Two members of the velvet family, *Vm*VeA and *Vm*VelB, affect conidiation, virulence and pectinase expression in *Valsa mali*

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SUMMARY

Velvet protein family members are important fungal-specific regulators that are involved in conidial development, secondary metabolism, and virulence. To gain broader insight into the physiological functions into the velvet protein family of *Valsa mali*, which causes a highly destructive canker disease on apple, we conducted a functional analysis of two Velvet protein family members (*Vm*VeA and *Vm*VelB) via gene replacement strategy. Deletion mutants of *VmVeA* and *VmVelB* showed increased melanin production, conidiation, and sensitivity to abiotic stresses, but exhibited reduced virulence on detached apple leaves and twigs. Further studies demonstrated that the regulation of conidiation by *Vm*VeA or *Vm*VelB was positively correlated with melanin synthesis transcription factor *Vm*Cmr1. More importantly, transcript levels of pectinase genes were shown to be decreased in deletion mutants compared to those of the wild type during infection. However, the expression of other cell wall-degrading enzymes including cellulase, hemi-cellulase, or ligninase genes was not affected in the deletion mutants. Furthermore, the determination of pectinase activity and immunogold labeling of pectin demonstrated that the capacity of pectin degradation was attenuated due to deletions of *VmVeA* and *VmVelB*. Finally, the interaction of *Vm*VeA with *Vm*VelB was identified through co-immunoprecipitation assays. *Vm*VeA and *Vm*VelB play critical roles in conidiation and virulence likely by regulating melanin synthesis transcription factor *Vm*Cmr1 and affecting pectinase gene expression in *V. mali*, respectively.

INTRODUCTION

Valsa mali, is an ascomycete which causes Apple Valsa Canker (AVC). The disease is very important for apple production in eastern Asia, especially in China, where yield losses can reach 100% (Wang *et al.*, 2014a, Li *et al.*, 2015). This necrotrophic pathogen mainly infects host bark by means of conidia entering through wounds (Wang *et al.*, 2014a, Ke *et al.*, 2013). After successful invasion in wounded tissue, infecting hyphae develop and colonize the bark tissue, leading to severe tissue maceration and necrosis (Yin *et al.*, 2015). To date, our understanding of the molecular mechanisms associated with pathogenicity of *V. mali* is very limited. Phytopathogenic fungi produce an array of cell wall-degrading enzymes (CWDEs) such as pectinases, cellulases, hemi-cellulases, and ligninases to overcome the barrier of the plant cell wall (Kubicek *et al.*, 2014). These hydrolases seem to be particularly important for pathogens without specialized penetration structures (Gibson *et al.*, 2011). Pectinase activities associated with host tissue maceration and virulence have been confirmed in various plant pathogenic fungi such as *Aspergillus flavus*, *Botrytis cinerea*, and *Colletotrichum gloeosporioides* (Valette-Collet *et al.*, 2003, Seiboth *et al.*, 2012, Shieh *et al.*, 1997). The important role of pectinases for virulence of *V. mali* has been demonstrated by targeted mutagenesis of five polygalacturonase genes and one pectate lyase gene (Yin *et al.*, 2015). Knockout mutants of each gene showed significantly reduced virulence on apple twigs compared to the wild-type strain. However, the overall biology and virulence mechanisms of this important fungal pathogen still remain poorly understood.

In filamentous fungi, members of the velvet protein family are key regulators of diverse cellular processes such as secondary metabolism, and asexual or sexual sporulation. The important role of VeA and VelB, two members of the velvet protein family, is to form heterotrimeric complexes with LaeA (Loss of aflR expression) and coordinate light signals with fungal development and secondary metabolism (Bayram *et al.*, 2008a). The founding member of this family is VeA whose truncated mutant produced more conidia and fewer fruiting bodies in the model fungus *Aspergillus nidulans* (Käfer, 1965). Further research in *A. nidulans* showed that deletion of *VeA* resulted in hyperactive asexual development, suggesting that it acts as a repressor of conidiation (Bayram *et al.*, 2008a; Mooney and Yager, 1990), while VelB acts as a positive regulator of asexual sporulation (Park *et al.*, 2012). In contrast, deletion of *VeA* leads to reduced asexual sporulation in *Dothistroma septosporum* (Chettri *et al.*, 2012), suggesting an opposite role of velvet family proteins in regulating asexual sporulation. The regulation of conidiation by velvet proteins also has been demonstrated in *Penicillium chrysogenum* (Hoff *et al.*, 2010), *Neurospora crassa* (Bayram *et al.*, 2008b), *Botrytis cinerea* (Yang *et al.*, 2013), *Magnaporthe oryzae* (Kim *et al.*, 2014), *Ustilago maydis* (Karakkat *et al.*, 2013), and *Cochliobolus sativus* (Wang *et al.*, 2016). In addition to the regulation of asexual sporulation, VeA controls the production of mycotoxins in fungi, including sterigmatocystin in *Aspergillus nidulans* (Kato *et al.*, 2003), aflatoxin in *Aspergillus flavus* (Cary *et al.*, 2007), ochratoxin in *Aspergillus carbonarius* (Crespo-Sempere *et al.*, 2013), fumonisin, deoxynivalenol, trichothecene and fusarins in *Fusarium spp.* (Myung *et al.*, 2009,

Jiang *et al.*, 2012, Merhej *et al.*, 2012, Lopez-Berges *et al.*, 2013), and dothistromin in *D. septosporum* (Chettri *et al.*, 2012). Most importantly, velvet proteins also have been reported to play a key role in virulence in plant pathogenic fungi such as *M. oryzae* (Kim *et al.*, 2014), *F. graminearum* (Jiang *et al.*, 2012, Merhej *et al.*, 2012), *B. cinerea* (Yang *et al.*, 2013), *F. oxysporum* (Lopez-Berges *et al.*, 2013), and *Histoplasma capsulatum* (Laskowski-Peak *et al.*, 2012). Recently, many studies have suggested velvet gene affects virulence likely through regulating the CWDE production. For example, protease activity in *A. fumigatus*, *A. flavus* and *B. cinerea* is regulated by VeA (Dhingra *et al.*, 2012, Duran *et al.*, 2014). In *Trichoderma reesei*, Vel1 and Vel2, the orthologs of VeA and VelB, are global regulators of cellulase gene expression (Aghcheh *et al.*, 2014). Furthermore, chitinase in *P. chrysogenum* and laccase and peroxidase in *M. oryzae* are down-regulated in *VeA* deletion mutants (Kamerewerd *et al.*, 2011, Kim *et al.*, 2014). Although many studies have examined velvet proteins in other fungi, their function in *V. mali* has not been analyzed, so far. Understanding the function of velvet proteins in *V. mali* might provide new tools to explore novel, sustainable disease management strategies against Apple Valsa Canker.

In this study, we constructed *ΔVmVeA* and *ΔVmVelB* strains to investigate the roles of *Vm*VeA and *Vm*VelB in pathogenicity. The functions of *Vm*VeA and *Vm*VelB and their involvement in conidiation, melanin production, and sensitivity to abiotic stresses have been elucidated. Our results suggest that these conserved *velvet* family genes in *V. mali* contribute to fungal development and pathogenicity mainly through the regulation of the melanin synthesis transcription factor *Vm*Cmr1 and pectinase

production.

RESULTS

Construction of *VmVeA* and *VmVelB* deletion strains

The *V. mali* genome contains only a single copy of all four *velvet* genes designated *VmVeA* (Accession number KUI67787.1), *VmVelB* (KUI66090.1), *VmVelC* (KUI64732.1), and *VmVosA* (KUI69826.1), respectively (Yin *et al.*, 2015). All four genes share the common velvet factor domain (Figure 1A). Analysis of the amino acid sequences revealed significant similarities to various velvet proteins across different fungal species (Figure 1B).

To investigate the roles of velvet genes in *V. mali*, we generated *VmVeA* and *VmVelB* deletion mutants (*ΔVmVeA* and *ΔVmVelB*) in which the entire open reading frame (ORF) was replaced with a hygromycin phosphotransferase gene (*hph*) by homologous recombination (Figure S1A). PCR analysis using primer pairs for the respective ORFs of the velvet and *hph* genes confirmed that *VmVeA* and *VmVelB* genes in the tranformants were deleted and replaced by the *hph* gene (Figure S1B). When hybridized with probes derived from the ORF of genes (Probe a or b), the fragment corresponding to each gene was present in the wild type, but absent in the respective deletion mutants. In addition, a band with the expected-size was present in the deletion mutants when hybridized with the hygromycin probe (Probe h), indicating that the two deletion mutants have a single locus homologous recombination at the location of their respective velvet gene (Figure S1C). Finally, our complementation study showed that the wild-type allele could be re-introduced to respective deletion mutants at an ectopic locus and generated *ΔVmVeA-C* and

ΔVmVelB-C complemented mutants.

_Vm_VeA and _Vm_VelB are dispensable for vegetative growth, but negatively regulate melanin production

To evaluate the roles of *Vm*VeA and *Vm*VelB in *V. mali* development, we measured mycelial growth of wild type and mutant strains on the PDA medium. The results showed that the deletion of these genes did not significantly affect the growth rate (Figure 2A; Table 1). However, the color of mycelium was significantly darker in *ΔVmVeA* and *ΔVmVelB* strains compared with the wild type. Quantitative real-time polymerase chain reaction (qRT-PCR) showed transcript levels of predicted melanin biosynthesis related genes such as VM1G_09944 (*VmCmr1*), VM1G_09945 (*VmVerA*), VM1G_09946 (*VmLanCl2*), VM1G_09947 (*VmGAL4*), VM1G_09948 (*VmPKS1*) and VM1G_09949 (*VmFet3*) (Table S1) were up-regulated in *ΔVmVeA* (2.2 - 5.7-fold) and in *ΔVmVelB* (3.8 - 8.4-fold) as compared to the wild type (Figure 2B). Similarly, the melanin content in hyphae of *ΔVmVeA* and *ΔVmVelB* strains was also higher (6.62, and 5.65 μg/g, respectively) compared to the wild type (2.81 μg/g). In addition, re-introducing the genes into the respective deletion mutants partially rescued the color of mycelia (Figure 2A). These results suggest that *Vm*VeA and *Vm*VelB are negative regulators of melanin synthesis.

_Vm_VeA and _Vm_VelB affect conidiation by regulating melanin synthesis transcription factor _Vm_Cmr1

To evaluate whether *Vm*VeA and *Vm*VelB affect conidiation in *V. mali*, the number of pycnidia was measured in wild type and deletion mutants. Under light conditions,

ΔVmVeA and *ΔVmVelB* strains produced 8 - 15 times more pycnidia than the wild type on PDA at 15 days post-inoculation (dpi). Under dark conditions, the wild type did not produced pycnidia on PDA at 15 dpi, but *ΔVmVeA* and *ΔVmVelB* strains exhibited a dramatic increase in conidiation. Again, these phenotypes could be reversed by re-introducing the genes into the respective deletion mutants (Figure 3; Table 1). Taken together, these results clearly show that *Vm*VeA and *Vm*VelB are negative regulators of conidiation regardless of light in *V. mali*.

To investigate the relationship between melanin biosynthesis and conidiation which seem both negatively regulated by *Vm*VeA and *Vm*VelB, we chose to delete *VmCmr1* (VM1G_09944), the homolog of the transcription factor *Cmr1* that regulates melanin biosynthesis (Tsuji et al., 2000, Cho *et al.*, 2012), in the wild type, *ΔVmVeA,* and *ΔVmVelB* strains (Figure S2). The *VmCmr1* deletion mutant exhibited the same growth rates, but produced a lower level of melanin than wild type (Figure S3; Table 1). Moreover, it failed to produce pycnidia on PDA at 15 dpi as compared to wild type that produced numerous pycnidia. However, pycnidia could be observed at 30 dpi. The double deletion mutants *ΔVmCmr1*/*ΔVmVeA* and *ΔVmCmr1*/*ΔVmVelB* showed the same phenotype with respect to growth rate, melanin synthesis and conidiation as the single deletion mutant (*ΔVmCmr1*) (Figure 3; Table 1). This result indicated *VmVeA* and *VmVelB* mutants lost the regulation of melanin synthesis and conidiation when *VmCmr1* was deleted.

_Vm_VeA and _Vm_VelB affect responses of _V. mali_ to different abiotic stresses

To test whether *Vm*VeA and *Vm*VelB are involved in abiotic stress responses, we

investigated the growth rate inhibition of wild type and mutant strains on PDA supplemented with KCl (osmotic pressure), H_2O_2 (oxidative stress), Congo red (cell wall inhibitor), or SDS (cell member damaging agent) (Figure 4A). In the presence of these inhibitors, vegetative growth of all strains was inhibited, however, to different levels. The inhibition of growth rate of *ΔVmVeA* and *ΔVmVelB* strains was higher than that of the wild type and complemented mutant strains under respective conditions. *ΔVmVeA* was more sensitive than *ΔVmVelB* (Figure 4B).

_Vm_VeA and _Vm_VelB are required for full virulence

To determine if *Vm*VeA and *Vm*VelB play a role in disease development, virulence assays were performed on detached leaves and twigs and lesion sizes were quantified. The results showed that inactivation of *VmVeA* led to significantly reduced lesion sizes on leaves and twigs as compared to those caused by the wild type strain (Figure 5). Similarly, the inactivation of *VmVelB* also led to significantly reduced lesion sizes on leaves and twigs, but to a lesser extent as compared to *VmVeA* (Figure 5). When *VmVeA* or *VmVeB* was re-introduced into their respective deletion mutants, the previously observed phenotype was rescued. We also tested the effect of *VmVelC* and *VmVosA* deletion mutants on virulence. Both of them did not show any changes in phenotype compared to the wild type (Figure S4).

_Vm_VeA and _Vm_VelB regulate the expression of pectinase genes

To test whether *Vm*VeA and *Vm*VelB play a role in the regulation of expression of CWDEs during infection, transcript levels of different genes, including twelve pectinase genes (six genes involved in virulence and six genes significantly

up-regulated during infection), five cellulase genes (three genes significantly up-regulated), five hemi-cellulase genes (three genes significantly up-regulated), and five ligninase genes were examined by qRT-PCR (Table S1) (Yin et al., 2015, Ke *et al.*, 2014).). Compared with the wild type, the inactivation of *VmVeA* led to a reduction in the expression of all pectinase genes tested. Eleven out of twelve pectinase genes were down-regulated in the *VmVelB* deletion mutant. However, the deletion of *VmVeA* and *VmVelB* did not significantly affect the transcript levels of hemi-cellulase, cellulase, and ligninase genes (Figure 6). These results suggest that *Vm*VeA and *Vm*VelB are positive regulators of pectinase genes expression in *V. mali*.

In order to further confirm the roles of velvet proteins in the regulation of CWDEs, we measured the enzymatic activity of *ΔVmVeA* and *ΔVmVelB* strains in induced medium. The enzymatic activity of a defined volume of culture supernatant of wild type and mutant strains were chosen as a velvet-independent factor in the presence of pectin, xylan, and carboxymethylcellulose. The calculation of enzyme activities was carried out with similar amounts of mycelia. All strains were not able to grow in lignin medium. Enzymatic activity of *ΔVmVeA* and *ΔVmVelB* strains was the same as the wild type in the presence of xylan, or carboxymethylcellulose (Figure S5A). However, pectinase activity of the two deletion mutants was significantly reduced in pectin medium compare to the wild type and complemented mutants (Figure 7A).

To test the effect of velvet proteins on CWDEs during infection, the enzymatic activity of a defined location of lesion border of apple tree bark was calculated with similar amounts of sample. Pectinase activities rather than xylanase and cellulase

activities of *ΔVmVeA* and *ΔVmVelB* strains were significantly reduced compared to the wild type as the one in induced medium. Enzyme activity of pectinase was found much higher than that of xylanases and cellulases (Figure 7B, Figure S5B).

To further test the capacity of pectin degradation of *ΔVmVeA* and *ΔVmVelB* strains, we observed the pectin content in cell walls of apple bark by immunogold labeling (Figure 7C). Uninfected apple bark showed a dense labeling with gold particles. In infected bark tissue, a reduced amount of gold particles was detected in host cell wall. However, *ΔVmVeA* and *ΔVmVelB* mutants showed more gold particles than wild type (Figure 7D). This result indicated that the inactivation of *VmVeA* and *VmVelB* led to a significant reduction in the capacity of pectin degradation during infection by *V. mali*. The results of this study confirmed that *Vm*VeA and *Vm*VelB may play an important role in the virulence of *V. mali* by up-regulating pectinase gene expression resulting in a faster degradation of the host cell wall.

_Vm_VeA interacts with _Vm_VelB

Because VeA was shown to physically interact with VelB in *A. nidulans* (Bayram et al., 2008a), we tested whether this interaction also occurs in *V. mali*. We first attempted to study this interaction using yeast two-hybrid assays. However, both *Vm*VeA and *Vm*VelB showed strong self-activation activities. Therefore, we generated *Vm*VeA-His and *Vm*VelB-Flag constructs and co-transformed them into the wild type strain. In total protein samples isolated from *Vm*VeA-His/*Vm*VelB-Flag co-transformants, the 63-kDa *Vm*VeA-His band and the 50-kDa *Vm*VelB-Flag band were detected with the anti-His and anti-Flag antibodies, respectively (Figure 8). In

proteins eluted from an anti-FLAG immuno-affinity column, the *Vm*VeA-His band could also be detected. This result clearly indicates that *Vm*VeA interacts with *Vm*VelB in *V. mali*.

DISCUSSION

Although velvet family proteins have been characterized in a number of species, their functions in *V. mali* remained unknown. Our results show that the *V. mali* genome contains four members of the velvet family. The four velvet proteins are evolutionarily conserved among different fungal species, suggesting that they have important functions in fungi. This study has also been indicated that *Vm*VeA and *Vm*VelB play a key role in conidiation, melanin synthesis, oxidative stress response, and disease development. As VelB and VeA are part of the trimeric VelB–VeA–LaeA complex critical to secondary metabolism and development, it is not surprising that both genes share a similar function in fungal development (Bayram *et al.*, 2008a). In the current study, a physical interaction of *Vm*VeA and *Vm*VelB was observed in co-immunoprecipitation assays (Figure 8). The interaction of *Vm*VeA with *Vm*VelB maybe contributes to the observation that *Vm*VeA and *Vm*VelB coordinate similar processes in the regulation of fungal development in *V. mali*. Similar interactions and functions were observed in *B. cinerea* (Yang *et al.*, 2013). However, in *F. graminearum*, *Fg*VeA did not interact with *Fg*VelB (Jiang *et al.*, 2011). The different interactions between VeA and VelB may lead to different functions of velvet proteins in different fungi. In our preliminary studies, deletion of the other two members, *VmVelC* and *VmVosA*, did not show any changes in phenotype compared to the wild type in all tests (Figure S4). Therefore, we did not conduct further test on these two deletion mutants.

The function of velvet proteins as regulators of secondary metabolism and

conidiation has been well studied in a number of fungi (Bayram & Braus, 2012, Calvo, 2008). The present study indicates that *Vm*VeA and *Vm*VelB affect melanin production in a negative way (Figure 2B, Table 1). It is known that the production of melanin, an important secondary metabolite, is regulated by velvet proteins in several fungal species. But how they regulate melanin production varies among different fungal species. Similar to the finding of this study, the production of melanin is also reported to be negatively regulated by velvet proteins in *C. heterostrophus*, *B. cinerea* and *Curvularia lunata* (Wu *et al.*, 2012, Yang *et al.*, 2013, Gao *et al.*, 2017). By contrast, the production of melanin appears to be positively regulated by velvet proteins in *M. graminicola* (Choi & Goodwin, 2011) and *C. sativus* (Wang *et al.*, 2016), respectively.

It is well established that conidia reproduction is important for fungi to survive in nature especially as *V. mali* mainly infects the host bark via conidia (Wang *et al.*, 2014a). Our results suggest that *Vm*VeA and *Vm*VelB negatively regulate conidiation (Figure 3A, Table 1), a finding similar to results obtained from *A. nidulans* (Kato *et al.*, 2003), *N. crassa* (Bayram et al., 2008b), *F. graminearum* (Jiang *et al.*, 2011), and *B. cinerea* (Yang *et al.*, 2013). By contrast, VeA appears to positively regulate conidiation in *M. oryzae* (Kim *et al.*, 2014), *P. chrysogenum* (Hoff *et al.*, 2010) and *A. parasiticus* (Calvo *et al.*, 2004). Therefore, the regulation of conidiation by velvet proteins varies depending on the fungal species. In the present study, the inactivation of *VmCmr1* further proved that conidiation is correlated with this transcription factor (Figure 3B, Table 1). Taken together, these results suggest that *Vm*VeA and *Vm*VelB affect conidiation via the regulation of melanin synthesis transcription factor *Vm*Cmr1.

This type of regulation is different from those in *A. nidulans* and *M. oryzae*, in which VeA directly regulates the expression of its conidiation-related genes such as *brlA*, *Mo*COS1, *Mo*CON6, or *Mo*CON7 (Kato *et al.*, 2003, Kim *et al.*, 2014). The joined regulation of secondary metabolism and conidiation by velvet proteins has also been reported for *A. nidulans* (Kato *et al.*, 2003), *F. fujikuroi* (Wiemann *et al.*, 2010), and *P. chrysogenum* (Hoff *et al.*, 2010). Our results show that this regulation mode also exists in *V. mali*.

Eukaryotic cells have stress-protective functions against a variety of stress conditions such as oxidative stresses (Lawrence *et al.*, 2007). Our stress tests indicated that deletion of *VmVeA* or *VmVelB* led to increased sensitivity to osmotic and oxidative stresses, and cell wall inhibitor. These studies suggested that *Vm*VeA and *Vm*VelB are involved in the regulation of the sensibility to osmotic pressure and oxidative stress, and maintenance of cell wall integrity in *V. mali* (Figure 4). The *ΔVmVeA* mutant was more sensitive than the *ΔVmVelB* mutant. Similar results were found in *F. verticillioides*, where *Fv*VE1 plays a more important role than other velvet proteins, and both *Fv*VE1 and *Fv*VelB positively regulate the transcription of a catalase-encoding gene, *Fv*CAT2, which results in reduced oxidant resistance (Lan *et al.*, 2014). Also, conidia of *AfuVelB* deletion mutants exhibited reduced tolerance to oxidative stress because of a deficiency in trehalose biogenesis in *A. fumigatus* (Park *et al.*, 2012). In *Curvularia lunata*, decreased resistance of *clvelB* mutants to stress agents may be attributed to a lower basal accumulation of glycerol (Gao *et al.*, 2017). In *A. nidulans* it was shown that VelB can bind to the promoter region of the ***β***-glucan

synthase gene *fsA* to regulate cell wall synthesis (Park *et al.*, 2015). However, the possible mechanism how velvet proteins regulate stress resistance in *V. mali* should be further be investigated.

Our pathogenicity assays indicated that deletion of *VmVeA* or *VmVelB* led to a significant reduction in virulence on detached leaves and twigs (Figure 5). The result clearly indicates that *Vm*VeA and *Vm*VelB play an important role in the virulence of *V. mali*. It is well known that velvet proteins are required for full virulence in a number of fungal pathogens and their roles in virulence differ among various fungal species (Yang *et al.*, 2013). In most fungi, velvet proteins contribute to virulence by mainly regulating secondary metabolism of the pathogen. For example, *Ff*Vel1 and *Ff*Vel2 both are virulence factors likely due to their roles as positive regulators of gibberellic gcid biosynthesis and negative regulators of bikaverin biosynthesis in *F. fujikuroi* (Wiemann *et al.*, 2010). In *F. graminearum*, the biosynthesis of deoxynivalenol and trichothecenes, two important virulence factors, are modulated by *Fg*VeA or *Fg*VelB (Jiang *et al.*, 2012, Jiang *et al.*, 2011, Merhej *et al.*, 2012). In the present study, the negative regulation of *VmCmr1* by *Vm*VeA and *Vm*VelB has been demonstrated. We also tested the virulence of *VmCmr1* deletion mutant and double deletion mutants *ΔVmCmr1/ΔVmVeA* and *ΔVmCmr1/ΔVmVelB*. The three mutants exhibited the same virulence as their parental strains (Figure S3B), suggesting melanin was not required for fungal virulence. This is different to many other *Ascomycetes* in which 1,8-dihydroxynaphthalene (DHN) melanin plays a role in virulence (Langfelder *et al.*, 1998; Ludwig *et al.*, 2014; Woo *et al.*, 2010). However, some other secondary

metabolites like 3-(p-hydroxyphenyl) propanoic acid and p-hydroxybenzoic acid have been shown to be involved in virulence in *V. mali* (Wang *et al.*, 2014b). Therefore, whether *Vm*VeA and *Vm*VelB are involved in virulence in connection with these secondary metabolites will need further research.

It is well known that reactive oxygen species (ROS) play an important role in pathogen–host interactions. We were able to show that *Vm*VeA and *Vm*VelB are involved in the regulation of the sensibility to oxidative stress. Under pathogen attack, plants use the oxidative burst as an early defense reaction. However, there is indeterminacy for necrotrophs. For example, in *B. cinerea* deletion of the AP-1 transcription factor did not lead to reduced virulence (Temme and Tudzynski, 2009). Thus, for *V. mali*, the relevance between abiotic stress responses and virulence needs further research.

An important discovery of our work was the finding that deletion of *VmVeA* and *VmVelB* significantly reduced the expression level of pectinase rather than hemi-cellulase, cellulase, and ligninase genes (Figure 6, Figure 7). Regulation of expression of CWDE genes by velvet proteins has so far been a subject of major interest in plant biomass degradation fungi such as *T. reesei* (Aghcheh *et al.*, 2014, Liu *et al.*, 2015). The regulation of pectinase by *Vm*VeA and *Vm*VelB is a very interesting finding since previous histological and cytological studies of the cell wall components demonstrated that pectinases, rather than cellulases or xylanases, were involved in the pathogenesis of *V. mali* (Ke *et al.*, 2013). Remarkably, genomic and transcriptomic analyses indicated that *V. mali* is more likely to derive nutrients from

the decomposition of pectin (Yin *et al.*, 2015, Ke *et al.*, 2014). Our results clearly indicate that *Vm*VeA and *Vm*VelB play an essential role in the virulence of *V. mali* mainly through the regulation of pectinase levels. Though the regulation of the expression hydrolase genes by velvet proteins has attracted a great deal of attention, the mode of action is undefined. Velvet proteins may act as direct regulators at the DNA level (Ahmed *et al.*, 2013; Beyhan *et al.*, 2013). However, whether the regulation of pectinase genes by *Vm*VeA and *Vm*VelB involves the above-mentioned direct regulation remains to be investigated.

In conclusion, we have shown that two members of the velvet protein family, *Vm*VeA and *Vm*VelB, are regulators of melanin production, conidiation, response to osmotic and oxidative stresses, and cell wall integrity in *V. mali*. More importantly, they are involved in virulence by selectively regulating pectinase expression.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

The *Valsa mali* wild type strain 03-8 was obtained from the Laboratory of Integrated Management of Plant Diseases in College of Plant Protection, Northwest A&F University (Yin *et al.*, 2015). The wild type and transformants generated in this study were cultured on PDA (20% potato extract, 2% glucose, 1.5% agar).

Growth rates on PDA were assayed by measuring colony diameters at 1 and 2 dpi at 25°C. The capacity of conidiation was compared by recording the number of pycnidia per plate after incubation in the dark or under the light at 25°C for 15 or 30 days on PDA medium. For selection of transformants, TB_3 medium (0.3% yeast extract, 0.3% casamino acids, 20% sucrose, 1.5% agar) supplemented with 100 μg/mL Hygromycin B (Calbiochem, LaJolla, CA), or 100 μg/mL Geneticin (Sigma, St. Louis, MO) was used. To assay stress responses, fungi were grown on PDA containing KCl (0.5 M), H_2O_2 (3 mM), Congo red (200 mg/L) or SDS (0.01%) (Song *et al.*, 2017). Colony diameter on KCl medium was measured after incubation for 7 days and others for 3 days, respectively. The inhibition of growth rate (%) was calculated as percentage of colony growth on media with the inhibitor compared to that on normal PDA media. For examining enzymatic activity, different cell wall substrates were used. Synthetic medium (SM) (0.5% $(NH_4)_2SO_4$, 0.05% yeast extract, salts (0.15% KH_2PO_4, 0.06% $MgSO_4$, and 0.06% $CaCl_2$), and trace amounts of metals (0.0005% FeSO4, 0.00016% $MnSO_4$, 0.00014% $ZnSO_4$, and 0.00037% $CoCl_2$)) (Srivastava *et al.*, 2012) supplemented with 1% pectin, xylan, carboxymethylcellulose, or lignin as

sole carbon source were used. All experiment was repeated three times. Data were analyzed by Fisher's least significant difference (LSD) using the SAS software package (SAS Institute), $p<0.05$.

Infection assays on twigs and leaves

Strains were cultured on PDA for 2 days. Agar plugs (5 mm each) were taken from the edge of a growing colony. Leaves (the fourth or fifth leaf from the top of a branch) or twigs (one-year old) of *Malus domestica* borkh. cv. 'Fuji' were inoculated by stab-inoculation (leaves) and an armature using the scald wounding method (twigs) (Wei *et al.*, 2010). Inoculated samples incubated at 25°C for 3 days (leaves), and 9 days (twigs). In leaves, the size of lesions was the diameter of lesions using the crossing method. In twigs, the total length of longitudinal lesions along twigs was recorded as the size of lesions. All treatments were performed with at least three replicates, and all experiments were repeated three times. Data were analyzed by LSD using the SAS software package (SAS Institute), $p<0.05$.

Phylogenetic analysis

Velvet genes were originally identified through homology searches of the *V. mali* genomic sequences (GenBank accession number JUIY00000001) (Yin *et al.*, 2015) using the velvet proteins of *A. nidulans* (Bayram *et al.*, 2008a) as a query. Phylogenetic trees were constructed using the neighbor-joining method (Tamura *et al.*, 2007). Alignments between genomic and transcriptomic sequences (Yin *et al.*, 2015, Ke *et al.*, 2014) were used to verify the existence and size of introns. Six melanin biosynthesis related genes and CWDEs genes are rooted by annotation in the *V. mali*

genome (Table S1).

Gene knockout and complementation

Gene disruption constructs were generated by replacing the complete ORFs of the Velvet and *Cmr1* genes (Figure S1A, S2A). Upstream and downstream flanking sequences of the target genes were amplified with primer pairs of VmVeA-, VmVelB-, or VmCmr1-1F/2R and 3F/4R, respectively (Table S2). Primers HYG/F and HYG/R were used to amplify the hygromycin resistance gene carrying fragment. Primers GEN/F and GEN/R were used to amplify the neomycin resistance gene carrying fragment. Deletion cassettes were constructed by the double-joint PCR method (Yu *et al.*, 2004). Resulting PCR products were transformed into protoplasts of the wild type strain 03-8 using the PEG method (Gao *et al.*, 2011). Hygromycin- or neomycin resistant transformants were screened by PCR with primer pairs of VmVeA-, VmVelB-, or VmCmr1-5F/6R, H (G) 850F/H (G) 852R, and VmVeA-, VmVelB-, or VmCmr1-7F/H (G) 856R, or H (G) 855F/ VmVeA-, VmVelB-, or VmCmr1-8R to confirm gene replacement events (Table S2). Putative gene deletion mutants were further confirmed by Southern blot analyses with respective probes: a for *VmVeA*, b for *VmVelB*, c for *VmVelC,* or d for *VmVosA*, h for *hph* gene, and g for *neo* gene according to the manufacturer's instructions (the DIG-High Prime DNA Labeling and Detection Starter Kit II Roche, Penzberg, Germany).

Complemented mutants were generated using a gap repair approach by co-transformation of velvet or *Cmr1* gene fragments amplified with primer pairs VmVeA-, VmVelB-, or VmCmr1-C-F/R, and a *Xho*I-digested plasmid pFL2 into yeast

strain XK1-25 (Bruno *et al.*, 2004). Resulting fusion constructs rescued from Trp^+ yeast transformants were confirmed by sequencing and transformed into the respective *V. mali* deletion mutant. Geneticin-resistant transformants expressing the complementing constructs were identified by PCR with primer pairs VmVeA-, VmVelB-, or VmCmr1-C-5F/6R.

Quantitative RT-PCR

Transcript levels of melanin biosynthesis genes, and CWDEs related genes were determined by qRT-PCR (Yin *et al.*, 2013). RNA was extracted from mycelia grown on PDA with cellophane on top for 4 days (for melanin biosynthesis genes) and from mycelium which formed at the lesion border of apple tree bark (for CWDE related genes) as described (Ke *et al.*, 2014). RNA was isolated with the TRIzol reagent (Invitrogen, Carlsbad, CA) as described (Yin *et al.*, 2013). For qRT-PCR assays, we used the Fermentas (Hanover, MD, USA) 1^{st} 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 $2^{-\Delta\Delta CT}$ method (Livak & 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), $p<0.05$.

Enzymatic activity and melanin content assays

For enzymatic activity assays, strains were cultured on PDA with cellophane on top for 2 days. Mycelial slices (5 mm in diameter) were taken from the edge of a

colony. Five mycelial slices were cultivated in flasks (250 mL) containing 100 mL PDA medium for 48 h. Then the plugs were transferred to same volume SM with 1% pectin, xylan, or carboxymethylcellulose as sole carbon source. The mycelia slices were cultured for 6, 12, 24, 48 and 72 h. Mycelia and supernatants were collected and mycelia dried at 105°C overnight. Measured enzyme activities of supernatants were correlated to the biomass (dry weight of mycelia). The experiment was repeated three times.

In order to assay the enzymatic activity in infected tissue, bark piece was sampled from a defined location of the lesion border of apple tree bark (three millimeters in lesion and two millimeters in healthy bark) to extract total protein. Intact bark tissues were used as control (CK). Total protein was extracted from the samples and the enzyme activity was determined according to the manufacturer's instructions (The Pectinase, xylanase or cellulase test kit, Solarbio, Beijing, China). The experiments were repeated six times.

Enzymatic activities of supernatants and extracts were quantified by the increase in absorbance at 540 nm due to the release of reducing sugars from pectin, xylan, or carboxymethylcellulose (The Pectinase, xylanase or cellulase test kit, Solarbio, Beijing, China). The incubation time of the reaction was 30 min for pectinase, 12 h for xylanase and cellulose. The reaction mixture was diluted to absorbance value 0.2 - 0.8 for detection. Determination of enzyme activity is expressed in units: one unit is equal to 1 mg of the corresponding sugar released after 1 h of incubation under standard conditions. The absorbance of D- galacturonic acid, xylose, and glucose (0 -

125 µg/mL each) at 540 nm were measured and standard curves generated.

For melanin extraction, mycelia were grown on PDA with cellophane on top for 4 days. Extraction and quantification of melanin was performed as described (Bashyal *et al.*, 2009). Melanin (Sigma Chemicals Co., St. Louis, USA) was first dissolved in 1 mL of 1 M NaOH and then the absorbance of melanin (0 - 50 µg/mL) at 405 nm was measured and a standard curve was generated. Data were analyzed by LSD using the SAS software package (SAS Institute), $p<0.05$.

Immunogold labeling of pectin

Apple tree bark samples were generated via the same method as for enzymatic activity determinations. Intact bark tissue was collected as control (CK). The labeling method of pectin was carried out as described (Ke *et al.*, 2013). The monoclonal antibody JIM7 (PlantProbes, Leeds, UK) and goat anti-rat immunoglobulin linked to 15 nm colloidal gold particles (A10706G-Gold, Solarbio, Beijing, China) were used according to the instructions of the manufacturers. Gold density was measured by recording the number of gold particles per square micron labeled cell wall. Data from fifteen micrographs were used to calculate mean and standard deviation. Data were analyzed by LSD using the SAS software package (SAS Institute), $p<0.05$.

Co-immunoprecipitation assays

VmVeA was amplified with primer pair *Vm*VeA-His-F/R, in which the His-tag was integrated in the forward primer. The resulting fragment was cloned into vector pDL2 by the yeast gap repair approach to generate the His-tag fusion constructs (Bruno *et al.*, 2004). A similar approach was used to generate the *Vm*VelB-Flag fusion

constructs with the pFL2 vector. Resulting fusion constructs were verified by sequencing. Plasmids were transformed separately or co-transformed into protoplasts of wild type strain 03-8. *Vm*VeA-His, *Vm*VelB-Flag, and *Vm*VeA-His/*Vm*VelB-Flag transformants were confirmed by Western blot analysis. For co-immunoprecipitation assays, total protein was isolated from mycelia grown on PDA with cellophane on top for 3 days according to the manufacturer's instructions (Protein Extraction Kits, BestBio Science, Beijing, China). The extract was incubated with the anti-FLAG M2 beads as described (Wang *et al.*, 2015). Western blots of proteins eluted from anti-FLAG beads were detected with monoclonal anti-His and anti-FLAG antibodies (Sigma, St. louis, MO, USA).

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SUPPORTING INFORMATION LEGENDS

Figure S1: Generation of *VmVeA* and *VmVelB* deletion mutants. (A) *VmVeA* and *VmVelB* gene replacement constructs were generated using the double-joint method. The small arrows mark the positions and directions of the primers used for PCR. 1F, 2R, 3F, and 4R primers were used to amplify the flanking sequences. *Hph:* hygromycin phosphotransferase gene. (B) For PCR detection of deletions, four primer pairs (5F/6R, H850F/H852R, 7F/H855F and H856F/8R) were used for each gene. (C) Southern blot analysis of wild type, deletion mutants, and complemented mutants of *VmVeA* and *VmVelB* by hybridization with probe: a (*VmVeA*), b (*VmVelB*), or h (*hph*).

Figure S2: Generation of *VmCmr1* deletion mutants. (A) Schematic representation of the *VmCmr1* gene deletion strategy. The small arrows mark the positions and directions of primers used for PCR. (B) For PCR conformation of deletions four primers pairs 5F/6R, H (G) 850F/852R, 7F/H (G) 855F, and H (G) 856F/8R were used. (C) Southern blot analyses using an *hph* probe (Probe h) for *ΔVmCmr1* and a neomycin probe (Probe g) for *ΔVmVeA/ΔVmCmr1,* and *ΔVmVelB/ΔVmCmr1*. (D) PCR confirmation of complementation using primer pair 5F/6R.

Figure S3: Phenotypes of mycelial growth and twigs inoculated with the *VmCmr1* deletion mutants of *V. mali*. (A) Mycelial growth on PDA for 2 days. (B) Apple twigs were inoculated with mycelium agar plugs from wild type, deletion mutants, and complemented mutant strains. Lesion sizes were quantified at 9 dpi. Different letters represent a statistically significant difference in respective lesion size ($P < 0.05$). Bars indicate standard deviations of the mean of three individual host plants.

The experiment was repeated three times.

Figure S4: *VmVelC* and *VmVosA* replacement constructs and phenotypes on growth rate, conidiation, response to different stresses, and virulence. (A) The generation of *VmVelC* and *VmVosA* deletion mutants was done accordingly to the generation of *VmVeA* and *VmVelB* mutants. (B) Mycelial growth on PDA for 2 days, and PDA supplemented with 3mM H_2O_2 for 3 days. (C) Quantification of pycnidia produced on PDA in the light or dark at 15 dpi of wild type and detection mutant strains. (D) Phenotypes of leaves and twigs inoculated with *VmVelC* and *VmVosA* deletion mutants. Apple leaves were inoculated with mycelium agar plugs from the wild type, deletion mutants, and complemented mutants. Photographs were taken at 3 dpi. Apple twigs were inoculated with mycelium agar plugs from the wild type, deletion mutants, and complemented mutants. Lesion sizes were determined 9 dpi.

Figure S5: Inactivation of *VmVeA* and *VmVelB* does not affect hemi-cellulase and cellulase formation. (A) Enzymatic activities of supernatants of the wild type, *VmVeA*, and *VmVelB* deletion mutants on SM supplemented with xylan or carboxymethylcellulose as a sole carbon source were assayed after 6, 12, 24, 48 and 72 h - after pre-growth in PDA for 48 h. Enzymatic activities are given in arbitrary units and related to the respective biomass dry weight of the sample at the respective time point. The five columns of each graph represent (from left to right) represent: wild type (white), *ΔVmVeA* (gray), *ΔVmVeA-C* (black), *ΔVmVelB* (white with bias) and *ΔVmVelB-C* (gray with cross grain). (B) Enzymatic activities of samples from a defined location of lesion (three millimeters in lesion and two millimeters in healthy

bark) were calculated with similar amounts of sample. Experiments are means of six biological replicates and the standard deviations are given by vertical bars.

Table S1. Melanin biosynthesis related genes and cell wall-degrading enzyme genes for expression.

Table S2. Primers used in this study.

FIGURE LEGENDS

Figure 1: Structure and sequence analyses of velvet proteins in *V. mali*. (A) The ORF of *VmVeA* consists of 1,799 bp which is interrupted by a single 77 bp intron and encodes a protein with 573 amino acids. *VmVelB* consists of a 1,836 bp ORF interrupted by 5 introns and encodes a protein with 459 amino acids. No introns were found in *VmVelC* and *VmVosA*. All of four velvet genes contain the common velvet domain. (B) A phylogenetic tree for the *V. mali* velvet genes was constructed using neighbor-joining analysis with 1,000 bootstrap replicates. Numbers on the branches represent the percentage of replicates supporting each branch. Labels on the right indicate the accession numbers in GenBank and the fungal species. Subclades containing velvet genes of *V. mali* and orthologs in other species are shaded. The bar represents 20% sequence divergence.

Figure 2: Color changes in *VmVeA* and *VmVelB* deletion mutants. (A) Mycelial growth on PDA for 2 days. (B) Up regulation of the melanin biosynthesis related genes (see also Table S1). RNA samples were isolated from 4 day old cultures of the wild type, deletion mutants and complemented mutants of *VmVeA* and *VmVelB* and the

transcript level of each gene was determined as described. The experment was carried out in triplicates and data were analyzed using the protected Fisher's least significant difference (LSD) test.

Figure 3: Effects of inactivation of *VmVeA*, *VmVelB*, or/and *VmCmr1* on conidiation (asexual reproduction). Quantification of pycnidia produced on PDA was carried out as described. Arrows indicate pycnidia on PDA medium.

Figure 4: Effects of inactivation of *VmVeA* and *VmVelB* on the response of *V. mali* to abiotic stresses. (A) Cultures of the wild-type, *VmVeA*, and *VmVelB* deletion mutants, and complemented mutants, grown on PDA supplemented with 0.5MKCl, 3mM H_2O_2, 200mg/L Congo red (CR) or 0.01% SDS. Images were taken after 7 days on KCl and 3 days on other inhibitors. (B) Inhibition of growth rate on PDA with inhibitor compared to PDA without stress (given in Table 1). Different letters represent a statistically significant difference ($P < 0.05$). Bars indicate standard deviations from the mean of three replicates.

Figure 5. Phenotypes of leaves and twigs inoculated with *VmVeA* and *VmVelB* deletion mutants. (A) Apple leaves were inoculated with mycelium agar plugs from the wild type, deletion mutants, and complemented mutants. Lesion sizes were determined 3 dpi. (B) Apple twigs were inoculated with mycelium agar plugs from the wild type, deletion mutants, and complemented mutants. Lesion sizes were determined 9 dpi. (C) Quantification of lesion sizes on apple leaves at 3 (twigs at 9) dpi. Different letters represent a statistically significant difference in respective lesion size ($P < 0.05$). Bars indicate standard deviations of the mean of three individual host

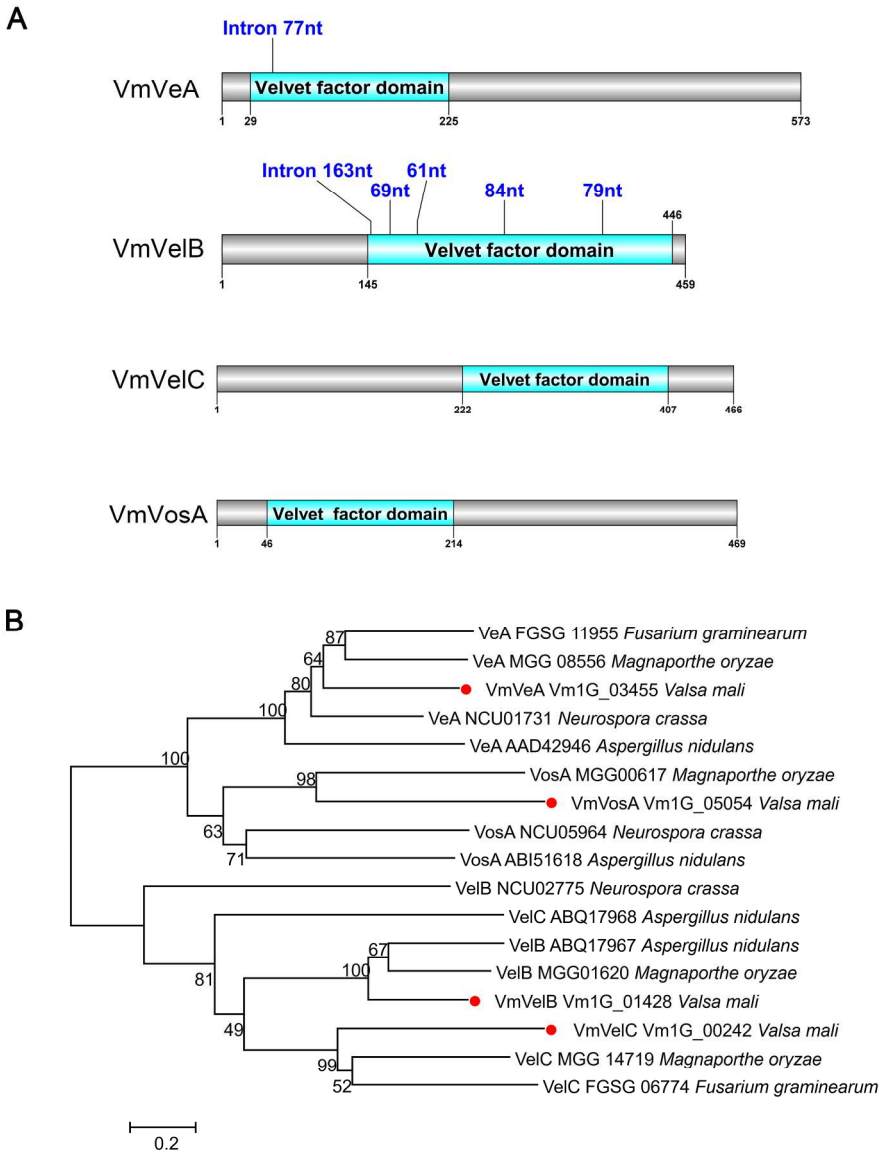
plants. The experiment was repeated three times.

Figure 6: Transcript levels of pectinase, hemi-cellulase, cellulase, and ligninase genes determined by qRT-PCR in the wild type, *VmVeA*, and *VmVelB* deletion mutants. RNA samples were isolated from the border of *V. mali* colonized apple tree barks at 3 dpi and transcript levels of pectinase, hemi-cellulase, cellulase and ligninase genes in the wild type, *VmVeA*, and *VmVelB* deletion mutants quantified by qRT-PCR. The transcript level of the *G6PDH* gene was used to normalize different samples. Transcript levels of wild type were set to 1. The mean and standard deviation were calculated with data from three independent biological replicates. Data from three replicates were analyzed with the Student's t-test. Asterisks represent a significant difference in transcript levels ($P < 0.05$).

Figure 7: Inactivation of *VmVeA* and *VmVelB* differentially affect pectinase formation in *V. mali*. (A) Pectinase activities of supernatants of wild type, *VmVeA*, and *VmVelB* deletion mutants on SM supplemented with pectin as sole carbon source were assayed at 6, 12, 24, 48 and 72 h - after pre-growth in PDA for 48 h. Enzymatic activities are given in arbitrary units and related to the respective mycelium dry weight of the strain at the respective time point. Bars indicate standard deviations of the mean of three replicates. (B) Pectinase activities of samples from a defined location of lesion (three millimeters in lesion and two millimeters in healthy bark) were calculated with similar amounts of sample. Experiments are means of six biological replicates and the standard deviations are given by vertical bars. (C) Immunogold labeling of pectin on infected and uninfected bark tissue. Pectin labeling

of host cell wall (HCW). Gold particles (arrows) were densely in intact bark, but reduced labeling was found in proximity to the hyphae (H). Bars = 0.5 μm. (D) Labeling density of pectin on healthy and infected tissues (3 dpi) using different strains. Density of labeling is expressed as the number of gold particles per μm^2. Bars indicate standard deviations of the mean from fifteen micrographs. Mean densities of different strains were analyzed using the protected Fisher's least significant difference (LSD) test. Different letters represent a statistically significant difference ($P < 0.05$).

Figure 8: Co-immunoprecipitation assays for the interactions between *Vm*VeA and *Vm*VelB. Total protein (Total) isolated from *Vm*VeA-His, *Vm*VelB-Flag and *Vm*VeA-His/*Vm*VelB-Flag transformants. Proteins eluted (Elution) from anti-FLAG immuno-affinity columns were visualized with anti-FLAG and anti-His antibodies.



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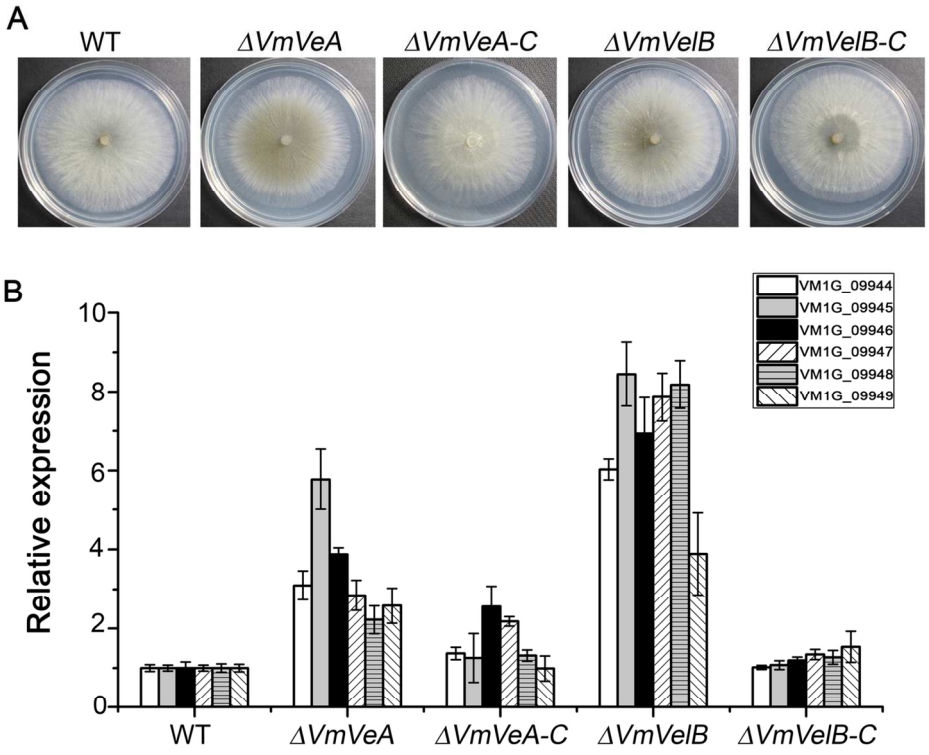

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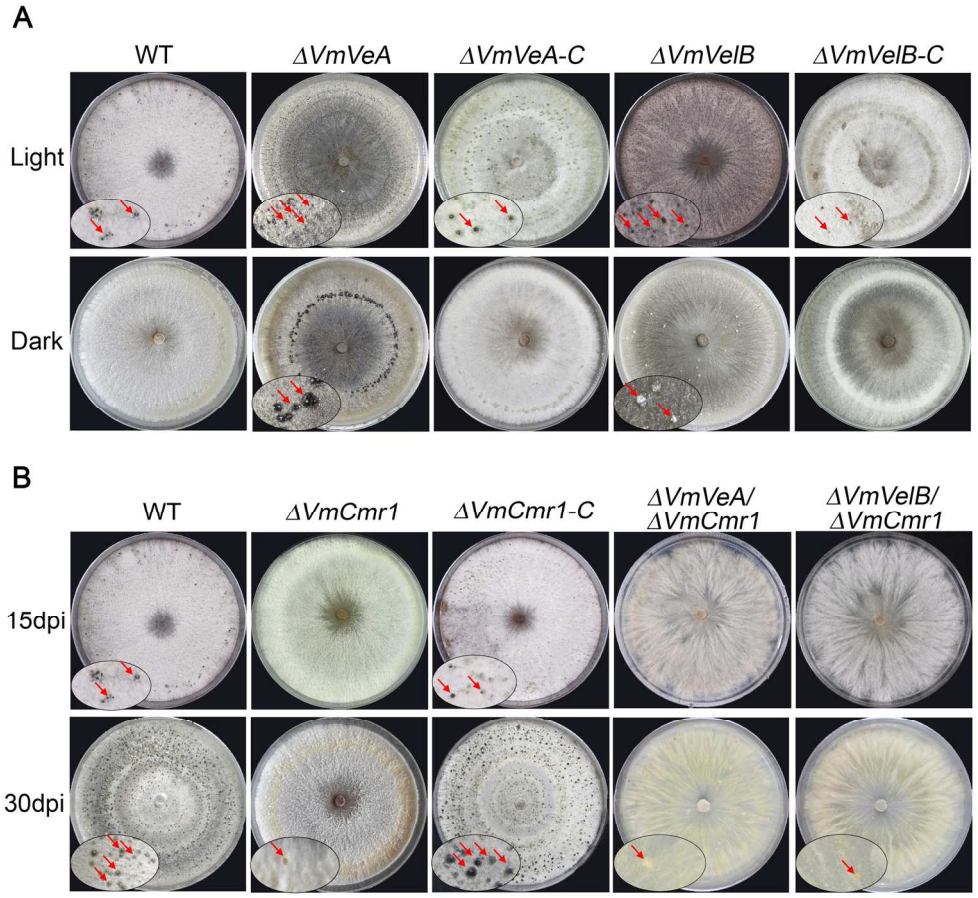

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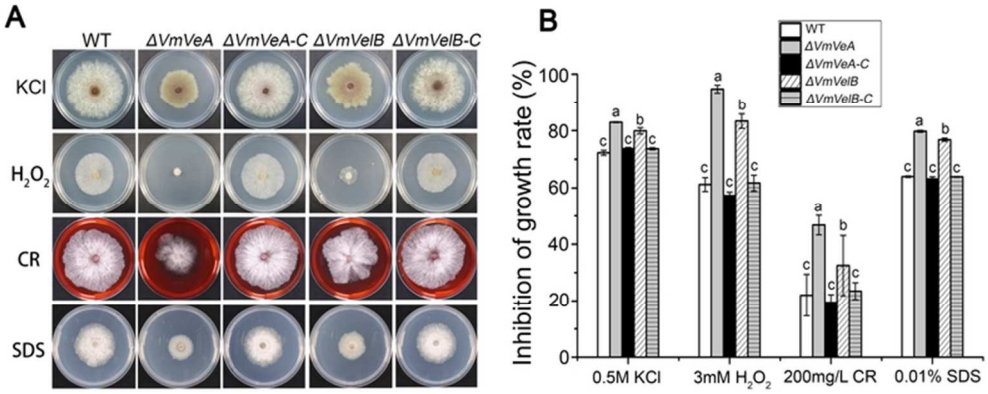

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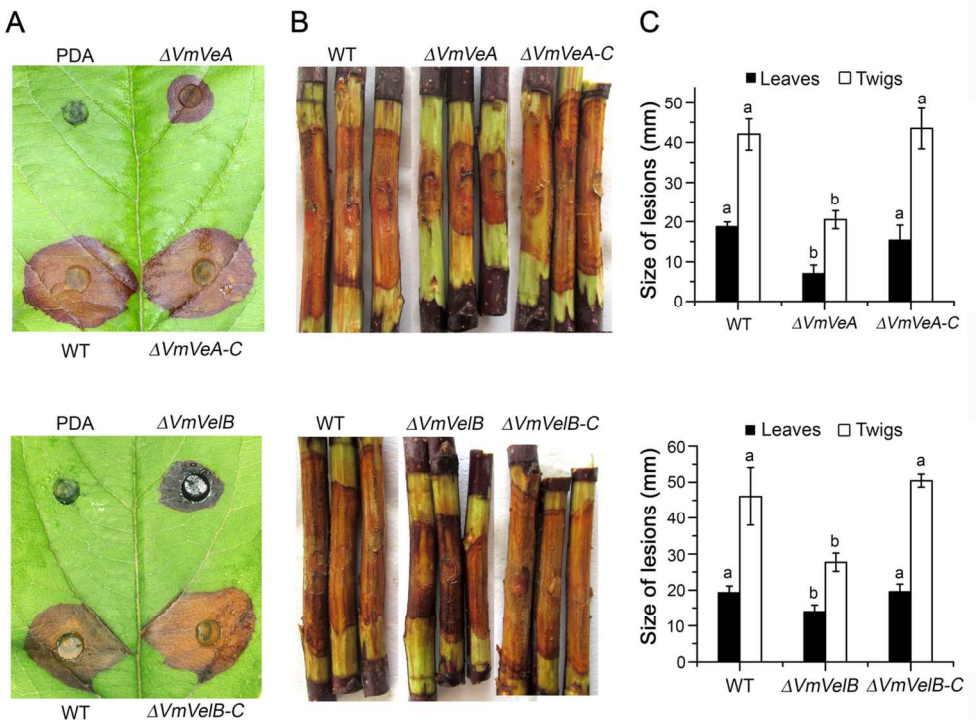

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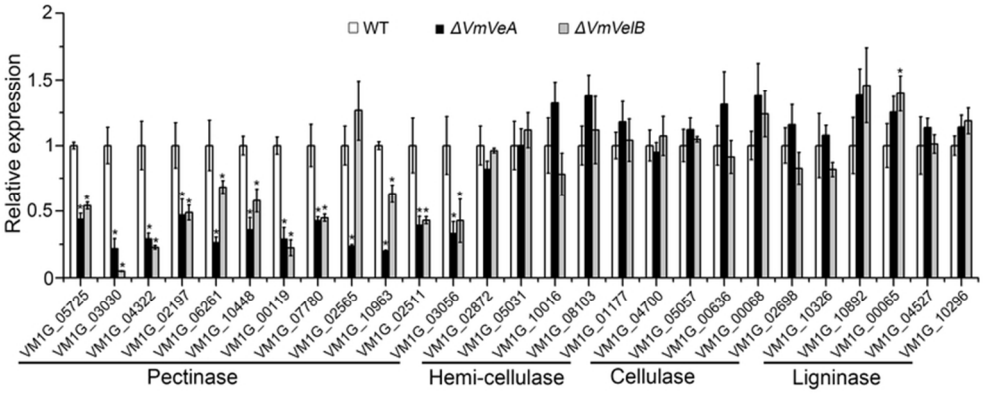

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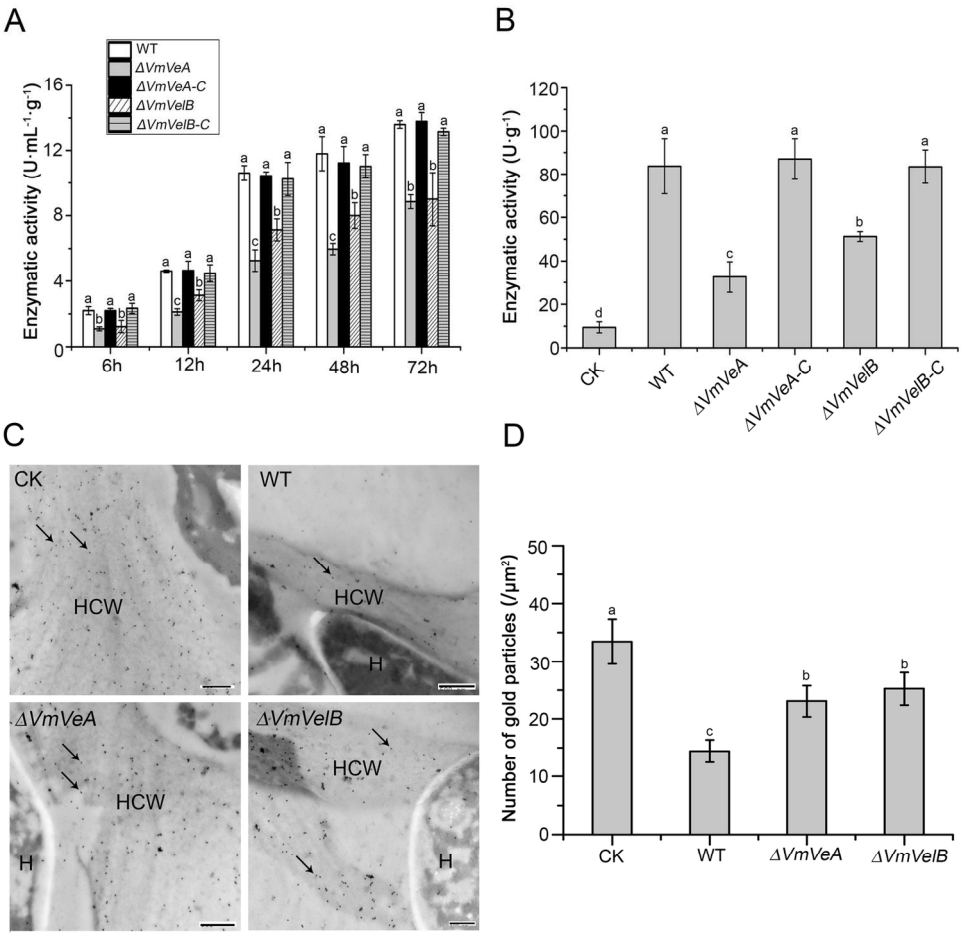

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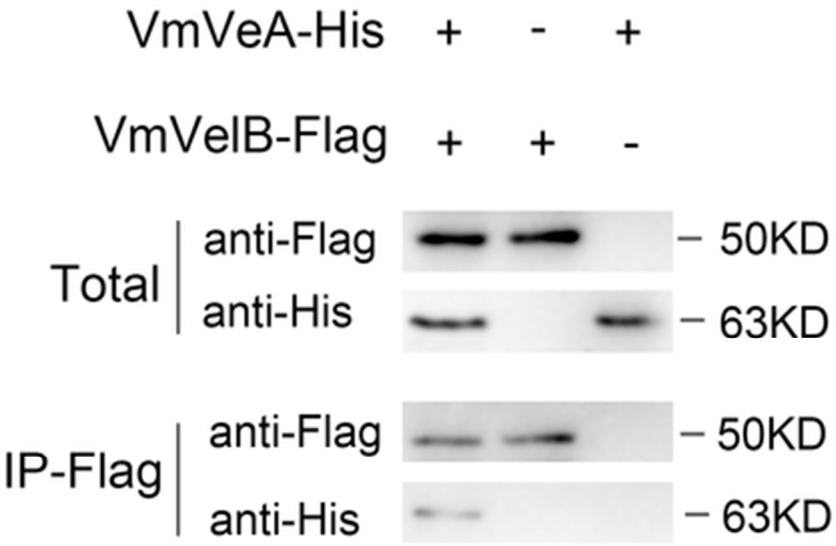

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37x24mm (300 x 300 DPI)

Table 1: Effects of deletion of *VmVeA*, *VmVelB*, and *VmCmr1* on growth, melanin biosynthesis and conidiation of *V. mali*

strain	Growth rate (mm/day)	Melanin content (μg/g)	Pycnidia amount (/plate)	
			Light 15 dpi	Dark 15 dpi
WT	15.9±0.4 [a*]	2.81±0.38 [c]	42±11 [c]	0
ΔVmVeA	15.4±0.4 [a]	6.63±0.51 [a]	638±52 [a]	223±23 [a]
ΔVmVeA-C	15.8±0.4 [a]	3.48±0.35 [c]	53±7 [c]	0
ΔVmVelB	15.5±0.3 [a]	5.65±0.73 [b]	342±37 [b]	77±6 [b]
ΔVmVelB-C	15.5±0.4 [a]	3.43±0.45[c]	47±7 [c]	0
ΔVmCmr1	15.5±0.6 [a]	0.88±0.24 [d]	0	0
ΔVmCmr1-C	15.6±0.6 [a]	2.48±0.42 [c]	38±9 [c]	0
ΔVmVeA/ΔVmCmr1	15.6±1.4 [a]	1.09±0.20 [d]	0	0
ΔVmVelB/ΔVmCmr1	14.5±1.8 [a]	0.84±0.06 [d]	0	0

Pycnidia (/plate) were counted from cultures grown on PDA.

Data are presented as means ± SD from three independent experiments. According to the protected Fisher's Least Significant Difference (LSD) test, the same letter indicates no significant difference ($P < 0.05$).