



VmPacC Is Required for Acidification and Virulence in *Valsa mali*

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The role of the transcription factor PacC has been characterised in several pathogenic fungi, and it affects virulence via several mechanisms. In this study, we examined the role of the PacC homolog VmPacC in *Valsa mali*, the causal agent of apple canker disease. We found that the expression of VmPacC was up-regulated in neutral and alkaline pH and during infection. At pH 6–10, the radial growth of a VmPacC deletion mutant decreased compared to wild-type. In addition, the sensitivity to oxidative stress of the VmPacC deletion mutant was impaired, as its growth was more severely inhibited by H₂O₂ than that of the wild-type. The lesion size caused by the VmPacC deletion mutant was smaller than that of the wild-type on apple leaves and twigs. Interestingly, expression of pectinase genes increased in deletion mutant during infection. To further confirm the negative regulation, we generated dominant activated C-27 allele mutants that constitutively express VmPacC. The pectinase activity of activated mutants was reduced at pH 4. We further observed that *V. mali* can acidify the pH during infection, and that the capacity for acidification was impaired after VmPacC deletion. Furthermore, VmPacC is involved in the generation of citric acid, which affects virulence. These results indicate that VmPacC is part of the fungal responses to neutral and alkaline pH and oxidative stress. More importantly, VmPacC is required for acidification of its environment and for full virulence in *V. mali*.

Keywords: *Malus domestica*, deletion mutant, pH regulation, pectinase, virulence

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INTRODUCTION

To adapt to changing environmental conditions, fungi require an intracellular pH homeostasis system and regulatory mechanism that ensures that directly exposed molecules such as secreted enzymes can function at optimal pH (Penalva and Arst, 2002). An important system to regulate ambient pH in fungi is the Pal signalling pathway. This pathway includes seven components: PalA, PalB, PalC, PalF, PalH, PalI, and PacC. PacC is a transcription factor that regulates pH-dependent gene expression at the end of the pH signalling pathway (Peñalva et al., 2008). In *Aspergillus nidulans*, the PacC polypeptide shows a closed conformation with a processing protease domain inaccessible at acidic conditions. However, the full-length polypeptide is cleaved after a shift to neutral or alkaline conditions, and an intermediate 53 kDa fragment is generated (Díez et al., 2002; Peñalva et al., 2008). The 53-kDa fragment is cleaved again by the proteasome, and results in the final active form of 27-kDa PacC27 (Hervas-Aguilar et al., 2007). In addition, the binding motif of the PacC transcription factor is GCCARG. Transcription of alkaline-expressed genes is activated by PacC at alkaline pH, whereas transcription of acid-expressed genes is repressed at the same condition (Tilburn et al., 1995; Penalva and Espeso, 1996).

In several plant pathogenic fungi, the involvement of PacC in virulence has been demonstrated. Deletion of *PacC* homologs reduces fungal virulence in *Colletotrichum gloeosporioides* (Yakoby et al., 2000), *Penicillium digitatum* (Zhang et al., 2013), *Magnaporthe oryzae* (Landraud et al., 2013), *Fusarium oxysporum* (Caracuel et al., 2003), and *Sclerotinia sclerotiorum* (Rollins, 2003). PacC not only detects environment conditions, but also modifies these conditions via acid or ammonia secretion. Some plant pathogens are therefore grouped into two categories. The first group can alkalise their host tissue and enhance virulence by producing ammonia. Examples include *Colletotrichum* spp. and *Alternaria alternata* (Eshel et al., 2002; Alkan et al., 2009). The other group of pathogens, including *Penicillium* spp., *Botrytis cinerea*, and *S. sclerotiorum*, can acidify the host tissue by producing acids (Wubben et al., 2000; Rollins and Dickman, 2001; Barad et al., 2012; Zhang et al., 2013). This modulation of host environment provides a suitable condition for the function of virulence factors like secreted enzymes. It may also serve as a signal to activate the production of these virulence factors. In addition to modify environment pH, PacC is also known to regulate some cell wall degrading enzymes such as polygalacturonase (Rollins, 2003), pectate lyase (Kramer-Haimovich et al., 2006), and endoglucanase to affect fungal virulence (Eshel et al., 2002).

Apple Valsa canker is a bark disease of apple trees (*Malus* sp.) that seriously imperils apple production in China (Wang et al., 2014), Korea (Lee et al., 2006), and Japan (Abe et al., 2007). This disease is caused by the parasite fungus *Valsa mali*. *V. mali* infects the bark of apple trees through various wounds such as pruning ends, sunscalds, frostbites, and some mechanical injuries (Ke et al., 2013). Following colonisation of the wounded tissue, the hyphae spread to all bark tissues, resulting in severe tissue maceration and necrosis. As a plant pathogenic fungus without specialised penetration structures, *V. mali* secretes hydrolytic enzymes, particularly pectinases, involved in disease development and pathogenesis (Ke et al., 2013, 2014; Feng et al., 2017b). Pectinases are considered virulence factors because the virulence of several *V. mali* pectinase gene deletion mutants is attenuated (Yin et al., 2015). However, these findings do not clarify the role of PacC in the regulation of virulence in this important woody plant fungal pathogens. Understanding the role of PacC during *V. mali* infection might allow the development of novel sustainable apple Valsa canker management strategies. In the present study, we have investigated the role of PacC in pH acidification, oxidative stress, and pectinase gene expression during *V. mali* infection.

MATERIALS AND METHODS

Strains and Culture Conditions

The wild-type strain of *V. mali* named 03-8 (Laboratory of Integrated Management of Plant Diseases in College of Plant Protection, Northwest A&F University) was used (Yin et al., 2015). All strains including the wild-type and the transformants generated in this work were propagated on potato dextrose agar (PDA, 20% potato extract, 2% dextrose, 1.5% agar) when

necessary. Potato dextrose broth (PDB, 20% potato extract, 2% dextrose) was used for measuring the dynamic pH change or for liquid culture.

Growth of wild-type and mutant strains on PDA media at different pH were assayed by measuring colony diameters 2 days post-inoculation (dpi) at 25°C. PDA medium was adjusted with HCl or NaOH to pH 3–10. The PDA media mended NaCl (0.1 M), H₂O₂ (3 mM), congo red (CR, 300 mg/L), or sodium dodecyl sulfonate (SDS 0.01%) were used for stress response assays. For selection of transformants during gene deletion, TB₃ medium (0.3% yeast extract, 0.3% casamino acids, 20% sucrose, 1.5% agar) supplemented with 100 μg/mL hygromycin B (Calbiochem, La Jolla, CA, United States) or 100 μg/mL geneticin (Sigma, St. Louis, MO, United States) was used. To determine protein concentration and pectinase activity, synthetic medium (SM) (Srivastava et al., 2012) with 1% pectin as the sole carbon source was used. Buffered SM medium was obtained using 0.05 M Na₂HPO₄ and 0.05 M C₄H₂O₇ to maintain pH 4 (38.5% volume ratio of Na₂HPO₄) or pH 7 (82.4% volume ratio of Na₂HPO₄).

Twig and Leave Infection Assays

Wild-type and mutant strains were cultured on PDA for 2 days. Five-mm agar plugs were taken from the edge of a colony and were inoculated in the armature wound on the fourth or fifth leave from the top of a branch at 25°C for 3 days. Lesion diameters were recorded. For twig assays, the agar plugs were inoculated in the scald wound on 1-year old twigs of *Malus domestica* borkh. 'Fuji' at 25°C for 9 days. The lesion lengths were recorded (Wu et al., 2017b).

Nucleic Acid Isolation and Manipulation

VmPacC genes were originally identified through homology searches in the *V. mali* genomic sequence (Accession No. JUIY00000000.1) using the PacC amino acid sequence of *A. nidulans* as query (Q002.2.1). The neighbour-joining method was used to construct phylogenetic trees of Pal pathway proteins (Tamura et al., 2007). **Supplementary Table S1** shows the pectinase genes annotated in the *V. mali* genome.

Gene disruption constructs were generated by replacing the complete open reading frame (ORF) of *VmPacC* gene (**Supplementary Figure S2A**). The primer pairs VmPacC-1F/2R and VmPacC-3F/4R were used to amplify the upstream and downstream flanking sequences, respectively, of the target gene (primer sequences are shown in **Supplementary Table S2**). The hygromycin resistance gene (*hph*) fragment was amplified with the primer pair HYG/F and HYG/R. Deletion cassettes were constructed using double-joint PCR as described (Yu et al., 2004). The deletion cassettes were transformed into protoplasts of the 03-8 strain using the polyethylene glycol (PEG) method as described (Gao et al., 2011). Following screening in hygromycin-containing medium, the transformants were tested using PCR with primer pairs VmPacC-5F/6R, VmPacC-7F/H855R, or H856F/VmPacC-8R to confirm gene replacement events (**Supplementary Table S2**). Putative gene deletion mutants were further confirmed using Southern blotting with target (probe p) and hygromycin (probe h) gene probes and

the DIG-High Prime DNA Labelling and Detection Starter Kit II (Roche, Penzberg, Germany).

Complementation mutants and activated mutants were generated using the gap repair approach by co-transformation of *VmPacC* gene or activated fragments (**Supplementary Figure S3A**) amplified with primer pairs VmPacC-C (27)-F/R and *Clal*-digested plasmid pFL2 into yeast strain XK1-25 (Zhou et al., 2012; Wu et al., 2017a). Trp⁺ yeast transformants were screened for the desired fusion constructs, which were confirmed by sequencing and transformed into the *VmPacC* deletion mutant. Geneticin-resistant transformants of complementation with the desired constructs were identified using PCR with primer pairs VmPacC-C-F/R and Southern blotting. The activated geneticin-resistant transformants were detected using PCR with primer pairs VmPacC27-F/R (**Supplementary Figure S3B**). The activated mutants were further identified by measuring the growth at pH 9 in unbuffered PDA for 3 days.

Transcript levels of *VmPacC* and pectinase-related genes were determined using real-time PCR. For expression of *VmPacC* in different pH, strains were cultured on PDA for 2 days. Five-mm agar plugs were taken from the edge of a colony and were cultured in a non-buffered PDB medium (pH 5.5) for 48 h. Mycelia were then transferred to fresh media buffered to pH 4, pH 7, or pH 9 for 6 h. The colonised apple tree bark was sampled as described (Ke et al., 2014). RNA was isolated with the TRIzol reagent (Invitrogen, Carlsbad, CA, United States) as described (Yin et al., 2013). For qRT-PCR assays, we used the Fermentas (Hanover, MD, United States) 1st strand cDNA synthesis kit following instructions of the manufacturer. The glyceraldehyde-6-phosphate dehydrogenase (*G6PDH*) gene of *V. mali* was used as internal control (Yin et al., 2013). Relative transcript levels of each gene were calculated by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Data from three replicates were used to calculate means and standard deviations. Statistical analysis was done using the Student's *t*-test implemented in the SAS software package (SAS Institute), $P < 0.05$. Primers used for gene expression are listed in **Supplementary Table S2**.

Pectinase Activity and Protein Concentration Assays

Five-mm agar plugs were taken from the edge of a colony and were cultured in PDB medium for 48 h. Mycelia were transferred to SM buffered to pH 4 or pH 7 and supplemented with 1% pectin as the sole carbon source for 12 h. Pectinase activity was quantified using the 3,5-dinitrosalicylic acid (DNS) method as described (Wu et al., 2017b).

Total extracellular protein content in culture supernatant was measured using the Bradford Protein Assay Kit (TIANGEN, China) with absorbance at 595 nm, and using bovine serum albumin as the standard.

pH and Citric Acid Measurements

The pH value of liquid PDB was measured with a pH electrode (Mettler Toledo, Shanghai, China) at different times after incubation. The pH of inoculation sites on the apple tree bark was

measured using a flathead pH electrode as described (Schmidt et al., 2001). Three replicates were tested for each treatment.

High-performance liquid chromatography (HPLC) was used to determine citric acid content (Chinnici et al., 2005). Five-mm agar plugs were taken from the edge of a colony and were cultured in PDB medium for 48 h. Mycelia were transferred to SM supplemented with 0.5% saccharose as the sole carbon source for 48 h. The supernatant was filtered through a 0.22 μ m cellulose acetate membrane before injection and measured using ion-exclusion chromatography. A Waters 600E HPLC apparatus with an attached refractive index detector (2414 RI) was used. Samples were separated in a Sepax Carbomix H-NP column under the following conditions: 2.5 mM H₂SO₄ mobile phase, 55°C column temperature, 0.6 mL/min flow-rate, and 30 min or less of analysis time. Peak data were collected with Empower LC solution. Citric acid standard (Solarbio, China) at 0.01–1 mg/mL was used for the standard curve.

RESULTS

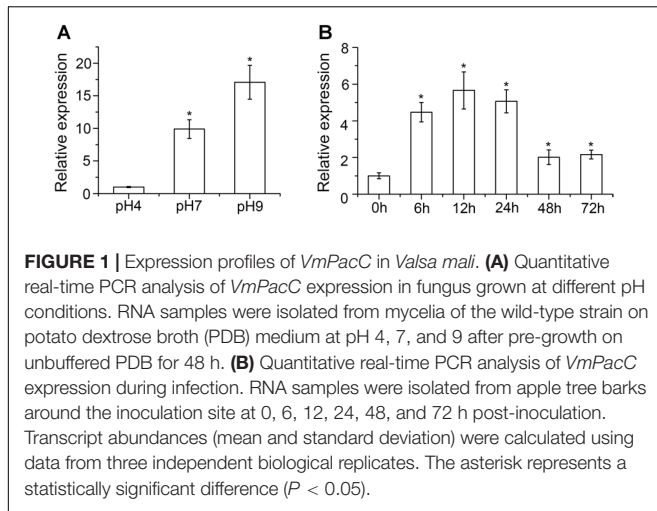
Gene Expression Analysis of *VmPacC* in *V. mali*

The *V. mali* genome contains single copy genes for pH-signalling pathway proteins designated VmPalA, VmPalB, VmPalC, VmPalF, VmPalI, VmPalH, and VmPacC. Analysis of the amino acid sequences revealed significant similarities to four fungi pH signalling pathway protein sequences (**Supplementary Figure S1A**). *VmPacC* codes for 560 amino acid residues interrupted by two introns, being smaller than the *PacC/Rim101* orthologs of *A. nidulans* (678 aa) and yeast (635 aa). In addition, *VmPacC* contains classical zinc-finger domain and Zinc-finger double domains at its N-terminal region (residues 116–138 and 102–127) (**Supplementary Figure S1B**).

Expression of *VmPacC* in different pH and infection conditions was determined. After transferring to different pH for 6 h, qRT-PCR analysis showed that *VmPacC* gene expression was upregulated 9.9- and 17-fold in pH 7 and pH 9, respectively, compared to that of pH 4 (**Figure 1A**). These results show the important regulatory role of this gene in alkaline conditions. Upregulated *VmPacC* expression was also observed during infection. After inoculating *V. mali* in 1 year-old twigs, the *VmPacC* gene expression increased and reached a peak 12 h post-inoculation, and then decreased and remained stable at twofold (**Figure 1B**). These results suggest that *VmPacC* might be involved in *V. mali* virulence.

VmPacC Is Required for Mycelial Growth at Alkaline pH

To investigate the roles of *VmPacC* genes in *V. mali*, we generated a deletion mutant in which the entire ORF of *VmPacC* was deleted. We constructed a gene replacement cassette by placing upstream and downstream flanking sequences next to *hph* resistance gene sequences (**Supplementary Figure S2A**). The cassette was introduced in protoplasts of the wild-type strain. Three *VmPacC* deletion mutants (Δ *VmPacC*) were



obtained from 215 hygromycin-resistant transformants and identified using PCR (**Supplementary Figure S2B**). When hybridised with the *VmPacC* ORF probe (probe p), a 5.6-kb *Clal* fragment was detected in the wild-type strain but not in the $\Delta VmPacC$ mutant. In addition, $\Delta VmPacC$ showed a single locus homologous recombination event after hybridisation with the hygromycin probe (probe h) (**Supplementary Figure S2C**). Complementation mutant strains ($\Delta VmPacC-C$) were also generated by re-introducing a wild-type allele of *VmPacC* in deletion mutants at an ectopic locus.

Wild-type, $\Delta VmPacC$, and $\Delta VmPacC-C$ strains were grown on unbuffered PDA at pH 3 to pH 10. The wild-type strain of

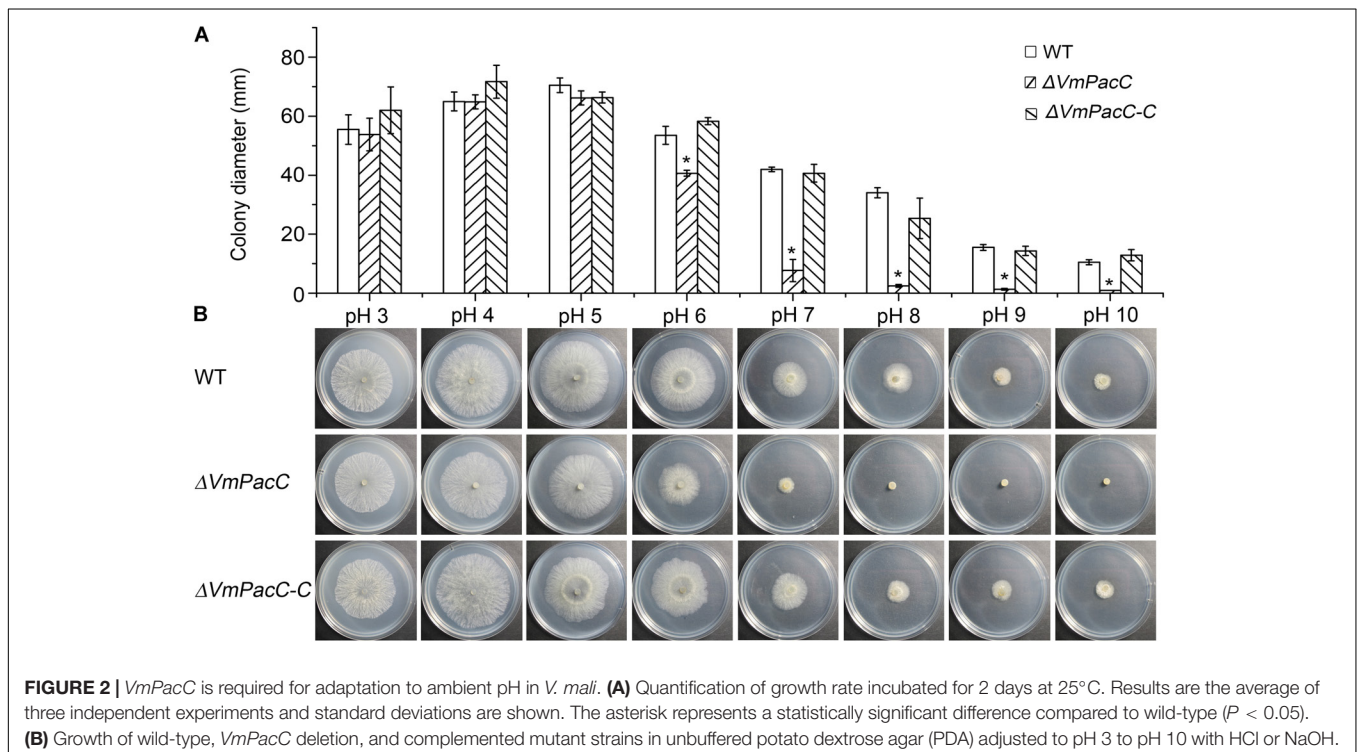
V. mali showed a more wide-range adaptation to pH 3 to pH 10. The colony diameters of the $\Delta VmPacC$ strain on PDA were similar to those of the wild-type at pH 3 to pH 5, but were reduced at pH 6 to pH 10. The deletion mutant hardly grew at pH 8 or higher (**Figure 2**). Normal growth was restored in the $\Delta VmPacC-C$ mutant, indicating that *VmPacC* regulates growth in alkaline conditions.

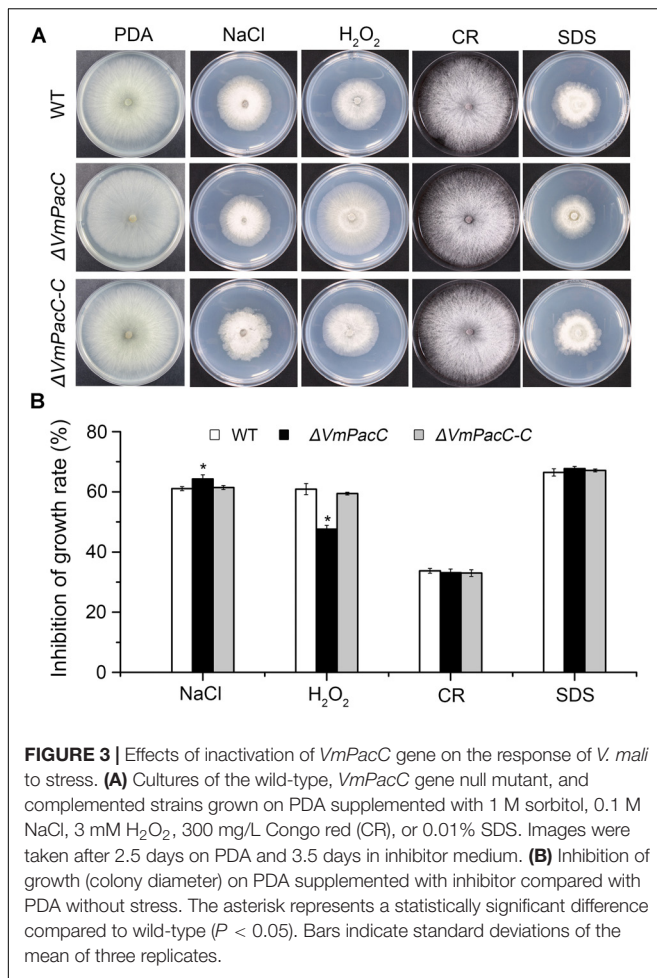
VmPacC Is Involved in the Oxidative Stress Response

To test whether *VmPacC* is involved in the response to abiotic stress, we measured the colony diameters of wild-type, $\Delta VmPacC$, and $\Delta VmPacC-C$ strains on PDA supplemented with NaCl, H_2O_2 , CR, or SDS. Results showed that the growth inhibition of NaCl on the $\Delta VmPacC$ mutant was higher than that on the wild-type or complemented mutant strains. However, the sensitivity of the *VmPacC* deletion mutant was reduced on PDA supplemented with H_2O_2 . In addition, no significant differences were observed between wild-type, $\Delta VmPacC$, and $\Delta VmPacC-C$ on PDA supplemented with CR or SDS (**Figure 3**). These results suggest that *VmPacC* is involved in the response to oxidative stress.

VmPacC Is Required for Virulence

To determine whether *VmPacC* plays an important role in disease development, virulence assays were performed on leaves and twigs using the wild-type, $\Delta VmPacC$, and $\Delta VmPacC-C$ strains. The diameter of the lesions in leaves inoculated with the wild-type was 34 mm at 3 dpi, whereas that of $\Delta VmPacC$ -inoculated leaves was 17 mm. When twigs were





used as host, the length of lesions was also smaller in samples infected with the $\Delta VmPacC$ strain. In addition, infection with the complementation strains restored the wild-type phenotype (Figure 4). These results clearly indicate that *VmPacC* plays a significant role in controlling virulence in *V. mali*.

VmPacC Suppresses the Production of Pectinase

To further evaluate the role of *VmPacC* on the virulence factor pectinase, we determined the transcript levels of a dozen pectinase genes in the deletion mutant and compared to those of the wild-type strain in colonised apple tree bark. Unexpectedly, transcript levels of most pectinase genes increased to different degrees (Figure 5). These results suggest that *VmPacC* negatively regulates pectinase expression.

To further confirm the repressive effect of *VmPacC* on pectinase expression, we generated dominant activated allele mutants, strains C-27, which constitutively express *VmPacC* in an acid environment. C-27 strains showed normal growth compared to wild-type on alkaline PDA indicating the *VmPacC* was expressive (Supplementary Figure S3C). However, C-27 strains showed significantly reduced virulence (Figure 6A). When inoculated on solid medium with pectin as the sole

carbon source, the growth rate of C-27 was reduced compared with the wild-type and $\Delta VmPacC$ strains (Figure 6B). To determine the requirement of *VmPacC* for pH-dependent expression of pectinase genes, pectinase transcript levels and activity in the wild-type, $\Delta VmPacC$, and C-27 strains were assayed (Figures 6C,D). The results show that pectinase activity in the supernatant of C-27 strains was reduced compared to that of wild-type and $\Delta VmPacC$ strains at pH 4 (Figure 6E). At pH 7, the pectinase genes of $\Delta VmPacC$ were up regulated 3- to 7-fold, and protein content was also increased (Figure 6F). However, pectinase activity was similar in all strains possible because the optimum pH value of pectinases is 3.5 (Figure 6E) (Feng et al., 2017a). These results indicate that pectinases are suppressed by *VmPacC*.

VmPacC Is Required for Acidification of the *V. mali* Environment

To investigate the involvement of *VmPacC* in the modification of the environment, the dynamic change in pH of the wild-type, $\Delta VmPacC$, and $\Delta VmPacC-C$ strains was measured in an unbuffered liquid medium. The pH value of the wild-type strain decreased from the initial pH 5.8 to pH 3.4 in 120 h. Even though the same decrease in pH units was observed after 96 h in the *VmPacC* deletion mutant, the rate of descent was slower than in the wild-type (Figure 7A).

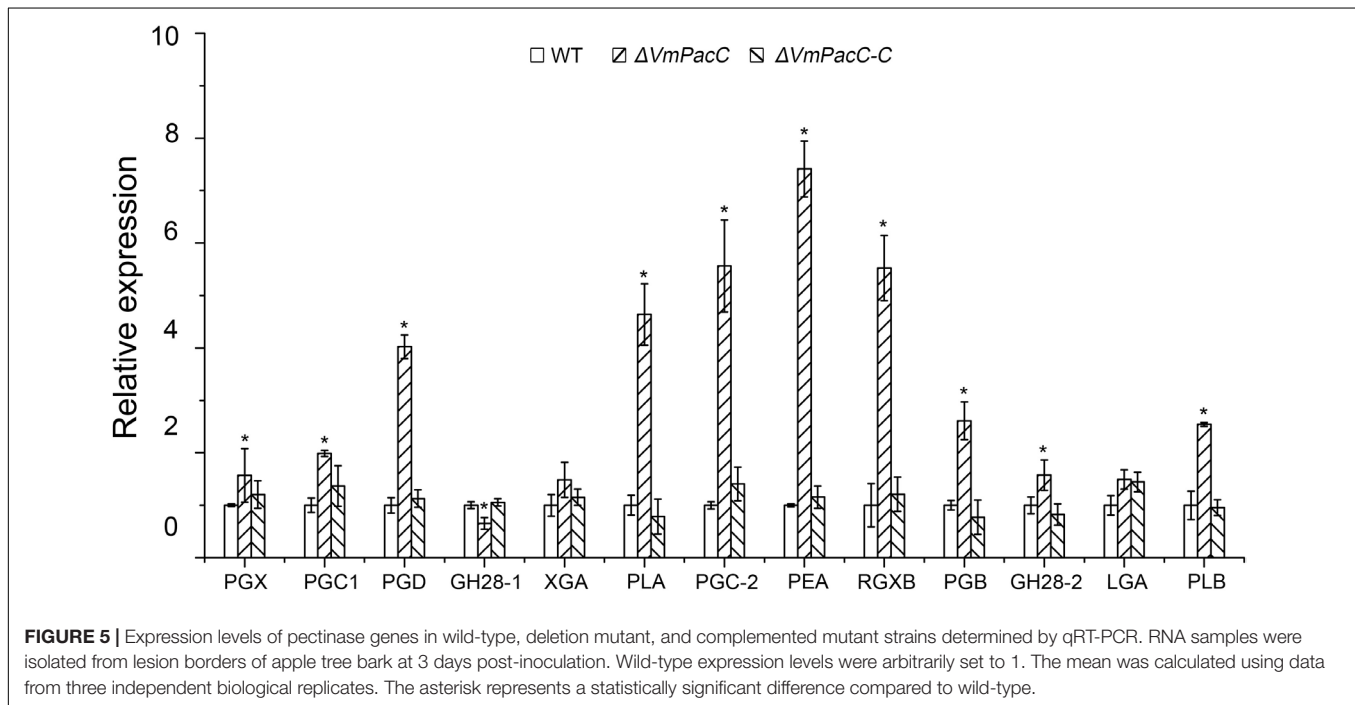
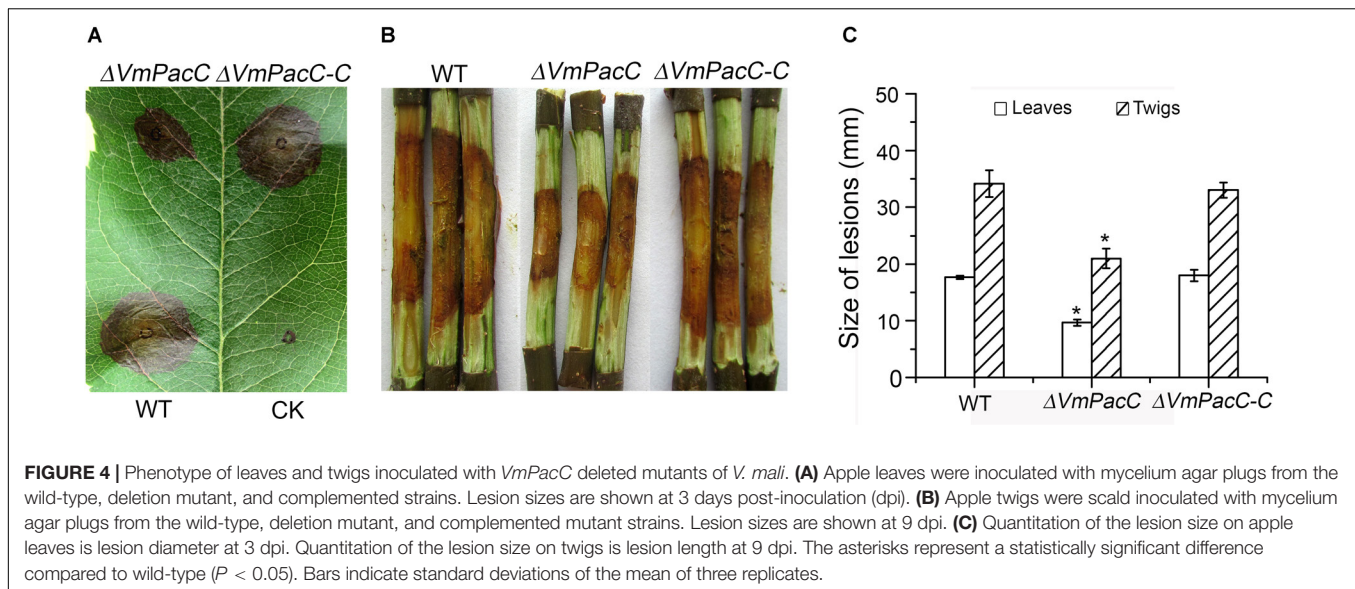
When the wild-type strain of *V. mali* was inoculated in detached apple twigs, the initial pH at the inoculation sites was 6.2. The pH decreased to 3.8 when canker symptoms appeared 24 h after inoculation. Thereafter, the pH remained relatively stable. However, the same pH value (pH 3.9) was determined 48 h after inoculation in the *VmPacC* deletion mutant. The pH value of the deletion mutant decreased more slowly than in the wild-type strain. Furthermore, the pH value at the border of the lesion in the deletion mutant was higher than in the wild-type strain. The acidification capacity was impaired when *VmPacC* was deleted, and this effect could be reversed by complementation of the deletions (Figures 7B,C).

High-performance liquid chromatography analysis of organic acids secreted by *V. mali* indicated that the accumulation of citric acid was reduced in the *VmPacC* deletion mutant compared to wild-type (Figure 7D). To further test whether acidification of the environment contributes to virulence, we have inoculated the deletion mutant and externally treated with citric acid at pH 3.5. As expected, the virulence of the *VmPacC* deletion mutant and the wild-type strain increased when externally treated with citric acid (Figure 7E).

Our results clearly show that *V. mali* requires acidification of its environment for growth. In addition, *VmPacC* is involved in virulence partly via regulating the generation of citric acid.

DISCUSSION

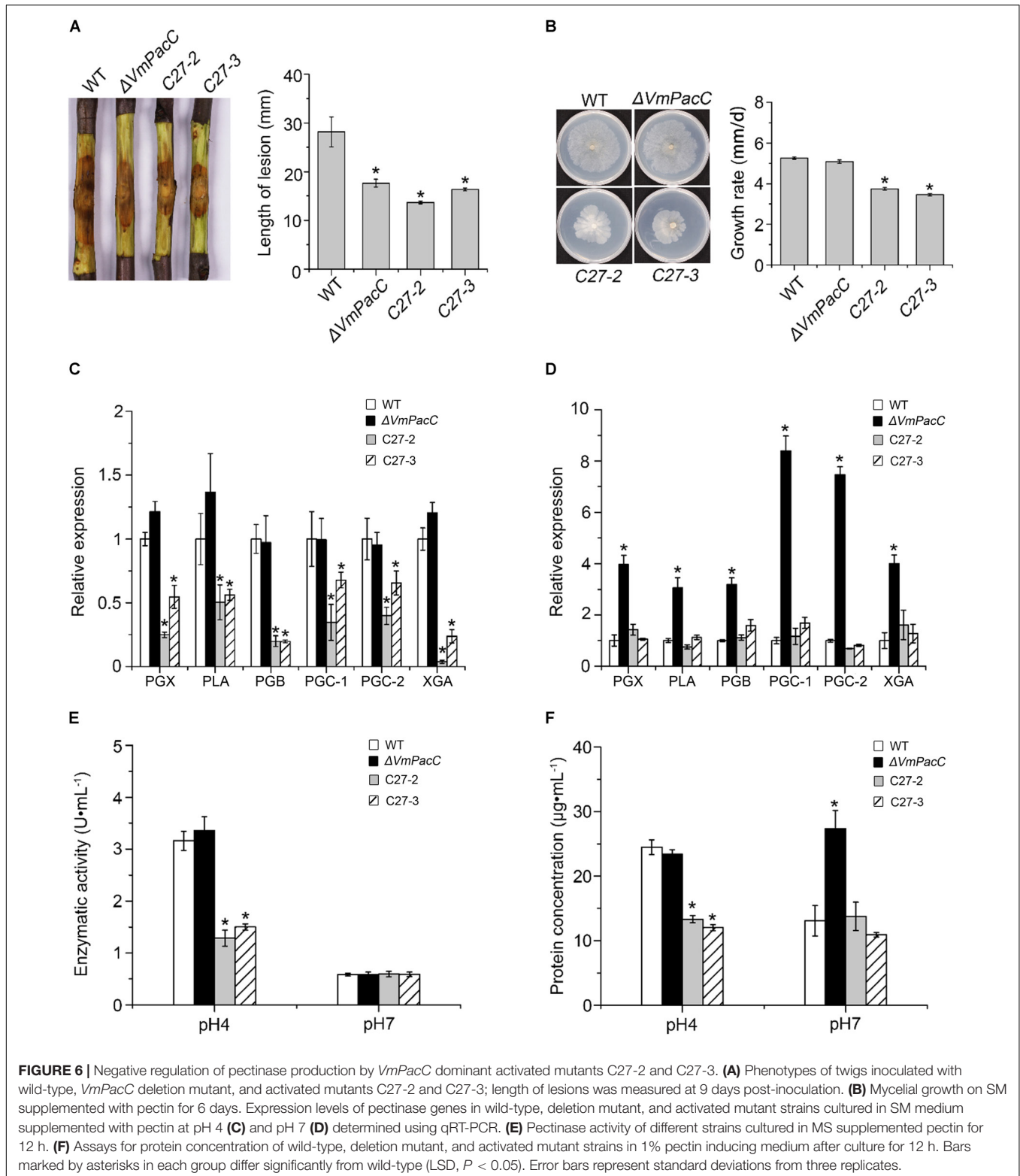
The Pal ambient pH response signalling pathways are essential for fungi to adapt to environmental and host conditions. This signalling cascade system ultimately activates the PacC transcription factor, which controls the environmental pH



sensing and response (Peñalva et al., 2008). In this study, we found that the optimum pH of the apple bark pathogenic fungus, *V. mali*, is acid. Neutral or alkaline conditions did not favour mycelial growth, as indicated by the impaired vegetative growth of deletion mutant *VmPacC* at neutral and alkaline conditions (Figures 2A,B). These results indicate that *VmPacC* is required for *V. mali* adaptation to alkaline pH. This is consistent with what has been described in other fungi such as *P. digitatum* (Zhang et al., 2013), *S. sclerotiorum* (Rollins, 2003), *F. graminearum* (Merhej et al., 2011), and *A. nidulans* (Tilburn et al., 1995). In contrast, in *Ustilago maydis*, the null *rim101* mutants show normal growth rate *in vitro*

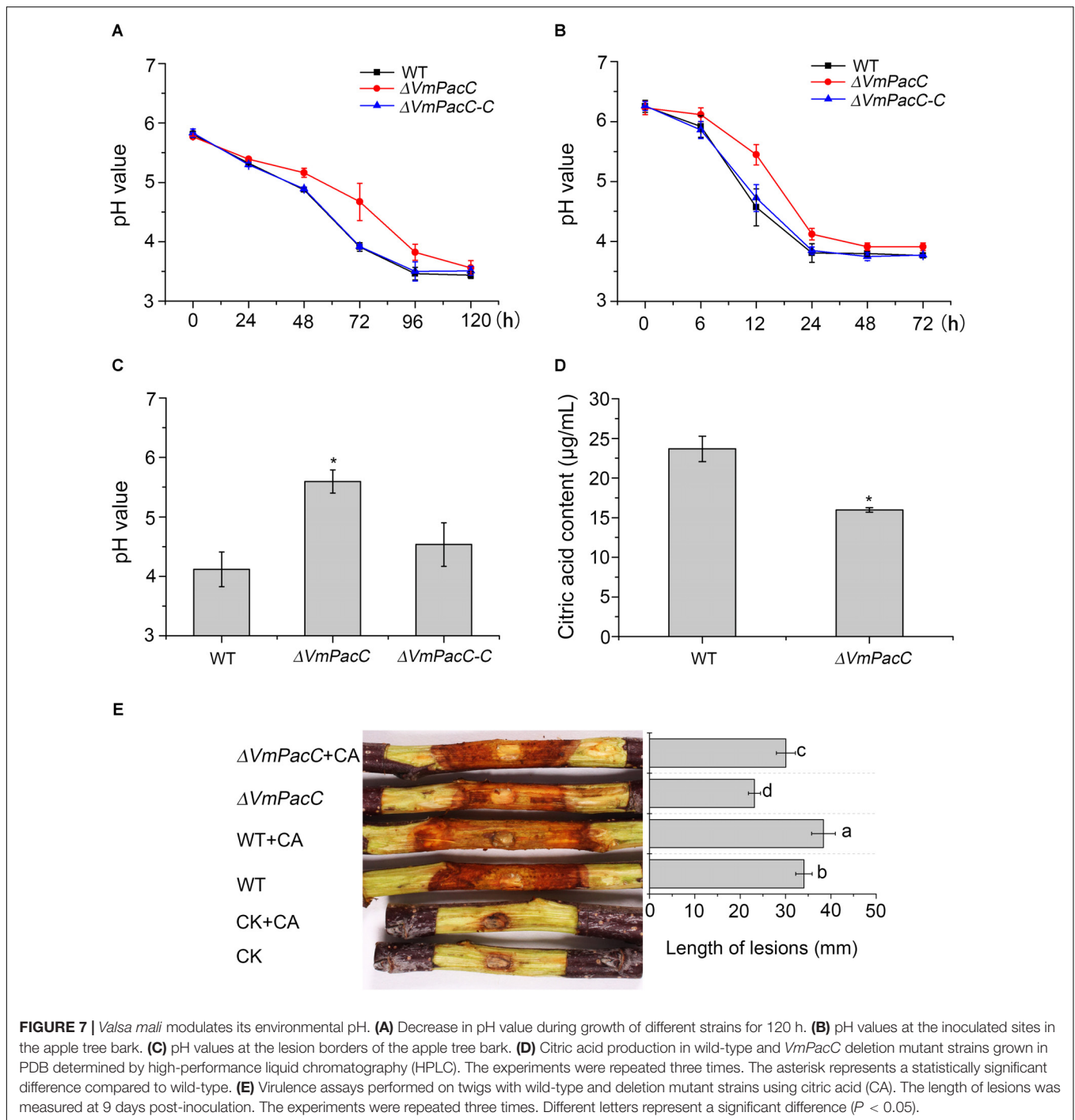
(Arechiga-Carvajal and Ruiz-Herrera, 2005). Moreover, in *Metarhizium robertsii* and *M. oryzae*, PacC affects growth under both acidic and alkaline conditions (Landraud et al., 2013; Huang et al., 2015). These results suggest that, in addition to adaptation to alkaline pH, alternative PacC signalling pathways may have evolved in some fungi.

We also found that *VmPacC* affected the sensitivity to salt and oxidative stress in *V. mali* (Figure 3). Similarly, deletion of PacC/Rim101 reduces ion tolerance because of repression of ion pump genes (Caracuel et al., 2003). In the insect pathogenic fungi *M. robertsii*, the reduced ion tolerance in $\Delta MrpacC$ results from smaller vacuoles



(Huang et al., 2015). In pathogen–host interactions, the reactive oxygen species (ROS) play an important role. Plants use the oxidative burst as an early defence reaction to pathogen attack. Here, we show that *VmPacC* is involved in the sensitivity

to oxidative stress. However, this issue is indeterminacy in necrotrophs. For example, in *B. cinerea*, deletion of the AP-1 transcription factor gene does not lead to impaired virulence (Temme and Tudzynski, 2009). Thus, for *V. mali*, the relation



between abiotic stress responses and virulence needs further research.

The present study showed that deletion of the *VmPacC* gene significantly impaired the virulence of *V. mali* (Figure 4). Similar to what has been reported for *PacC* mutant strains of *P. digitatum* (Zhang et al., 2013), *S. sclerotiorum* (Rollins, 2003), *C. gloeosporioides* (Miyara et al., 2008), and *M. oryzae* (Landraud et al., 2013), *PacC* regulates the virulence in a positive manner in *V. mali* (Figure 3). However, *PacC* is a negative regulator

of virulence in *F. oxysporum* and *F. graminearum* (Caracuel et al., 2003; Merhej et al., 2011). In addition, the pathogenicity of null *rim101* mutants of *U. maydis* is not affected (Arechiga-Carvajal and Ruiz-Herrera, 2005). In phytopathogenic fungi, cell wall-degrading enzymes are important virulence factors during plant infection (Kubicek et al., 2014). The *PacC* is also a key regulator of secretion of these hydrolytic enzymes in several filamentous fungi (Miyara et al., 2010). In *C. gloeosporioides*, the reduced expression of a pectate lyase gene decreases virulence in

a *CgpacC*-disrupted mutant (Miyara et al., 2008). In *P. digitatum*, the expression of cell wall degradation enzyme genes such as *Pdpg2* and *Pdpnl1* is regulated by PdPacC. In addition, this PdPacC regulation is associated with pathogenesis (Zhang et al., 2013). In *S. sclerotiorum*, the induced virulence of a PacC deletion mutant results from impaired expression of endopolygalacturonase (*pg1*) in higher ambient pH (Rollins, 2003). Pectinase is an important virulence factor in *V. mali* (Ke et al., 2013; Yin et al., 2015). Therefore, we determined the transcript levels of six pathogenesis-related pectinase genes and six up-regulated genes during infection (Yin et al., 2015). However, unexpectedly, the expression of most pectinase genes tested was up-regulated to different degrees in *VmPacC* deletion mutants (Figure 5). These results show that *VmPacC* is involved in repressing pectinase genes. This regulation model of virulence and pectinase expression is distinct from the above-mentioned mechanism. Therefore, *VmPacC* should not impact virulence via regulating the expression of pectinase genes.

To further investigate the repressive effect of *VmPacC* on pectinase gene expression, we generated dominant activated allele mutants. The mutants constitutively express PacC independent of pH. In agreement with our hypothesis, we found that dominant activated allele mutants showed significantly reduced growth on solid media supplemented with pectin as the sole carbon source. In addition, both the expression and activity of pectinase genes decreased and led to a reduction in the size of lesions on twigs. These results are consistent with the expression of two polygalacturonase genes, *pg1* and *pg5*, being negatively regulated by PacC in *F. oxysporum*, although PacC functions as a negative regulator of virulence in this organism (Caracuel et al., 2003). In several fungi, PacC is responsible for up-regulation of alkaline-expressed genes and suppression of acid-expressed genes in alkaline pH (Alkan et al., 2013). Proteins need to be selectively expressed at the optimal external host pH environment to function correctly. We have demonstrated that the optimum pH value of pectinase activity is 3.5 (Feng et al., 2017a). The pectinase gene should be inhibited by PacC in neutral and alkaline conditions. Although transcript and protein levels in $\Delta VmPacC$ significantly increase at pH 7, the pectinase activity was the same as in the wild-type. These results indicate that the secreted pectinases do not contribute to pectinase activity in neutral and alkaline conditions.

In plant pathogens, the capacity to modify the ambient pH seems to be associated with infection of the host plants. Several phytopathogenic fungi acidify or alkalinise their surrounding pH during infection. Some plant pathogenic fungi such as *S. sclerotiorum* and *P. digitatum* are known to acidify their environment during infection via the secretion of oxalic acid or other organic acids (Rollins, 2003; Zhang et al., 2013). Other fungi, such as *M. oryzae* and *Colletotrichum* species, tend to secrete ammonia to alkalinise the invaded plant tissues (Landraud et al., 2013; Ment et al., 2015). In the present study, *V. mali* showed similar behaviour to *S. sclerotiorum* and *P. digitatum*. *V. mali* can acidify the surrounding or host pH, and the acidification capacity was impaired after deletion of *VmPacC*. We further demonstrated that *VmPacC* is required for

the accumulation of citric acid in *V. mali*, and that the virulence of the wild-type strain and deletion mutant can be increased by externally adding citric acid to twigs. These results show that *VmPacC* is involved in virulence partly owing to the regulation of citric acid generation. However, whether citric acid contributes to virulence via another pathway remains to be elucidated.

CONCLUSION

We determined that the optimum pH of *V. mali* is acid, and that this organism can acidify its surrounding pH. In addition, the capacity to acidify the environmental pH is regulated by *VmPacC*, which is also involved in the response to oxidative stress and modulates expression of pectinase genes. More importantly, *VmPacC* is involved in virulence partly owing to the regulation of citric acid generation. In the future, comparison of the transcriptomes of the wild-type strain and the *VmPacC* deletion mutant of *V. mali* at different pH or during infection of apple bark will contribute to a better understanding of pathways involved in PacC-related pathogenesis.

AUTHOR CONTRIBUTIONS

YW and LH conceived and designed the experiments and wrote the paper. YW and LX performed the experiments. YW, ZY, and HF analyzed the experiment data. ZY and LH contributed reagents, materials, and analysis tools. All authors have read and approved the final manuscript.

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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.2018.01981/full#supplementary-material>

FIGURE S1 | Structure and sequence analysis of pH-signalling pathway proteins in *Valsa mali*. **(A)** Phylogenetic analysis of pH-signalling pathway proteins of *V. mali* and their homologs from *Magnaporthe oryzae*, *Aspergillus nidulans*, and *Neurospora crassa*. The amino acid sequences of Pal pathway proteins were analysed using MEGA version 6 and neighbour-joining analysis with 1,000 bootstrap replicates. Numbers on the branches represent the percentage of replicates supporting each branch. Subclades containing pH-signalling pathway proteins of *V. mali* and orthologs from other species are shaded. The bar represents 20% sequence divergence. **(B)** The open reading frame (ORF) of *VmPacC* consists of 1,825 bp, interrupted by two introns, and encodes a predicted protein of 560 amino acids with classical zinc-finger domain and zinc-finger double domain at its N-terminal region.

FIGURE S2 | Generation of *VmPacC* gene deletion mutants. **(A)** Generation of gene replacement constructs. The small arrows mark the position and direction of primers used for PCR. **(B)** For PCR detection of deletion transformants, four

primer pairs (VmPacC-5F/6R, H850F/H852R, VmPacC-7F/H855F, and H856F/VmPacC-8R) were used to detect *VmPacC* gene deletion mutants. **(C)** Southern blots of *Clal*-digested genomic DNA of wild-type, deletion mutant (Δ VmPacC), and complemented mutant (Δ VmPacC-C) strains hybridised with probe p (*VmPacC* ORF or probe h (*hph* ORF)).

FIGURE S3 | Construction of dominant activated mutant of *VmPacC27*. **(A)** Prediction of activated sequence with *A. nidulans*. **(B)** PCR detection of positive

transformants of activated mutants with primer pairs VmPacC27-F/R. **(C)** Wild-type (WT) deletion and activated mutants grow at pH 9 in unbuffered potato dextrose agar for 3 days. The normal growth was restored in the activated mutants, indicating the *VmPacC* was expressive.

TABLE S1 | Pectinase genes for expression.

TABLE S2 | Primers used for deletion, complementation, and gene expression.

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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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