extracellular proteins [13,14]. Moreover, proteomics analysis demonstrates that SOD3 is present in many fungal secretomes, such as *Aspergillus fumigatus* and *Fusarium graminearum* [15]. However, there is no concrete experimental evidence for the unconventional secretion of fungal SOD3.

Verticillium dahliae Kleb. is a destructive phytopathogenic fungus that attacks a wide range of hosts and targets the xylem tissue, causing Verticillium wilt disease on many economically important crops, such as cotton, potato and lettuce [16]. During the battle between *V. dahliae* and hosts, ROS are accumulated at the infected site, suggesting ROS are also involved in the fungus–plant interaction [17]. Recently, to identify new factors facilitating *V. dahliae* infection, we examined the exoproteome of *V. dahliae* induced in the medium containing tissues of susceptible host cotton by iTRAQ. From this, we identified a predicted signal-peptide-lacking Mn-SOD (VdSOD3) with seven unique matching peptides and 41.95% coverage of the full length of the VdSOD3 protein [18], suggesting that VdSOD3 is probably secreted out and functions extracellularly. However, the function of VdSOD3 during infection in host plants is yet to be understood.

Thus, the main objectives of the current study were: (1) to investigate whether VdSOD3 is a secretory protein; (2) to determine the SOD activity of VdSOD3; (3) to determine the role of VdSOD3 in pathogenicity.

2. Materials and Methods

2.1. Growth of Microbial and Plant Material

The highly virulent isolate *V. dahliae* wild-type (WT) strain Vd991 was cultured on potato dextrose agar (PDA, potato, 200 g/L; glucose, 20 g/L; agar, 15 g/L) or in Czapek medium (CM, NaNO₃, 3.0 g/L; MgSO₄·7H₂O, 0.5 g/L; KCl, 0.5 g/L; FeSO₄·7H₂O, 0.01 g/L; K₂HPO₄, 1.0 g/L; sucrose, 30.0 g/L) at 25 °C. Cotton (*Gossypium hirsutum* Linn. cv. Junmian No. 1) and *Nicotiana benthamiana* Domin. plants were grown over a 14 h light/10 h dark photoperiod in a greenhouse, at 28 °C for two weeks and at 25 °C for four weeks, respectively.

2.2. Gene Cloning and Bioinformatics Analysis

The coding sequence of VdSOD3 (VEDA_05933) was amplified from cDNA samples of Vd991 with primers SOD3-F/R based on *V. dahliae* genome database [19]. All primers used for vector construction are listed in Table S1. Multiple sequence alignment was performed by Clustal X2. The N-terminal peptide of VdSOD3 was predicted using SignalP 5.0 [20]. Unconventionally secreted protein prediction was performed using OutCyte 1.0 [21].

2.3. Yeast Signal Sequence Trap System

The secretion function of the N-terminal signal peptide of VdSOD3 was tested by the yeast invertase secretion assay as previously described [22]. The coding region of the first 35 amino acids (aas) in the N-terminal peptide of VdSOD3 was amplified with primers N35-F/R and fused into the pSUC2 vector. The reconstruct plasmid was transformed into the yeast strain YTK12, and the positive clones were incubated on YPRAA medium (1% yeast extract, 2% peptone, 2% raffinose and 2 µg/mL antimycin A) to test the secretion ability of N-terminal peptide of VdSOD3. The known secretion function of the N-terminal peptide from Avr1b was set as the control.

2.4. Fungal Transformation

Targeted gene deletion plasmid was generated based on a previously described method with modifications [19]. Briefly, a 1242 bp upstream sequence, a 1178 bp downstream sequence of *VdSOD3* and the hygromycin phosphotransferase gene (*Hpt*) cassette were fused to one DNA fragment via fusion PCR. Subsequently, the fusion product was then cloned into a *Hind* III/*Xba* I-linearized pGKO2-Gateway vector to generate the targeted gene deletion plasmid using a homologous recombination reaction. The complementation DNA fragment, containing the *VdSOD3* wild-type gene with its native promoters and terminator regions, was fused into the binary vector pCOM with geneticin resistance to generate the complementation plasmid. *A. tumefaciens*-mediated fungal transformation was performed as described previously [23]. Gene knockout mutants were selected on PDA medium supplemented with hygromycin (50 µg/mL), while the complemented strains were selected in the presence of geneticin (50 µg/mL). Single spore isolations were performed for all transformants followed by PCR verification.

For expression of the *VdSOD3*-green fluorescent protein (GFP) fusion protein, the *VdSOD3* ORF was fused into the *Kpn* I sites of pCOM-GFP (integrating the GFP ORF into pCOM) using the recombination method. The recombined plasmid was transformed into *Vd991* to create WT::*VdSOD3*-GFP.

2.5. Confocal Microscopy Analysis

To examine the subcellular localization of *VdSOD3*, the coding sequence of *VdSOD3* was recombined with pBin::GFP to construct pBin::*VdSOD3*-GFP. The plasmid was transformed into *A. tumefaciens* GV3101, and agroinfiltrated into 4-week-old *N. benthamiana* leaves. To observe green fluorescence, the infiltrated tobacco leaves were harvested 2 days post-agro-infiltration and then imaged under a laser scanning confocal microscope (LSM T-PMT) with excitation and emission wavelengths of 488 and 510 nm, respectively. To observe the localization of *VdSOD3* in host–pathogen interactions, the inner layer of onion epidermal cells imitated the host plants to inoculate with conidial suspension (1×10^7 conidia/mL⁻¹) of strain WT::*VdSOD3*-GFP. After incubating on a water agar (1%) plate at room temperature for 2 days, laser scanning confocal microscopy was used to view green fluorescence in onion epidermal cells.

2.6. *VdSOD3* Expression Analysis

The *V. dahliae* *Vd991* strain was cultured in liquid CM plus 1 mM MnCl₂ from 3 to 9 days. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Potential genomic DNA was digested with DNase I (TaKaRa Bio, Shiga, Japan). First-strand cDNA was synthesized using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) with an oligo(dT)18 primer. The expression levels of *VdSOD3* were measured by qRT-PCR using FastFire qPCR premix (SYBR Green, TianGen, Beijing, China). The real-time PCR program consisted of an initial denaturation step at 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C with the primer qSOD3-F/R for 1 min. The expression level of *VdSOD3* was normalized to that of *V. dahliae* elongation factor 1-α (*EF-1α*). Each reaction was carried out in triplicate.

2.7. Measurement of SOD Enzyme Activity

The wild-type strain, Δ *SOD3*, and complemented strains were cultured in liquid CM plus 1 mM MnCl₂ for 7 days. The supernatant and fungal tissue were separated by centrifugation and ultrafiltration membrane filtration. The total SOD activity of each sample was measured following the nitro blue tetrazolium (NBT) reduction method [24]. The SOD unit was defined as the amount of crude enzyme required to inhibit the reduction in NBT by 50% and was expressed as units per mg protein (U/mg protein).

2.8. Fungal Morphology and Pathogenicity

For colony morphology and radial growth rate assay, a 2 μL conidial suspension with a concentration of 2×10^6 conidia/ mL^{-1} of the wild-type strain Vd991, *VdSOD3* deletion mutants and complemented strains was placed in the center of a PDA plate and CM plates containing different carbon sources (sucrose, starch, pectin and sodium carboxymethyl cellulose, 1% w/v), respectively. After incubating at 25 °C for 9 days, fungal colonies were measured and photographed. To evaluate conidial production, agar plugs were collected from the edges of 5- and 7-day-old fungal colonies using a 5 mm diameter cork borer. After shaking in 1 mL of sterile water, the number of conidia was quantified using a hemocytometer.

Pathogenicity assays were carried on *N. benthamiana* and cotton seedlings using a root-dip method as previously described [19]. Briefly, *N. benthamiana* and cotton seedlings were gently uprooted, immersed in 20 mL of 5×10^6 conidia/ mL^{-1} suspension from *V. dahliae* strain for 5 min and then replanted into new pots with three replicates. In each replicate, 12 *N. benthamiana* plants or six pots of cotton seedlings (five plants for each pot) were used for each transformant. After maintaining at 28 °C (14 h/10 h, day/night cycle) for three weeks, disease phenotypes and vascular discoloration were photographed. After extraction of the genomic DNA of shoots of the infected plants, the fungal biomass was quantified by qPCR with *V. dahliae* *EF-1 α* for quantifying fungal colonization by standardization with endogenous plant control of cotton *18S* gene or *N. benthamiana* *EF-1 α* .

3. Results

3.1. Bioinformatics Analysis of *VdSOD3*

The *V. dahliae* genome possesses two divergent class of Mn-SODs: *VdSOD2* with a predicted mitochondria-targeting signal (Gene-ID in Vd991 genome: VEDA_08263, Gene-ID in VdLs.17 genome: XP_009658205.1) and cytosolic *VdSOD3* without this targeting signal (Gene-ID in Vd991 genome: VEDA_05933, Gene-ID in VdLs.17 genome: EGY16066.1) [16,19]. Proteomic analysis of the *V. dahliae* Vd991 extracellular proteins mimicking host–pathogen interactions identified *VdSOD3* in the exoproteome of *V. dahliae* [18]. The *VdSOD3* ORF was predicted to encode a polypeptide of 205 aas with a predicted molecular weight of 22.69 kDa and an isoelectric point of 6.60. Sequence alignment of *VdSOD3* with other plant fungal pathogens' SOD3 displayed high homology, with 65.85% identity to *Fusarium graminearum* and 70.24% to *Magnaporthe oryzae* (Figure 1A), respectively. *VdSOD3* has four predicted metal binding sites for manganese (H27, H71, D156 and H160), a conserved DMWEHAYY region (156–163 aa) and Mn-SOD specific residues (G66, G67, F74, Q143 and D144) (Figure 1A). Sequence analysis with SignalP 5.0 predicted that the *N*-terminus of *VdSOD3* would not display signal peptide characteristics with high probability (0.9988) (Figure 1B). However, *VdSOD3* was predicted to be a potential unconventionally secreted protein by OutCyte 1.0 with a score of 0.6323, which is above the default threshold of 0.50 (Figure 1C). Together, these results indicated that *VdSOD3* is likely a signal-peptide-lacking Mn-SOD exported into the extracellular space unconventionally.

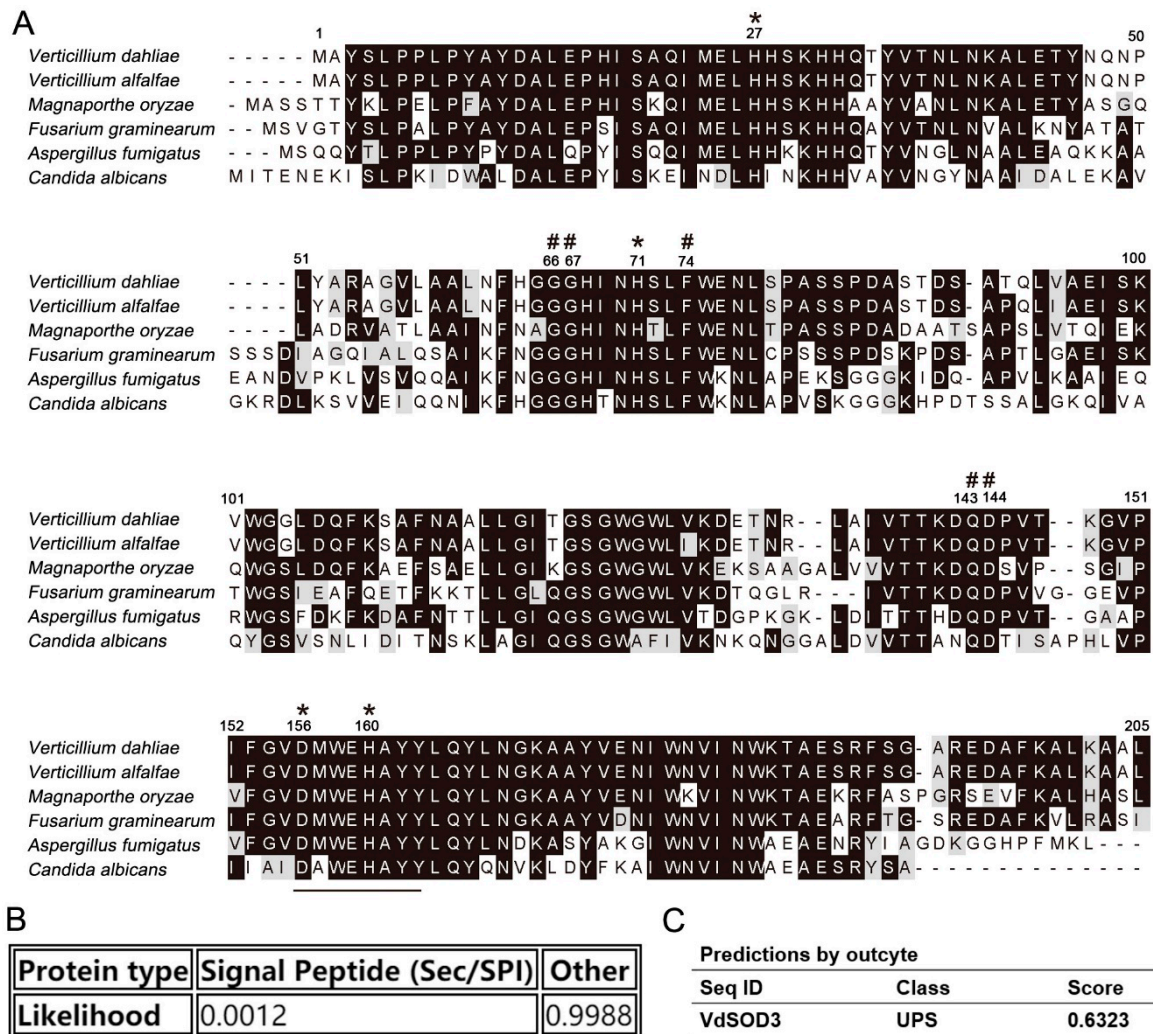


Figure 1. Bioinformatics analysis of *Verticillium dahliae* signal-peptide-lacking Manganese (Mn)-superoxide dismutase (SOD) (VdSOD3). (A) Alignment of protein sequence of VdSOD3 and its homologs from other fungi. Highly conserved residues are marked by a black background. The conserved SOD motif is underlined, Mn²⁺ binding sites are marked by asterisks and specific residues for Mn-SOD are labeled by hashtags. The Genbank accession numbers of aligned sequences are listed as follows: *Verticillium dahliae* (VEDA_05933); *Verticillium alfalfae* (XP_003000585.1); *Fusarium graminearum* (ESU07439.1); *Magnaporthe oryzae* (EHA51830.1); *Aspergillus fumigatus* (XP_752824.1); *Candida albicans* (AAL08560.1). (B) Signal peptide prediction of VdSOD3 using SignalP 5.0 program. (C) Unconventional secretion of VdSOD3 predicted by OutCyte 1.0. UPS is short for unconventional protein secretions.

3.2. Signal-Peptide-Lacking VdSOD3 Can Be Secreted

Subsequently, the secretory ability of the N-terminus of VdSOD3 was determined by the yeast signal trap assay to confirm the prediction of SignalP 5.0. Yeast needs to secrete invertase to grow on the YPRAA medium, which contains raffinose as the sole carbon source. The 35 aas of the VdSOD3 N-terminal peptide (VdSOD3^{N35}), where the signal peptide is generally found, were fused to the vector pSUC2, which carries a signal-peptide-lacking invertase gene. The recombined plasmid was then transformed into the invertase negative yeast strain YTK12 to create YTK12::VdSOD3^{N35}. In the CMD-W medium with sucrose as the carbon source, YTK12::VdSOD3^{N35} grew well. However, unlike the known signal peptide from Avr1b, VdSOD3^{N35} cannot mediate invertase secretion to provide the viability of YTK12::VdSOD3^{N35} in the YPRAA medium (Figure 2A). Thus, these results indicated that VdSOD3 does not contain a functional signal peptide, as predicted by SignalP 5.0.

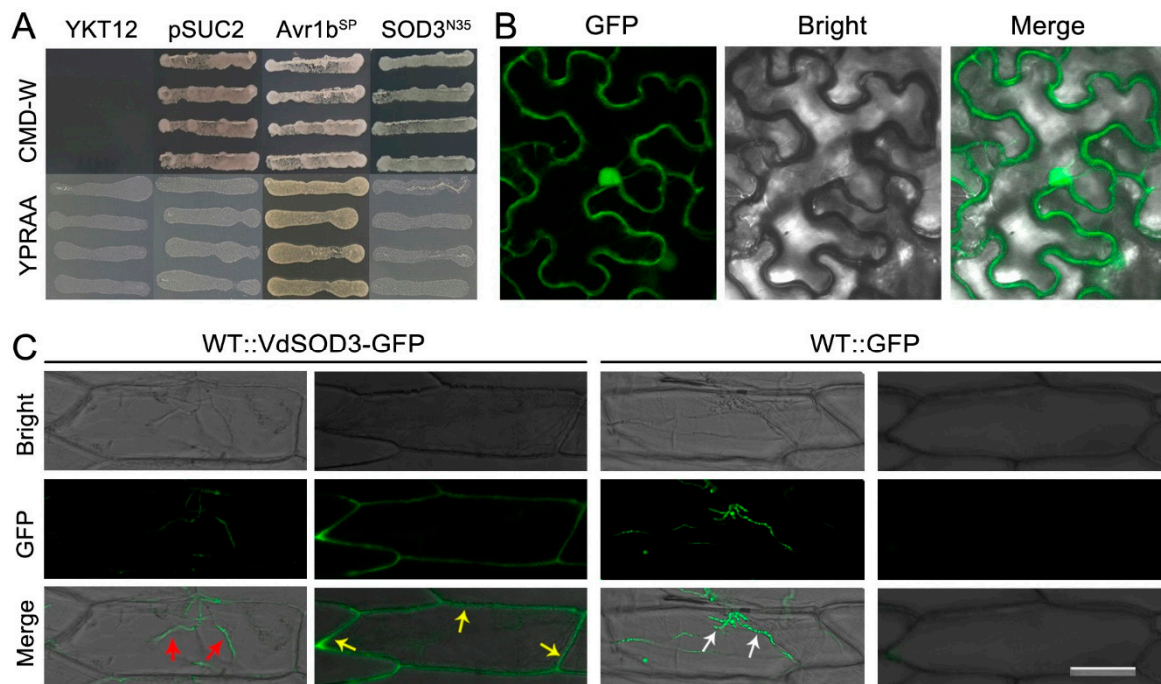


Figure 2. Signal-peptide-lacking VdSOD3 can be secreted. (A) Validation of the nonsecretion function of the 35-aa N-terminal peptide of VdSOD3 using the yeast signal trap assay. The known functional signal peptide Avr1b was used as a positive control. (B) Subcellular localization of VdSOD3 was determined by transient expression of VdSOD3-green fluorescent protein (GFP) in *N. benthamiana* leaves. (C) VdSOD3 can be secreted extracellularly during infection. *V. dahliae* strain wild-type (WT)::VdSOD3-GFP was used to infect onion epidermal cells, and confocal microscopic images were taken at 2 days postinoculation. WT::GFP inoculated onion cells were used as a negative control. The red arrow indicates green fluorescence of VdSOD3-GFP observed in the fungal hyphae of WT::VdSOD3-GFP, while the white arrow indicates green fluorescence of free cytosolic GFP observed in the fungal hyphae of WT::GFP. The yellow arrow indicates green fluorescence of secretory VdSOD3-GFP around onion epidermal cells. Bar, 50 μ m.

To investigate whether VdSOD3 is secreted, the coding region of VdSOD3 was fused with the green fluorescent protein (GFP) and transiently expressed in *N. benthamiana* leaves by agroinfiltration. The results showed that, in addition to the cytoplasm, VdSOD3-GFP also aggregated at the periphery of *N. benthamiana* cells (Figure 2B), indicating that VdSOD3 can be translocated extracellularly. Further, the onion epidermal cells, an advantage material to capture the GFP signal, imitated the host plant to determine the secretion characteristic of VdSOD3 when inoculated with *V. dahliae*. We constructed WT::VdSOD3-GFP, which expresses the VdSOD3-GFP fusion protein under the *TrpC* promoter in the wild-type strain background. WT::GFP, a *V. dahliae* wild-type strain expressing free cytosolic GFP, was used as a negative control. Conidial spores of WT::VdSOD3-GFP and WT::GFP were inoculated on onion epidermal cells for 2 days. As expected, the green fluorescence was observed in the fungal hyphae for both WT::VdSOD3-GFP and WT::GFP (Figure 2C), indicating the cytosolic localization of VdSOD3. However, only the invaded onion epidermal cells inoculated with WT::VdSOD3-GFP showed strong green fluorescence around onion epidermal cells, while no fluorescence was observed in the onion tissues infected by WT::GFP (Figure 2C), demonstrating that VdSOD3 is also a secreted protein, which translocates extracellularly during infection. Therefore, our results support *V. dahliae* VdSOD3 can be secreted extracellularly, although it lacks a functional signal peptide.

3.3. VdSOD3 Expression Pattern Analysis

Since the expression of *SOD3* of *Candida albicans* was strongly stimulated upon entering the stationary phase in continuous cultures [25], the gene expression pattern of *VdSOD3* was detected in time course (3–9 days) in continuous cultures. Similar to the *SOD3* of

C. albicans, expression of *VdSOD3* was also induced as the cells entered the stationary phase, reaching the maximum level at 7 days after culturing (Figure 3).

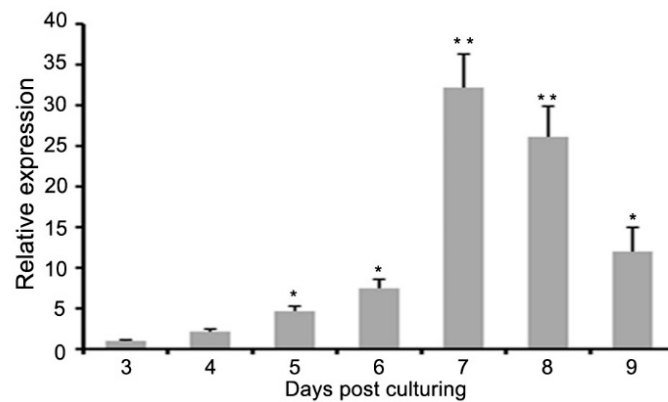


Figure 3. Transcript levels of *VdSOD3* during culturing in Czapek medium (CM) from 3 to 9 days. The expression levels of *VdSOD3* were calculated by the comparative Ct method with *EF-1 α* of *V. dahliae* as an endogenous control. The values are averaged and error bars represent standard deviations ($n = 3$). Asterisks ** and * represent significant differences $p < 0.01$ and $p < 0.05$ by Student's *t*-test, respectively.

3.4. *VdSOD3* Contributes to Both Intra- and Extracellular SOD Activity of *V. Dahliae*

To further explore the function of *VdSOD3*, gene knockout mutant of *VdSOD3* was constructed by replacing the coding sequence of the wild-type strain with hygromycin phosphotransferase gene (*Hpt*) cassette via homologous recombination. Five transformants were verified by PCR, of which two deletion mutants ($\Delta SOD3-1$ and $SOD3-2$) were selected randomly for further research (Figure 4A). Two randomly complemented strains (EC-1 and EC-2) were selected by reintroducing *VdSOD3* with its native promoter and the neomycin resistance gene to $\Delta SOD3-1$ (Figure 4B).

Due to *VdSOD3* being located both intra- and extracellularly in *V. dahliae*, as detected by a confocal microscope, the total SOD activity was tested for both the fungal hyphal and culture filtrates of the wild-type strain and $\Delta SOD3-1/2$ 7 days after culturing (strongly expression stage) in liquid CM plus Mn^{2+} (for inducing Mn-SOD activity). As expected, we found that the total SOD activity in the hyphal extract decreased significantly by 58.23% in *VdSOD3* deletion mutants compared to that of the wild-type strain, indicating that *VdSOD3* contributed to the majority of fungal tissue SOD activity of *V. dahliae* under the tested conditions (Figure 4C). Meanwhile, the SOD activity in fungal culture filtrates also decreased by 17.37% as a consequence of *VdSOD3* deletion (Figure 4C), suggesting that *VdSOD3* also contributed to a portion of the SOD activity of the culture filtrates, probably due to its secretion to the extracellular. Complemented strains restored culture filtrates and fungal tissue SOD activities, which were comparable to those of the wild-type strain (Figure 4C).

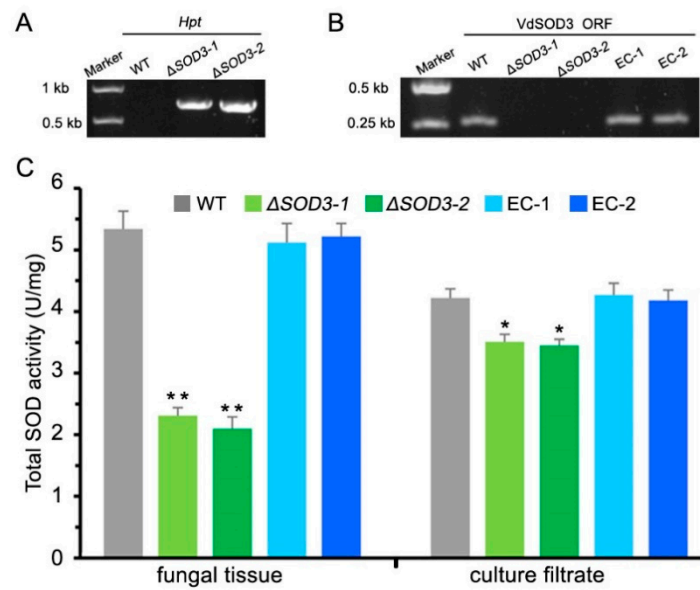


Figure 4. VdSOD3 contributes to both fungal intra- and extracellular SOD activity upon *Verticillium dahliae* entering the stationary phase. (A) Detection of the positive targeted *VdSOD3* deletion strains by detection of the *Hpt* fragment. (B) Detection of the positive targeted *VdSOD3* deletion strains and complementation strains by amplifying a *VdSOD3* internal fragment. (C) Total superoxide dismutase activities of fungal tissue and extracellular culture filtrate of indicated strains collected from 7-day-old culture. SOD activities were determined by the nitroblue tetrazolium (NBT) reduction method. Error bars represent standard deviations ($n = 3$). Asterisks ** and * represent significant differences $p < 0.01$ and $p < 0.05$ by Student's *t*-test, respectively.

3.5. Fungal Morphology and Pathogenicity

The colony morphology and growth rate of *VdSOD3* deletion mutants were comparable to those of the wild-type strain on PDA plates. Moreover, the mutants' growth rates were also identical with those of the wild-type strain on medium with sucrose, starch, pectin and cellulose as sole carbon sources (Figure 5A). Additionally, the number of conidia produced by the two *VdSOD3* deletion strains was similar to that of the wild-type strain (Figure 5B). Thus, these results suggested that *VdSOD3* is not required for fungal viability.

Further, to assess the possible role of *VdSOD3* to virulence in *V. dahliae*, the susceptible cotton *Gossypium hirsutum* cv. Junmian No.1 was inoculated with wild-type strain Vd991, *VdSOD3* deletion mutants and complemented strains, respectively, using a root-dip method. As expected, cotton inoculated with the wild-type strain showed leaf wilting, necrosis and vascular discoloration phenomenon, which are typical *Verticillium* wilt symptoms. In contrast, cotton inoculated with two *VdSOD3* deletion strains displayed significantly alleviated symptoms of the disease (Figure 6A), along with reduced fungal biomass in planta relative to cotton inoculated with the wild-type strain (Figure 6C). Similarly, Δ *SOD3*-inoculated *N. benthamiana* plants also displayed attenuated *Verticillium* wilt symptoms (Figure 6B) and reduced fungal biomass compared with plants inoculated with the wild-type strain (Figure 6D). Complemented strains showed that the disease symptoms and fungal biomass were comparable to those of the wild-type strain (Figure 6A–D). Taken together, these results suggested that the unconventionally secreted protein *VdSOD3* was required for full virulence of *V. dahliae* on the host plant of cotton and tobacco.

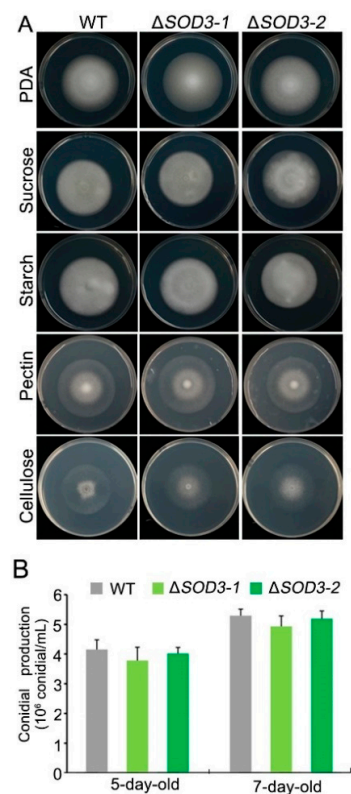


Figure 5. *VdSOD3* is not required for fungal growth, carbon source utilization and conidia production in vitro. (A) The wild-type strain and $\Delta SOD3-1/2$ were grown on potato dextrose agar (PDA) plates and CM plates containing different carbon sources (sucrose, starch, pectin and cellulose) for 9 days. (B) Quantification of conidia production of the wild-type strain and $\Delta SOD3-1/2$ from 5- and 7-day-old fungal colonies, respectively.

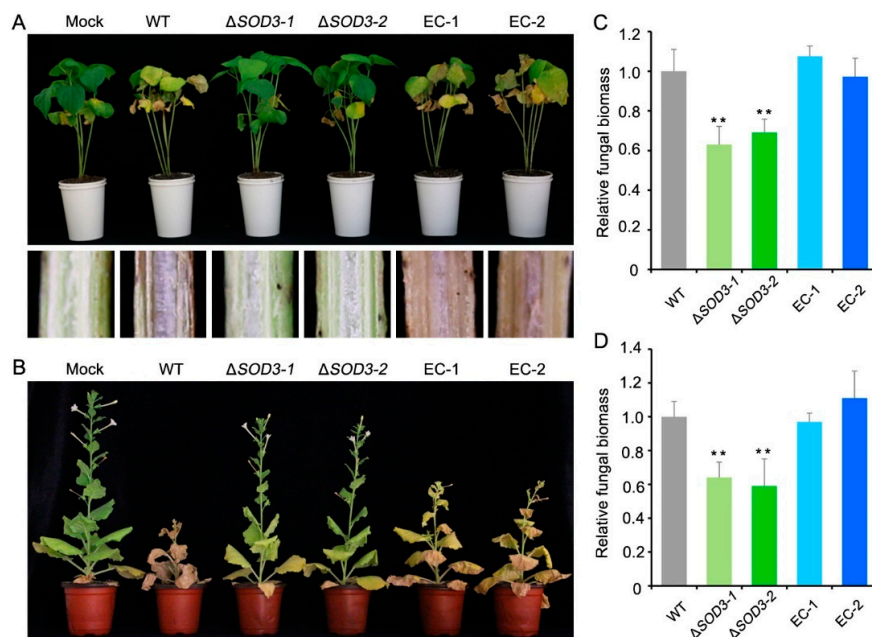


Figure 6. *VdSOD3* contributes to virulence of *V. dahliae* on host plants. (A) The disease symptoms of cotton seedlings inoculated with the indicated strains (Top) and the discoloration of the inoculation shoot longitudinal sections (bottom). (B) Phenotypes of *N. benthamiana* plants inoculated with the indicated strains. The fungal biomasses of each fungal strain in cotton (C) and *N. benthamiana* (D) were determined by RT-qPCR. Error bars represent standard deviations ($n = 3$). Asterisks ** indicates significant differences ($p < 0.01$) by Student's *t*-test.

4. Discussion

Despite extensive studies on the secretory characteristics of signal-peptide-lacking Mn-SOD in bacteria [12], few studies have referred to the extracellular distribution of Mn-SOD in fungi, except for the extracellular fungal Mn-SODs detected by proteomics analysis [15]. In addition, Mn-SOD enzymatic activity has only been identified from cell-free extracts of the filamentous fungus *Penicillium chrysogenum* [26]. In this study, bioinformatics analysis suggested that cytosolic VdSOD3 in *V. dahliae* could be secreted unconventionally (Figure 1). Although the yeast signal trap assay proved that VdSOD3 has no signal peptide, its translocation to host apoplastic spaces in vivo was observed by confocal microscopy (Figure 2), providing conclusive proof that VdSOD3 is an unconventionally secreted protein. Not surprisingly, VdSOD3 contributes to the SOD activity of the culture filtrate of *V. dahliae* (Figure 4). To our knowledge, this is the first study to identify both the secretory characteristics and extracellular catalytic function of Mn-SOD simultaneously in a fungal phytopathogen and offer a possible explanation of how Mn-SOD is involved in detoxifying extracellular ROS produced by the host.

Unconventionally secreted proteins involved in the virulence of fungal phytopathogens is an emerging theme in plant–microbe interactions. Secretion of plant cytoplasmic effectors in *Magnaporthe oryzae* is independent of the Golgi complex [27]. Signal-peptide-lacking sterol carrier protein 2 (Scp2) of *Ustilago maydis* was detected in the apoplastic fluid of infected maize, which plays a role in the inhibition of competitors in the apoplast [28]. Our previous work identified 99 signal-peptide-lacking proteins in *V. dahliae* secretome, implying that many unconventional secreted proteins may play essential roles during host–pathogen interactions [18]. However, only one signal-peptide-lacking protein, VdIscl, a plant cytoplasmic effector regulating the hydrolysis of salicylate’s precursor, has been extensively studied [29]. In this study, we identified another signal-peptide-lacking protein with plant apoplastic localization, furthering the understanding of how unconventionally secreted proteins contribute to the virulence of *V. dahliae*. We speculate that VdSOD3 following an unconventional secretion pathway may be a pathogen strategy to maintain Mn-SOD function both inside and outside of the *V. dahliae* cells.

We found that the expression of VdSOD3 was induced as the cells entered the stationary phase, similar to the SOD3 of *C. albicans*, which was also stimulated upon approaching the stationary phase in continuous cultures [25]. Recently, Culotta lab found that intracellular copper decreases during stationary phase of *C. albicans* and as part of a copper starvation response, *C. albicans* represses Cu/Zn SOD1, which requires Cu²⁺ for SOD activity and switches to the noncopper alternative SOD3 [30]. Interestingly, the *V. dahliae* genome also harbors a putative Cu/Zn VdSOD1 (Gene-ID in Vd991 genome: VEDA_03436, Gene-ID in VdLs.17 genome: VDAG_02630) [16,19] and this principle may also apply to *V. dahliae*. VdSOD3 may substitute for VdSOD1 when copper is low to ensure constant SOD activity of *V. dahliae* during different stages of infection.

Mn-SODs are important virulence factors in nearly all phytopathogens, with Mn-SOD of *C. graminicola* being the exception [6]. In this study, deletion of VdSOD3 did not affect the viability of *V. dahliae*, but alleviated the disease symptoms and reduced *V. dahliae* biomass in both cotton and *N. benthamiana*, suggesting that VdSOD3 was required for the virulence of *V. dahliae* (Figure 6). Two possible reasons may explain this phenomenon. First, the significantly impaired intracellular SOD activity in Δ VdSOD3 may lead to fungal inability to manage its metabolic superoxide to promote pathogen survival within the host. Second, the extracellular distribution of VdSOD3 may function in scavenging extracellular host-derived superoxide, and Δ VdSOD3 may have difficulty in surviving the ROS storm generated by the host.

5. Conclusions

In addition to being localized inside the cell, signal-peptide-lacking manganese superoxide dismutase VdSOD3 can also be secreted to extracellular spaces and possesses the SOD activity inside and outside of the cells for host–*V. dahliae* interactions. While being nonessential for

growth or conidiation, VdSOD3 contributes to the virulence of *V. dahliae* during infection in host plants.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2073-4395/11/1/13/s1>, Table S1: Primers used in this study.

Author Contributions: Conceptualization, N.Q. and D.Z.; methodology, L.T., W.S. and J.L.; software, L.T.; validation, L.T.; formal analysis, L.T.; investigation, L.T., W.S. and J.L.; writing—original draft preparation, L.T.; writing—review and editing, J.C., X.D., N.Q. and D.Z.; supervision, D.Z.; funding acquisition, L.T. and D.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Key Research and Development Program of China (2018YFE0112500), the National Natural Science Foundation of China (31501588, 31972228, 31970142).

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Acknowledgments: We thank Jin-long Wang and Yu-feng Xin for helpful discussions.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Warris, A.; Ballou, E.R. Oxidative responses and fungal infection biology. *Semin. Cell Dev. Biol.* **2019**, *89*, 34–46. [CrossRef]
2. Imlay, J.A. Pathways of Oxidative Damage. *Annu. Rev. Microbiol.* **2003**, *57*, 395–418. [CrossRef] [PubMed]
3. Broxton, C.N.; Culotta, V.C. SOD Enzymes and Microbial Pathogens: Surviving the Oxidative Storm of Infection. *PLoS Pathog.* **2016**, *12*, e1005295. [CrossRef] [PubMed]
4. Fridovich, I. Superoxide Radical and Superoxide Dismutases. *Annu. Rev. Biochem.* **1995**, *64*, 97–112. [CrossRef] [PubMed]
5. Fréalle, E.; Noël, C.; Nolard, N.; Symoens, F.; Felipe, M.-S.; Dei-Cas, E.; Camus, D.; Viscogliosi, E.; Delhaes, L. Manganese superoxide dismutase based phylogeny of pathogenic fungi. *Mol. Phylogenetics Evol.* **2006**, *41*, 28–39. [CrossRef] [PubMed]
6. Staerck, C.; Gastebois, A.; Vandeputte, P.; Calenda, A.; Larcher, G.; Gillmann, L.; Papon, N.; Bouchara, J.-P.; Fleury, M.J.J. Microbial antioxidant defense enzymes. *Microb. Pathog.* **2017**, *110*, 56–65. [CrossRef] [PubMed]
7. Frealle, E.; Noel, C.; Viscogliosi, E.; Camus, D.; Dei-Cas, E.; Delhaes, L. Manganese superoxide dismutase in pathogenic fungi: An issue with pathophysiological and phylogenetic involvements. *FEMS Immunol. Med Microbiol.* **2005**, *45*, 411–422. [CrossRef]
8. Leclère, V.; Bechet, M.; Blondeau, R. Functional significance of a periplasmic Mn-superoxide dismutase from *Aeromonas hydrophila*. *J. Appl. Microbiol.* **2004**, *96*, 828–833. [CrossRef]
9. Krehenbrink, M.; Edwards, A.; Downie, J.A. The superoxide dismutase SodA is targeted to the periplasm in a SecA-dependent manner by a novel mechanism. *Mol. Microbiol.* **2011**, *82*, 164–179. [CrossRef]
10. Schraw, W.; McClain, M.S.; Cover, T.L. Kinetics and Mechanisms of Extracellular Protein Release by *Helicobacter pylori*. *Infect. Immun.* **1999**, *67*, 5247–5252. [CrossRef]
11. Chen, X.; Kodama, T.; Iida, T.; Honda, T. Demonstration and Characterization of Manganese Superoxide Dismutase of *Providencia alcalifaciens*. *Microbiol. Immunol.* **2007**, *51*, 951–961. [CrossRef] [PubMed]
12. Ebner, P.; Götz, F. Bacterial Excretion of Cytoplasmic Proteins (ECP): Occurrence, Mechanism, and Function. *Trends Microbiol.* **2019**, *27*, 176–187. [CrossRef] [PubMed]
13. Andersson, A.; Rasool, O.; Schmidt, M.; Kodzius, R.; Flückiger, S.; Zargari, A.; Cramer, R.; Scheynius, A. Cloning, expression and characterization of two new IgE-binding proteins from the yeast *Malassezia sympodialis* with sequence similarities to heat shock proteins and manganese superoxide dismutase. *JBC J. Biol. Inorg. Chem.* **2004**, *271*, 1885–1894. [CrossRef] [PubMed]
14. Miura, N.; Ueda, M. Evaluation of Unconventional Protein Secretion in *Saccharomyces cerevisiae*. *Bioinform. MicroRNA Res.* **2014**, *1270*, 51–70. [CrossRef]
15. Caccia, D.; Dugo, M.; Callari, M.; Bongarzone, I. Bioinformatics tools for secretome analysis. *Biochim. Biophys. Acta Proteins Proteom.* **2013**, *1834*, 2442–2453. [CrossRef]
16. Klosterman, S.J.; Subbarao, K.V.; Kang, S.; Veronese, P.; Gold, S.E.; Thomma, B.P.H.J.; Chen, Z.; Henrissat, B.; Lee, Y.-H.; Park, J.; et al. Comparative Genomics Yields Insights into Niche Adaptation of Plant Vascular Wilt Pathogens. *PLoS Pathog.* **2011**, *7*, e1002137. [CrossRef]
17. Zhang, Y.; Wang, X.; Rong, W.; Yan, Z.; Li, Z.; Wu, L.; Zhang, G.; Ma, Z. Histochemical Analyses Reveal That Stronger Intrinsic Defenses in *Gossypium barbadense* Than in *G. hirsutum* Are Associated with Resistance to *Verticillium dahliae*. *Mol. Plant Microbe Interact.* **2017**, *30*, 984–996. [CrossRef]
18. Chen, J.-Y.; Xiao, H.-L.; Gui, Y.-J.; Zhang, D.-D.; Li, L.; Bao, Y.-M.; Dai, X. Characterization of the *Verticillium dahliae* Exoproteome Involves in Pathogenicity from Cotton-Containing Medium. *Front. Microbiol.* **2016**, *7*, 1709. [CrossRef]

19. Chen, J.-Y.; Liu, C.; Gui, Y.-J.; Si, K.-W.; Zhang, D.-D.; Wang, J.; Short, D.P.G.; Huang, J.-Q.; Li, N.-Y.; Liang, Y.; et al. Comparative genomics reveals cotton-specific virulence factors in flexible genomic regions in *Verticillium dahliae* and evidence of horizontal gene transfer from *Fusarium*. *New Phytol.* **2018**, *217*, 756–770. [[CrossRef](#)]
20. Armenteros, J.J.A.; Tsirigos, K.D.; Sønderby, C.K.; Petersen, T.N.; Winther, O.; Brunak, S.; Von Heijne, G.; Nielsen, H. SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat. Biotechnol.* **2019**, *37*, 420–423. [[CrossRef](#)]
21. Zhao, L.; Poschmann, G.; Waldera-Lupa, D.; Rafiee, N.; Kollmann, M.; Stühler, K. OutCyte: A novel tool for predicting unconventional protein secretion. *Sci. Rep.* **2019**, *9*, 1–9. [[CrossRef](#)]
22. Jacobs, K.A.; Collins-Racie, L.A.; Colbert, M.; Duckett, M.; Golden-Fleet, M.; Kelleher, K.; Kriz, R.; LaVallie, E.R.; Merberg, D.; Spaulding, V.; et al. A genetic selection for isolating cDNAs encoding secreted proteins. *Gene* **1997**, *198*, 289–296. [[CrossRef](#)]
23. Mullins, E.D.; Chen, X.; Romaine, P.; Raina, R.; Geiser, D.M.; Kang, S. Agrobacterium-Mediated Transformation of *Fusarium oxysporum*: An Efficient Tool for Insertional Mutagenesis and Gene Transfer. *Phytopathology* **2001**, *91*, 173–180. [[CrossRef](#)] [[PubMed](#)]
24. Beauchamp, C.; Fridovich, I. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **1971**, *44*, 276–287. [[CrossRef](#)]
25. Lamarre, C.; Lemay, J.-D.; DesLauriers, N.; Bourbonnais, Y. *Candida albicans* Expresses an Unusual Cytoplasmic Manganese-containing Superoxide Dismutase (SOD3 Gene Product) upon the Entry and during the Stationary Phase. *J. Biol. Chem.* **2001**, *276*, 43784–43791. [[CrossRef](#)]
26. Díez, B.; Schleissner, C.; Moreno, M.A.; Rodríguez, M.; Collados, A.; Barredo, J.L. The manganese superoxide dismutase from the penicillin producer *Penicillium chrysogenum*. *Curr. Genet.* **1998**, *33*, 387–394. [[CrossRef](#)]
27. Giraldo, M.C.; Dagdas, Y.F.; Gupta, Y.K.; Mentlak, T.A.; Yi, M.; Martinez-Rocha, A.L.; Saitoh, H.; Terauchi, R.; Talbot, N.J.; Valent, B. Two distinct secretion systems facilitate tissue invasion by the rice blast fungus *Magnaporthe oryzae*. *Nat. Commun.* **2013**, *4*, 1996. [[CrossRef](#)]
28. Krombach, S.; Reissmann, S.; Kreibich, S.; Bochen, F.; Kahmann, R. Virulence function of the *Ustilago maydis* sterol carrier protein 2. *New Phytol.* **2018**, *220*, 553–566. [[CrossRef](#)]
29. Liu, T.; Song, T.; Zhang, X.; Yuan, H.; Su, L.; Li, W.; Xu, J.; Liu, S.; Chen, L.; Chen, T.; et al. Unconventionally secreted effectors of two filamentous pathogens target plant salicylate biosynthesis. *Nat. Commun.* **2014**, *5*, 4686. [[CrossRef](#)]
30. Li, C.X.; Gleason, J.E.; Zhang, S.X.; Bruno, V.M.; Cormack, B.P.; Culotta, V.C. *Candida albicans* adapts to host copper during infection by swapping metal cofactors for superoxide dismutase. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E5336–E5342. [[CrossRef](#)]