



Valsa mali Pathogenic Effector VmPxE1 Contributes to Full Virulence and Interacts With the Host Peroxidase MdAPX1 as a Potential Target

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The *Valsa* canker, caused by *Valsa mali* (*V. mali*), is a destructive disease of apple in Eastern Asia. Effector proteins are important for fungal pathogenicity. We studied a candidate effector VmPxE1 isolated based on the genome information of *V. mali*. By using the yeast invertase secretion assay system, VmPxE1 was shown to contain a signal peptide with secretory functions. VmPxE1 can suppress BCL-2-associated X protein (BAX)-induced cell death with a high efficacy of 92% in *Nicotiana benthamiana*. The expression of *VmPxE1* was upregulated during the early infection stage and deletion of *VmPxE1* led to significant reductions in virulence on both apple twigs and leaves. VmPxE1 was also shown to target an apple ascorbate peroxidase (MdAPX1) by the yeast two-hybrid screening, bimolecular fluorescence complementation and *in vivo* co-immunoprecipitation. Sequence phylogenetic analysis suggested that MdAPX1 was an ascorbate peroxidase belonging to a subgroup of heme-dependent peroxidases of the plant superfamily. The ectopic expression of *MdAPX1* in the mutant of *VmPxE1* significantly enhanced resistance to H₂O₂, while the presence of *VmPxE1* seems to disturb *MdAPX1* function. The present results provide insights into the functions of VmPxE1 as a candidate effector of *V. mali* in causing apple canker.

Keywords: Apple *Valsa* canker, effector protein, cell death suppressor, virulence factor, peroxidase

INTRODUCTION

Secreted by bacteria, oomycetes, and fungi, effectors are defined as small rich cysteine secreted proteins, contributing to the pathogen virulence (Vleeshouwers and Oliver, 2014). Pathogen and host have been co-evolving, resulting in the establishment of multi-layered pathogen offense and host defense systems. Pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs), and then PAMP-triggered immunity (PTI) of the host is induced (Boller and He, 2009; Albert, 2013). Under the pressure from host defense, pathogens secrete effectors to suppress host defense, leading to effector-triggered susceptibility (ETS). ETS results in production of plant resistance proteins (R proteins) and leads to the second layer of immune response, the effector-triggered immunity (ETI), leading to heavy oxidative burst and hypersensitive reaction (HR) of the host (Jones and Dangl, 2006; Dodds and Rathjen, 2010).

Filamentous pathogenic effectors have been reported to interfere with several aspects of host immunity (Rovenich et al., 2014). Some act as inhibitors of proteases, such as Pit2 from *Ustilago maydis* and EPI10 from *Phytophthora infestans* (Tian et al., 2005; Mueller et al., 2013). Some influence enzymes related to the ROS pathway. For example, two cytoplasmic effectors of *Phytophthora sojae* interact with catalases to regulate H₂O₂ concentration (Zhang et al., 2015). An *U. maydis* effector Pep1 targets a maize peroxidase POX12 *in vivo* and suppresses the early immune responses of maize (Hemetsberger et al., 2012). Some effectors may competitively bind defense-related proteins to disturb the host-recognition system, such as PsXLP1, from *P. sojae*, acting as a decoy to shield the true virulence factor (Ma et al., 2017), and Ecp6 from *Cladosporium fulvum*, targeting chitin to suppress the chitin-induced immunity (de Jonge et al., 2010). Moreover, the resistance signal pathway could also be disturbed by pathogenic effectors. For instance, the effector HaRxL44 from *Hyaloperonospora arabidopsidis* breaks down the SA-triggered immunity (Caillaud et al., 2013) and the *U. maydis* effector Cmu1 interdicts SA biosynthesis (Djamei et al., 2011). Thus, pathogenic fungi have effectors that could function via several mechanisms to defeat/avoid host defense systems.

Valsa mali is a necrotrophic fungus belonging to Ascomycete and causes *Valsa* canker on apple, a destructive disease of apple in the Eastern Asia. In China, this disease resulted in significant economic losses (Lee et al., 2006; Wang et al., 2011). Previous research identified 193 candidate effector proteins (CEPs) with unknown functions and predicted 779 secreted proteins of *V. mali* with rich cysteine residues (average length of 233 amino acids) (Yin et al., 2015). The ability to suppress BAX triggered PCD is an important initial criterion for screening pathogenic effectors (Wang et al., 2011a). BAX is a member of the Bcl-2 family proteins, triggering cell death when expressed in plants. The cell death-promoting function of BAX in plants correlated with the upregulated expression of the defense-related protein PR1, which strongly suggests that BAX activates an endogenous cell-death program, one specific host defense reaction (Lacomme and Santa Cruz, 1999). Using this initial screening system for BAX responses, eight effectors have been identified and one of them (VmEP1) shown to be an important virulence factor of *V. mali* (Li et al., 2015). There are probably other *V. mali* effectors yet to be identified (Ke et al., 2014; Yin et al., 2015). Further research is needed to identify other effectors and study their functions.

In this study, we identified VmPxE1 as a cell death suppressor, contributing to the full virulence of *V. mali* and directly targeting a peroxidase of apple tree.

MATERIALS AND METHODS

Strains and Culture Conditions

Valsa mali strain 03-8 was cultured at 25°C in the dark on potato dextrose agar (PDA) medium. *Nicotiana benthamiana* plants were maintained at 25°C with a daily 13 h:11 h light:dark regime. *Agrobacterium tumefaciens* strain GV3101 used for agro-infiltration experiments was cultured on Luria-Bertani medium

at 28°C. *Escherichia coli* strain JM109 used for storing and propagating plasmids was cultured on Luria-Bertani medium at 37°C. All the strains are stored in the Laboratory of Integrated Management of Plant Diseases at the College of Plant Protection, Northwest A&F University, Yangling, China.

Construction of *V. mali* cDNA Libraries

Total RNA was extracted using the RNeasy Micro kit (Qiagen, Shenzhen, China) according to the manufacturer's protocol from (a) *V. mali* mycelia grown on PDA medium for 3 days, and (b) apple twig tissues of *Malus domestica* borkh. cv. 'Fuji' inoculated with *V. mali* mycelium plugs [0 h (i.e. immediately before inoculation), 6, 12, 18, 24, 36, 48, 72 h post inoculation (hpi)]. First-strand cDNA was synthesized by the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Shenzhen, China) according to the manufacturer's protocol.

Plasmid Constructs

Targeted genes were amplified from the cDNA library using the Ex-taq mix DNA polymerase (Takara, Dalian, China). For *A. tumefaciens* infiltration assays in *N. benthamiana*, PCR products were cloned into the corresponding vectors (Giraldo and Valent, 2013) with the restriction enzyme digestion and T4 DNA ligase (Takara, Dalian, China). Primers used for plasmid constructs are listed in **Supplementary Table S1**. Sequences of all plasmids were confirmed by Sangon Biotech, Shanghai.

Sequence Analyses

In our previous study, the whole genome shotgun sequences of *V. mali* were deposited at DDBJ/EMBL/GenBank under the accession JUIY01000000. The secretome and CEPs of *V. mali* were predicted from materials obtained in previous studies (Li et al., 2015; Yin et al., 2015). Pfam¹ was used to predict protein domain structure, and SignalP 4.1² to signal peptides. An unrooted phylogenetic tree was constructed by MEGA5 with the neighbor-joining method.

Agrobacterium tumefaciens Infiltration Assays

Agro-infiltration assays were carried out following the previously described procedure (Dou et al., 2008). Strain GV3101 carrying an expression plasmids was grown in LB medium containing kanamycin (50 µg/ml) for transient expression; cells were re-suspended in 10 mM MgCl₂ (pH 5.6) with the suspension adjusted to an OD₆₀₀ of 0.5. Bacteria were infiltrated with a syringe to the upper leaves of 5-week-old *N. benthamiana* plants. As a control, plants were infiltrated with bacteria carrying an empty pGR106 vector. Symptoms were assessed 4–5 days after infiltration. Each assay was performed three times.

Secretory Function Validation of Putative N-Terminal Signal Peptide of VmPxE1

The yeast invertase secretion assay was performed to validate the function of the putative N-terminal signal peptide of

¹<http://pfam.xfam.org/>

²<http://www.cbs.dtu.dk/services/SignalP/>

VmPxE1. The predicted signal peptide of VmPxE1 was cloned to pSUC2 and transformed into yeast strain YTK12 with the lithium acetate method (Geitz et al., 1995). The CMD-W medium (0.08% tryptophan dropout supplement, 2.5% sucrose, 0.65% yeast N base without amino acids, 0.1% glucose, and 2% agar) was used to select YTK12 colonies with pSUC2 empty vector or pSUC2-VmPxE1sigp. For validating invertase secretion, positive colonies on the CMD-W medium were transferred to YPRAA plates (1% yeast extract, 2% raffinose, 2% peptone, and 2 mg/ml antimycin A) containing raffinose as the only carbohydrate source. The untransformed YTK12 colonies, YTK12 colonies transformed with an empty pSUC2 vector, and the non-secreted Mg87 protein from *Magnaporthe oryzae* were used as negative controls (Gu et al., 2011); the YTK12 colonies carrying the signal peptide of Avr1b from oomycete was used as a positive control (Shan et al., 2004; Gu et al., 2011).

RNA Extraction and Transcript Level Analysis

VmPxE1 transcript level was measured by qRT-PCR in apple twig tissues inoculated with *V. mali* strain 03-8 sampled at 0 (immediately before inoculation), 6, 12, 18, 24, 36, 48, and 72 hpi. Total RNA was extracted using the RNeasy R Plant Mini Kit (Qiagen, Shenzhen, China) following the recommended protocol. First-strand cDNA was synthesized using a RT-PCR system (Promega, Madison, WI, United States) following the manufacturer's instructions. SYBR green qRT-PCR assays were performed to quantify transcript levels; *G6PDH* of *V. mali* was chosen as a housekeeping gene (Yin et al., 2013). There were three biological replicates for each treatment. Primers used for qRT-PCR are given in **Supplementary Table S2**.

Generation of VmPxE1 Mutants

A reaction with three components using the *Neo* gene as a selective marker was performed for single gene deletion. The *Neo* gene was amplified with primers Neo-F and Neo-R from PFL2. The upstream and downstream flanking sequences of *VmPxE1* were amplified using primer pairs 1F/2R, 3F/4R, respectively. Then, deletion cassette for homologous recombination was generated by double-joint PCR as described previously (Yu et al., 2004). The primer pair CF/CR was used for nest-PCR and produced the gene-replacement construct. Protoplasts of *V. mali* were prepared and then the gene-replacement construct was transformed into the protoplasts as previously described (Gao et al., 2011). Each putative single gene deletion mutant was verified by PCR using four primer pairs (5F/6R, 7F/NeoR, NeoF/8R, and NeoF/NeoR) to detect the target gene, upstream-neo fusion segment, neo-downstream fusion segment, and the neo gene, respectively. Southern blot hybridization using the DIG DNA Labeling and Detection Kit II (Roche, Mannheim, Germany) was performed to confirm deletion mutants. All primers used for gene deletion are given in **Supplementary Table S1**.

Complementation of the Deletion Mutants

The entire *VmPxE1* gene with upstream 2000 bp was amplified with primers pDL2-VmPxE1-F and pDL2-VmPxE1-R cloned into plasmid pDL2 with the yeast gap repair approach (Bruno et al., 2004; Zhou et al., 2012). The resulting construct, pDL2-VmPxE1, was transformed into protoplasts of the *VmPxE1* gene deletion mutants. Complementation was selected by geneticin (G418) and hygromycin, and confirmed by PCR using the primer pair 5F/6R.

Pathogenicity, Conidiation, and Vegetative Growth of Mutants

For pathogenicity assay, detached apple (*M. domestica* borkh. cv. 'Fuji') twigs and leaves were inoculated with the deletion mutant Δ VmPxE1, complementation mutant Δ VmPxE1/VmPxE1 and wild type following the method previously described (Oliva et al., 2010). The experiments were repeated twice with fifteen replicates in each repeat experiment. Vegetative growth and conidiation was examined at 3 and 40 days, respectively. Data were analyzed by Student's *t*-test using the SAS software package (SAS Institute, Cary, NC, United States).

Yeast Two-Hybrid Screening

The yeast two-hybrid system was performed for screening *VmPxE1* interacting proteins (Ito et al., 2001). The *VmPxE1* coding region was cloned into the bait vector pGBKT7 without the signal peptide sequence and pGBKT7-VmPxE1 was transformed into yeast strain AH109. Yeast cells carrying pGBKT7-VmPxE1 were transformed with cDNA library that was constructed into the prey vector pGADT7 using mRNA isolated from the junction of diseased (infected by *V. mali* wild type strain 03-8) and healthy twigs. Candidate clones growing on the SD/-Leu-Trp-His medium were picked to SD/-Leu-Trp-His+X- α -galactosidase medium for confirmation of the interaction.

Bimolecular Fluorescence Complementation

A published BiFC procedure (Waadt et al., 2008) was used. *VmPxE1* and *MdAPX1* were cloned into the vector pSPYNE(R)173 and the vector pSPYCE(M), respectively. *A. tumefaciens* strain GV3101 carrying the expression vector pSPYNE(R)173-VmPxE1 was co-injected with *A. tumefaciens* strain GV3101 carrying pSPYCE(M)-MdAPX1 at the 1:1 ratio into 4-week-old *N. benthamiana* plants. About 48–60 h after co-agroinfection, live-cell imaging was taken with a two-photon confocal laser scanning microscopy (Olympus FV1000MPE) with the FV10-ASW 3.1 software suite. The assay was repeated twice.

Protein Extraction and Western Blots

About 60–72 h after agro-infection, the treated *N. benthamiana* leaves were rapidly frozen in liquid nitrogen and triturated in mortar, then total proteins of the leaf tissues were extracted with Plant Total Protein Extraction Kit, following the manufacturer's instruction (P0028, Beyotime technologies, Shanghai, China).

For the western blot analysis, total proteins from leaves were separated using SDS-PAGE (40V, 8 mA) and transferred onto nitrocellulose membranes (60V, 150 mA). The blot was blocked with Western Quick Block mixture (P0023B, Beyotime Technologies, Shanghai, China) for 1 h at ambient temperature and followed by (1) incubation with the monoclonal antibody (1:500) in WB primary antibody diluent (P0103, Beyotime technologies, Shanghai, China) for 2 h at ambient temperature; (2) being washed with TBS containing 0.1% Tween-20 (TBST), and (3) reacting with goat anti-mouse IgG horseradish peroxidase (HRP) (1:500) (A0216, Beyotime Technologies, Shanghai, China) for 1 h at ambient temperature. Finally, the nitrocellulose membrane was immunostained with 3,3'-diaminobenzidine (DAB) for 10 min in the dark before visualization.

In Vivo Co-immunoprecipitation

For the *in vivo* Co-IP assay, *A. tumefaciens* strain GV3101 carrying the expression vectors PICH86988-*HA-MdAPX1* and pBin-*GFP-VmPxE1* was co-agroinfiltrated into *N. benthamiana* leaves. Harvest proteins 500 μ L was added to equilibrated GFP-trap beads (Chromotek) and fully mixed at 4°C overnight on a slow shake incubator, and then centrifuged with the supernatant discarded. Re-suspended GFP-trap beads were washed by pre-cold wash buffer twice and then transferred to 100 μ L 2 \times SDS loading buffer. The sample was boiled for 10 min to dissociate for immunoblot analysis using the anti-GFP or anti-HA antibody.

Ectopic Expression of *MdAPX1* in *V. mali*

The entire *MdAPX1* gene sequence was cloned into plasmid pDL2 with the yeast gap repair approach (Bruno et al., 2004; Zhou et al., 2012). Then PEG-mediate protoplast transformation was performed to transferred pDL2-*MdAPX1* into *V. mali* wild type 03-8 and the *VmPxE1* gene deletion mutant Δ *VmPxE1* with the hygromycin B phosphotransferase gene (*hph*) as a selective marker. Transformed colonies were selected by hygromycin B. Then the stress resistance against H₂O₂ was measured by culturing wild type 03-8, transformed colonies of 03-8/*MdAPX1* and Δ *VmPxE1*/*MdAPX1* on PDA with 0.06% H₂O₂ at 25°C. Fungal colony size was assessed 3 days after treatment. There were 30 petri dishes per treatment. The expression of *MdAPX1* was identified with RT-PCR. The experiment was repeated twice.

RESULTS

VmPxE1 From *V. mali* Suppressed BAX-Induced Cell Death in *N. benthamiana*

A protein of unknown functions, *VmPxE1* (KUI70334.1) residing on chromosome 6 of *V. mali*, was found to contain 213 amino acid residues with a predicted signal peptide. Transient expression assay in *N. benthamiana* leaves with BAX suggested that *VmPxE1* is an effective cell death suppressor effector with a cell death suppression ratio of 92% (46/50) (Figure 1). Subsequent RT-PCR and western blot with an anti-eGFP and anti-HA antibody confirmed that *VmPxE1*, BAX, and

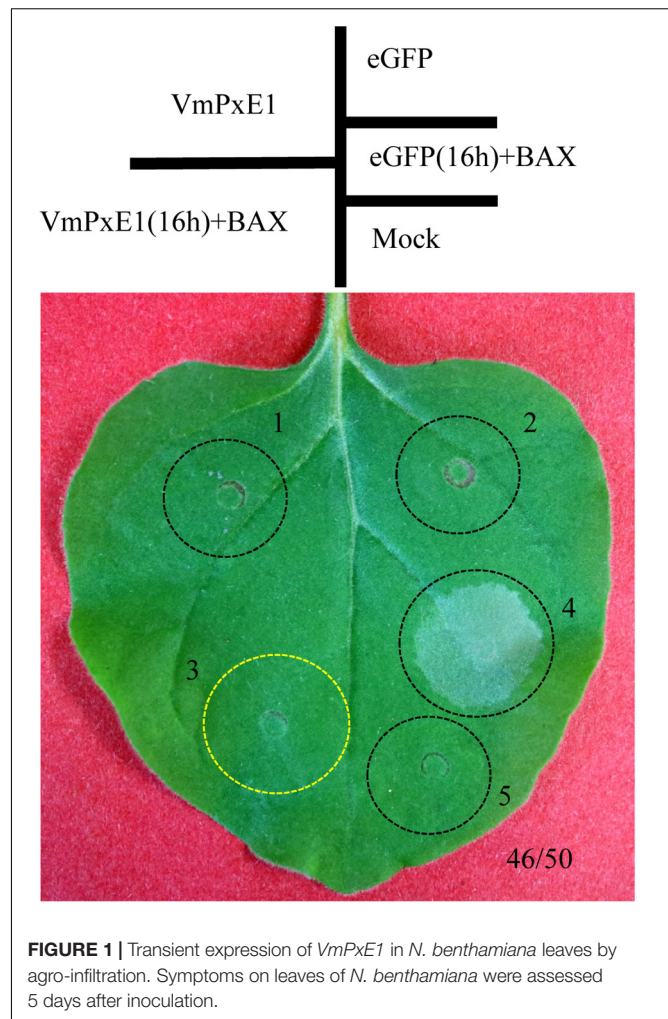


FIGURE 1 | Transient expression of *VmPxE1* in *N. benthamiana* leaves by agro-infiltration. Symptoms on leaves of *N. benthamiana* were assessed 5 days after inoculation.

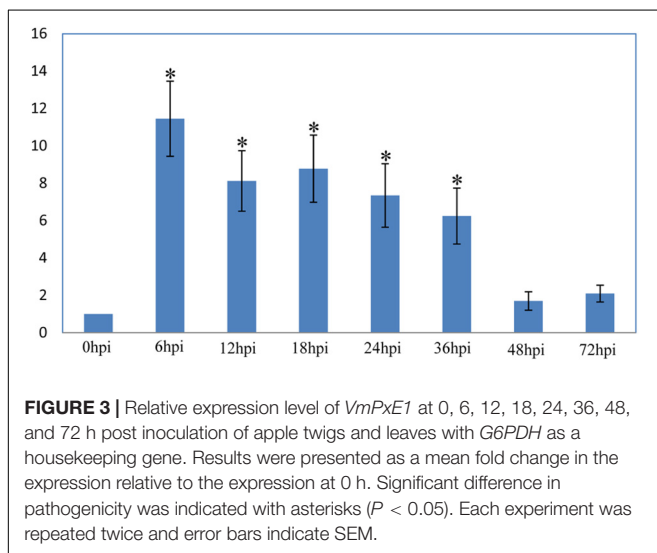
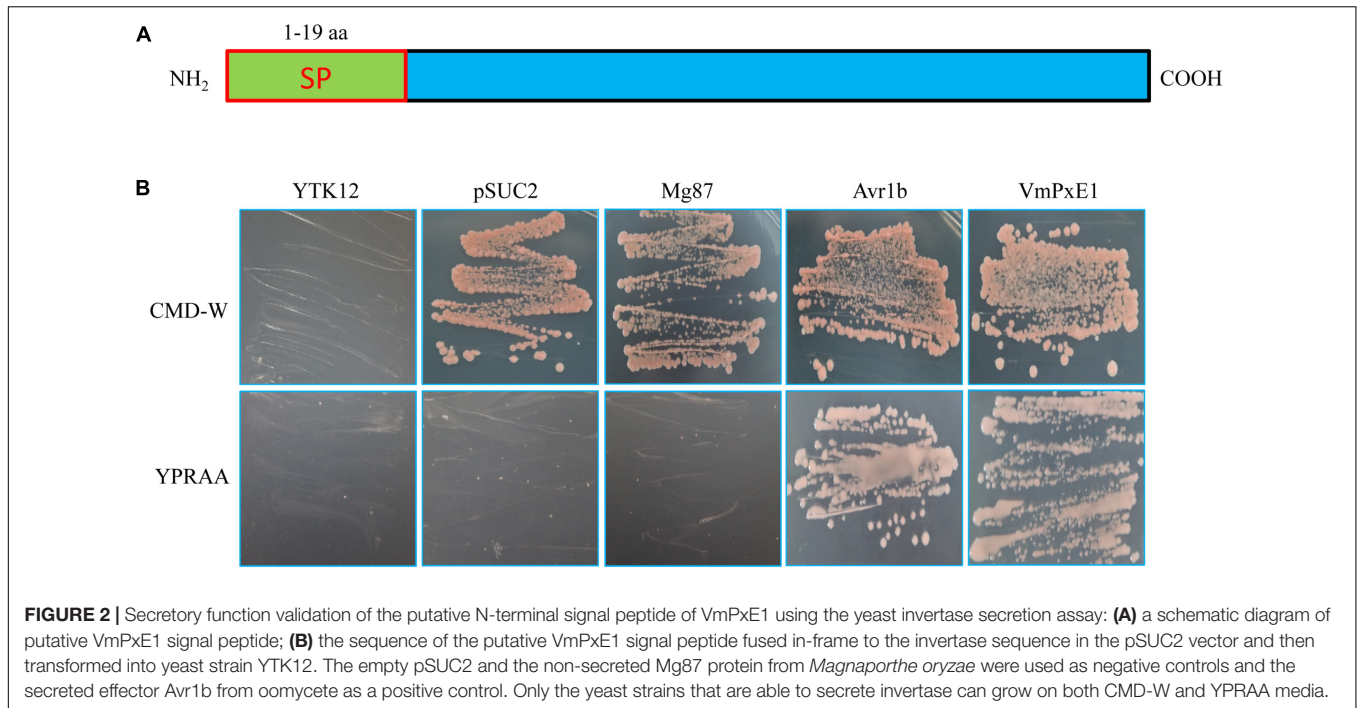
eGFP were all expressed in *N. benthamiana* (Supplementary Figures S1A,B).

Secretory Function Validation of the Putative N-Terminal Signal Peptide of *VmPxE1*

The *VmPxE1* amino acid sequence contained a predicted signal peptide at the position of 1-19 aa (Figure 2A). The invertase mutant yeast strain YTK12 containing *VmPxE1* signal peptide recombinant plasmids could grow on YPRAA medium (with raffinose instead of sucrose) indicating that the invertase was secreted (Figure 2B). This result indicates that the putative N-terminal signal peptide of *VmPxE1* was a functional secretion signal peptide.

Transcription Level of Effector Gene *VmPxE1*

VmPxE1 expression was quantified at 0 (immediately before inoculation), 6, 12, 18, 24, 36, 48, and 72 hpi on apple twigs with *G6PDH* as a housekeeping gene. *VmPxE1* was upregulated for



all sampling points. The expression of *VmPxE1* was increased significantly at 6, 12, 18, 24, and 36 hpi (**Figure 3**).

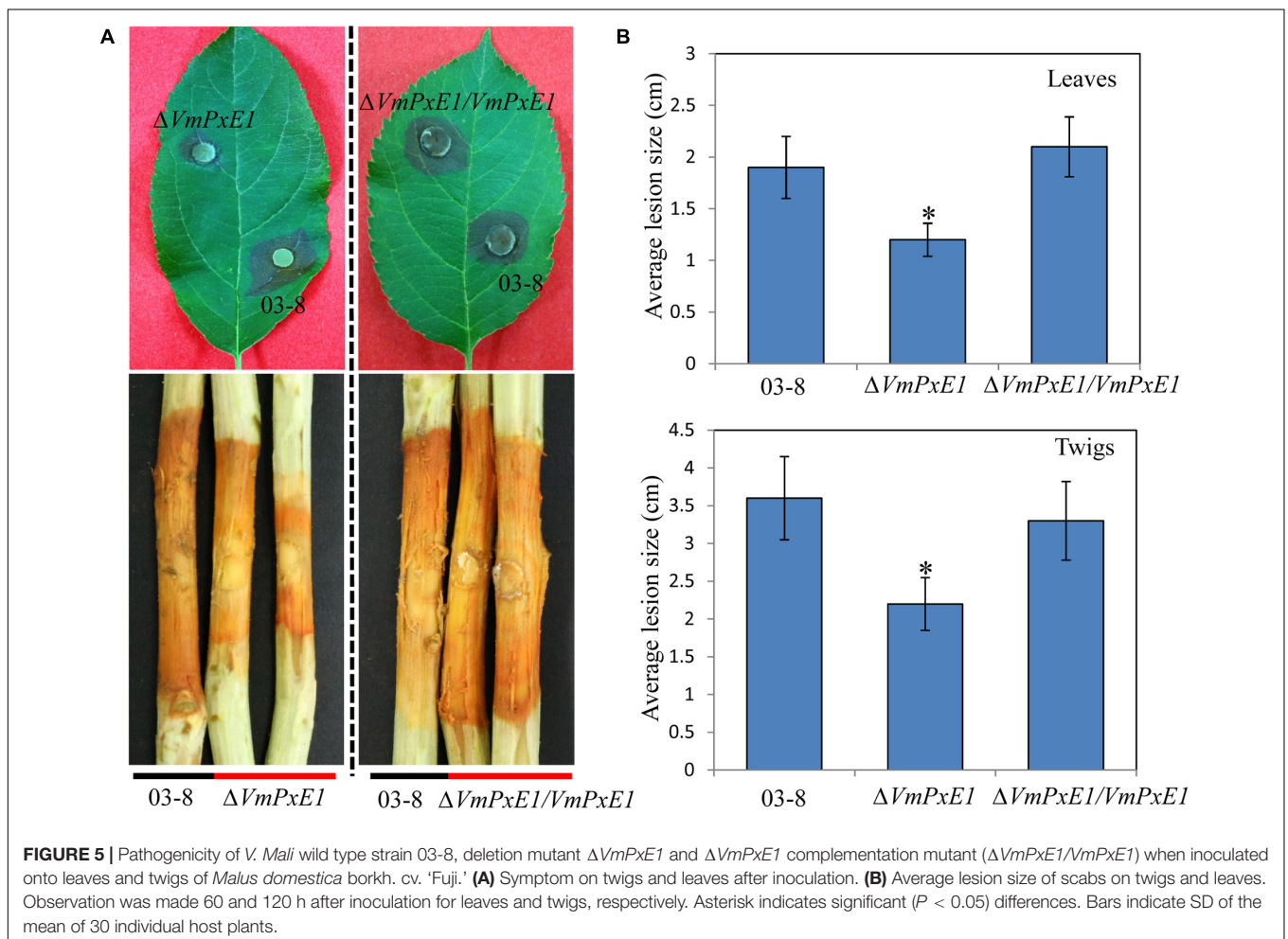
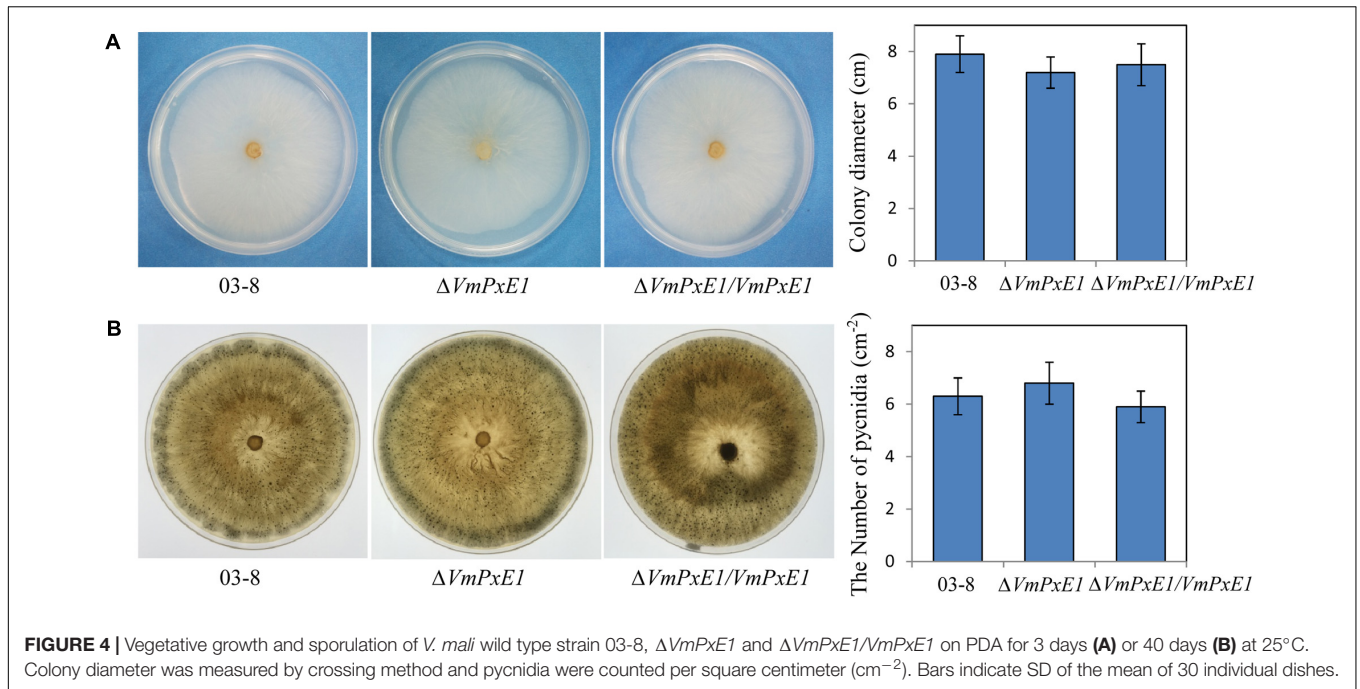
The *VmPxE1* Is Not a Necessary Growth Factor but a Virulence Factor of *V. mali*

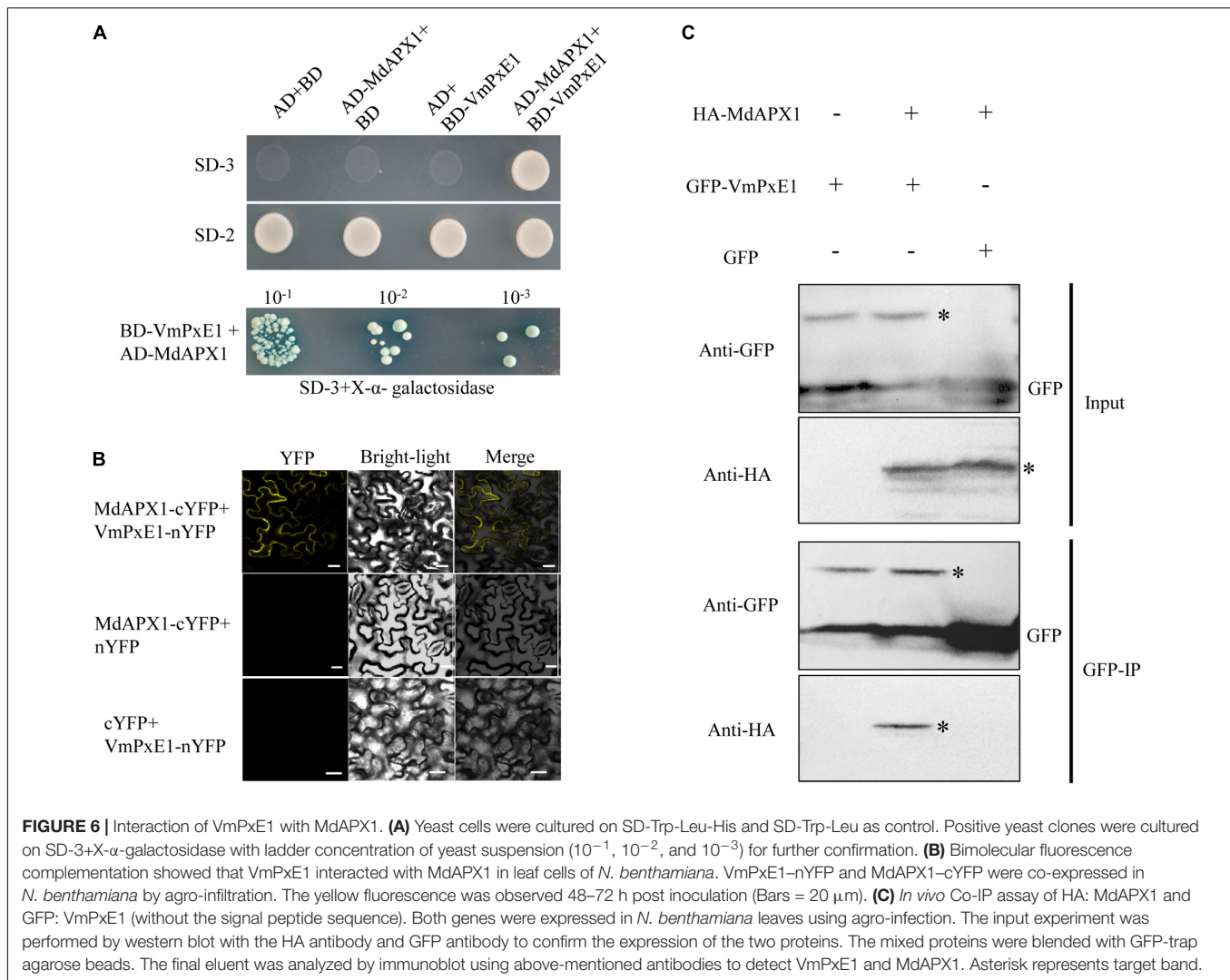
To estimate the contribution of *VmPxE1* to virulence, this gene was deleted (**Supplementary Figure S2A**). The deletion mutant $\Delta VmPxE1$ was obtained and further confirmed by PCR analysis (**Supplementary Figure S2C**) and Southern hybridization (**Supplementary Figure S2B**). The complementation mutant $\Delta VmPxE1/VmPxE1$ was generated and confirmed by PCR analysis (**Supplementary Figure S2D**).

In vitro inoculation showed that deletion of *VmPxE1* did not affect vegetative growth (**Figure 4A**) and sporulation of *V. mali* (**Figure 4B**). However, $\Delta VmPxE1$ showed significant reduction in virulence on both apple twigs and leaves (**Figure 5A**), about 42.1 and 38.9% on leaves and twigs, respectively (**Figure 5B**). The complementation mutant $\Delta VmPxE1/VmPxE1$ had the similar level of infection as the wild type 03-8.

VmPxE1 Interacted With MdAPX1, an Apple Ascorbate Peroxidase

To identify the target of *VmPxE1*, the GAL4 yeast two-hybrid system was performed using a *V. mali*-apple cDNA library. A potential gene segment was captured after screening tests on SD-Trp-Leu-His medium. The sequence was predicted as an apple ascorbate peroxidase protein, named as MdAPX1 (*Malus domestica* ascorbate peroxidase 1). Its intact coding sequence was constructed into the prey vector pGADT7. Auxotroph yeast strain AH109 was able to recover growth on SD-Trp-Leu-His when synchronously transformed with pGBKT7-*VmPxE1* and pGADT7-*MdAPX1* (**Figure 6A**). The dichotomous YFP segments were recovered and yellow fluorescence signal was detected in leaf cells of *N. benthamiana*, indicating that MdAPX1 could interact with *VmPxE1* (**Figure 6B**). HA-MdAPX1 and GFP-*VmPxE1* were transiently co-expressed in *N. benthamiana*, and total proteins went through GFP-trap gel beads for the specific adsorption. The immunoblotting showed that HA-MdAPX1 was present in the final GFP-*VmPxE1*-precipitated immunocomplex (**Figure 6C**), suggesting that MdAPX1 is a potential target of *VmPxE1*. Phylogenetic analysis showed that MdAPX1 (XP008344846.1) is an L-ascorbic peroxidase, belonging to





a subgroup of heme-dependent peroxidases of the plant superfamily (Figure 7).

Ectopic Expression of MdAPX1

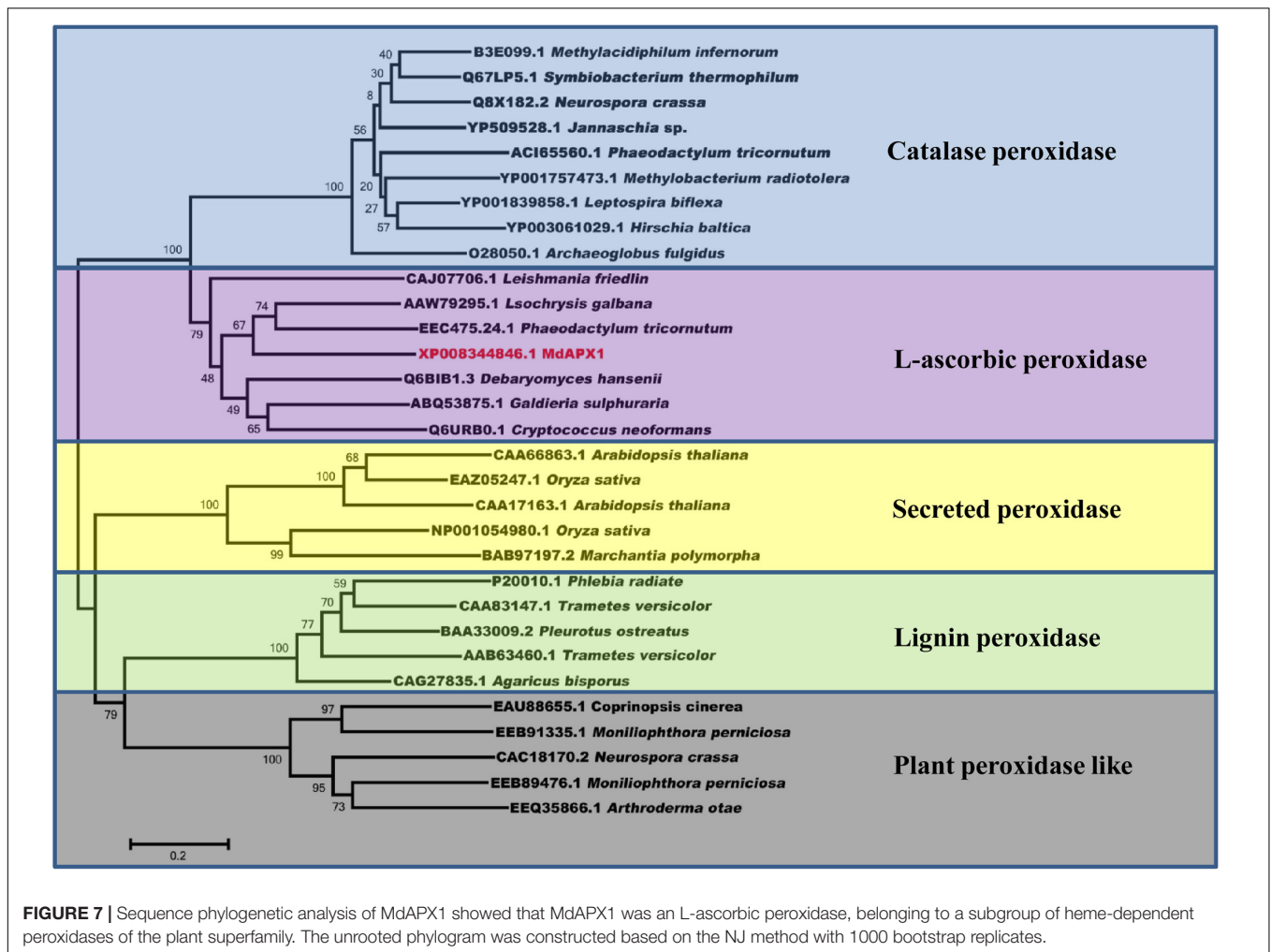
The ectopic expression of *MdAPX1* in wild type 03-8 (03-8/*MdAPX1*) and *VmPxE1* gene deletion mutant strain (Δ *VmPxE1*/*MdAPX1*) was successful (Supplementary Figure S3). The growth of both the strains was significantly suppressed on PDA containing 0.06% H₂O₂. The transformed strain Δ *VmPxE1*/*MdAPX1* exhibited stronger resistance to H₂O₂ than the 03-8/*MdAPX1* strain. These results suggested that *MdAPX1* enhanced the resistance of only Δ *VmPxE1* to H₂O₂ and that the presence of *VmPxE1* seems to have disturbed *MdAPX1* function (Figure 8).

DISCUSSION

We demonstrated that a cell death suppressor effector of *V. mali*, VmPxE1, was upregulated during the early stage of fungal

infection of apple twigs and leaves, and identified one potential target of VmPxE1.

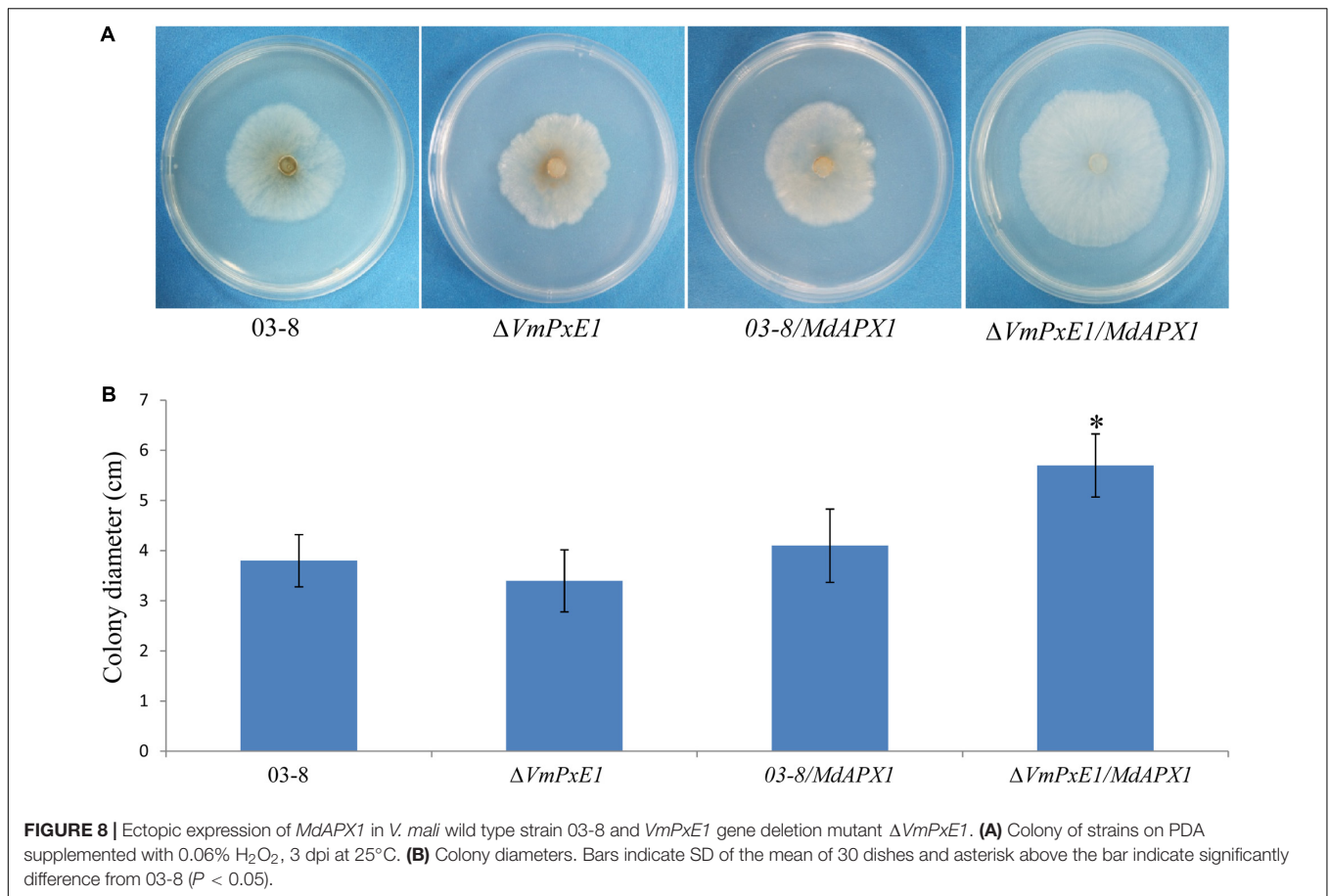
VmPxE1 from *V. mali*, a necrotrophic pathogen, could suppress the BAX-induced PCD, which implies that VmPxE1 is involved in plant immune response, resulting in weakened host defense. This is supported by the fact that deletion of *VmPxE1* reduced virulence of *V. mali* but did not affect fungal vegetative growth and sporulation. Plant cell death induced by hypersensitive responses is believed to be detrimental for biotrophic pathogens, because of the reduction of vivosphere and restriction of hyphae extension (Gilchrist, 1998). On the other hand, plant cell death could facilitate the infection of necrotrophic pathogens and hence presence of effectors inducing cell death may be important for necrotrophic pathogens during the early infection process (Govrin and Levine, 2000; Wang et al., 2014). On the contrary, cell death suppressor effectors are vitally important for biotrophic and hemibiotrophic pathogens (Sharpee et al., 2017). However, the present study suggested that suppressing cell death may also play an important role in the infection by *V. mali*. Therefore, *V. mali* may not need to kill host cells rapidly but control cell



death via these suppressors to enable successful colonization over time.

The target of VmPxE1 was identified to be an ascorbate peroxidase MdAPX1 from apple. Ascorbate peroxidase utilizes ascorbic acid as a specific electron donor and scavenges H₂O₂. During the plant–pathogen interaction, oxidative burst is concurrent and accompanied with the production of reactive oxygen species (ROS) (Apel and Hirt, 2004; Daudi et al., 2012). The ROS act as antimicrobial molecules and are also important as a signal related to plant disease resistance (Suzuki et al., 2011; Gilroy et al., 2014). However, excessive ROS would be phytotoxic. Plants possess a complex mechanism to balance the pernicious and beneficial effects of ROS (Daudi et al., 2012). Peroxidases act as an equalizer beam in the defense system of plant by using phenols, amine, or heterocyclic compound as hydrogen donor and preferring H₂O₂ as reaction substrates. Therefore, peroxidases could regulate H₂O₂ and concentrations of hydrogen donors (Welinder, 1992; Hiner et al., 2002; Yoshida et al., 2003; Camejo et al., 2016). Changes in H₂O₂ concentration could induce pathogenesis-related proteins and affect plant signals of disease resistance such as salicylic acid (Klessig and Malamy, 1994; Passardi et al., 2005), and is closely associated

with hypersensitive cell death (Yoda et al., 2003). Apple tree possesses a large and complete peroxidase system that is well documented to be involved in its defense against pathogens (Camejo et al., 2016). The increased peroxidase activity in apple enhances resistance to *Glomerella* leaf spot (Araujo and Stadnik, 2013). Peroxidases increased activity associated with increased apple resistance to apple ring spot, blue mold, and apple scab (Valentines et al., 2005; Rachenko et al., 2014). These evidences suggest that this group of enzymes may play an important role in plant resistance to pathogens. Thus, it is not surprising that pathogens have to deal with peroxidases of its host. For instance *Ustilago maydis* effector Pep1 targets a maize peroxidase POX12 *in vivo* and suppresses the early immune responses of maize (Hemetsberger et al., 2012). Moreover, ascorbate peroxidase is reported to be closely related to plant resistance (Sarowar et al., 2005) and to act as a defense enzyme, enhancing systemic acquired resistance (Kvaratskhelia et al., 1997). We may, therefore, speculate that *MdAPX1* is involved in the apple defense system against pathogens. For successful infection, *MdAPX1* needs to be suppressed. Ectopic expression of *MdAPX1* in $\Delta VmPxE1$ ($\Delta VmPxE1/MdAPX1$) showed stronger resistance to H₂O₂ than ectopic expression of *MdAPX1* in wild



type 03-8 (03-8/*MdAPX1*), indicating that *VmPxE1* interfered the function of *MdAPX1*. Further research is needed to confirm whether and, if so, how *VmPxE1* disrupts the function of *MdAPX1*.

CONCLUSION

We identified a cell death suppressor effector *VmPxE1* from necrotrophic pathogen *V. mali* that contributes to fungal virulence and targets a peroxidase *MdAPX1* of apple. Although, the putative effector is highly expressed during the apple infective process, *VmPxE1* was also expressed in basal conditions and an endogenous function could not be excluded. This data indirectly pointed that *VmPxE1* disturbs the function of *MdAPX1*. Further research is needed to confirm whether and, if so, how *VmPxE1* disrupts the function of *MdAPX1*.

AUTHOR CONTRIBUTIONS

LH, MZ, and HF contributed to the design of the work. MZ, HF, YZ, LS, and CG performed the experiments. MZ and YZ analyzed the sequencing data. MZ, HF, and XX wrote and revised the manuscript. LH was responsible for all aspects of this study.

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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.2018.00821/full#supplementary-material>

FIGURE S1 | **(A)** RT-PCR detected *BAX*, *VmPxE1* and *eGFP* expression in *N. benthamiana* tissues at different locations: *VmPxE1* in location 1; *eGFP* in location 2; *VmPxE1* in location 3; *BAX* in location 3; *eGFP* in location 4; *BAX* in location 4; *GAPDH*. Total RNA was extracted 48 h after the second infiltration. **(B)** Validation of proteins expression of *BAX*, HA: *VmPxE1* by western blot with respective monoclonal murine antibodies injected in location 3.

FIGURE S2 | Generation and identification of gene *VmPxE1* deletion and complementation mutants. **(A)** Flanks homologous recombination facilitated target gene replacement. The resistance gene cassette denoted by a gray arrow with homologous flank arms replaced the target gene denoted by a blue arrow based on homologous recombination. **(B)** Southern blot hybridization analysis of *VmPxE1* gene deletion mutants using primer NeoF/NeoR with digoxin marked nucleic fragment of *Neo* gene as hybridization probe. **(C)** Confirmation of *VmPxE1* knockout mutants by PCR analysis with four pairs of primers. The wild type 03-8 was as control. 1: 7F/Neo-CR detected upstream fusion segment. 2: Neo-CF/8R detected downstream fusion segment. 3: 5F/6R detected targeted gene and 4: Neo-CF/Neo-CR detected incoming resistant gene *Neo*. M: Maker. **(D)** PCR analysis was performed with primers 5F/6R to identify $\Delta VmPxE1/VmPxE1$

complementation mutant. 1: Wild type 03-8. 2: *VmPxE1* gene deletion mutants. 3: $\Delta VmPxE1/VmPxE1$ complementation mutant. M: Maker.

FIGURE S3 | RT-PCR detects MdAPX1 and *VmPxE1* in transformed strains 03-8/MdAPX1 and $\Delta VmPxE1/MdAPX1$ after cultured on PDA supplemented with 0.06% H₂O₂ 3 days at 25°C.

TABLE S1 | Primers for cloning gene *VmPxE1* to vectors, *VmPxE1* gene deletion and PCR analysis in this study. The red color stands for corresponding directing sequences for homologous recombination and yellow color stands for corresponding restriction enzyme cutting site.

TABLE S2 | primers for RT-PCR and qRT-PCR in this study.

REFERENCES

- Albert, M. (2013). Peptides as triggers of plant defence. *J. Exp. Bot.* 64, 5269–5279. doi: 10.1093/jxb/ert275
- Apel, K., and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55, 373–399. doi: 10.1146/annurev.arplant.55.031903.141701
- Araujo, L., and Stadnik, M. J. (2013). Cultivar-specific and ulvan-induced resistance of apple plants to Glomerella leaf spot are associated with enhanced activity of peroxidases. *Acta Sci. Agric.* 35, 287–293.
- Boller, T., and He, S. Y. (2009). Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science* 324, 742–744. doi: 10.1126/science.1171647
- Bruno, K. S., Tenjo, F., Li, L., Hamer, J. E., and Xu, J.-R. (2004). Cellular localization and role of kinase activity of *PMK1* in *Magnaporthe grisea*. *Eukaryot. Cell* 3, 1525–1532. doi: 10.1128/EC.3.6.1525-1532.2004
- Caillaud, M. C., Asai, S., Rallapalli, G., Piquerez, S., Fabro, G., and Jones, J. D. (2013). A downy mildew effector attenuates salicylic acid-triggered immunity in Arabidopsis by interacting with the host mediator complex. *PLoS Biol.* 11:e1001732. doi: 10.1371/journal.pbio.1001909
- Camejo, D., Guzmán-Cedeño, A., and Moreno, A. (2016). Reactive oxygen species, essential molecules, during plant-pathogen interactions. *Plant Physiol. Biochem.* 103, 10–23. doi: 10.1016/j.plaphy.2016.02.035
- Daudi, A., Cheng, Z., O'Brien, J. A., Mammarella, N., Khan, S., Ausubel, F. M., et al. (2012). The apoplastic oxidative burst peroxidase in *Arabidopsis* a major component of pattern-triggered immunity. *Plant Cell* 24, 275–287. doi: 10.1105/tpc.111.093039
- de Jonge, R., van Esse, H. P., Kombrink, A., Shinya, T., Desaki, Y., and Bours, R. (2010). Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science* 329, 953–955. doi: 10.1126/science.1190859
- Djamei, A., Schipper, K., Rabe, F., Ghosh, A., Vincon, V., Kahnt, J., et al. (2011). Metabolic priming by a secreted fungal effector. *Nature* 478, 395–398. doi: 10.1038/nature10454
- Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* 11, 539–548. doi: 10.1038/nrg2812
- Dou, D., Kale, S. D., Wang, X., Jiang, R. H., Bruce, N. A., Arredondo, F. D., et al. (2008). RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen-encoded machinery. *Plant Cell* 20, 1930–1947. doi: 10.1105/tpc.107.056093
- Gao, J., Li, Y., Ke, X., Kang, Z., and Huang, L. (2011). Development of genetic transformation system of *Valsa mali* of apple mediated by PEG. *Acta Microbiol. Sin.* 51, 1194–1199.
- Geitz, R. D., Scheistl, R. H., Wellems, A. R., and Woods, R. A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* 11, 355–360. doi: 10.1002/yea.320110408
- Gilchrist, D. (1998). Programmed cell death in plant disease: the purpose and promise of cellular suicide. *Annu. Rev. Phytopathol.* 36, 393–414. doi: 10.1146/annurev.phyto.36.1.393
- Gilroy, S., Suzuki, N., Miller, G., Choi, W.-G., Toyota, M., Deviredy, A. R., et al. (2014). A tidal wave of signals: calcium and ROS at the forefront of rapid systemic signaling. *Trends Plant Sci.* 19, 623–630. doi: 10.1016/j.tplants.2014.06.013
- Giraldo, M. C., and Valent, B. (2013). Filamentous plant pathogen effectors in action. *Nat. Rev. Microbiol.* 11, 800–814. doi: 10.1038/nrmicro3119
- Govrin, E. M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol.* 10, 751–757. doi: 10.1016/S0960-9822(00)00560-1
- Gu, B. A., Kale, S. D., Wang, Q. H., Wang, D. H., Pan, Q. N., Cao, H., et al. (2011). Rust secreted protein Ps87 is conserved in diverse fungal pathogens and contains a RXLR-like motif sufficient for translocation into plant cells. *PLoS One* 6:e27217. doi: 10.1371/journal.pone.0027217
- Hemetsberger, C., Herrberger, C., Zechmann, B., Hillmer, M., and Doehlemann, G. (2012). The *Ustilago maydis* effector Pep1 suppresses plant immunity by inhibition of host peroxidase activity. *PLoS Pathog.* 8:e1002684. doi: 10.1371/journal.ppat.1002684
- Hiner, A. N., Raven, E. L., Thorneley, R. N., García-Cánovas, F., and Rodríguez-López, J. N. (2002). Mechanisms of compound I formation in heme peroxidases. *J. Inorg. Biochem.* 91, 27–34. doi: 10.1016/S0162-0134(02)00390-2
- Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M., and Sakaki, Y. (2001). A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4569–4574. doi: 10.1073/pnas.061034498
- Jones, J. D., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286
- Ke, X., Yin, Z., Song, N., Dai, Q., Voegelé, R. T., Liu, Y., et al. (2014). Transcriptome profiling to identify genes involved in pathogenicity of *Valsa mali* on apple tree. *Fungal Genet. Biol.* 68, 31–38. doi: 10.1016/j.fgb.2014.04.004
- Klessig, D. F., and Malamy, J. (1994). The salicylic acid signal in plants. *Plant Mol. Biol.* 26, 1439–1458. doi: 10.1007/BF00016484
- Kvaratskhelia, M., George, S. J., and Thorneley, R. N. (1997). Salicylic acid is a reducing substrate and not an effective inhibitor of ascorbate peroxidase. *J. Biol. Chem.* 272, 20998–21001. doi: 10.1074/jbc.272.34.20998
- Lacomme, C., and Santa Cruz, S. (1999). BAX-induced cell death in tobacco is similar to the hypersensitive response. *Proc. Natl. Acad. Sci. U.S.A.* 96, 7956–7961. doi: 10.1073/pnas.96.14.7956
- Lee, D. H., Lee, S. W., Choi, K. H., Kim, D. A., and Uhm, J. Y. (2006). Survey on the occurrence of apple diseases in Korea from 1992 to 2000. *Plant Pathol. J.* 22, 375–380. doi: 10.5423/PPJ.2006.22.4.375
- Li, Z., Yin, Z., Fan, Y., Xu, M., Kang, Z., and Huang, L. (2015). Candidate effector proteins of the necrotrophic apple canker pathogen *Valsa mali* can suppress BAX-induced PCD. *Front. Plant Sci.* 6:579. doi: 10.3389/fpls.2015.00579
- Ma, Z., Zhu, L., Song, T., Wang, Y., Zhang, Q., Xia, Y., et al. (2017). A paralogous decoy protects *Phytophthora sojae* apoplastic effector PsXEG1 from a host inhibitor. *Science* 355, 710–714. doi: 10.1126/science.aai7919
- Mueller, A. N., Ziemann, S., Treitschke, S., Assmann, D., and Doehlemann, G. (2013). Compatibility in the *Ustilago maydis*-maize interaction requires inhibition of host cysteine proteases by the fungal effector Pit2. *PLoS Pathog.* 9:e1003177. doi: 10.1371/journal.ppat.1003177
- Oliva, R., Win, J., Raffaele, S., Boutemy, L., Bozkurt, T. O., Chaparro-García, A., et al. (2010). Recent developments in effector biology of filamentous plant pathogens. *Cell. Microbiol.* 12, 705–715. doi: 10.1111/j.1462-5822.2010.01471.x

- Passardi, F., Cosio, C., Penel, C., and Dunand, C. (2005). Peroxidases have more functions than a Swiss army knife. *Plant Cell Rep.* 24, 255–265. doi: 10.1007/s00299-005-0972-6
- Rachenko, M. A., Rachenko, E. I., Romanova, I. M., Zhivet'yev, M. A., and Graskova, I. A. (2014). Peroxidase activity and isoforms in the leaves of apple tree varieties differing in scab resistance. *J. Stress Physiol. Biochem.* 10, 78–83.
- Rovenich, H., Boshoven, J. C., and Thomma, B. P. (2014). Filamentous pathogen effector functions: of pathogens, hosts and microbiomes. *Curr. Opin. Plant Biol.* 20, 96–103. doi: 10.1016/j.pbi.2014.05.001
- Sarowar, S., Kim, E. N., Kim, Y. J., Ok, S. H., Kim, K. D., Hwang, B. K., et al. (2005). Overexpression of a pepper ascorbate peroxidase-like 1 gene in tobacco plants enhances tolerance to oxidative stress and pathogens. *Plant Sci.* 169, 55–63. doi: 10.1016/j.plantsci.2005.02.025
- Shan, W. X., Cao, M., Dan, L. U., and Tyler, B. M. (2004). The *Avr1b* locus of *Phytophthora sojae* encodes an elicitor and a regulator required for Avirulence on soybean plants carrying resistance gene Rps1b. *Mol. Plant Microbe Interact.* 17, 394–403. doi: 10.1094/MPMI.2004.17.4.394
- Sharpee, W., Oh, Y., Yi, M., Franck, W., Eyre, A., Okagaki, L. H., et al. (2017). Identification and characterization of suppressors of plant cell death (SPD) effectors from *Magnaporthe oryzae*. *Mol. Plant Pathol.* 18, 850–863. doi: 10.1111/mpp.12449
- Suzuki, N., Miller, G., Morales, J., Shulaev, V., Torres, M. A., and Mittler, R. (2011). Respiratory burst oxidases: the engines of ROS signaling. *Curr. Opin. Plant Biol.* 14, 691–699. doi: 10.1111/mpp.12449
- Tian, M., Benedetti, B., and Kamoun, S. (2005). A second Kazal-like protease inhibitor from *Phytophthora infestans* inhibits and interacts with the apoplastic pathogenesis-related protease P69B of tomato. *Plant Physiol.* 138, 1785–1793. doi: 10.1104/pp.105.061226
- Valentines, M. C., Vilaplana, R., Usall, J., and Larrigaudière, C. (2005). Involvement of enzymatic browning and peroxidase activity as resistance mechanisms in 'golden delicious' apples. *Acta Hort.* 682, 2041–2048. doi: 10.17660/ActaHortic.2005.682.277
- Vleeshouwers, V. G., and Oliver, R. P. (2014). Effectors as tools in disease resistance breeding against biotrophic, hemibiotrophic, and necrotrophic plant pathogens. *Mol. Plant Microbe Interact.* 27, 196–206. doi: 10.1094/MPMI-10-13-0313-IA
- Waadt, R., Schmidt, L. K., Lohse, M., Hashimoto, K., Bock, R., and Kudla, J. (2008). Multicolor bimolecular fluorescence complementation reveals simultaneous formation of alternative CBL/CIPK complexes in planta. *Plant J.* 56, 505–516. doi: 10.1111/j.1365-313X.2008.03612.x
- Wang, Q., Han, C., Ferreira, A. O., Yu, X., Ye, W., Tripathy, S., et al. (2011a). Transcriptional programming and functional interactions within the *Phytophthora sojae* RXLR effector repertoire. *Plant Cell* 23, 2064–2086. doi: 10.1105/tpc.111.086082
- Wang, X., Jiang, N., Liu, J., Liu, W., and Wang, G.-L. (2014). The role of effectors and host immunity in plant–necrotrophic fungal interactions. *Virulence* 5, 722–732. doi: 10.4161/viru.29798
- Wang, X., Wei, J., Huang, L., and Kang, Z. (2011). Re-evaluation of pathogens causing Valsa canker on apple in China. *Mycologia* 103, 317–324. doi: 10.3852/09-165
- Welinder, K. G. (1992). Superfamily of plant, fungal and bacterial peroxidases. *Curr. Opin. Plant Biol.* 2, 388–393. doi: 10.1016/0959-440X(92)90230-5
- Yin, Z., Ke, X., Huang, D., Gao, X., Voegelé, R. T., Kang, Z., et al. (2013). Validation of reference genes for gene expression analysis in *Valsa mali* var. *mali* using real-time quantitative PCR. *World J. Microbiol. Biotechnol.* 29, 1563–1571. doi: 10.1007/s11274-013-1320-6
- Yin, Z., Liu, H., Li, Z., Ke, X., Dou, D., Gao, X., et al. (2015). Genome sequence of *Valsa* canker pathogens uncovers a potential adaptation of colonization of woody bark. *New Phytol.* 208, 1202–1216. doi: 10.1111/nph.13544
- Yoda, H., Yamaguchi, Y., and Sano, H. (2003). Induction of hypersensitive cell death by hydrogen peroxide produced through polyamine degradation in tobacco plants. *Plant Physiol.* 132, 1973–1981. doi: 10.1104/pp.103.02.4737
- Yoshida, K., Kaothien, P., Matsui, T., Kawaoka, A., and Shinmyo, A. (2003). Molecular biology and application of plant peroxidase genes. *Appl. Microbiol. Biotechnol.* 60, 665–670. doi: 10.1007/s00253-002-1157-7
- Yu, J.-H., Hamari, Z., Han, K.-H., Seo, J.-A., Reyes-Domínguez, Y., and Scazzocchio, C. (2004). Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet. Biol.* 41, 973–981. doi: 10.1016/j.fgb.2004.08.001
- Zhang, M., Li, Q., Liu, T., Liu, L., Shen, D., Zhu, Y., et al. (2015). Two cytoplasmic effectors of *Phytophthora sojae* regulate plant cell death via interactions with plant catalases. *Plant Physiol.* 167, 164–175. doi: 10.1104/pp.114.252437
- Zhou, X., Zhang, H., Li, G., Shaw, B., and Xu, J.-R. (2012). The Cyclase-associated protein Cap1 is important for proper regulation of infection-related morphogenesis in *Magnaporthe oryzae*. *PLoS Pathog.* 8:e1002911. doi: 10.1371/journal.ppat.1002911

Conflict of Interest Statement: 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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