



Regular Articles

Secreted peroxidases VmPODs play critical roles in the conidiation, H₂O₂ sensitivity and pathogenicity of *Valsa mali*Hao Feng¹, Mian Zhang¹, Yuhuan Zhao, Chen Li, Linlin Song, Lili Huang*

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ABSTRACT

Apple *Valsa* canker, caused by the necrotrophic pathogen *Valsa mali*, is a devastating disease of apples and causes great financial loss in East Asia. Improving the understanding of apple - *V. mali* interactions will contribute to disease management. In this study, three predicted secreted peroxidases (VmPOD1, VmPOD2 and VmPOD3) were uncovered based on the secretome and genome information of *V. mali*. Phylogenetic analysis showed that VmPOD1 is a catalase peroxidase, VmPOD2 is a chloroperoxidase, and VmPOD3 is a plant peroxidase-like peroxidase. The secretion function of the corresponding genes was confirmed using the yeast invertase secretion system. The deletion of *VmPODs* did not affect the vegetative growth when the mutants (Δ VmPOD1, Δ VmPOD2 and Δ VmPOD3) and the wild-type strain 03-8 were grown on PDA medium at 25 °C in the dark. However, the respective mutants showed impaired conidiation ability with fewer pycnidia, and all gene deletion mutants grew more slowly than 03-8 on PDA supplemented with H₂O₂ (Final concentration: 0.06 mol/L H₂O₂). In addition, VmPOD1 and VmPOD2 were found to be significantly up-regulated at an early infection stage, and VmPOD3 showed sustained high expression during the whole infection progress of *V. mali*. In addition, the virulence of Δ VmPOD3 was significantly reduced, implying that VmPOD3 plays a critical role during the interaction between *V. mali* and apple. All of the defective phenotypes could be nearly restored by re-introducing the wild-type VmPOD1, VmPOD2 or VmPOD3 allele. The results enhanced our understanding of the secreted peroxidase, which could also act as a type of virulence factor from the necrotrophic pathogen *V. mali* and provided new insight into the role of the pathogen-secreted peroxidase.

1. Introduction

Peroxidases, mainly involved in the reduction of hydrogen peroxide (H₂O₂) to water, are widely distributed in plants, animals and microorganisms. There has been no uniform classification of peroxidases, and most of them are determined based on their biological function. On the basis of sequence similarity, peroxidases are categorized into two super families: the mammalian peroxidase superfamily and the plant peroxidase superfamily (Dunford, 1999; Welinder, 1992). Only the heme-containing peroxidases, which are widely found in plants, fungi and bacteria, are generally classified into three subclasses based on their primary structures (Welinder, 1992). To scavenge H₂O₂, peroxidases need substrates to offer hydrogen, and their function could vary with different hydrogen donors (Zámocký et al., 2001). Different peroxidases prefer different substrates. For example, ascorbate peroxidases prefer ascorbate, while the cytochrome c peroxidases prefer cytochrome c as a substrate

(Sundaramoorthy et al., 1995). In particular, catalase peroxidases can directly reduce H₂O₂ (Patterson and Poulos, 1995).

Peroxidases are closely related to a wide array of physiological processes, especially the response to oxidative stress during the whole life process (Asada, 1997; Shigeoka et al., 2002; Zámocký, 2004). In plants, peroxidases are widely involved in the biosynthesis and degradation of lignin (Grisebach, 1981), protection from wounding (Espelie et al., 1986), regulation of auxin (Hinnman and Lang, 1965) and vegetative growth (Kawaoka et al., 2003). In addition, one of the most well known and most important functions is its role in biotic stress resistance. During pathogen infection, the reactive oxygen species (ROS) typically burst in the apoplastic compartment within minutes, and a series of resistance signals are induced and amplified (Bolwell and Wojtaszek, 1997; Wojtaszek, 1997), which finally lead to pathogenesis-related (PR) gene expression and hypersensitive responses (Huckelhoven, 2007; Jones and Dangl, 2006; Torres et al., 2005). Several lines of evidence have shown that different peroxidases play

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various roles in different defense pathways to enhance plant resistance in the interactions between the plants and their corresponding pathogens (Camejo López, 2016). Examples include the peroxidases that contribute to the resistance of tomato to several pathogens (Lagrimini et al., 1993) and regulate the apoplastic oxidative burst in *Arabidopsis* required for pathogen resistance (Bindschedler et al., 2006). The increased activity of peroxidases could induce wheat resistance to *Fusarium graminearum* (Mohammadi and Kazemi, 2002) and could also induce cassava resistance to *Tetranychus urticae* (Liang et al., 2017). Thus, the host-driven ROS production and its cascade reactions are clearly important threats to pathogenic fungi.

To succeed in the infection process, a pathogen has to effectively incapacitate the ROS-induced resistance of its host (Mir et al., 2015). During this process, pathogen peroxidases play a crucial role in scavenging ROS to sever the resistance signal and suppress the subsequent immunoreactions of the host. Several studies have demonstrated that peroxidases can contribute to the full virulence of pathogens (Dietz et al., 2006; Missall et al., 2004; Huang et al., 2011). For example, a glutathione peroxidase was recently reported to contribute to the virulence of the citrus fungal pathogen *Alternaria alternata* (Yang et al., 2016). Thus, the peroxidases of pathogens can play essential roles in the interactions between the pathogen and the host.

Valsa mali is a necrotrophic pathogen that causes apple *Valsa* canker in East Asia, which leads to severe economic loss in China (Wang et al., 2011). Accelerating knowledge of the molecular pathogenic mechanism of *V. mali* will contribute to the understanding of the apple-*V. mali* interaction and disease management. The pathogen mainly infects apple trunk by conidia and causes severe tissue necrosis and maceration during the later development of hyphae in tissues (Wang et al., 2011; Ke et al., 2013). Several genes involved in plant cell wall degradation and toxin synthesis are widely excavated based on the *V. mali* genome, transcriptome and functional genomics (Ke et al., 2014; Yin et al., 2015). Further, some genes such as Gα proteins coding genes *Gvm2* and *Gvm3* (Song et al., 2017), velvet genes *VmVeA* and *VmVeB* (Wu et al., 2017), feruloyl esterases gene family (Xu et al., 2018), and dicer-like genes *VmDCL1* and *VmDCL2* (Feng et al., 2017) were already confirmed to be related with the virulence and survival of *V. mali*. However, understanding of mechanism is still limited. Peroxidases exist widely in organisms and participate in various important life activities. In previous study, secretome containing 779 secreted proteins was predicted from the *V. mali* genome (Yin et al., 2015). Intriguing, we found that there were all only tree peroxidases with signal peptide in the secretome. However, the peroxidase genes of *V. mali* have not yet been functionally characterized.

In this study, three secreted peroxidases (VmPOD1, VmPOD2 and VmPOD3) were identified based on secretome and genome information of *V. mali*. VmPODs were essential for conidiation and H₂O₂ stress resistance. In addition, the VmPODs were significantly up-regulated during the infection progress, and VmPOD3 was necessary for full virulence. This result is the first to demonstrate that VmPODs are involved in the physiological metabolism and pathogenicity of *V. mali*.

2. Materials and methods

2.1. Strains and growth conditions

The wild-type strain 03-8 and mutant strains were cultured on potato dextrose agar (PDA, 20% potato, 2% glucose, 1.5% agar) medium at 25 °C in the dark. All the strains were deposited in the Laboratory of Pathogen Biology and Integrated Control of Fruit Tree Diseases, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi, PR China.

2.2. Sequence data analyses

Three sequences were isolated and identified as candidate secreted peroxidases based on secretome (data not published) and genome (Accession JUIY01000000) information regarding *V. mali*. The signal peptides were predicted using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>). The protein domain structure was predicted on the basis of Pfam analysis (<http://pfam.sanger.ac.uk/>). The amino acid sequence was analysed using BLASTP and compared with non-redundant databases (NCBI) to identify homologous proteins. Some homologous proteins of VmPODs (Identity > 50%, Query cover > 85%) from other fungi were selected and further compared using ClustalW. The unrooted phylogenetic tree was constructed using the neighbor-joining method of MEGA5.

2.3. Function validation of putative N-terminal signal peptides of VmPODs

To validate the secretion function of the putative N-terminal signal peptide of VmPODs, the yeast invertase secretion assay was designed. The predicted signal peptide of the VmPODs was cloned into pSUC2. The transformation was performed using auxotrophic and invertase mutant strain YTK12 and the lithium acetate method (Gietz et al., 1995). The tryptophan deficiency medium CMD-W (0.08% tryptophan dropout supplement, 2.5% sucrose, 0.65% yeast N base without amino acids, 0.1% glucose and 2% agar) was used for selecting strains containing constructed or empty pSUC2 vector. YPRAA plates (1% yeast extract, 2% raffinose, 2% peptone and 2 mg/ml antimycin A) containing raffinose as the only carbohydrate source was used as for selecting functional secretion signal peptides. The untransformed YTK12 strain and YTK12 strains transformed with an empty pSUC2 vector were considered to be the negative control and the secreted effector Avr1b of oomycete was regard as positive controls. The auxotrophic and invertase mutant strain YTK12, which was transformed with constructed or empty pSUC2 vector, could grow on tryptophan deficiency medium CMD-W and only the yeast strains that are able to secrete invertase can grow on both CMD-W and YPRAA media.

2.4. Nucleic acid isolation and manipulation

Apple twigs (*Malus domestica* borkh. cv. ‘Fuji’) were inoculated with wild-type strain 03-8, and samples were collected at 6, 12, 18, 24, 36, 48 and 72 hours post inoculation (hpi). Mycelium was collected after 03-8 was cultured on PDA medium for 4 days. The fungal genomic DNA was extracted using the CTAB method (Möller et al., 1992). For RNA isolation, total samples together with the mycelium of 03-8 were extracted using an RNAeasy R Plant mini kit (Qiagen, Shenzhen, PRC) according to recommended protocol. Contaminating genomic DNA was removed using DNase I (Invitrogen, Carlsbad, CA). A NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA) was used to check the DNA/RNA purity, concentration and integrity.

2.5. cDNA synthesis and transcription level analysis

First-strand cDNA was synthesized using a Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, Shenzhen, PRC) according to the manufacturer's instructions. Transcription levels were analyzed by qRT-PCR assays. qRT-PCR was performed with a CFX96 Real-Time System (Bio-Rad) with SYBR Green (Invitrogen). The Glucose 6 Phosphate Dehydrogenase (*G6PDH*) of *V. mali* was used as an internal control gene. Relative expression levels of each gene were calculated by the 2^{−ΔΔCT} method (Livak and Schmittgen, 2001). The experiment was repeated three times, and the data was used to calculate the mean and standard deviation. The primers used for qRT-PCR are listed in Supplementary Table S1.

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