Phytopathology Research

RESEARCH

Open Access

β-Glucosidase VmGlu1 is required for toxin production and pathogenicity of *Valsa mali*

Xinyue Cui[1,2†], Dewan Zhang[1†], Liyong Gao[1], Na Liu[1], Sen Lian[1], Weichao Ren[1], Baohua Li[1,2*] and Caixia Wang[1*]

Abstract

Flavonoids, serving as crucial secondary metabolites, are widely distributed in plants and play a key role in inhibiting microbial growth, protecting plants from pathogen invasion, and conserving energy. Our previous study revealed that the β-glucosidase VmGlu2 effectively catalyzes the degradation of phloridzin, a typical flavonoid in apples, and regulates the pathogenic process of *Valsa mali*. However, VmGlu2 exhibits a lower efficiency in degrading rutin, another characteristic flavonoid in apples. In this study, we found that the expression level of the β-glucosidase gene *VmGlu1* was induced by 9.42-fold following rutin treatment, and VmGlu1 possessed the capability to degrade both rutin and phloridzin, but under the same conditions, VmGlu1 showed a distinct preference for rutin degradation, with an initial degradation rate 8.66 times higher than that of VmGlu2. Targeted deletion of *VmGlu1* resulted in a 99.32% reduction in pycnidia production, a nearly 33% decrease in toxin production, and a significant decrease in pathogenicity on apple twigs and leaves. Furthermore, we verified that VmGlu1 and VmGlu2 exhibit functional redundancy in pycnidia production, toxin-related β-glucosidase activity, and pathogenicity by generating double-deletion mutants. The results of this study provide valuable insights into the roles of VmGlus in the interaction of *V. mali* and apple during the infection process.

Keywords *Valsa mali*, β-glucosidase, Flavonoids, Pathogenicity

Background

Apple tree canker caused by *Valsa mali* leads to severe damage to the tree trunk, which can extend to the entire tree, potentially resulting in orchard destruction and significant reductions in apple yield, posing a substantial threat to the apple industry (Wang et al. 2014). The pathogen infects the woody parts of apple branches and rapidly proliferates in the xylem vessels, making complete removal of the pathogen from the wood during the treatment challenging, which contributes to the recurrence of old lesions (Wang et al. 2012; Li et al. 2017; Wang et al. 2018).

Necrotrophic fungi, including *V. mali*, are capable of infecting living cells and tissues, as well as thriving within host tissues. A key pathogenic strategy involves secreting enzymes and synthesizing toxins that weaken host tissue by degrading the cell wall, facilitating fungal invasion (McCombe et al. 2022; Fei and Liu 2023). Therefore, the pathogenic mechanism of *V. mali* is considered complex. Previous studies have shown that the cell wall-degrading enzymes (CWDEs), such as xylanase, pectinase and β-glucosidase, along with toxins like protocatechuic acid

†Xinyue Cui and Dewan Zhang these authors have contributed equally to this work.

*Correspondence:
Baohua Li
baohuali@qau.edu.cn
Caixia Wang
cxwang@qau.edu.cn
[1] Shandong Engineering Research Center for Environment-Friendly Agricultural Pest Management, College of Plant Health and Medicine, Qingdao Agricultural University, Qingdao 266000, Shandong, China
[2] College of Horticulture, Qingdao Agricultural University, Qingdao 266000, Shandong, China

and p-hydroxybenzoic acid, plays an important role in the pathogenesis of *V. mali* (Wang et al. 2014; Yin et al. 2015; Xu et al. 2018; Yu et al. 2018).

Flavonoids, the most abundant non-nitrogenous phytochemicals in vascular plants, comprise over 10,000 known secondary metabolites in the plant kingdom (Liu et al. 2021; Tan et al. 2022). They regulate plant development and pigmentation, defense against ultraviolet radiation, insects, and pathogens (Shen et al. 2022; Wang et al. 2022). Apple flavanols (catechins, epicatechins, proanthocyanidins and quercetin glycosides), dihydrochalcones (phloretin glycosides) such as phloridzin and rutin are among the flavonoids present in apples (Rana and Bhushan 2016).

Phloridzin, a glucoside of phloretin, is a dihydrochalcone found in various tissues of the apple tree, including roots, leaves, fruit, and seeds. It serves as a storage form of carbohydrates and is thought to be involved in resistance to various pathogens (Táborský et al. 2021). Studies have shown that apple cultivars resistant to rot showed similar or higher levels of phloridzin compared to susceptible varieties. The biosynthesis of phloridzin mediated by MdUGT88F1 plays an important role in apple tree development and resistance to *V. mali* (Zhou et al. 2019). Rutin, another key flavonoid, has important functions such as enhancing plant stress tolerance, antioxidant activity, scavenging free radicals, and antibacterial effects (Adamcová et al. 2022). As a secondary metabolite, rutin can act as an elicitor of plant disease resistance, inducing and regulating plant resistance to various diseases (Shafi et al. 2019). Rutin can act on bacteria and fungi at multiple targets, inhibiting the production of bacterial biofilms, competitively inhibiting the expression of β-lactamase, interfering with the action of fungal cell walls and membranes, and showing synergistic effects when used in combination with traditional antibiotics (Mandal et al. 2017). Rutin, as an ATP competitive inhibitor, specifically fills the Hsp90 ATP binding site in the N-terminal domain of the red ringworm model structure, thereby exerting antibacterial effects, which can inhibit fungal growth by interfering with Hsp90, which can be considered as a potential novel antifungal cell wall strategy (Gaziano et al. 2018). Although flavonoids have antimicrobial properties, pathogens such as *V. mali* can still infect tissues rich in these compounds. Whether flavonoids play a direct role in apple defense against *V. mali* infection or whether the pathogen can exploit these compounds for infection remains unclear.

Our previous research revealed that the β-glucosidase VmGlu2 can effectively degrade phloridzin in apples and regulate the pathogenic process of *V. mali* (Huang et al. 2021). However, VmGlu2 has a lower degradation rate for rutin. In this study, we found that the expression level of another β-glucosidase gene *VmGlu1* was induced by rutin, increasing 9.42-fold upon exogenous addition of rutin. Further studies have found that VmGlu1 degrades rutin and phloridzin, though it preferentially degrades quercetin under the same conditions. Functional analysis revealed that the Δ*VmGlu1* mutant exhibited decreased pycnidia production and toxin synthesis, significantly reducing its pathogenicity on apple twigs and leaves. These results indicate that VmGlu1 is a key pathogenic factor in *V. mali*, which is involved in canker disease by degrading rutin. VmGlu1 is the first reported CWDE in plant pathogenic fungi that can specifically degrade rutin, providing insights into the VmGlu1-mediated development and pathogenicity of *V. mali*.

Results

Exogenous addition of rutin promoted the expression of *VmGlu1*

In previous studies, we found that VmGlu2 in *V. mali* can specifically degrade phloridzin, contributing to fungal growth, development, and pathogenesis (Huang et al. 2021). However, the degree of degradation of the specific flavonoid compound rutin in apples is relatively low. In the gene annotation of *V. mali* on the National Center for Biotechnology Information (NCBI), we found another β-glucosidase called VmGlu1. VmGlu1 belongs to glycoside hydrolase family 1 and was predicted to carry a 24-aa N-terminal signal peptide.

The wild-type strain LXS080901 was grown on minimal medium supplemented with only 0.2% rutin or 0.1% phloridzin as the sole carbon source, and then detected gene expression levels of *VmGlu1* and *VmGlu2*. Gene expression analysis showed that *VmGlu1* expression increased 9.42-fold under rutin treatment, while *VmGlu2* expression increased 2.83-fold. Under phloridzin treatment, *VmGlu1* expression increased 2.23-fold, whereas *VmGlu2* expression increased 13.42-fold (Fig. 1). These results indicate that *VmGlu1*, rather than *VmGlu2*, plays a more prominent role in rutin degradation in *V. mali*.

Overexpression of *VmGlu1* increased *V. mali* infection in apple trees

Given the high expression of *VmGlu1* in the pathogenic process of *V. mali*, we overexpressed *VmGlu1* in the wild-type strain and confirmed by polymerase chain reaction (PCR) and Western blot (Additional file 1: Figure S1). The wild-type strain and overexpression strains *VmGlu1-OE-1* and *VmGlu1-OE-2* were inoculated on potato dextrose agar (PDA) and apple bark agar (APA) to investigate the influence of *VmGlu1* on the growth of *V. mali*. No significant differences in growth rate or colony

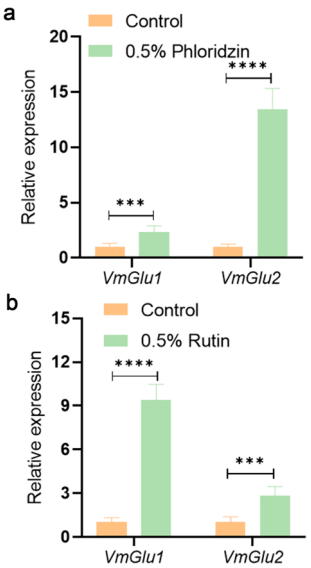

Fig. 1 Transcript levels of *VmGlu1* and *VmGlu2* at exogenous addition of 0.2% rutin or 0.1% phloridzin by quantitative reverse transcription PCR. The transcript level of *V. mali EF1-a* was used as an endogenous control, and the transcript level of each *VmGlu1* and *VmGlu2* gene in the mycelia grown on PDA was normalized to 1. The means and standard deviation of the relative expression levels were calculated from three independent biological replicates. Asterisks represent significant differences (***$P < 0.001$; ****$P < 0.0001$) in transcript levels as compared to those grown at PDA

morphology were observed between *VmGlu1-OE-1*, *VmGlu1-OE-2* and the wild-type strain (Fig. 2a). However when 0.2% salicin was added, the *VmGlu1-OE* strains exhibited faster growth rates than the wild-type strain (Fig. 2a). To further explore whether the overexpression of the VmGlu1 affects the pathogenicity of *V. mali*, the wild-type strain and overexpression strains *VmGlu1-OE-1* and *VmGlu1-OE-2* were inoculated to detached leaves and wounded branches of 'Fuji'. The *VmGlu1-OE-1* and *VmGlu1-OE-2* strain exhibited an increase of 93.2% and 39.3% in the average lesion size on apple leaves and twigs, respectively (Fig. 2b, c). These findings suggest that *VmGlu1* is involved in the pathogenic process of *V. mali*.

VmGlu1 is involved in the pycnidia formation of *V. mali*

Given the enhanced pathogenicity displayed by the *VmGlu1* overexpression in *V. mali*, we proceeded to perform targeted knockout of *VmGlu1* to further investigate its role in the development and pathogenic process of *V. mali*. Mutant strains with a targeted deletion of *VmGlu1* were generated and confirmed via PCR using the primer pairs listed in Table S1 (Additional file 1: Figure S1). The results from these assays confirmed that *VmGlu1* was successfully replaced by the hygromycin B phosphotransferase gene (*HPH*). In addition, the complementation strain Δ*VmGlu1-C* was created by introducing the native promoter and coding sequence of *VmGlu1* into the *ΔVmGlu1* mutant. The complementation strain Δ*VmGlu1-C* was verified by PCR and its phenotype was consistent with that of the wild-type strain (Additional file 1: Figures S1, S2). Additionally, double knockout strains of *VmGlu1* and *VmGlu2* ΔΔ*VmGlu2/VmGlu1* were further obtained to detect the functional redundancy of *VmGlu1* and *VmGlu2*, and the identification PCR electrophoretogram was shown in Additional file 1: FigureS1d. Then, we examined vegetative growth and pathogenicity on apple branches and leaves to confirm that ectopic expression of *VmGlu1* can restore the phenotype missing of Δ*VmGlu1*.

To analyze the roles of *VmGlu1* and *VmGlu2* in *V. mali* growth and development, we evaluated the colony morphology, growth rate, and pycnidia formation of the gene deletion mutant Δ*VmGlu1*, Δ*VmGlu2*, ΔΔ*VmGlu2/VmGlu1* and the wild-type strain. No significant growth reduction in Δ*VmGlu1*, Δ*VmGlu2*, ΔΔ*VmGlu2/VmGlu1* compared to the wild-type strain (Fig. 3a, c and d). All strains were cultured on bark culture medium for 3 days at 25℃, followed by induction for 30 days under UV light (365 nm), and the number of pycnidia was counted. The Δ*VmGlu1* and Δ*VmGlu2* strain produced fewer than 3 pycnidia per plate, ΔΔ*VmGlu2/VmGlu1* produced fewer pycnidia that are close to 0 than Δ*VmGlu1* and Δ*VmGlu2* strains, whereas the wild-type strain produced over 110 and pycnidia per plate. (Fig. 3b, e). These results indicate that while *VmGlu1* is not involved in vegetative growth, it plays a crucial role in the pycnidia formation of *V. mali*.

Preference of VmGlu1 and VmGlu2 for flavonoid degradation

In view of the differences in *VmGlu1* and *VmGlu2* expression induced by rutin, we hypothesized that VmGlu1 and VmGlu2 may differ in their abilities to degrade rutin and phloridzin. To test this, the wild-type strain, Δ*VmGlu1*, Δ*VmGlu2* and ΔΔ*VmGlu2/VmGlu1* mutant were cultured on minimal medium supplemented with rutin or phloridzin as the sole carbon source. We then observed the colony growth of the strains. For phloridzin, the colony diameter of Δ*VmGlu2* was significantly decreased compared with the wild-type strain and Δ*VmGlu1*, while ΔΔ*VmGlu2/VmGlu1* exhibited an even greater reduction compared to Δ*VmGlu2* (Fig. 4a, b). In contrast, for rutin, the colony diameter of Δ*VmGlu1* was significantly reduced compared with the wild-type strain and Δ*VmGlu2*, while ΔΔ*VmGlu2/VmGlu1* showed an even greater reduction compared to Δ*VmGlu1* (Fig. 4a, c). To further quantify the utilization of phloridzin and rutin by each strain,

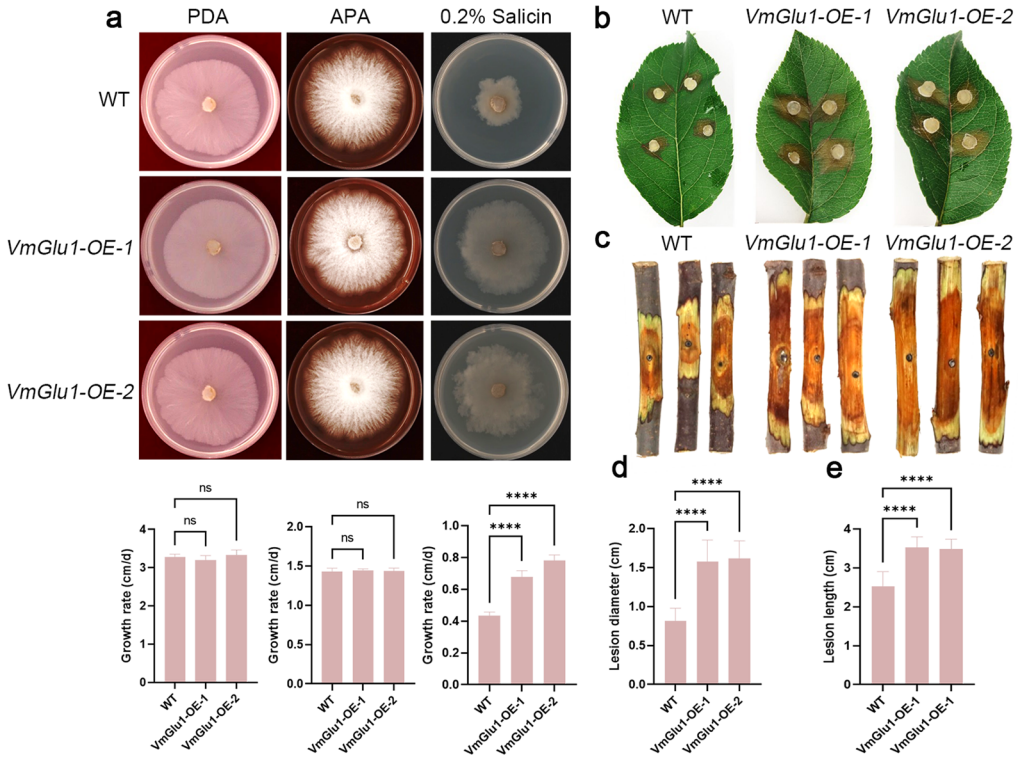

Fig. 2 Colony morphology, vegetative growth and pathogenicity of the wild-type strain and *VmGlu1-OE-1*, *VmGlu1-OE-2* mutants. **a** Colony phenotypes of different strains grown on PDA, APA and 0.2% salicin at 25 °C in the dark for 3 days, and the mycelial growth rate of different strains on PDA, APA and 0.2% salicin at 25 °C for 3 days. **b, c** The infected phenotype of apple leaves and twigs inoculated with different strains.**d, e** Lesion sizes produced by different strains on apple leaves and twigs. Asterisks on the bars indicate a significant difference with the wild-type strain (****$P < 0.001$). The experiments were repeated thrice

the contents of phloridzin and rutin in the remaining medium were measured by high-performance liquid chromatography (HPLC). The results revealed that Δ*VmGlu2* had a significantly reduced capacity to utilize phloridzin, while Δ*VmGlu1* had a significantly reduced capacity to utilize rutin (Fig. 4d, e).

VmGlu1 and VmGlu2 were expressed in a prokaryotic expression system and purified by glutathione S-transferase (GST)-tag affinity chromatography to obtain the purified proteins of VmGlu1 and VmGlu2. In a system containing either VmGlu1 or VmGlu2 proteins, 0.1 mg/mL rutin and phloridzin were added, and degradation was assessed by HPLC. During the first 0–12 h, the degradation rate of phloridzin by VmGlu2 was 2.7-fold higher than that of VmGlu1, with phloridzin being nearly completely degraded after 36 h (Fig. 4f). Meanwhile, the degradation rate of rutin by VmGlu1 was 8.66-fold higher than that of VmGlu2 in the first 0–12 h, with a significant difference persisting until 48 h, with the the final degradation rate of rutin being about 80% (Fig. 4g). Based on these results, we conclude that VmGlu1 preferentially degrades rutin, while VmGlu2 preferentially degrades phloridzin. VmGlu1 and VmGlu2 exhibit some redundancy in the degradation of both flavonoids in the absence of other carbon sources.

VmGlu1 is required for pathogenicity

Pathogenicity assays on both the detached apple leaves and twigs were conducted to investigate the role of *VmGlu1* and *VmGlu2* in disease infection. The Δ*VmGlu1* and Δ*VmGlu2* strains exhibited significantly reduced pathogenicity on apple leaves and twigs compared to the wild-type strain, which typically causes necrosis and canker (Fig. 5). The Δ*VmGlu1* and Δ*VmGlu2* strains exhibited a reduction of over 38.24% and 38.82% in the average lesion size on apple leaves and 43.28% and 40.89% on twigs. Moreover, the ΔΔ*VmGlu2/VmGlu1* showed a milder virulent phenotype compared with Δ*VmGlu1* and Δ*VmGlu2*, with lesion sizes by 65.29% on apple leaves and 70.21% on branches compared to the wild-type strain. These results indicated that *VmGlu1* and *VmGlu2* play a crucial role in the pathogenicity of *V. mali*. Moreover, *VmGlu1* and *VmGlu2* are redundant in regulating *V. mali* pathogenicity.

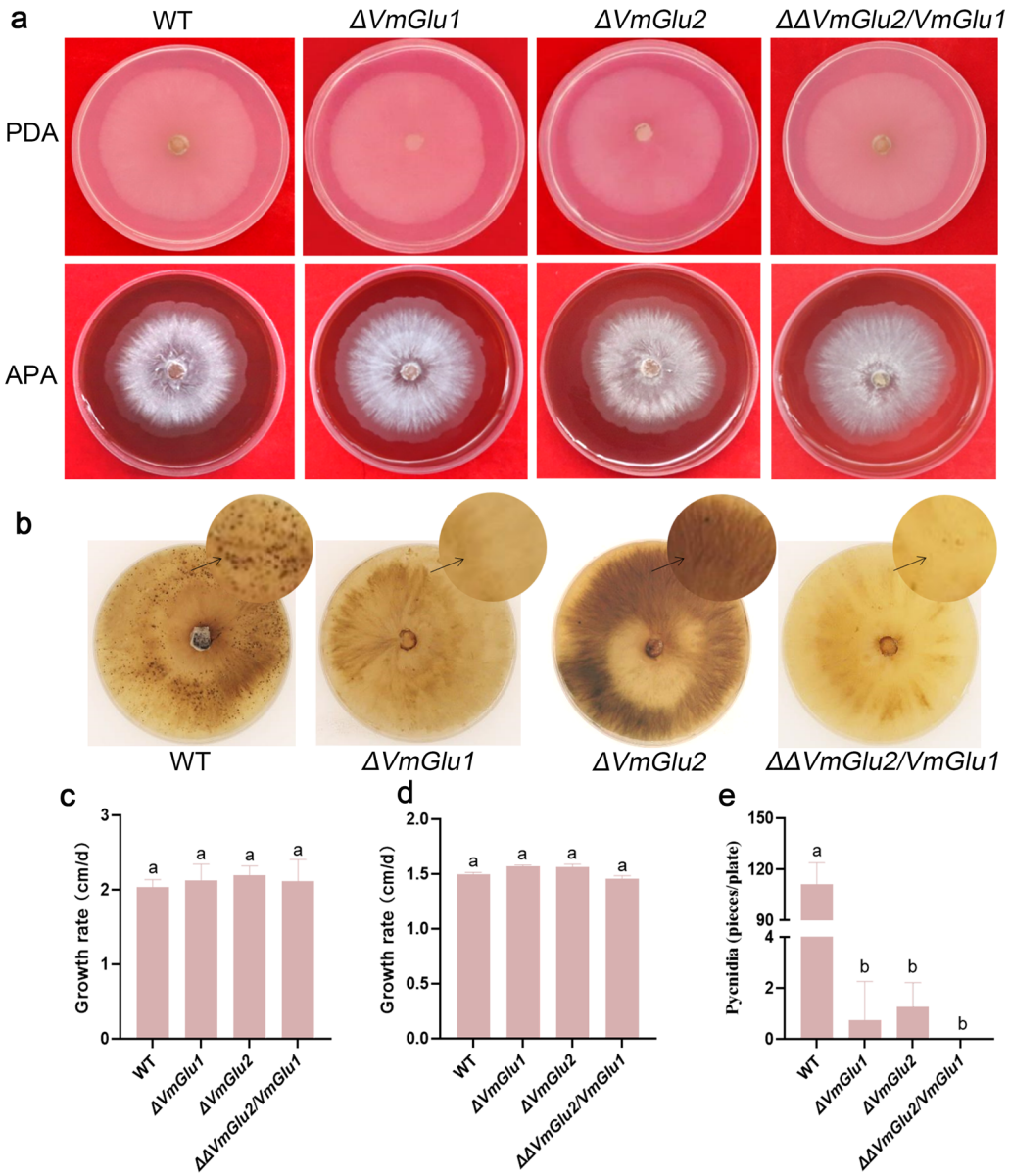

Fig. 3 Colony morphology, vegetative growth and sporulation of the wild-type strain, *VmGlu1* and *VmGlu2* deletion mutants. **a** Colony phenotypes of different strains grown on PDA and APA at 25 °C in the dark for 3 days. **b** Colony phenotypes of different strains grown on bark culture medium for 3 days at 25 °C and then induced for 30 days under UV light (365 nm). **c** The mycelial growth rate of different strains on PDA at 25 °C for 3 days. **d** The mycelial growth rate of different strains on APA at 25 °C for 3 days. **e** The number of pycnidia of the different strains was counted. The bars represent the standard deviations, and the different letters indicate significant differences (ANOVA test with Tukey's comparisons, $P < 0.01$). The experiments were repeated thrice

VmGlu1 deletion affects the activity of β-glucosidases in *V. mali*

We further examined the effect of VmGlu1 deletion on β-glucosidases activity in the culture filtrates of the wild-type and mutant strains. Sucrose was found to induce the production and secretion of β-glucosidases. The β-glucosidases activity of the Δ*VmGlu1* and Δ*VmGlu2* mutants was reduced by 34.57% and 28.97%, respectively, compared to the wild-type strain (Fig. 6a). ΔΔ*VmGlu2*/*VmGlu1* exhibited an even further reduction, with β-glucosidase activity reaching only 61.21% of that in the wild-type strain. These results indicated that VmGlu1 has β-glucosidases activity in *V. mali*. In addition, VmGlu1 and VmGlu2 have redundancy in controlling the β-glucosidases activity of *V. mali*.

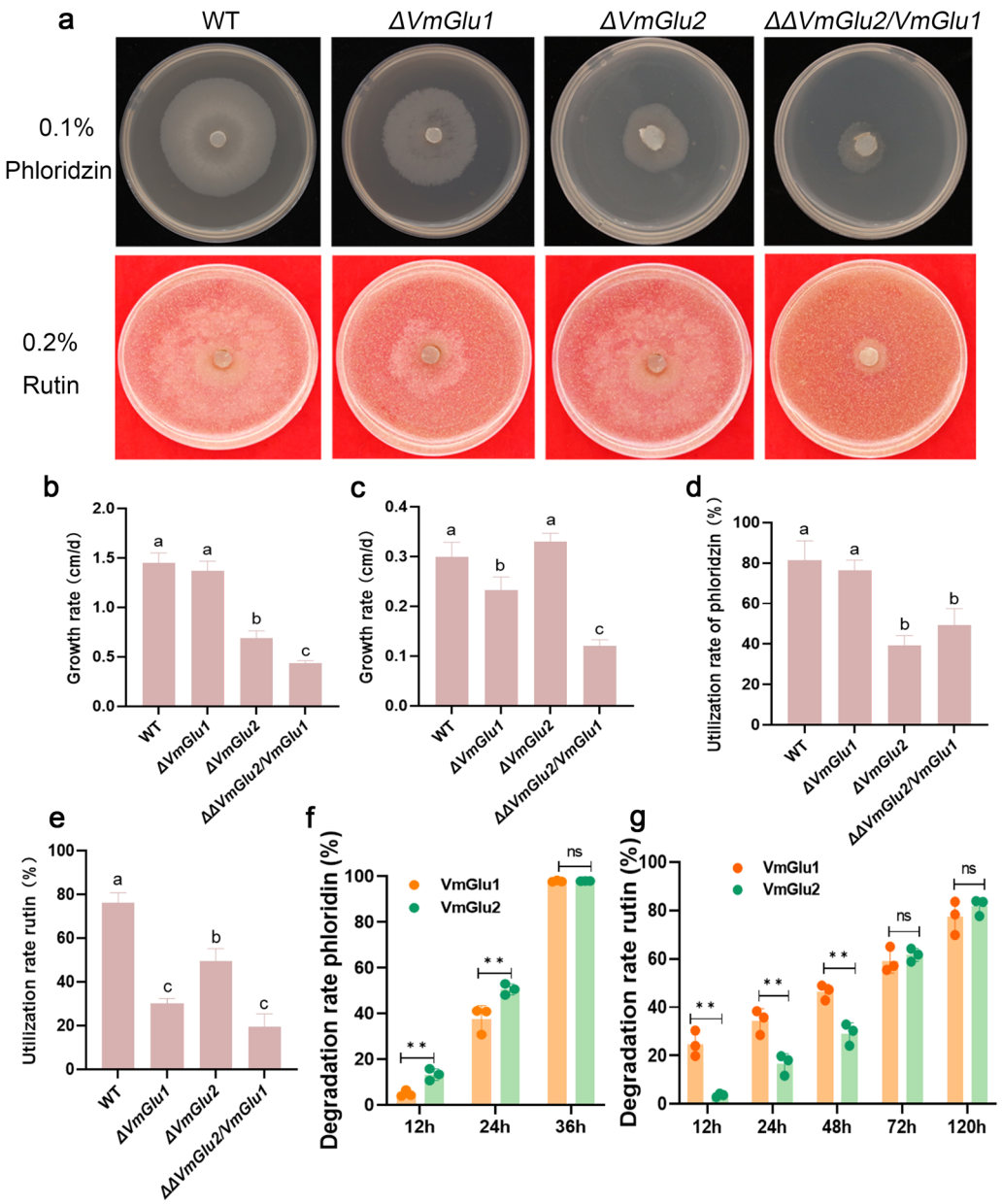

Fig. 4 Utilization and degradation of rutin and phloridzin of each strain. **a** Colony phenotypes of different strains grown on PDA with 0.1% phloridzin (upper half part) and 0.2% rutin (bottom half part) at 25 °C in the dark for 3 or 6 days. **b** The mycelial growth rate of different strains on PDA with 0.1% phloridzin at 25 °C for 3 days. **c** Mycelial growth rate of different strains on PDA with 0.2% rutin at 25 °C for 6 days. **d** The degradation rate of phloretin in different strains on minimal medium supplemented with phloridzin was determined by HPLC. **e** The degradation rate of phloretin in different strains on minimal medium supplemented with containing rutin was determined by HPLC. **f** The degradation rate of purified proteins VmGlu1 and VmGlu2 under phloretin-containing PBS buffer was determined by HPLC. **g** The degradation rate of purified proteins VmGlu1 and VmGlu2 under rutin-containing PBS buffer was determined by HPLC. Asterisks on the bars indicate a significant difference with the wild-type strain (**$P < 0.01$). Different letters indicate significant differences (ANOVA test with Tukey's comparisons, $P < 0.01$). The experiments were repeated thrice

Sequence alignment revealed that *VmGlu1* contains two potentially highly conserved catalytic residues (E248 and E473), which are crucial for its β-glucosidases activity. Site-directed mutagenesis was performed to substitute these glutamic acid residues with aspartate (Additional file 1: Figure S3). In PBS buffer containing 0.2% rutin, the degradation rate of VmGlu1 protein was 59.22% in 72 h, while the degradation rate of the

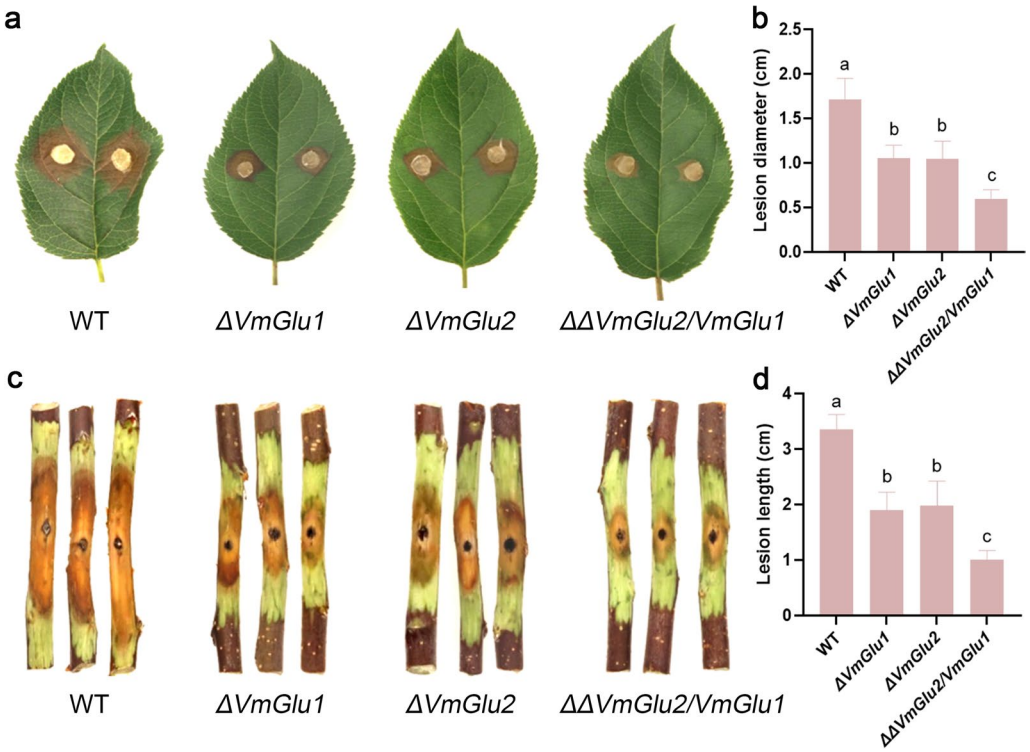

Fig. 5 Pathogenicity assays of the wild-type strain, *VmGlu1* and *VmGlu2* deletion mutants against leaves and twigs of apple (*M. domestica* 'Fuji'). **a, c** The infected phenotype of apple leaves and twigs inoculated with different strains. **b, d** Lesion sizes produced by different strains on apple leaves and twigs. Different letters indicate significant differences (ANOVA test with Tukey's comparisons, $P < 0.01$) The experiments were repeated thrice

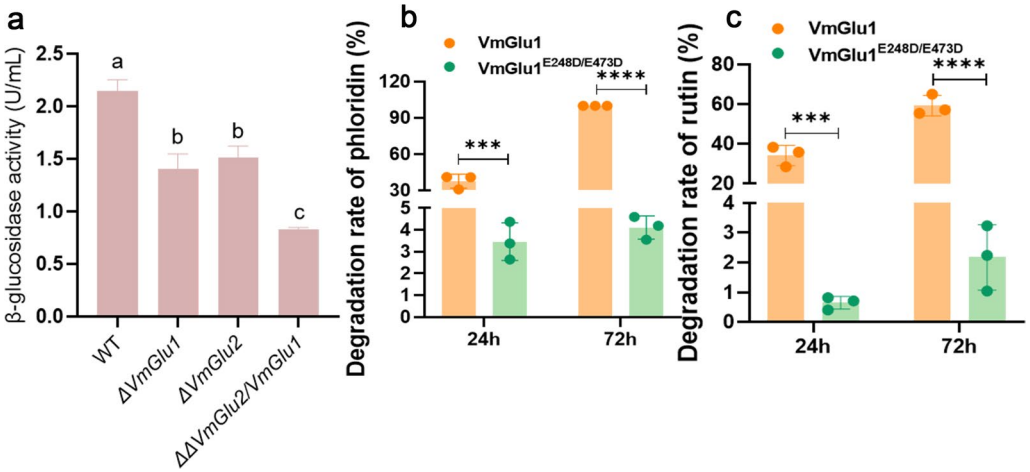

Fig. 6 β-glucosidases activity assays of the wild-type strain, *VmGlu1* and *VmGlu2* deletion mutants. **a** Effect of *VmGlu1* and *VmGlu2* deletion on the β-glucosidases activity. β-glucosidases activity was determined in the infestation of isolated 'Fuji' twigs for the wild-type strain, *VmGlu1* deletion strain, and complementation strain after 5 days. The β-glucosidases activity was expressed as units per min per ml (U/mL). The bars represent the standard deviations, and the asterisks indicate significant differences from the wild-type and complementation strains (***$P < 0.001$; ****$P < 0.0001$). **b** The degradation rate of purified site-directed mutant protein of VmGlu1 for phloridzin at 24 and 72 h. **c** The degradation rate of purified site-directed mutant protein of VmGlu1 for rutin at 24 and 72 h. Asterisks on the bars indicate a significant difference with the wild-type strain ($P < 0.01$). Different letters indicate significant differences (ANOVA test with Tukey's comparisons, $P < 0.01$). The experiments were repeated thrice

site-directed mutant protein was only 2.17%. Similarly, when treated with 0.1% phloridzin, the degradation rate of VmGlu1 protein was 99.96% in 72 h, while the degradation rate of the site-directed mutant protein was only 4.11% (Fig. 6b, c). These results confirmed that the conserved catalytic residues (E248 and E473) are the key to the degradation of rutin and phloridzin.

VmGlu1 is involved in the production of toxins in *V. mali*

To examine the differences in toxin content among Δ*VmGlu1*, Δ*VmGlu2*, and ΔΔ*VmGlu2/VmGlu1* by HPLC, we measured the concentrations of protocatechuic acid and 3-hydroxybenzoic acid, the main toxins produced by *V. mali*, using HPLC. Therefore, we detected the content of these two toxins produced by the different strains in liquid APA medium, and the results showed that for 3-hydroxybenzoic acid, the content of 3-hydroxybenzoic acid in the Δ*VmGlu1* was about 81% of that in the wild-type strain, the content of 3-hydroxybenzoic acid in the Δ*VmGlu2* was about 70% of that in the wild-type strain, and the content of 3-hydroxybenzoic acid in the ΔΔ*VmGlu2/VmGlu1* was about 40% of that in the wild-type strain (Fig. 7a). For protocatechuic acid, the content of protocatechuic acid in Δ*VmGlu1* was about 65% of that in the wild-type strain, the protocatechuic acid in Δ*VmGlu2* was about 55% of that in the wild-type strain, and the content of protocatechuic acid in the ΔΔ*VmGlu2/VmGlu1* was about 32% of that in the wild-type strain (Fig. 7b). This indicates that *VmGlu1* knockout can significantly reduce the level of protocatechuic acid and 3-hydroxybenzoic acid produced by the *V. mali*, and that the absence of both *VmGlu1* and *VmGlu2* further reduces the level of protocatechuic acid and 3-hydroxybenzoic acid produced by the *V. mali*. It also indicates that the absence of VmGlu1 and VmGlu2 affects the level of toxin production by *V. mali*, and that *VmGlu1* and *VmGlu2* have redundant effects on the production of 3-hydroxybenzoic acid and protocatechuic acid by the *V. mali*.

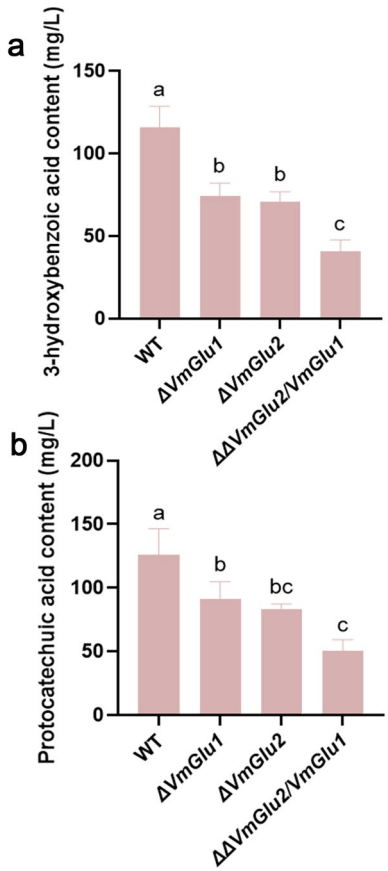

Fig. 7 Toxin-producing level of the wild-type strain, *VmGlu1* and *VmGlu2* deletion mutants. **a** The concentration of 3-hydroxybenzoic acid produced by different strains. **b** The concentration produced by different strains. Different letters indicate significant differences (ANOVA test with Tukey's comparisons, $P < 0.01$)

Discussion

In this study, we characterized *VmGlu1* and elucidated its involvement in *V. mali* sporulation, pathogenicity, and toxin production, along with its β-glucosidase activity during the pathogenic process. We also performed a comparative analysis of the substrate specificity of VmGlu1 and VmGlu2. Exogenous application of rutin significantly increased the expression levels of both genes, with *VmGlu1* showing expression levels 3.35 times higher than *VmGlu2*. Interestingly, VmGlu1 and VmGlu2 exhibited distinct functions in the selective degradation of rutin and phloridzin. In the presence of both compounds, VmGlu1 preferentially degraded rutin before phloridzin, while VmGlu2 prioritized the degradation of phloridzin over rutin. The double knockout mutant ΔΔ*VmGlu2/Glu1* revealed that *VmGlu1* and *VmGlu2* have redundant functions in toxin production and β-glucosidase secretion.

CWDEs are vital enzymes secreted by pathogenic fungi, playing crucial roles in pathogen invasion, establishment, and reproduction processes (Rafiei et al. 2021). For example, in *Botrytis cinerea*, it was found that the BcXyl1 gene is highly expressed during the infection of tobacco and tomato, and the strains lacking *BcXyl1* severely weakened the virulence of *B. cinerea* (Yang et al. 2018). Moreover, the disruption of *Xyn11A* in *B. cinerea* led to a decrease in extracellular endo-beta-1–4-xylanase activity and reduced the size of disease spots by more than 70% (Brito et al. 2006). In *V. mali*, it was also found that knocking out the gene encoding xylanase VmXyl1 resulted in normal growth rates but significantly lower sporulation levels and

virulence compared to the wild-type (Yu et al. 2018). β-glycoside hydrolase, as one of the primary CWDEs, has been shown to play a role in the pathogenicity of phytopathogen. In this study, we targeted the key CWDEs β-glucosidase in *V. mali*, and found that *VmGlu1* also participates in the pathogenic process of *V. mali*. Moreover, the double knockout strain *ΔΔVmGlu1/Glu2* exhibits lower levels of pathogenicity, providing strong evidence for the involvement of CWDEs in the infection process of pathogenic fungi.

In this study, we observed that *VmGlu1* deletion did not affect morphology and mycelial growth rate, but it was found to be essential for pycnidia formation in *V. mali*. Similar results have been demonstrated in other pathogenicity factors of pathogenic fungi (Xu et al. 2018). For example, the deletion mutant of *VmXyl2*, which is another gene encoding xylanase, displayed a normal growth rate and reduction in pycnidia formation (Cui et al. 2024). Moreover, the deletion of *BcKMO*, which encodes kynurenine 3-monooyxgenase, in *B. cinerea* is known to play a crucial role in fungal development. The mutant lacking *BcKMO* exhibits impaired conidia and sclerotia production (Zhang et al. 2018). Conidia production is a vital phase in the pathogens' life cycle (Huang et al. 2021). Therefore, it is widely believed that inhibiting the pycnidia formation of *V. mali* could alleviate or effectively control the occurrence of Valsa canker in apple trees.

Flavonoid compounds, such as rutin, play key roles in plant defense against biotic and abiotic stresses. Rutin enhances stress tolerance, has antioxidant properties, scavenges free radicals, and inhibits bacterial growth. It also interferes with bacterial biofilm formation, inhibits β-lactamase expression, and disrupts fungal cell walls and membranes, showing synergistic effects with traditional antibiotics. In this study, purified VmGlu1 in vitro targeted and degraded rutin, which may explain the weakened pathogenicity of the Δ*VmGlu1* strain.

The main pathogenic factors of *V. mali* involve various CWDEs and toxins (Castillo et al. 2017; Chen et al. 2012; Wang et al. 2014). Therefore, this study investigated the activity of β-glucosidase VmGlu1 and toxin production. Compared to the wild-type strain, the *VmGlu1* mutant strain exhibited significantly lower β-glucosidase activity, while *VmGlu1* overexpression transformants had significantly higher enzyme activity, consistent with the decreased and increased pathogenicity of gene deletion and overexpression strains, respectively. Similarly, the deletion mutant of *VmXyl1* showing significantly reduced virulence also had significantly lower enzyme activities than the wild-type strain. Additionally, our data also indicate that the toxin production in the *VmGlu1* mutant strain was significantly lower than in the wild-type strain, corresponding to the results of reduced β-glucosidase activity and pathogenicity.

In summary, our study provides new evidence that *VmGlu1* is essential for pycnidia formation and pathogenicity in *V. mali*. *VmGlu1* regulates glucosidase activity and selectively degrades rutin, playing a key role in counteracting plant defenses. The distinct degradation functions of VmGlu1 and VmGlu2 will help further explore their roles in fungal biology and provide potential targets for developing new fungicidal strategies against *V. mali*. This research offers important theoretical insights for the sustainable, green control of *V. mali*.

Conclusions

In summary, our research provides new evidence demonstrating that VmGlu1 as CWDEs is essential for the pathogenicity of *V. mali*. VmGlu1 is involved in pycnidia production, regulates β-glucosidase activity, and participates in the production of key toxins 3-hydroxybenzoic acid and catechin. VmGlu1 can specifically degrade rutin. Substrate preference analysis revealed that VmGlu1 primarily targets rutin, while VmGlu2 predominantly degrades phloridzin. Furthermore, VmGlu1 and VmGlu2 exhibit functional redundancy in pycnidia formation, β-glucosidase activity, toxin production, and the pathogenic processes of *V. mali*.

Methods

Strains and culture conditions

The wild-type *V. mali* strain LXS080901 isolated and preserved by our laboratory was grown on PDA at 25℃ in the dark. The gene deletion mutants and complementation strains were cultured on PDA supplemented with 100 mg/mL hygromycin B or geneticin G418 (Sigma, St. Louis, MO, USA). *Escherichia coli* strains were grown in Luria–Bertani (LB) with appropriate antibiotics at 37 °C.

Identification and sequence analysis of *VmGlu1* in *V. mali*

Total RNA was isolated from fresh mycelia using RNAiso Plus Kit (TaKaRa, Dalian, China). Subsequently, 5 μg of total RNA from each sample was reverse transcribed to cDNA using a *HiScript* II 1st-Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, Nanjing, China). The gene *VmGlu1*, which is predicted to have xylanase activity and shows high transcript levels during *V. mali* infection, was cloned and sequenced.

DNA and RNA extraction and RT-PCR

The genomic DNA of all tested strains was extracted from the mycelia following the SDS-CTAB method. The resulting genomic DNA was used for PCR. Total RNA

was isolated from fresh mycelia using an RNeasy Mini Kit (Qiagen, 74104, Hilden, Germany). Subsequently, 5 mg of total RNA from each sample was reverse transcribed to cDNA using a HiScript II 1st-Strand cDNA Synthesis Kit (+ gDNA wiper) (Vazyme, China). RT–PCR was performed to confirm the deletion and complementation of the targeted gene with gene-specific primers (Additional file 2: Table S1).

The wild-type strain was cultured on minimal liquid medium supplemented with only 0.2% rutin or 0.1% phloridzin added as a sole carbon source for 24 h. The RNA was extracted from mycelium, and then the cDNA was synthesized. All RT-qPCR experiments were conducted with SYBR Master Mix (TaKaRa, Dalian, China), following the manufacturer's protocol. The *EF1-a* gene was used as an endogenous reference. The whole experiment was repeated twice, and three replicates were included in each experiment. All primers used in these assays are listed in Additional file 2: Table S1.

Generation of gene deletion and complementation strains

To obtain *VmGlu1* gene deletion mutants, PEG-mediated protoplast transformation was carried out to get homologous recombination, as described previously (Meng et al. 2021). The gene deletion cassette with three components used the *HPH* as a selective marker for gene deletion (Additional file 1: Figure S1). Upstream and downstream fragments of *VmGlu1* genes were amplified from the genomic DNA of the wild-type strain LXS080901 using the gene-specific primers (Additional file 2: Table S1). The *HPH* gene was amplified from the vector pBS. The gene deletion cassette was generated by double-joint PCR, and the result was confirmed by sequencing. The cassettes were later transformed into the protoplasts of *V. mali* LXS080901, and the transformants were screened by culturing on a medium with 100 μg/ml hygromycin B. The putative gene deletion mutants were validated by PCR using four primer pairs (Additional file 1: Figure S1 and Additional file 2: Table S1). For double-knockout mutants, the screening gene was replaced with G418, and the *VmGlu2* gene was knocked out in the *ΔVmGlu1* background. For generating the *VmGlu1* complementation strains, the fragment containing the full-length coding of *VmGlu1* and its native promoter region are constructed into the pYF11 vector by homologous recombination, which was then introduced into the *VmGlu1* deletion mutant through PEG-mediated transformation. The transformants were confirmed by PCR.

Vegetative growth, pycnidia formation, and pathogenicity assays

Mycelial plugs (diameter of 5 mm) cut from actively growing colony edges of the wild-type strain, gene deletion mutants, and complemented mutants were transferred to PDA plates. The plates were then incubated at 25℃ before the colony shape, color, and diameters were assessed. For the pycnidia formation experiment, the *V. mali* strains were cultured on PDA plates for 3 days at 25℃ and then induced for 30 days under UV light (365 nm), and the number of pycnidia was counted.

Pathogenicity assays were performed using apple leaves and 1-year-old twigs (*Malus. domestica* 'Fuji') were collected from the greenhouse at Qingdao Agricultural University, Qingdao, China. The detached leaves and twigs were sterilized with 75% ethanol, and wounds were made as described by Yu et al. (2018). Mycelial plugs were used to inoculate the wounds. The inoculated leaves and twigs were placed in trays, maintained under high humidity, 25℃ and darkness. The lesion length was measured, and the development of the lesions was photographed at several time points. The assays were repeated three times, and at least 15 leaves and twigs were included in each treatment.

Recombinant protein expression and purification.

Full-length VmGlu1 cDNA and site-directed VmGlu1 fragment was amplified and cloned into was amplified and cloned and inserted into the *Ecor* I and *BssH* II sites of the pET-32a vector. VmGlu1 recombinant protein was expressed in *E. coli* strain DE3 cells. Expression was induced by incubation with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 24 h at 16 °C. Cells were collected by centrifugation at 5000×*g* for 10 min. For protein extraction, cells were resuspended in lysis buffer (20 mM sodium hydrogen phosphate, 300 mM NaCl, pH 7.4) supplemented with 1 mg/ml lysozyme, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1.98 mM β-mercaptoethanol and then subjected to sonication and centrifugation at 10,000×*g* for 10 min. VmGlu1 was purified by affinity chromatography using Ni–NTA resin (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions.

β-glucosidase activity assay

The β-glucosidase activity was evaluated following the 3,5-dinitrosalicylic acid (DNS) method described by Yu et al. (2018). This process involved a reaction mixture comprising the purified recombinant protein or culture filtrate and 0.5% beechwood sucrose dissolved in 50 mM sodium citrate buffer at pH 5.0. The mixture was incubated at 50 °C for 30 min. Subsequently, DNS

solution was added to the mixture, which was then boiled for 5 min. Absorbance was measured at 540 nm. β-glucosidase activity was defined as the enzyme quantity necessary to catalyze the release of 1.0 mM of sucrose per minute under the conditions of pH 5.0 and 50 °C. The activity was quantified in units per minute per milligram of protein (U/mg).

Toxigenic assay

The wild-type LXS080901, Δ*VmGlu1*, Δ*VmGlu2* and ΔΔ*VmGlu2*/*VmGlu1* strains were taken and inoculated into the apple bark medium. Each medium was 40 mL, each bottle was inoculated with 6 hyphal pegs, and 3 bottles were repeated for each strain. The inoculated medium was placed in a shaking table of 180 rpm at 25℃ for 11 d, the supernatant of the medium was absorbed, diluted twice with methanol, and then filtered with 0.45 μm filter membrane. The toxigenic levels of the four strains were detected by HPLC.

Bioinformatics and data analysis

The molecular weight of VmGlu1 protein was analyzed and predicted using https://prosite.expasy.org/prosite.html. Signal peptides were analyzed with DetaiBio (http://www.detaibio.com/tools/index.php?r=signal-peptide/index). The hydrophobic amino acid sequence was analyzed using https://web.expasy.org/protparam/.

Statistical analysis

All treatments were performed in three independent biological experiments with three replicates. All statistical analysis was conducted using GraphPad Prism software (Version 9.5). All the data collected were subjected to analysis of variance (ANOVA). The asterisks indicate a statistically significant difference with the wild-type (**$P < 0.01$; ***$P < 0.001$; ****$P < 0.001$).

Abbreviations

APA	Apple bark agar
CWDEs	Cell wall-degrading enzymes
DNS	3,5-Dinitrosalicylic acid
GST-tag	Glutathione S-transferase tag
HPLC	High-performance liquid chromatography
HPH	Hygromycin B phosphotransferase gene
IPTG	Isopropyl-β-D-thiogalactopyranoside
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PMSF	Phenylmethanesulfonyl fluoride

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-024-00296-z.

Additional file 1: **Figure S1**. Identification of gene deletion, complementation, and overexpressed mutants. **Figure S2**. The phenotype of ΔVmGlu1-C was consistent with that of the wild-type strain. **Figure S3**. The Western blot result of the expression of VmGlu1 and site-directed mutagenesis of VmGlu1.

Additional file 2: **Table S1**. Primers used in this study.

Acknowledgements

We thank all the other members of Shandong Province Key Laboratory of Applied Mycology, Qingdao Agricultural University for their helpful suggestions and constructive criticism.

Author contributions

XC wrote the manuscript, XC, DZ and LG performed the experiments. WR, NL and SL discussed the results. BL and CW helped with the design of the experiments and revised the manuscript. All authors read and approved the final manuscript.

Funding

This study was funded by grants from the National Natural Science Foundation of China (Grant no. 32072367), Shandong Provincial Natural Science Foundation (Grant no. ZR2023MC020), Key Research and Development Program of Shandong Province (2022CXGC020709), and Chinese Modern Agricultural Industry Technology System (Grant no. CARS-27).

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 8 July 2024 Accepted: 5 December 2024
Published online: 23 January 2025

References

Abeliovich H, Darsow T, Emr SD. Cytoplasm to vacuole trafficking of aminopeptidase I requires a t-SNARE-Sec1p complex composed of Tlg2p and Vps45p. Embo J. 1999;18(21):6005–16. https://doi.org/10.1093/emboj/18.21.6005.

Adamcová A, Horna A, Šatínský D. Determination of phloridzin and other phenolic compounds in apple tree leaves, bark, and buds using liquid chromatography with multilayered column technology and evaluation of the total antioxidant activity. Pharmaceuticals (Basel). 2022;15(2):56. https://doi.org/10.3390/ph15020244.

Branda SS, Gonzalez-Pastor JE, Ben-Yehuda S, Losick R, Kolter R. Fruiting body formation by Bacillus subtilis. Proc Natl Acad Sci USA. 2001;98(20):11621–6. https://doi.org/10.1073/pnas.191384198.

Brito N, Espino JJ, González C. The endo-beta-1,4-xylanase xyn11A is required for virulence in Botrytis cinerea. Mol Plant Microbe Interact. 2006;19(1):25–32. https://doi.org/10.1094/mpmi-19-0025.

Castillo L, Plaza V, Larrondo LF, Canessa P. Recent advances in the study of the plant pathogenic fungus *Botrytis cinerea* and its interaction with the environment. Curr Protein Pept Sci. 2017;18(10):976–89. https://doi.org/10.2174/1389203717666160809160915.

Cui X, Li X, Li S, Huang Y, Liu N, Lian S, et al. Xylanase VmXyl2 is involved in the pathogenicity of *Valsa mali* by regulating xylanase activity and inducing

cell necrosis. Front Plant Sci. 2024;15:1342714. https://doi.org/10.3389/fpls.2024.1342714.
Fei W, Liu Y. Biotrophic fungal pathogens: a critical overview. Appl Biochem Biotechnol. 2023;195(1):1–16. https://doi.org/10.1007/s12010-022-04087-0.
Gaziano R, Campione E, Iacovelli F, Marino D, Pica F, Di Francesco P, et al. Antifungal activity of *Cardiospermum halicacabum* L. (Sapindaceae) against Trichophyton rubrum occurs through molecular interaction with fungal Hsp90. Drug Des Devel Ther. 2018;12:2185–93. https://doi.org/10.2147/dddt.S155610.
Huang Y, Yu C, Sun C, Saleem M, Li P, Li B, et al. β-Glucosidase VmGlu2 contributes to the virulence of *Valsa mali* in apple tree. Front Microbiol. 2021;12:695112. https://doi.org/10.3389/fmicb.2021.695112.
Ivanov M, Kannan A, Stojković DS, Glamočlija J, Calhelha RC, Ferreira I, et al. Flavones, flavonols, and glycosylated derivatives-impact on candida albicans growth and virulence, expression of CDR1 and ERG11, Cytotoxicity. Pharmaceuticals (Basel). 2020;14(1):63. https://doi.org/10.3390/ph14010027.
Jourdan S, Francis IM, Deflandre B, Tenconi E, Riley J, Planckaert S, et al. Contribution of the β-glucosidase BglC to the onset of the pathogenic lifestyle of Streptomyces scabies. Mol Plant Pathol. 2018;19(6):1480–90. https://doi.org/10.1111/mpp.12631.
Liu W, Feng Y, Yu S, Fan Z, Li X, Li J, Yin H. The flavonoid biosynthesis network in plants. Int J Mol Sci. 2021;22(23):520. https://doi.org/10.3390/ijms222312824.
Mandal SM, Dias RO, Franco OL. Phenolic compounds in antimicrobial therapy. J Med Food. 2017;20(10):1031–8. https://doi.org/10.1089/jmf.2017.0017.
McCombe CL, Greenwood JR, Solomon PS, Williams SJ. Molecular plant immunity against biotrophic, hemibiotrophic, and necrotrophic fungi. Essays Biochem. 2022;66(5):581–93. https://doi.org/10.1042/ebc20210073.
Meng L, Sun C, Gao L, Saleem M, Li B, Wang C. Hydroxybenzoate hydroxylase genes underlying protocatechuic acid production in *Valsa mali* are required for full pathogenicity in apple trees. Mol Plant Pathol. 2021;22(11):1370–82. https://doi.org/10.1111/mpp.13119.
Musetti R, Favali MA, Pressacco L. Histopathology and polyphenol content in plants infected by phytoplasmas. Cytobios. 2000;102(401):133–47.
Pérez-Hernández A, González M, González C, van Kan JA, Brito N. BcSUN1, a *B. cinerea* SUN-family protein, is involved in virulence. Front Microbiol. 2017;8:35. https://doi.org/10.3389/fmicb.2017.00035.
Rafiei V, Vélëz H, Tzelepis G. The role of glycoside hydrolases in phytopathogenic fungi and oomycetes virulence. Int J Mol Sci. 2021;22(17):52. https://doi.org/10.3390/ijms22179359.
Rana S, Bhushan S. Apple phenolics as nutraceuticals: assessment, analysis and application. J Food Sci Technol. 2016;53(4):1727–38. https://doi.org/10.1007/s13197-015-2093-8.
Shafi W, Mansoor S, Jan S, Singh DB, Kazi M, Raish M, et al. Variability in catechin and rutin contents and their antioxidant potential in diverse apple genotypes. Molecules. 2019;24(5):52. https://doi.org/10.3390/molecules24050943.
Shen N, Wang T, Gan Q, Liu S, Wang L, Jin B. Plant flavonoids: classification, distribution, biosynthesis, and antioxidant activity. Food Chem. 2022;383:132531. https://doi.org/10.1016/j.foodchem.2022.132531.
Táborský J, Sus J, Lachman J, Šebková B, Adamcová A, Šatínský D. Dynamics of phloridzin and related compounds in four cultivars of apple trees during the vegetation period. Molecules. 2021. https://doi.org/10.3390/molecules26133816.
Tan Z, Deng J, Ye Q, Zhang Z. The antibacterial activity of natural-derived flavonoids. Curr Top Med Chem. 2022;22(12):1009–19. https://doi.org/10.2174/1568026622666220221110506.
Wang C, Li C, Li B, Li G, Dong X, Wang G, Zhang Q. Toxins produced by *Valsa mali var. mali* and their relationship with pathogenicity. Toxins (Basel). 2014;6(3):1139–54. https://doi.org/10.3390/toxins6031139.
Wang L, Chen M, Lam PY, Dini-Andreote F, Dai L, Wei Z. Multifaceted roles of flavonoids mediating plant-microbe interactions. Microbiome. 2022;10(1):233. https://doi.org/10.1186/s40168-022-01420-x.
Xu M, Gao X, Chen J, Yin Z, Feng H, Huang L. The feruloyl esterase genes are required for full pathogenicity of the apple tree canker pathogen *Valsa mali*. Mol Plant Pathol. 2018;19(6):1353–63. https://doi.org/10.1111/mpp.12619.
Yang Y, Yang X, Dong Y, Qiu D. The *Botrytis cinerea* Xylanase BcXyl1 modulates plant immunity. Front Microbiol. 2018;9:2535. https://doi.org/10.3389/fmicb.2018.02535.
Yin Z, Liu H, Li Z, Ke X, Dou D, Gao X, Huang L. Genome sequence of Valsa canker pathogens uncovers a potential adaptation of colonization of woody bark. New Phytol. 2015;208(4):1202–16. https://doi.org/10.1111/nph.13544.
Yu C, Li T, Shi X, Saleem M, Li B, Liang W, Wang C. Deletion of Endo-β-1,4-Xylanase VmXyl1 impacts the virulence of *Valsa mali* in apple tree. Front Plant Sci. 2018;9:663. https://doi.org/10.3389/fpls.2018.00663.
Zhang K, Yuan X, Zang J, Wang M, Zhao F, Li P, et al. The kynurenine 3-monooxygenase encoding gene, BcKMO, is involved in the growth, development, and pathogenicity of botrytis cinerea. Front Microbiol. 2018. https://doi.org/10.3389/fmicb.2018.01039.
Zhou K, Hu L, Li Y, Chen X, Zhang Z, Liu B, et al. MdUGT88F1-mediated phloridzin biosynthesis regulates apple development and valsa canker resistance. Plant Physiol. 2019;180(4):2290–305. https://doi.org/10.1104/pp.19.00494.