INTRODUCTION

Valsa mali is a necrotrophic fungal pathogen that mainly infects branches and trunks of apple trees, causing significant losses to apple production in eastern Asia, especially in China (Li et al., 2013; Wang et al., 2012). It is characterized by abundant pycnidia on cankers that can release conidia throughout the year (Li et al., 2013). This pathogen infects apple trees with conidia through wounds, and may rapidly expand into the xylem, making its control difficult (Abe et al., 2007; Chen et al., 2016). To date, our strategies to effectively prevent and control plant diseases remain limited. Thus, understanding the pathogenicity mechanisms of V. mali is crucially important to develop more effective disease management strategies.

Similar to other necrotrophic fungi, V. mali kills host cells by producing toxins and secreting cell wall-degrading enzymes (CWDEs), which are considered the major pathogenicity factors (Chen et al., 2012; Wang et al., 2014; Xu et al., 2016). A whole-genome analysis has suggested that V. mali has a large number of pathogenicity-related genes underlying the production of protocatechuic acid. In this study, we identified four hydroxybenzoate hydroxylase genes (VmHbh1, VmHbh2, VmHbh3, and VmHbh4) from the transcriptome of V. mali. The VmHbh protein had high enzymatic activities of hydroxybenzoate hydroxylase, which could convert 4-hydroxybenzoate to protocatechuic acid. These four VmHbh genes all had highly elevated transcript levels during the V. mali infection process, especially VmHbh1 and VmHbh4, with 26.0- and 53.4-fold increases, respectively. Mutants of the four genes were generated to study whether VmHbhs are required for V. mali pathogenicity. Of the four genes, the VmHbh1 and VmHbh4 deletion mutants considerably attenuated V. mali virulence in apple leaves and in twigs, coupled with much reduced toxin levels. The VmHbh2 and VmHbh3 deletion mutants promoted the transcript levels of the other VmHbhs, suggesting functional redundancies of VmHbhs in V. mali virulence. The results provide insights into the functions of VmHbhs in the production of protocatechuic acid by V. mali during its infection of apple trees.
genes involved in plant cell wall degradation and biosynthesis of secondary metabolites (Feng et al., 2020; Yin et al., 2015). Previous studies have reported that genes encoding the CWDEs play important roles during the pathogenicity of *V. mali*. For instance, pectinase secreted by *V. mali* causes apple bark tissue maceration and is thus essential to pathogen virulence (Xu et al., 2016). The role in the virulence played by xylanase, involved in the hydrolysis of xylan, was confirmed in plant pathogens such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Brito et al., 2006; Yu et al., 2016). One xylanase gene, *VmXyl1*, contributes to the production of pycnidia and is required for the full pathogenicity of *V. mali* (Yu et al., 2018). Inactivation of feruloyl esterases in *V. mali* leads to reduced virulence (Xu et al., 2018). *V. mali* can produce several kinds of nonspecific toxic compounds, both in vivo and in vitro, that are associated with the pathogenicity (Natsume et al., 1982; Wang et al., 2014). However, the genes involved in the production of those toxins remain unknown.

Among the five toxic compounds produced by *V. mali*, protocatechuic acid exhibits the greatest phytotoxic activity (Wang et al., 2014). According to the hypothesized toxin production pathway in *V. mali*, 4-hydroxybenzoate is hydroxylated and then converted to protocatechuic acid (Natsume et al., 1982). Previous studies in bacteria suggest that 4-hydroxybenzoate is predominantly hydroxylated at the third position by 4-hydroxybenzoate 3-hydroxylase to yield 3,4-dihydroxybenzoic acid (protocatechuic acid) (Huang et al., 2008). In *Xanthomonas campestris*, 4-hydroxybenzoate is hydroxylated by 4-hydroxybenzoate 3-hydroxylase for conversion to protocatechuic acid, which can be further metabolized into other products. The 4-hydroxybenzoate degradation pathway in *X. campestris* is required for its full pathogenicity (Wang et al., 2015). In *Pseudomonas* sp. and *Alcaligenes* sp., 4-hydroxybenzoate is also degraded by 4-hydroxybenzoate 3-hydroxylase via the protocatechuic acid route (Deveryshetty et al., 2007).

The aim of the present study was to identify those hydroxybenzoate hydroxylase (*Hbh*) genes that convert 4-hydroxybenzoate to protocatechuic acid, and to evaluate their potential functions during infection of apple trees by *V. mali*. The published genomic sequence predicted 10 candidate genes that encode hydroxybenzoate hydroxylase (*VmHbh*) genes in the host tissues with those of mycelia grown on potato dextrose agar (PDA). The quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis showed that the four *VmHbh* genes were all closely related to the 3-hydroxybenzoate hydroxylase (Montersino et al., 2011, 2017). A phylogenetic tree was constructed with the characterized proteins of 3-hydroxybenzoate 6-hydroxylases and 4-hydroxybenzoate 3-hydroxylases from other strains, and confirmed that the four *VmHbh* genes were all closely related to the 3-hydroxybenzoate 6-hydroxylases (Figure 1a). The sequence alignment of *VmHbh* genes and four characterized 3-hydroxybenzoate 6-hydroxylases are given in Figure 1b. *VmHbh1*, *VmHbh3*, *VmHbh4*, and the four reference sequences all have the three flavin-binding motifs, such as GxGxGG, DG, and GD, whilst *VmHbh2* has the two conserved motifs DG and GD (Eppink et al., 1997).

### 2.2 Conversion of 4-hydroxybenzoate to 3,4-dihydroxybenzoic acid by *VmHbh1*

The purified *VmHbh1* protein was obtained using a prokaryotic expression technique (Figure 2a). High-performance liquid chromatography (HPLC) analysis showed that 4-hydroxybenzoate decreased with the corresponding increase in protocatechuic acid (Figure 2b). Further analysis based on the liquid chromatography-mass spectrometry (LC-MS) confirmed that 4-hydroxybenzoate and protocatechuic acid are the substrate and product, respectively (Figure 2c,d). During the conversion of 4-hydroxybenzoate by *VmHbh1*, 2,4-dihydroxybenzoic acid, which has the same molecular weight as protocatechuic acid, was not detected. Similarly, 2,4-dihydroxybenzoic acid was not detected in *V. mali* culture on apple branch medium nor in *V. mali*-infected twigs (Figure 2d). These results suggest that *VmHbh1* encodes *V. mali* Hbh, which converts 4-hydroxybenzoate to protocatechuic acid.

### 2.3 Transcription levels of *VmHbh* genes

Infected apple phloem and xylem tissues were sampled at 72 hr postinoculation (hpi) to compare the transcript levels of the four *VmHbh* in the host tissues with those of mycelia grown on potato dextrose agar (PDA). The quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis showed that the transcript levels of the four *VmHbh* genes were significantly upregulated in the apple phloem compared to their respective expression levels in the apple xylem (Figure 3a). This was particularly true for *VmHbh1*: the transcription level in apple phloem was 17.1-fold higher than that in apple xylem.

The transcript levels of *VmHbh* during infection of apple twigs by *V. mali* were determined for infected apple phloem tissues sampled at seven time points (0, 6, 12, 24, 48, 72, and 120 hpi) as well as for mycelia grown on PDA (Figure 3b). The transcript level of *VmHbh1* was upregulated from 24 to 120 hpi with 16.2- to 26.0-fold
increases. Similarly, VmHbh4 was upregulated from 12 to 120 hpi with 7.5- to 53.4-fold increases. For VmHbh2, upregulation was observed only at 48 and 72 hpi with 2.7- and 5.3-fold increases, respectively. The transcript level of VmHbh3 was upregulated from 6 to 120 hpi with 2.0- to 3.1-fold increases. Overall, the high induction of VmHbhs during infection suggested their potential role in the pathogenicity of V. mali.

2.4 | VmHbhs are not required for growth and pycnidia formation of V. mali

For functional analysis of the four VmHbhs in V. mali, we knocked out each of the four genes by the polyethylene glycol (PEG)-mediated protoplast transformation method. The mutants were initially examined via PCR assays, and then were confirmed by
Southern blotting (Figure S2). For the complementation of each VmHbh deletion mutant, a complementary construct was generated and transformed into the corresponding mutants. All the complementation transformants were selected using geneticin G-418 and confirmed by PCR assays with primer pairs ID-F/ID-R (Table S1). In addition, RT-PCR analysis was performed to confirm that the target VmHbh was knocked out in the gene deletion mutants, and the complementation strains contained their corresponding target genes (Figure S4).

Colony morphology, growth rate, dry mycelia weight, and pycnidia formation of the VmHbh deletion mutants and the wildtype strain were measured to determine whether any VmHbh played a role in V. mali development. The VmHbh deletion mutants did not differ statistically from the wildtype strain in colony morphology (Figure 4a), growth rate (Figure 4b), and dry mycelia weight after 7 days’ culturing in potato dextrose broth (PDB) (Figure 4c). All strains were able to form pycnidia and did not differ in the number of pycnidia produced (Figure 4d). These results indicated that VmHbhs did not affect the vegetative growth or pycnidia formation of V. mali.

2.5 | Deletion of VmHbh1 or VmHbh4 reduce V. mali virulence

Pathogenicity assays on the detached apple leaves and twigs were conducted to investigate whether the four VmHbh genes play roles in disease development. Pathogenicity was measured in terms of the lesion size following inoculation (Figure 5). Deletion of VmHbh1 led to at least 65% reduction in lesion sizes on the apple leaves and twigs compared to the wildtype strain. Similarly, the ΔVmHbh4 mutant also reduced the lesion size on apple leaves and twigs, but to a lesser extent (about 40%), compared to ΔVmHbh1. In contrast, the ΔVmHbh2 and ΔVmHbh3 mutants did not differ from the wildtype strain in lesion size. To confirm that the reduced pathogenicity of the mutants was caused by the deletion of VmHbhs, each complementary construct was generated and transformed into their respective deletion mutants. The previously observed phenotype was rescued in the complementation strains. These results suggested that VmHbh1 and VmHbh4 are essential for the pathogenicity of V. mali in apple leaves and twigs.

2.6 | Deletion of VmHbh1 and VmHbh4 reduce toxin production by V. mali

To identify possible mechanisms underlying the reduced pathogenicity of the ΔVmHbh1 and ΔVmHbh4 mutants, toxins in the apple branch extract medium under the exposure of the wildtype and mutant strains were quantified. All strains produced five types of toxins, but the ΔVmHbh1 mutant produced 79% and 31% less protocatechueic acid and 4-hydroxybenzoate, respectively, in the apple branch extract medium (Figure 6a). Similarly, the ΔVmHbh4 mutant produced less protocatechueic acid (43% less) and 4-hydroxybenzoate (38% less) compared to the wildtype strain. Toxins during fungal infection of apple twigs with the wildtype and the deletion strains were also quantified (Figure 6b). The ΔVmHbh1 and ΔVmHbh4 mutants had 72% and 48% reductions in protocatechueic acid, respectively; the corresponding values were 42% and 34% for 4-hydroxybenzoate. There were no significant differences in the quantities of the other three toxic compounds between the wildtype and mutant strains. When VmHbh1 or VmHbh4 was reintroduced into the gene deletion mutants, the phenotype was rescued. Neither the ΔVmHbh2 nor ΔVmHbh3 mutants differed from the wildtype strain in the toxin levels (data not shown).

2.7 | Redundancies of VmHbhs in the virulence of V. mali

The RT-qPCR analysis showed that, when grown on PDA, the transcript levels of VmHbhs did not differ between the deletion mutants and the wildtype strain (Figure S5). During infection, the transcript levels of VmHbh2, VmHbh3, and VmHbh4 remained at a similar level in the ΔVmHbh1 mutant relative to that of the wildtype strain (Figure 7a). However, VmHbh1, VmHbh3, and VmHbh4 were significantly upregulated (with 4.7-, 3.2-, and 6.0-fold increases, respectively) in the ΔVmHbh2 mutant relative to the wildtype strain (Figure 7b). Similarly, VmHbh1 and VmHbh4 were upregulated in the ΔVmHbh3 mutant (Figure 7c) as well as the transcript level of VmHbh1 in the ΔVmHbh4 mutant (Figure 7d). These data indicate that a deficiency in VmHbh2, VmHbh3, or VmHbh4 could stimulate the upregulation of the other genes to different degrees.
3 | DISCUSSION

Protocatechuic acid, 4-hydroxybenzoate, 4-hydroxyacetophenone, 4-hydrobenzene propanoic acid, and phloroglucinol produced by *V. mali* have been described as phytotoxins that can cause necrosis in plants (Natsume et al., 1982; Wang et al., 2014). In *V. mali*, strains producing more toxins are more virulent than strains producing fewer toxins (Li et al., 2014; Wang et al., 2014). The five studied toxic compounds are believed to be the degradation products of the phloridzin, but little is known about functional genes that are involved in the phloridzin degradation process. Among these toxic compounds, protocatechuic acid is the most phytotoxic and the last toxic compound in the production pathway of these toxins. Previous studies in bacteria have shown that 4-hydroxybenzoate is hydroxylated by 4-hydroxybenzoate 3-hydroxylase to yield protocatechuic acid (Devershyetty et al., 2007; Donoso et al., 2011; Romero-Silva et al., 2013). In the present study, we identified four *VmHbh* genes and demonstrated the role of hydroxybenzoate hydroxylase in the

![FIGURE 2](image_url)

**FIGURE 2** Purification and functional verification of the *VmHbh1* recombinant protein. (a) Detection and purification of the *VmHbh1* recombinant protein by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Lane M, protein molecular weight marker (low); lane 1, the purified recombinant protein; lane 2, the supernatant of the induced cells at 15 °C. (b) Enzymatic activity of the *VmHbh1* recombinant protein, showing the conversion of 4-hydroxybenzoate into 3,4-dihydroxybenzoic acid (protocatechuic acid) as determined by high-performance liquid chromatography. (c) The substrate and conversion product identified by liquid chromatography-mass spectrometry. (d) The extracted ion chromatograms of the conversion product of 4-hydroxybenzoate, and toxins produced by *Valsa mali* in apple branch extract medium and apple twigs.
production of toxins and virulence of *V. mali* in apple leaves and twigs.

Interestingly, amino acid sequence analysis suggested that these four hydroxybenzoate hydroxylases from *V. mali* are more closely related to the 3-hydroxybenzoate 6-hydroxylases than to the 4-hydroxybenzoate 3-hydroxylases. Moreover, these four VmHbh1s have similar conserved motifs to those of 3-hydroxybenzoate 6-hydroxylases, namely GxGxGG, DG, and GD (Chen et al., 2018). Previous studies demonstrated that 3-hydroxybenzoate 6-hydroxylases can convert 3-hydroxybenzoate to 2,5-dihydroxybenzoic acid and show activity when salicylate or 4-hydroxybenzoate is used as the substrate (Gao et al., 2005; Montersino & van Berkel, 2012). By sequence analysis, we found few functional annotations of 4-hydroxybenzoate 3-hydroxylases in fungi in the NCBI database. Until now, the functional analysis and crystal structure of 4-hydroxybenzoate 3-hydroxylases and 3-hydroxybenzoate 6-hydroxylase are all from bacteria or yeast (van Berkel et al., 1994; Hiromoto et al., 2006; Holesova et al., 2011; Montersino et al., 2013; Schreuder et al., 1988). This is the first report that hydroxybenzoate hydroxylase (VmHbh) from *V. mali* can convert 4-hydroxybenzoate to protocatechuic acid but does not have enzymatic activities when the substrate is 3-hydroxybenzoate (data not shown). Therefore, the present results indicate that VmHbh1 performs the function of 4-hydroxybenzoate 3-hydroxylase in *V. mali*.

During infection by *V. mali*, all four VmHbh1s genes showed high transcript levels, especially VmHbh1 and VmHbh4, indicating that these genes may play a role in pathogenicity toward apples. Furthermore, RT-qPCR analysis also demonstrated that the greatest upregulation of their expression occurred at different time points: VmHbh1, VmHbh3, and VmHbh4 were upregulated at 6–24 hpi, whereas VmHbh2 was.

**FIGURE 3** Transcript levels of VmHbh1s at different conditions and time points postinoculation of apple tissues or twigs with *Valsa mali* as determined by quantitative reverse transcription PCR. The transcript level of *V. mali* EF1-α was used as an endogenous control, and the transcript level of each VmHbh gene in the mycelia grown on potato dextrose agar was normalized to 1. The means and standard deviation of the relative expression levels were calculated from three independent biological replicates. (a) Relative expression levels of VmHbh1s in apple tissues at 72 hr postinoculation (hpi). Asterisks represent significant differences (*p* < 0.05) in transcript levels in the apple phloem and xylem tissues. (b) Relative expression levels of VmHbh1s at 0, 6, 12, 24, 48, 72, and 120 hpi of apple twigs. Asterisks represent significant differences (*p* < 0.05) in transcript levels as compared to that at 0 hpi.
upregulated at 48 hpi. The differential temporal dynamics in the transcripts may reflect that these genes play important roles at different time points during infection. In addition, the four VmHbh genes showed significantly higher expression levels in phloem than in xylem tissues. Previous studies indicated that V. mali can grow rapidly and survive for a long time in xylem, but it could not develop disease symptoms until the pathogen reached the phloem (Chen et al., 2016; Wang et al., 2018). Thus, the transcript levels of the VmHbh genes may reflect the pathogenicity level of V. mali in apple tissues.

In this study, we generated gene deletion mutants of all four VmHbh genes and demonstrated that VmHbh are not necessary for mycelial growth and pycnidia formation in V. mali. In contrast, the pathogenicity-related gene BcKMO in B. cinerea, encoding kynurenine 3-monooxygenase (KMO) with a monooxygenase FAD-binding domain, is known to be important for fungal development in B. cinerea, and its mutant does not produce conidia or sclerotia, and has a lower rate of growth (Zhang et al., 2018). These data indicate that the contribution of monooxygenases to fungal growth and development may depend on the pathogen species.

VmHbh2 or VmHbh3 did not influence the virulence of V. mali, but the pathogenicity of the deletion mutants of VmHbh1 or VmHbh4 showed large reductions compared to the wildtype strain. This may
imply functional redundancies of the genes in *V. mali* pathogenicity. Such a functional redundancy also exists in other gene families of *V. mali*, for instance the abundant effectors and CWDEs that act as virulence factors (Xu et al., 2018; Zhang et al., 2019). Moreover, the RT-qPCR analysis of each gene, with the exception of *VmHbh1*, showed a complementary effect in the deletion mutants. When *VmHbh1* was knocked out, the expression of other three *VmHbh* genes was not upregulated, which indicates that these genes are unlikely to complement the function of *VmHbh1*. For the deletion of *VmHbh4*, only *VmHbh1* was upregulated with a lower increase, which may partly complement the function of the deleted *VmHbh4*. However, in the gene deletion mutants of *VmHbh2* and *VmHbh3*, the expression levels of the other three *VmHbh* genes were significantly upregulated. These results indicate that the upregulated *VmHbh1* and *VmHbh4* could rescue the function of the four deleted genes, and hence the deletion of *VmHbh2* or *VmHbh3* did not impair the virulence of *V. mali*. A similar result has been found previously with regard to Hce2-containing effectors of *V. mali* such as *VmHEP1* and *VmHEP2* (Zhang et al., 2019). The deletion of one *VmHEP* promoted the expression of the other one, meanwhile the deletion of each single *VmHEP* did not lead to a reduction in the

**FIGURE 5** Pathogenicity assays of the wildtype strain, *VmHbh* deletion, and complementation mutants on apple leaves and twigs of *Malus domestica* 'Fuji'. (a) Infected phenotypes of apple leaves by different strains at 3 days postinoculation (dpi). (b) Infected phenotypes of apple twigs by different strains at 5 dpi. (c) Lesion sizes caused by different strains on apple leaves at 3 dpi and apple twigs at 5 dpi. The mean lesion length was calculated from nine apple leaves and 15 apple twigs. Bars represent the standard deviation. Asterisks indicate significant differences with the wildtype strain (*p* < 0.05)
virulence. In another study of polygalacturonase (PG), the transcript levels of three genes in the PG family were significantly induced in strains with the double-deletion of Vmpg7 and Vmpg8 (Xu et al., 2016).

An important discovery of our work is the finding that the deletion of VmHbh1 and VmHbh4 significantly reduced levels of toxin, which may explain the corresponding decrease in the virulence of V. mali. This is consistent with the previous results of the V. mali xylanase gene VmXyl1, which contributes significantly to both the total xylanase activity and virulence (Yu et al., 2018). Similarly, the deletion of feruloyl esterase genes decreases the capacity of V. mali to utilize ethyl ferulate, which is consistent with reduced virulence of the gene deletion mutants (Xu et al., 2018). In contrast, polyketide synthase BcPKS6 and BcPKS9 are key enzymes for the biosynthesis of botcinic acid, an important phytotoxin during infection by B. cinerea: when one of these two genes is deleted, the loss of botcinic acid production does not affect the virulence of B. cinerea in plant tissues (Dalmais et al., 2011). In this study, the fact that deletion of VmHbh2 or VmHbh3 did not affect the toxin levels demonstrated redundant functions in toxin production.

In the present study, we demonstrated that VmHbh1 can convert 4-hydroxybenzoate to protocatechuic acid. VmHbhs are required for full pathogenicity of V. mali against apple trees. The present findings provide a new perspective for the contribution of Hbhs to the growth, development, and virulence of phytopathogenic fungi.

4 | EXPERIMENTAL PROCEDURES

4.1 | Strains and culture conditions

The V. mali wildtype strain LXS080601 was grown on potato dextrose agar (PDA, 200 g potato, 20 g dextrose, 15 g agar per L) at 25 °C in the dark. The gene deletion and complemented mutants were cultured on PDA supplemented with 100 mg/ml hygromycin B or gentamicin G-418 (Sigma). Escherichia coli strains were grown in lysogeny broth (LB) with appropriate antibiotics at 37 °C. Colony diameter was measured to calculate the growth rate of different strains on PDA.
4.2 | Identification of Hbh genes in V. mali

To identify the four candidate Hbh genes in V. mali genome, BLAST searches with well-characterized Hbh genes were conducted. The conserved domains of the candidate Hbh proteins were confirmed using the BLAST-based NCBI conserved domain search engine (Marchler-Bauer et al., 2017). Primer pairs for the cloning of the four candidate Hbh genes were synthesized by TsingKe (Beijing, China) (Table S1). Total RNA was extracted from fungal mycelia with the RNAiso Plus Kit, and cDNA was synthesized with the Prime Script RT reagent kit with the gDNA Eraser (TaKaRa) with an oligo (dT) 12–18 primer. PCRs were performed with PrimerSTAR Max DNA polymerase and the PCR products were cloned to T-Vector pMD 19 Simple (TaKaRa), according to the manufacturer’s instructions.

4.3 | Sequence alignment and phylogenetic analysis

The amino acid sequences of Hbhs from other microorganisms in this study were obtained from the NCBI GenBank. All the homology searches were carried out on the NCBI BLAST server. These obtained sequences were compared with the sequence from V. mali (MT890625, MT890626, MT890627, and MT890628). The maximum-likelihood (ML) method, as implemented in MEGA 7, was used to infer the phylogenetic tree with 1,000 bootstrapping replicates. Multiple sequence alignments of VmHbhs and other published Hbh genes (Klebsiella pneumoniae, AAW63416; Polaromonas naphthalenivorans, Q3S4B7; Corynebacterium glutamicum, Q8NLB6; Rhodococcus jostii, ABG93680) were performed using DNAMAN v. 6.0 with all parameters set at the default values.

4.4 | Hydroxybenzoate hydroxylase activity assay

To assay the hydroxybenzoate hydroxylase activity of VmHbhs, the cDNA fragments were subcloned into pET-32a or pMal-c2× by homologous recombination using the ClonExpress II One Step Cloning Kit (Vazyme). The vector constructs were transformed into E. coli Rosetta whilst the soluble recombinant proteins of VmHbh1 and VmHbh4 were obtained after induction with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 16 hr at 15 ºC (Shi et al., 2015). We used amylose resin (New England Biolabs) or Ni-NTA spin column (Qiagen) to purify the recombinant proteins.

The hydroxybenzoate hydroxylase activity was measured following Chen et al. (2018) with some modifications. Activity was assayed in 1 ml of the reaction mixture containing 400 µM
4-hydroxybenzoate, 400 μM NADH, and about 15 μg of purified recombinant protein in 50 mM phosphate buffer (pH 8.0) at 25 °C. During the conversion, the concentrations of 4-hydroxybenzoate and protocatechuic acid were determined by HPLC and LC-MS (1200 series, 1290 Infinity/6460 ultra HPLC-MS; Agilent Technologies).

4.5 | Detection of gene expression by RT-qPCR

Mycelia grown on PDA for 3 days were used to inoculate apple twigs. Apple bark around the lesion margin was sampled at 0, 6, 12, 24, 48, 72, and 120 hpi. For samples at 0 hpi, the bark tissues around inoculation sites containing mycelium plugs were collected (Yu et al., 2018). The RNA was extracted from bark tissues using the RNAiso Plus Kit (TaKaRa), and then the cDNA was synthesized. All RT-qPCR experiments were conducted in a LightCycler 480II PCR detection system (Roche) with SYBR Master Mix (TaKaRa), following the manufacturer’s protocol. The V. mali EF1-a gene was used as an endogenous control, and the primers are given in Table S1. The relative expression levels of each gene were calculated using the 2-ΔΔCt method. Data from three biological replicates were used to calculate the means and standard deviation. The whole experiment was repeated twice.

4.6 | Generation of gene deletion and complementation strains

To obtain the VmHbh gene deletion mutants, the PEG-mediated homologous recombination was performed as described previously (Yu et al., 2018). The gene deletion cassette with three components used the hygromycin B phosphotransferase gene (HPH) as a selective marker for the gene deletion (Figure S1). Upstream and downstream fragments of VmHbh genes were amplified from genomic DNA of the wildtype strain LXS080601 using the gene-specific primer pairs Up-F/Up-R and Down-F/Down-R (Table S1), respectively. The HPH gene was amplified with primers HPH-F and HPH-R from the vector pBS (Table S1). The gene deletion cassette was generated by double-joint PCR, which was confirmed by the sequencing. The cassettes were later transformed into the protoplasts of V. mali LXS080601, and the transformants were screened by culturing on medium with 100 μg/ml hygromycin (Yu et al., 2018). The putative gene deletion mutants were validated by the PCR using four primer pairs (Figure S1 and Table S1). Primers I-F and I-R were used to detect the fusion segment; HPH-P-F and HPH-P-R to detect the resistance gene (HPH). The Up-F and Full-R were used to confirm if the upstream sequence of the replaced resistance gene was fused at the right position, and Full-F and Down-R to confirm whether the downstream sequence of the introduced resistance gene was fused at the right position. The gene deletion was finally verified by Southern hybridization with a DIG DNA Labeling and Detection Kit II (Roche), following the instruction manual. Genomic DNA was digested with appropriate restriction enzymes and separated via agarose gel electrophoresis. The HPH gene used for the replacement cassette served as probe.

To construct the gene complement vector, the entire VmHbh genes and predicted promoter sequences were amplified from genomic DNA using the primer pair CM-F/CM-R (Table S1). The PCR products were cloned into pYF11 using yeast gap repair, as described previously (Yu et al., 2014). Fusion constructs were verified by the sequencing analysis, and plasmids were transformed into the corresponding gene deletion mutant using the PEG-mediated method. The transformants were selected using G-418 and confirmed by PCR with the corresponding primers (Table S1).

4.7 | RT-PCR detection of VmHbhs in the wildtype, gene deletion mutants, and complementation mutants

The RNA extraction from cultured mycelia of the wildtype strain, VmHbh deletion mutants, and complementation mutants were performed as described previously. The cDNA was synthesized and the primers Full-F/Full-R were employed to detect the VmHbh genes (Table S1). The PCR conditions were as follows: 30 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 60 s; with a final extension at 72 °C for 5 min. The housekeeping gene EF1-a was used as a control.

4.8 | Vegetative growth, pycnidia formation, and pathogenicity assays

Mycelium plugs (5 mm diameter) cut from actively growing colony edges of the wildtype strain, gene deletion, and complemented mutants were transferred to new PDA plates. The plates were then incubated at 25 °C before colony shape, colour, and colony diameters were assessed. For the dry weight of mycelia, plugs were inoculated into potato dextrose broth at 150 rpm and 25 °C for 7 days.

Pathogenicity assays were performed on the apple leaves and 1-year-old twigs (Malus domestica ‘Fuji’) were taken from the greenhouse at Qingdao Agricultural University, Qingdao, China. The detached leaves and twigs were sterilized with 75% ethanol and the wounds were made as described by Yu et al. (2018). Mycelium plugs were used to inoculate the wounds. The inoculated leaves and twigs were placed in trays to maintain high humidity at 25 °C in the dark. Then, the lesion length was measured and lesion development was photographed at several time points. The assays were repeated three times, with at least 15 leaves and twigs per treatment.

4.9 | Determination of toxins production

For toxins production in the liquid media, mycelium plugs of the wildtype strain, gene deletion mutants, and complemented mutants were inoculated into apple branch extract medium. After culturing for 7 days under 150 rpm/min and 25 °C with a 12-hr photoperiod, the supernatant was collected. The medium inoculated with PDA
was used as a control. For toxin production in apple tissues, detached twigs were inoculated according to the method described by Wang et al. (2014). The final samples were filtered through a 0.22-μm pore membrane for the HPLC analysis. Each experiment was repeated two times.

ACKNOWLEDGEMENTS

We thank Professor Xiangming Xu at NIAB East Malling Research for helpful suggestions and revising the manuscript. This study was funded by grants from the National Natural Science Foundation of China (grant no. 32072367), the Shandong Provincial Natural Science Foundation (grant nos. ZR2020MC116 and ZR2018MC020), the Chinese Modern Agricultural Industry Technology System (grant no. CARS-27), and the Graduate Student Innovation Program of Qingdao Agricultural University (QYC2018110).

CONFLICT OF INTEREST

The authors declare that no competing interests exist.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Caixia Wang https://orcid.org/0000-0003-2533-1319

REFERENCES

Abe, K., Kotoda, N., Kato, H. & Soejima, J. (2007) Resistance sources to Valsa canker (Valsa ceratosperma) in a germplasm collection of diverse Malus species. Plant Breeding, 126, 449–453.

van Berkel, W.J.H.V., Eppink, M.H.M. & Schreuder, H.A. (1994) Crystal structure of p-hydroxybenzoate hydroxylase reconstituted with the modified fad present in alcohol oxidase from methylotrophic yeasts: evidence for an arabinofuranos. Protein Science, 3, 2245–2253.

Brito, N., Espino, J.J. & González, C. (2006) The endo-β-1,4-xylanase Xyn11A is required for virulence in Botrytis cinerea. Molecular Plant-Microbe Interactions, 19, 25–32.

Chen, C., Li, B.H., Dong, X.L., Wang, C.X., Lian, S. & Liang, W.X. (2016) Effects of temperature, humidity, and wound age on Valsa mali infection of apple shoot pruning wound. Plant Disease, 100, 2394–2401.

Chen, X.L., Niu, C.W., Li, B.H., Li, G.F. & Wang, C.X. (2012) The kinds and activities of cell wall-degrading enzymes produced by Valsa mali. Acta Agriculturae Boreali Sinica, 27, 207–212.

Chen, X., Tang, H., Liu, Y., Xu, P., Xue, Y., Lin, K. et al. (2018) Purification and initial characterization of 3-hydroxybenzoate 6-hydroxylase from a halophilic Marteillia strain AD-3. Frontiers in Microbiology, 9, 1335.

Dalmais, B., Schumacher, J., Moraga, J., Le Pécheur, P., Tudyński, B., Collado, I.G. et al. (2011) The Botrytis cinerea phytotoxin botcinic acid requires two polyketide synthases for production and has a redundant role in virulence with botrydial. Molecular Plant Pathology, 12, 564–579.

Deveryshetty, J., Suvekbala, V., Varadhamshetty, G. & Phale, P. (2007) Metabolism of 2-, 3- and 4-hydroxybenzoates by soil isolates Alcaligenes sp. strain PPH and Pseudomonas sp. strain PPD. FEMS Microbiology Letters, 268, 59–66.

Donoso, R.A., Pérez-Pantoja, D. & González, B. (2011) Strict and direct transcriptional repression of the pobA gene by benzoate avoids 4-hydroxybenzoate degradation in the pollutant degrader bacterium Cupriavidus necator JMP134. Environmental Microbiology, 13, 1590–1600.

Eppink, M.H., Schreuder, H.A. & van Berkel, W.J. (1997) Identification of a novel conserved sequence motif in flavoprotein hydroxylases with a putative dual function in FAD/NAD(P)H binding. Protein Science, 6, 2454–2458.

Feng, Y., Yin, Z., Wu, Y., Xu, L., Du, H., Wang, N. et al. (2020) LoeA controls virulence and secondary metabolism in apple canker pathogen Valsa mali. Frontiers in Microbiology, 11, 581203.

Gao, X., Tan, C.L., Yeo, C.C. & Poh, C.L. (2005) Molecular and biochemical characterization of the xlnD-encoded 3-hydroxybenzoate 6-hydroxylase involved in the degradation of 2,5-xylitol via the gentisate pathway in Pseudomonas alcaligenes NCIMB 9867. Journal of Bacteriology, 187, 7696–7702.

Hiromoto, T., Fujiwara, S., Hosokawa, K. & Yamaguchi, H. (2006) Crystal structure of 3-hydroxybenzoate hydroxylase from Comamonas testosteroni has a large tunnel for substrate and oxygen access to the active site. Journal of Molecular Biology, 364, 878–896.

Holesova, Z., Jakubkova, M., Zavadiakova, I., Zeman, I., Tomaska, L. & Nosek, J. (2011) Gentisate and 3-oxoadipate pathways in the yeast Candida parapsilosis: identification and functional analysis of the genes coding for 3-hydroxybenzoate 6-hydroxylase and 4-hydroxybenzoate 1-hydroxylase. Microbiology, 157, 2152–2163.

Huang, Y., Zhao, K.X., Shen, X.H., Jiang, C.Y. & Liu, S.J. (2008) Genetic and biochemical characterization of a 4-hydroxybenzoate hydroxylase from Corynebacterium glutamicum. Applied Microbiology and Biotechnology, 78, 75–83.

Li, B.H., Wang, C.X. & Dong, X.L. (2013) Research progress in apple diseases and problems in the disease management in China. Plant Protection, 39, 46–54.

Li, C., Li, B.H., Li, G.F. & Wang, C.X. (2014) Pathogenic factors produced by Valsa mali var. mali and their relationship with pathogenicity of different strains. Northern Horticulture, 13, 118–122.

Marchler-Bauer, A., Bo, Y.U., Han, L., He, J., Lanczycki, C.J., Lu, S. et al. (2017) CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucleic Acids Research, 45, D200–D203.

Montersino, S., Orru, R., Barendregt, A., Westphal, A.H., van Duijn, E., Mattevi, A. et al. (2013) Crystal structure of 3-hydroxybenzoate 6-hydroxylase uncovers lipid-assisted flavoprotein strategy for regioselective aromatic hydroxylation. Journal of Biological Chemistry, 288, 26235–26245.

Montersino, S., te Poele, E., Orru, R., Westphal, A.H., Barendregt, A., Heck, A.J.R. et al. (2017) 3-hydroxybenzoate 6-hydroxylase from Rhodococcus jostii RHA1 contains a phosphatidylinositol cofactor. Frontiers in Microbiology, 8, 1110.

Montersino, S., Tischler, D., Gassner, G. & van Berkel, W.J.H. (2011) Catalytic and structural features of flavoprotein hydroxylases and epoxidases. Advanced Synthesis & Catalysis, 353, 2301–2319.

Montersino, S. & van Berkel, W.J.H. (2012) Functional annotation and characterization of 3-hydroxybenzoate 6-hydroxylase from Rhodococcus jostii RHA1. Biochimica et Biophysica Acta, 1824, 433–442.

Natsume, H., Seto, H. & Ŭtake, N. (1982) Studies on apple canker disease. The necrotic toxins produced by Valsa ceratosperma. Agricultural and Biological Chemistry, 46, 2101–2106.

Romero-Silva, M.J., Méndez, V., Agulló, L. & Seeger, M. (2013) Genomic and functional analyses of the gentisate and protocatechuic ring-cleavage pathways and related 3-hydroxybenzoate and 4-hydroxybenzoate peripheral pathways in Burkholderia xenovorans LB400. PLoS One, 8, e56038.

Schreuder, H.A., van der Laan, J.M., Hol, W.G.J. & Drenth, J. (1988) Crystal structure of p-hydroxybenzoate hydroxylase complexed with
its reaction product 3,4-dihydroxybenzoate. *Journal of Molecular Biology*, 199, 637–648.

Shi, X.P., Yu, D.D., Kong, Q.Q., Li, B.H., Liang, W.X. & Wang, C.X. (2015) Optimizing prokaryotic expression of a xylanase gene from the apple pathogen *Valsa mali* var. *mali*. *Advance Journal Food Science and Technology*, 9, 701–705.

Wang, C.X., Dong, X.L., Zhang, Z.F., Li, G.F. & Li, B.H. (2012) Outbreak and the reasons of apple Valsa canker in Yantai apple production area in 2011. *Plant Protection*, 38, 136–138.

Wang, C., Li, C., Li, B., Li, G., Dong, X., Wang, G. et al. (2014) Toxins produced by *Valsa mali* var. *mali* and their relationship with pathogenicity. *Toxins*, 6, 1139–1154.

Wang, J.-Y., Zhou, L., Chen, B.o., Sun, S., Zhang, W., Li, M. et al. (2015) A functional 4-hydroxybenzoate degradation pathway in the phytopathogen *Xanthomonas campestris* is required for full pathogenicity. *Scientific Reports*, 5, 18456.

Wang, X.H., Pan, T.T., Lian, S., Wang, C.X. & Li, B.H. (2018) Effects of environmental factors on the growth and extension of *Valsa mali* in the xylem of apple branches. *Scientia Agricultura Sinica*, 51, 3291–3301.

Xu, C.J., Wu, Y.X., Dai, Q.Q., Li, Z.P., Gao, X.N. & Huang, L.L. (2016) Function of polygalacturonase genes Vmpg7 and Vmpg8 of *Valsa mali*. *Scientia Agricultura Sinica*, 8, 1489–1498.

Xu, M., Gao, X., Chen, J., Yin, Z., Feng, H. & Huang, L. (2018) The feruloyl esterase genes are required for full pathogenicity of the apple tree canker pathogen *Valsa mali*. *Molecular Plant Pathology*, 19, 1353–1363.

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 Phytopathology*, 208, 1202–1216.

Yu, C., Li, T., Shi, X., Saleem, M., Li, B., Liang, W. et al. (2018) Deletion of endo-β-1,4-xylanase *VmXyl1* impacts the virulence of *Valsa mali* in apple tree. *Frontiers in Plant Science*, 9, 663.

Yu, F.W., Gu, Q., Yun, Y.Z., Yin, Y.N., Xu, J.R., Shim, W.B. et al. (2014) The TOR signaling pathway regulates vegetative development and virulence in *Fusarium graminearum*. *New Phytologist*, 203, 219–232.

Yu, Y., Xiao, J.F., Du, J., Yang, Y.H., Bi, C.W. & Qing, L. (2016) Disruption of the gene encoding endo-β-1,4-xylanase affects the growth and virulence of *Sclerotinia sclerotiorum*. *Frontiers in Microbiology*, 7, 1787.

Zhang, K., Yuan, X., Zang, J., Wang, M., Zhao, F., Li, P. et al. (2018) The kynurenine 3-monoxygenase encoding gene, BcKMO, is involved in the growth, development, and pathogenicity of *Botrytis cinerea*. *Frontiers in Microbiology*, 9, 1039.

Zhang, M., Xie, S., Zhao, Y., Meng, X., Song, L., Feng, H. et al. (2019) Hce2 domain-containing effectors contribute to the full virulence of *Valsa mali* in a redundant manner. *Molecular Plant Pathology*, 20, 843–856.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Meng, L., Sun, C., Gao, L., Saleem, M., Li, B. & Wang, C. (2021) Hydroxybenzoate hydroxylase genes underlying protocatechuic acid production in *Valsa mali* are required for full pathogenicity in apple trees. *Molecular Plant Pathology*, 22, 1370–1382. [https://doi.org/10.1111/mpp.13119](https://doi.org/10.1111/mpp.13119)