The Putative Protein Phosphatase MoYvh1 Functions Upstream of MoPdeH to Regulate the Development and Pathogenicity in *Magnaporthe oryzae*

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Protein phosphatases are critical regulators in eukaryotic cells. For example, the budding yeast *Saccharomyces cerevisiae* dual specificity protein phosphatase (DSP) ScYvh1 regulates growth, sporulation, and glycogen accumulation. Despite such importance, functions of Yvh1 proteins in filamentous fungi are not well understood. In this study, we characterized putative protein phosphatase MoYvh1, an Yvh1 homolog in the rice blast fungus *Magnaporthe oryzae*. Deletion of the *MoYVH1* gene resulted in significant reductions in vegetative growth, conidial production, and virulence. The Δ *Moyvh1* mutant also displayed defects in cell-wall integrity and was hyposensitive to the exogenous osmotic stress. Further examination revealed that the Δ *Moyvh1* mutant had defects in appressorium function and invasive hyphae growth, resulting attenuated pathogenicity. Interestingly, we found that MoYvh1 affects the scavenging of host-derived reactive oxygen species that promotes *M. oryzae* infection. Finally, overexpression of the phosphodiesterase *MoPDEH* suppressed the defects in conidia formation and pathogenicity of the Δ *Moyvh1* mutant, suggesting MoYvh1 could regulate *MoPDEH* for its function. Our study reveals not only the importance of MoYvh1 proteins in growth, differentiation, and virulence of the rice blast fungus but, also, a genetic link between MoYvh1 and *MoPDEH*-cAMP signaling in this fungus.

Eukaryotic cells utilize protein phosphorylation and dephosphorylation as one of the regulatory mechanisms for gene expression, signal transduction, and cell-cycle progression (Zolnierowicz and Bollen 2000). Protein phosphatases hydrolyze phosphorylated proteins resulting in dephosphorylation with consequent effects on downstream signaling pathways. Protein phosphatases are divided into three classes according to their substrates, protein tyrosine phosphatases that hydrolyze phosphotyrosine residues, protein serine/threonine phosphatases that hydrolyze phosphoserine/threonine residues, and dual-specificity protein phosphatases (DSPs) that hydrolyze phosphotyrosine and phosphoserine/threonine residues (Keyse 1995).

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The first eukaryotic member of the DSP family, ScYvh1, was identified as a vaccinia virus VH1 phosphatase homolog in *Saccharomyces cerevisiae* (Guan et al. 1992). Alignment of YVH1 phosphatases from multiple species showed that all contain the conserved domain characteristic of DSPs in the N-terminal. At the core of this domain, there is the HCXXGXXR sequence that represents the catalytic core of tryptophan phosphatases. And the essential cysteine of the sequence forms a thiophosphate intermediate in DSP-catalyzed dephosphorylation (Denu and Dixon 1995; Kumar et al. 2004). Substitution of this cysteine by serine abolished its hydrolytic activity in vitro (Alessi et al. 1993; Ishibashi et al. 1992; Sun et al. 1993) and function in vivo (Aroca et al. 1995; Doi et al. 1994; Sun et al. 1993). The C-terminal domain of Yvh1 is also conserved, containing seven invariant cysteines, and one histidine reminiscent of sequences represented the zinc-binding domain. The zinc-binding domain is known to mediate protein-protein or DNA-protein interactions. As demonstrated in budding yeast and in human fungal pathogens, the zinc finger proteins function in a wide range of processes, including amino acid and vitamin synthesis, carbon and nitrogen metabolism, meiosis, and morphogenesis (Fang et al. 2003; Liu and Chang 2009). Other roles of zinc-binding domain include regulation of genes involved in the stress response and pleiotropic drug resistance (MacPherson et al. 2006). Deletion of the *ScYVH1* gene caused defects not only in vegetative growth but also in spore maturation, glycogen metabolism, and ribosome biogenesis (Beeser and Cooper 2000; Guan et al. 1992). In addition, the *ScYVH1* transcript level increased significantly in response to nitrogen starvation at low temperatures (Sakumoto et al. 1999). In another report, the decreased sporulation of a Δ *Scyvh1* strain was associated with the reduction in the transcription levels of a pair of sporulation-related genes (Ma et al. 1999). Moreover, an assay of the inhibitory effect to Congo Red (CR), which is a cell-wall stress agent, on ScYvh1 demonstrated that ScYvh1 functions in the maintenance of cell-wall integrity (CWI) by influencing the Mck1-Mkk1-Mps1 mitogen-activated protein kinase (MAPK) pathway (Sacristán-Reviriego et al. 2015).

Deletion of YVH1 also affected cyclic adenosine monophosphate (cAMP)-mediated signaling (Beeser and Cooper 2000). cAMP, a second messenger molecule produced in response to nutrients, is involved in the inhibition of downstream signaling components such as the phosphorylating enzyme protein kinase A (PKA) (Daniel et al. 1998). In fungi, the balance of intracellular cAMP levels is regulated by the activities of adenylyl cyclase and

phosphodiesterase (PDEase). In *S. cerevisiae*, overexpression of *ScPDE2*, which encodes a high-affinity PDEase modulating the basal cAMP level, suppressed the defect in sporulation caused by deletion of *ScYVH1* (Beeser and Cooper 2000). This suggests that ScYvh1 possibly functions upstream of the ScPde2-mediated cAMP pathway. In the opportunistic fungal pathogen *Candida albicans*, disruption of the *CaYVH1* gene resulted in slowed growth and altered cell morphology as well as virulence and CaYvh1 was also found to participate in nuclear division and septum formation (Hanaoka et al. 2005). Despite these findings, functions of Yvh1 proteins in filamentous fungi remain obscure.

Magnaporthe oryzae, the causal agent of rice blast disease, is widely used to study plant-pathogen interactions (Wilson and Talbot 2009). In this fungus, cAMP signaling is required for many aspects of development and pathogenicity. Deletion of *MoMAC1*, which encodes adenylyl cyclase, resulted in a defect in virulence, and the defect could be suppressed by addition of exogenous cAMP or obliteration of the protein kinase PKA regulatory subunit. MoRgs1, one of the main regulators of G-protein signaling, also participates in the regulation of the cAMP pathway (Liu et al. 2007; Zhang et al. 2011b). Similarly, MoPdeH, a high-affinity PDEase, regulates conidia formation, pathogenicity, and invasive growth. Deletion of *MoPDEH* not only increased the intracellular cAMP level but also reduced conidiation and virulence (Zhang et al. 2011a). Furthermore, recent studies reveal that MoPdeH interacts with MoMck1, which is one of the components of the MoMck1-MoMkk1-MoMps1 MAPK cascade and plays critical roles for CWI and pathogenicity (Jeon et al. 2008; Xu et al. 1998). Overexpression of *MoMCK1* could suppress defects in hyphal autolysis and pathogenicity of the Δ *MopdeH* mutant that suggested that MoPdeH involves CWI and might be modulated by the MAPK cascade. Moreover, we found that MoMck1 regulates *MoPDEH* expression through a feedback loop, to impact conidial development and pathogenicity through cellular cAMP levels (Yin et al. 2015).

In this study, we continued our studies by demonstrating that DSP MoYvh1 also plays an important role in vegetative growth, conidia formation, and CWI. MoYvh1 affects the turgor pressure in appressoria and scavenging of host-derived reactive oxygen species (ROS) to promote *M. oryzae* infection. Furthermore, overexpression of *MoPDEH* suppressed the defects of the Δ *Moyvhl* mutant in conidia formation and pathogenicity, indicating that MoYvh1 functions upstream of MoPdeH. Finally, we have analyzed MoYvh1 domain structures and showed that its C-terminal zinc-binding domain is required for its characterized functions.

RESULTS

Identification of MoYVH1 from *M. oryzae* and functional complementation of a *S. cerevisiae* Δ *Scyvh1* mutant.

Examination of the *M. oryzae* genome database maintained at the Broad Institute revealed that MGG_09700 shares high amino acid sequence homology (29% similarity) with *S. cerevisiae* ScYvh1, a member of the dual-specificity family of protein phosphatases, and we named it MoYvh1. MoYvh1 consists of 394 amino acids and contains a conserved tyrosine phosphatase catalytic domain within a dual-specificity phosphatase domain at its N-terminus and a zinc-binding domain consisting of one histidine and eight cysteine residues at its C-terminus (Supplementary Fig. S1A). Comparison of conserved tyrosine phosphatase catalytic domains in fungi showed that the MoYvh1 phosphatase catalytic domain contains an HCXXGXSR sequence similar to that found in other filamentous fungi. We expressed *MoYVH1* in a Δ *Scyvh1* mutant, using the yeast expression vector pYES2, and

analyzed the transformants. The transformants showed suppressed defects in sensitivity to CR, glycogen accumulation, cold sensitivity, and dityrosine production when compared with the original Δ *Scyvh1* mutant (Beeser and Cooper 1999, 2000; Sacristán-Reviriego et al. 2015; Sakumoto et al. 1999), indicating that MoYvh1 functions heterologous in *S. cerevisiae* (Supplementary Fig. S2).

To further understand the functional roles of MoYvh1, we examined its transcript levels at various developmental stages. *MoYVH1* transcript levels were relatively high from 24 to 36 h postinoculation (hpi) as determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Supplementary Fig. S3). During these periods, *MoYVH1* transcript levels were almost tenfold higher than those in conidia, indicating that *MoYVH1* was highly expressed during infection.

MoYvh1 is involved in vegetative growth and conidia formation.

Gene-targeted replacement was used to investigate the function of MoYvh1. Following the methods described by Zhang et al. (2009b) and following transformation, we generated and verified the Δ *Moyvhl* deletion mutants (Supplementary Fig. S4).

We examined the vegetative growth of the Δ *Moyvhl* mutant on complete medium (CM), minimal medium, straw decoction and corn agar (SDC), and oatmeal medium plates. The Δ *Moyvhl* mutant exhibited significantly reduced vegetative growth after incubation at 28°C for 7 days in the dark (Fig. 1A and B). We also examined conidiation and found that, after seven days on SDC medium, conidia formation was significantly reduced. The Δ *Moyvhl* mutant produced approximately 50% fewer conidia than Guy11 (Fig. 2A and B). This result indicated that MoYvh1 has a role in the regulation of both vegetative growth and conidia formation.

MoYvh1 contributes to the pathogenicity of *M. oryzae*.

To examine the involvement of MoYvh1 in pathogenicity, conidial suspensions of the Δ *Moyvhl* mutant, Guy11, and the complemented strain were sprayed onto susceptible rice seedlings (cv. CO-39) as described by Wang et al. (2013). After seven days, the Δ *Moyvhl* mutant caused disease lesions on the rice (Fig. 3A and B); however, the Δ *Moyvhl* mutant failed to cause severe type 4 or 5 lesions and most of the lesions were type 1 and 2 and a very few were type 3 (Fig. 3C). On barley leaves inoculated with 5×10^4 spores per milliliter, the Δ *Moyvhl* mutant caused less-severe lesions with restricted expansion of the yellow halo around the lesions (Fig. 4D).

MoYvh1 is required for appressorium turgor pressure and invasive hyphae growth.

To further understand the mechanism underlying reduced pathogenicity of the Δ *Moyvhl* mutant, we first examined appressorium formation on the Δ *Moyvhl* mutant, and there is no significant difference with the wild type (Supplementary Table S1). The appressoria turgor of the Δ *Moyvhl* mutants showed decreased collapse rates in the presence of 1, 2, and 3 M glycerol (Fig. 4A), which was measured by a method described previously (Zhang et al. 2009b). We also examined the invasive hyphal growth on barley leaves and rice leaf sheaths 48 hpi with conidia suspensions based on the following four types: type 1, no penetration; type 2, with penetration peg; type 3, with a single invasive hypha; type 4, with extensive hyphal growth (Chen et al. 2014b; Qi et al. 2016). In Guy11 and the complemented strains, more than 60% of cells showed type 4 ($73.96\% \pm 1.31\%$ and $77.43\% \pm 1.74\%$) hyphae and only 20 to 25% were type 1 ($4.66\% \pm 0.14\%$ and $3.93\% \pm 0.03\%$), type 2 ($8.36\% \pm 0.23\%$ and $5.93\% \pm 0.41\%$), or type 3 ($13.02\% \pm 0.83\%$ and $12.71\% \pm 0.77\%$) hyphae. In contrast, less than 5%

of the ΔMoYvh1 cells were type 4 ($2.92\% \pm 0.14\%$) hyphae and the rates of type 1 ($12.11\% \pm 0.13\%$), type 2 ($58.73\% \pm 1.84\%$), and type 3 ($26.24\% \pm 0.97\%$) hyphae were greater than 90% (Fig. 4B and C). Similar results were observed in infected rice leaf sheaths. After incubation with spore suspensions for 48 h, most invasive ΔMoYvh1 hyphae were restricted to the primary infected rice leaf sheath cells, while invasive hyphae spread freely in the Guy11 and complemented strains (Fig. 4D). These results suggested that MoYvh1 plays an important role in turgor pressure and infectious hyphal growth.

MoYvh1 is involved in the transfer of the intracellular glycogen.

M. oryzae conidia contain several storage carbohydrates, including mannitol, trehalose, and glycogen (Dixon et al. 1999). Since MoYvh1 was required for appressorium turgor, we suspected that the cellular distribution of glycogen during appressorium formation was altered in the ΔMoYvh1 mutant. Conidia from

Guy11, the ΔMoYvh1 mutant strains, and the complemented strains were allowed to germinate in water and to form appressoria on hydrophobic plastic cover slips (Supplementary Fig. S5). At various time points, they were incubated in a glycogen staining solution (60 mg KI and 10 mg I_2), according to Weber et al. (1998). Brown glycogen deposits became visible immediately. In the first 4 h prior to the formation of nascent appressoria, all samples showed an identical phenotype, but the glycogen contents of the topmost cells of conidia of Guy11 and the complemented strain reduced significantly. In contrast, the conidia of the ΔMoYvh1 mutant contained high levels of glycogen after 2 h. Over the next 12 h, glycogen was transferred gradually into mature appressoria; at 20 h, most ($71.33\% \pm 5.77\%$) conidia in ΔMoYvh1 mutant were still stained by the solution. Nevertheless, the Guy11 and complemented strain showed few conidia ($3.96\% \pm 0.83\%$). These results indicated that the deletion of *MoYVH1* disrupted transfer of the intracellular glycogen.

MoYvh1 has a role in mediating oxidative stress and scavenging ROS generated by the host plant.

Phosphorylation can be greatly enhanced by treatment with the inhibitor sodium orthovanadate or peroxy derivatives (Gordon 1991; Posner et al. 1994). To confirm the involvement of MoYvh1 in the oxidative stress response, we treated Guy11, the ΔMoYvh1 mutants, and the complemented strains with various oxidative stress reagents, including butanediamide, Rose Bengal sodium salt, potassium peroxide (KO_2), and hydrogen peroxide (H_2O_2). Mycelial growth of the ΔMoYvh1 mutant was reduced significantly compared with Guy11. Moreover, ΔMoYvh1 did not grow in the presence of high concentrations of oxidative stress-inducing agents (Supplementary Fig. S6A and B).

ROS accumulation functions as a secondary signal that mediates plant defense (Alvarez et al. 1998; Gechev and Hille 2005; Lamb and Dixon 1997; Nurnberger et al. 2004), and the activation of ROS scavenging by phytopathogens is a mechanism for coping with plant defenses to facilitate infection (Chi et al. 2009; Guo et al. 2010). We examined host-derived ROS levels by staining with 3,3'-diaminobenzidine (DAB) at 24 hpi. Rice cells with Guy11 infectious hyphae were not stained by DAB and no reddish-brown precipitate was observed in infected cells. The primary infected rice cells with infectious hyphae of the ΔMoYvh1 mutant were stained intensely by DAB, with reddish-brown precipitate around the appressoria and infected cells, indicating that the ΔMoYvh1 mutant failed to scavenge accumulated H_2O_2 at the site of infection (Fig. 5A).

Fig. 1. MoYvh1 is required for normal mycelial growth on various medium. **A**, Comparison of ΔMoYvh1 and wild-type strain in colony morphology. Wild type, the ΔMoYvh1 mutant, and the complementation strain were cultured at 28°C in darkness for 7 days. **B**, Statistical analyses of the diameter of hypha of wild-type Guy11, the ΔMoYvh1 mutant, and the complemented strain on different medium. Error bars represent the standard deviations; asterisks denote statistical significances ($P < 0.01$).

Fig. 2. The ΔMoYvh1 deletion mutant showed reduced conidiation. **A**, The conidia were developed on straw decoction and corn agar (SDC) medium for 7 days and were examined by light microscopy. **B**, Statistical analysis of conidia production. The conidia produced by the wild type, mutant, and complemented strain grown on SDC medium for 10 days were collected, counted, and analyzed; asterisks denote statistical significances ($P < 0.01$).

We assumed that the decreased rate of hyphal growth in infected cells and reduced virulence of the $\Delta MoYvh1$ mutant might be due to a lack of ROS scavenger production by the host plant. To evaluate this hypothesis, an excised leaf-sheath assay was performed using diphenyleneiodonium (DPI) to inhibit the activity of plant NADPH oxidases, which are important generators of ROS (Bolwell et al. 1998; Grant et al. 2000; Jones and Dangl 2006; Zhang et al. 2009a). After incubation at 28°C for 36 h, cells were observed under a light microscope. Without DPI treatment, the $\Delta MoYvh1$ mutant infectious hyphae in plant cells appeared swollen and were restricted to primary infected cells, while those of Guy11 spread into adjacent cells. Upon treatment with 0.5 μ M DPI to inhibit ROS generation, infectious hyphae of the $\Delta MoYvh1$ mutant spread into neighboring cells. In *M. oryzae*, conidia suspension carried elicitor activity that induced the production of H_2O_2 in rice cells that was dependent on fungal isolates and catalase activity in the suspension (Tanabe et al. 2009). Conidia suspensions harvested from Guy11, the $\Delta MoYvh1$ mutants, and the complemented strains were mixed with catalase of *Aspergillus niger* (CAG, Sigma) and were inoculated on the leaf sheath to control H_2O_2 around the infection site. After 36 h, the defects of infection in $\Delta MoYvh1$ mutants appeared suppressed (Fig. 5A). This observation suggested that scavenging of host-derived ROS at the infection site was important for virulence of the $\Delta MoYvh1$ mutant and that deletion of *MoYVH1* might explain the absence of infectious growth.

Therefore, we determined whether the $\Delta MoYvh1$ strains elicited stronger defense responses than Guy11 during infection of rice. Transcript levels of pathogenesis-related (PR) genes were higher in rice plants infected with the $\Delta MoYvh1$ mutants than with Guy11. *PR1a*, *PR4*, *PBZ1*, *PAL1*, and *LOX1* expression levels at 24 hpi during infection by the $\Delta MoYvh1$ strains were higher than during infection by Guy11 as determined by real-time qPCR analysis (Fig. 5C). At 24 hpi, Guy11 had already prevented the accumulated H_2O_2 at the infection site. Moreover, Guy11 was spreading into adjacent cells at this time. Based on these results, we concluded that restriction of the $\Delta MoYvh1$ mutant in rice cells might be due to host-derived ROS at the infection site and the subsequent activation of strong plant defense responses.

MoYvh1 reduces the activities of extracellular peroxidases and laccases.

CR binds to the cell-wall component β -1,4-glucan and is often used to assess CWI (Chi et al. 2009; Wood and Fulcher 1983). CR can also be used to detect secreted peroxidases, because it can be hydrolyzed by fungal peroxidases (Chi et al. 2009; Zhang et al. 2011b). To investigate whether MoYvh1 plays a role in CWI, CR (200 μ g/ml) was added to CM medium. Inhibition of mycelial growth was significantly higher in the $\Delta MoYvh1$ mutant (17%) than in Guy11 (8%), and the golden halo (which indicates CR degradation) was smaller around the $\Delta MoYvh1$ mutant than around Guy11 (Supplementary Fig. S7A). These results indicated that the *MoYvh1* mutant lost the peroxidase activity. Similarly, the extracellular laccase activity was also reduced in the $\Delta MoYvh1$ mutant compared with Guy11. We hypothesized that the decreased extracellular peroxidase and laccase activity in the $\Delta MoYvh1$ mutant might be due to reduced transcription of genes encoding these enzymes, so we then examined the expression of several extracellular peroxidase and laccase genes in the $\Delta MoYvh1$ mutant, by qRT-PCR. We found that the transcript levels of six peroxidase-encoding genes but not MGG_11856 and all nine genes encoding putative laccases (Guo et al. 2010) were significantly reduced in the $\Delta MoYvh1$ mutant. These results suggested that deletion of *MoYVH1* resulted in reduced peroxidase and laccase activities.

MoYvh1 disruption suppresses conidia formation and pathogenicity by reducing cAMP levels.

Overexpression of *ScPDE2* in the $\Delta Scyvh1$ mutant could suppress the defects caused by deletion of *ScYVH1* in *S. cerevisiae* (Beeser and Cooper 2000). To examine whether MoYvh1 is also linked to cAMP signaling, we measured intracellular cAMP levels in mycelia of the $\Delta MoYvh1$ mutant and found that the $\Delta MoYvh1$ mutant exhibited a 1.5-fold higher intracellular cAMP level than the wild-type Guy11 and the complemented strain (Fig. 6A). Examination of the *MoPDEH* expression level also showed decreased expression in the $\Delta MoYvh1$ mutant (approximately 0.5-fold). We further over-expressed *MoPDEH* in the $\Delta MoYvh1$ mutant to determine if MoPdeH could restore defects caused by *MoYVH1* deletion.

Fig. 3. *MoYVH1* deletion decreased pathogenicity. **A**, The pathogenicity on rice leaves. **B**, Quantification of lesion type (0 = no lesion, 1 = pinhead-sized brown specks, 2 = 1.5-mm brown spots, 3 = 2- to 3-mm gray spots with brown margins, 4 = many elliptical gray spots longer than 3 mm, 5 = coalesced lesions infecting 50% or more of the leaf area). Lesions were photographed and were measured at 7 days postinoculation, numbers within an area of 4 cm² were counted, and experiments were repeated three times with similar results. Asterisks indicate significant differences at *P* = 0.01. **C**, Leaves from 4-week-old rice seedlings were injected with conidial suspension (5×10^4 spores/ml) of Guy11, the $\Delta MoYvh1$ mutant, and the complemented strain. Diseased leaves were photographed 7 days after inoculation. **D**, Pathogenicity test on barley leaves.

Several transformants were obtained and tested by qRT (Fig. 6B). One of these, $\Delta MoYvh1/oxMoPDEH-5$, in which the *MoPDEH* expression level increased to 160% that in the wild type, was able to decrease intracellular cAMP levels (Fig. 6A) and suppress the defects in conidia formation and pathogenicity, although partially. However, the transformant $\Delta MoYvh1/oxMoPDEH-3$, in which *MoPDEH* expression level was almost the same as that of the $\Delta MoYvh1$ mutant, could not complement the cAMP levels and defects (Fig. 6C and D). Overexpressed *MoPdeH* suppressed the defects in sporulation and pathogenicity of *MoYVH1* deletion, the growth defect was not restored in both $\Delta MoYvh1/oxMoPDEH-3$ and $\Delta MoYvh1/oxMoPDEH-5$ strains (Supplementary Fig. S8A). Since higher intracellular cAMP levels and defects in conidia formation and pathogenicity were attributed to low levels of *MoPDEH* expression in the $\Delta MoYvh1$ mutant, these results were consistent with that of *MoYvh1* involved in the regulation of intracellular cAMP levels mediated by *MoPdeH*.

***MoYVH1* is required for the maintenance of CWI and the regulation of the MAPK pathway.**

Recently, we found that *MoPdeH* functions upstream of the *MoMck1-MoMkk1-MoMps1* MAPK pathway to regulate CWI (Yin et al. 2015). We overexpressed the *MoMCK1* in the $\Delta MoYvh1$ mutant (Supplementary Fig. S9) and found that it could suppress the defects in conidiation and virulence caused by *MoYVH1* deletion (Fig. 6C and D). To test whether *MoYvh1* functions upstream of *MoPdeH* to regulate this MAPK pathway, we measured mycelial growth on CM medium containing various concentrations of CR. After cultured for 7 days, the $\Delta MoYvh1$ mutant was significantly more sensitive to all CR concentrations tested (Fig. 7C). However, the $\Delta MoYvh1/oxMoMCK1$ transformants showed more tolerance than the $\Delta MoYvh1$ mutant.

We then tested the sensitivity of the strains to cell wall-degrading enzymes. Equal amounts of mycelia from the $\Delta MoYvh1$ mutant, Guy11, and the complemented strain were treated with cell wall-degrading enzymes. After incubation at 30°C, the $\Delta MoYvh1$ mutants were more readily digested and

released more protoplasts than the Guy11 and complemented strain (Fig. 7A and B). We further quantified the chitin contents of these cell types and found that the chitin content of the $\Delta MoYvh1$ mutant was reduced by 50% compared with Guy11 (Fig. 7D). These results indicated that *MoYvh1* is required for maintenance of CWI.

The MAPK cascade is thought to act upstream of CWI signaling in *S. cerevisiae*. In *M. oryzae*, the *MoMck1* and *MoMps1* proteins, which participate in MAPK signaling, have been reported to be crucial for CWI and pathogenicity. Therefore, we assayed *Mps1* activation in the $\Delta MoYvh1$ mutant and found that the phosphorylation of *Mps1* increased in the mutant (Fig. 7E). Taken together, these results demonstrated that *MoYvh1* functions in the maintenance of CWI via the MAPK pathway.

Functional characterization of different domains of *MoYvh1*.

MoYvh1 has conserved N-terminal phosphatase and C-terminal zinc-binding domains. To assess whether the phosphatase domain or the zinc-binding domain is important for the growth, conidiation, and pathogenicity of *MoYvh1*, several plasmids harboring *MoYVH1* fused to a green fluorescent protein (GFP) coding sequence were constructed, including *MoYvh1*^{ΔC}-GFP (deletion of the C-terminal region), *MoYvh1*^{ΔN}-GFP (deletion of the N-terminal region), and *MoYvh1*^{C123S}-GFP (point mutation of the active site). Expression of *MoYvh1*^{ΔN}-GFP and *MoYvh1*^{C123S}-GFP complemented the defects in the $\Delta MoYvh1$ strains. In contrast, *MoYvh1*^{ΔC}-GFP caused phenotypes similar to those of the $\Delta MoYvh1$ mutant in vegetative growth, conidia formation, and pathogenicity (Fig. 8). Further experiments in cAMP, oxidative stress, osmotic stress, the chitin content, protoplast release, DAB assay, and also, the transfer of glycogen showed similar results that only C-terminal domain complement the defects in the $\Delta MoYvh1$ mutant strains (Supplementary Fig. S11). Taken together, these findings suggest that the C-terminal zinc-binding domain is essential for the roles of *MoYvh1*.

Fig. 4. *MoYVH1* deletion decreased the infection on barley and rice. **A**, Different concentrations of glycerol (1 to 4 M) were used for cytorrhysis analysis. For each concentration, at least 100 appressoria were counted and the experiment was repeated three times with the same result; asterisks denote statistical significances ($P < 0.01$). **B**, Excised barley leaves from 7-day-old barley seedlings were inoculated with conidial suspension (1×10^5 spores/ml). Infectious growth was observed at 24 h postinoculation (hpi). **C**, Statistical analysis for each type of infectious hyphal shape. For each tested strain, 100 infecting hyphae were counted per replicate and the experiment was repeated three times. **D**, The conidial suspension (1×10^5 spores/ml) was inoculated in excised rice sheath of 4-week-old rice seedlings. The invasive hyphae growth was observed at 48 hpi.

DISCUSSION

In this study, we examined the conserved putative protein phosphatase MoYvh1 from *M. oryzae*, which is homologous to the *S. cerevisiae* YVH1 protein and participates in vegetative growth, conidia formation, pathogenicity, and the cAMP-dependent protein kinase cascade. Our data further understanding of the PKA-mediated cAMP signaling pathway by showing that the regulator is involved in adjusting intracellular cAMP levels. Our findings provide important insights into the molecular mechanisms underlying the pathogenicity of rice blast fungus.

In plants, the generation of ROS is regarded as one of the first responses to fungal invasion (Mellersh et al. 2002). For plant pathogens, ROS also play an important role and loss of the ability to eliminate intracellular ROS can result in functional defects (Egan et al. 2007). In *M. oryzae*, $\Delta Moatf1$ mutant was absent in scavenging the ROS around invasion hyphae and inhibition of the plant NADPH oxidase by DPI restored infectious hyphal growth of the mutants in rice cells (Guo et al. 2010). Moreover, deletion of the bZIP transcription factor *MoAP1* that mediates the oxidative stress response showed accumulation of ROS at the infection site and lack of invasive hyphae growth (Guo et al. 2011). In this study, we found that the $\Delta MoYvh1$

mutants had no apparent defects in appressorium formation but showed restricted infectious hyphae growth and reduced virulence on the host (Fig. 4D). In view of the sensitivity of the mutant strain to H_2O_2 , we suspected that such a result may be due to the loss of ROS-degrading ability in the mutants, and this hypothesis was substantiated by the DAB staining assay. Furthermore, after treatment with DPI and catalase, the defect in invasion was suppressed, which is agreed with *MoAtf1* and *MoAp1*. Taken together with our findings on the induced expression of PR genes in rice inoculated with $\Delta MoYvh1$ mutants (Fig. 5C), these observations suggested that MoYvh1 participates in inhibition of the plant defense response. We concluded that *MoYVH1* is involved in the disruption of extracellular ROS and avoiding host immune response is responsible for the virulence.

cAMP signaling plays an important role in regulating *M. oryzae* growth and differentiation (Ramanujam and Naqvi 2010), and our previous study demonstrated that deletion of *MoPDEH* resulted in a high intracellular cAMP concentration and significantly reduced sporulation and pathogenicity (Zhang et al. 2011a). Independently, Ramanujam and colleagues (2010) harvested more conidia from a $\Delta MoPdeH$ mutant than from a wild-type strain (B157) and they suggested that a high cAMP level contributes to conidiation. *MoRGS1*, regarded as one of

Fig. 5. The MoYVH1 deletion mutant induced strong plant defense responses. **A**, 3,3'-Diaminobenzidine (DAB) staining of the excised leaf sheath of rice infected by Guy11, the $\Delta MoYvh1$ mutant, and the complemented strain 24 h after inoculation. The excised sheath of rice was inoculated with conidial suspension after treatment with or without 0.5 μ M diphenyleneiodonium (DPI) dissolved in dimethyl sulfoxide (DMSO) by three independent experiments. The rice sheath was also inoculated with conidial suspension after treatment with or without 0.2 U catalase of *Aspergillus niger* (CAG, Sigma) dissolved in 10 mM $(NH_4)_2SO_4$. Samples were harvested and were observed 36 h after inoculation. This experiment was performed with three independent experiments and representative results from one of these experiments are presented. **B**, The infected cell stained by DAB. Three independent biological experiments were performed, with three replicates each time, yielding similar results in each independent biological experiment. Error bars represent standard deviation; asterisks represent significant difference between the different strains ($P < 0.01$). **C**, The transcription of *PBZ1*, *PR1a*, *PR4*, *PAL1*, and *LOX1* in the infected rice was assayed using quantitative reverse transcription-polymerase chain reaction. RNA samples were collected from rice plants 24 h after inoculation with the $\Delta MoYvh1$ mutant or wild-type strain. The average threshold cycle of triplicate reactions was normalized by the stable-expressions gene elongation factor 1 α (Os03g08020) in *Oryza sativa*. Three independent biological experiments were performed and yielded similar results. Error bars in the figure represent standard deviation; asterisks denote statistical significances ($P < 0.01$).

the major regulators of G protein signaling, also regulates cAMP levels in *M. oryzae*, and the Δ *Morgs1* mutant strains exhibit a higher cAMP level but reduced conidial production (Liu et al. 2007; Zhang et al. 2011b). In this study, we found that the Δ *Moyvh1* mutants produced smaller and restricted lesions and that the cAMP levels of the Δ *Moyvh1* mutants were markedly higher than that of Guy11, these results agreed with the deletion of *MoPDEH* or *MoRGS1* in *M. oryzae*. Moreover, overexpressed *MoPDEH* in the Δ *Moyvh1* mutant resulted in recovery of conidiation, pathogenicity (Fig. 6C and D) and, also, the ability to scavenge the ROS, which indicated that MoYvh1 influences the downstream PKA-cAMP pathway and the cAMP pathway plays important roles in regulating conidiation and virulence in *M. oryzae*.

Although the overexpression of MoPdeH suppressed the defects in sporulation and pathogenicity of Δ *Moyvh1*, the growth defect was not restored. cAMP plays roles in conidiation and pathogenicity of *M. oryzae*, however, it showed no evidence in regulating vegetative growth. The Δ *MopdeH* mutants that lead to threefold higher levels cAMP compared with the wild type showed no significant difference in colony morphology or growth rate on a variety of mediums (Zhang et al. 2011a). In addition, the MoPdeH-cAMP signaling pathway showed cross-talks with the CWI, and all of the MoMck1-MoMkk1-MoMps1 MAPK cascade components are involved in regulating intracellular cAMP levels; however, the vegetative growth of all these mutants showed no difference from the wild type (Jeon et al. 2008; Xu et al. 1998; Yin et al. 2015).

Moreover, MoGti1, which possesses a PKA phosphorylation site that regulates the MoCpkA- and MoPmk1-mediated cAMP pathway, exhibited a slightly larger colony diameter than the wild-type strain (Chen et al. 2014a). According to these results, we indicated that cAMP might not have a direct relationship with vegetative growth and, also, the defects in growth of Δ *Moyvh1* mutant strains might not account for the cAMP-PKA signaling pathway.

The CWI signaling pathway allows *M. oryzae* to sense and respond to cell-wall stresses that arise during growth, differentiation, and pathogenicity (Jeon et al. 2008; Xu et al. 1998). In our study, the Δ *Moyvh1* mutant was sensitive to the CWI inhibitor CR and the phosphorylation ability of Mps1 of Δ *Moyvh1* decreased, and also, the Δ *Moyvh1/oxMoMCK1* transformants suppressed the defects on the CWI of the Δ *Moyvh1* mutant. In the Δ *Momck1* mutant, the intracellular cAMP level increased and the expression of *MoPDEH* was down-regulated, which suggests a feedback regulatory mechanism of MoPdeH that cross-talks between cAMP signaling and the MAPK cascade (Yin et al. 2015). Here, we showed that the intracellular cAMP level in the Δ *Moyvh1* mutant increased, which probably resulted from the downregulated expression of *MoPDEH* (Fig. 6A and B). This type of regulation suggests that MoYvh1 functions upstream of MoPdeH-mediated cAMP-signaling and MoMck1-mediated CWI to affect asexual development and pathogenicity (Fig. 9).

In *S. cerevisiae*, the reduced growth rate of the Δ *Scyvh1* mutants was completely restored in the Δ *Scyvh1/ScMRT4^{G69D}* strains (Kemmler et al. 2009; Lo et al. 2009; Sugiyama et al.

Fig. 6. MoYvh1 is involved in the cAMP signal pathway and is located upstream to MoPdeH. **A**, Quantification of intracellular cAMP in the mycelia stage of all the strains. The error bars represent standard deviation (SD) of three replicates. **B**, Expression analysis of the *MoPDE2* gene in wild type and mutant strains. The error bars indicate SD of three replicates; asterisks indicate significant difference ($P < 0.01$). **C**, Conidia formation assay. Each strain was cultured in straw decoction and corn agar medium in the dark for 7 days; asterisks indicate significant difference ($P < 0.01$). **D**, Spraying assay. Conidial suspensions of each strain were sprayed on rice seedlings. Diseased leaves were photographed 7 days after inoculation.

2011). ScMrt4 is thought to be involved in the early stages of ribosome synthesis, and signal transduction is affected by delayed ribosomal synthesis, due to damage to the protein splicing and processing machinery (Harnpicharnchai et al. 2001; Kemmler et al. 2009; Lo et al. 2009; Zuk et al. 1999). These data showed that ScYvh1-mediated release of ScMrt4 precedes cytoplasmic loading of ScRpp0 on pre-60S particles and the assembly of ribosome stalk requires the ScYvh1 for the exchange of ScMrt4 with ScRpp0 in *S. cerevisiae* (Kemmler et al. 2009; Lo et al. 2009). Therefore, we speculate that MoYvh1 might participate in the early stage of ribosomal synthesis that subsequently influences vegetative growth in *M. oryzae*; further studies focusing on this hypothesis are warranted.

MoYvh1 is a modular two-domain protein that contains a conserved phosphatase domain and a C-terminal zinc-binding domain, and our results indicated that MoYvh1 function is independent of the phosphatase domain but the C-terminal zinc-binding domain can completely complement the defects caused by the Δ MoYvh1 mutation, including those affecting growth, conidia formation, pathogenicity, infection, and CWI (Fig. 8). In *S. cerevisiae*, inactivation of ScYvh1 phosphatase activity (Yvh1-C117A) or deletion of the entire N-terminal phosphatase domain did not cause a slow-growth phenotype (Beeser and Cooper 2000; Muda et al. 1999). Furthermore, mutations in which the entire C-terminal zinc-binding domain was deleted were found to be responsible for the slow growth of Δ Scyvh1 strains (Beeser and Cooper 1999; Liu and Chang 2009; Sakumoto et al. 1999), which is agreed with our results.

VH1 phosphatase was shown to have activity against tyrosine- and serine-phosphorylated substrates; mutagenesis of an essential cysteine in the vaccinia phosphatase abolishes catalytic activity directed toward both substrates (Guan et al. 1992). Although the levels of phosphatase activity are very low, Muda et al. (1999) observed ScYVH1 that have activity against several tyrosine-phosphorylated substrates. While no evidence has established how the N-terminal phosphatase domain of MoYvh1 functions in *M. oryzae*, we speculate that MoYVH1 might have a more restricted activity toward specific proteins under certain conditions in vivo and that the phenotype we have examined may be not reflective of the “true” function of the phosphatase domain. Since the C-terminal region of MoYvh1 complemented the defects, we assume that the zinc-binding domain is largely dispensable for the intrinsic enzymatic activity and its putative interaction with the endogenous target is essential for the vivo function. Roles for the zinc-binding domain might include correct localization of the catalytic core within cellular compartments or is involved in protein-protein interactions.

Our results revealed that the putative protein phosphatase MoYvh1 plays a critical role in *M. oryzae*. MoYvh1 is a multifunctional protein required for vegetative growth, conidiation, the CWI pathway, and pathogenicity. We confirmed that MoYvh1 functions upstream of the cAMP-PKA signaling pathway, and further studies investigating other signaling pathways influenced by MoYvh1 and its interacting proteins would help to further explore the importance of protein phosphorylation in growth and pathogenicity of *M. oryzae*.

Fig. 7. MoYvh1 has a role in cell-wall integrity. **A**, Light microscopic examination of protoplasts release after treatment for 60 min and photographed. **B**, Protoplast production of Guy11, the Δ MoYvh1 mutant, and the complemented strain treated by cell-wall degrading enzyme. Asterisks indicate a significant difference between the mutant and wild-type strain at $P < 0.01$. **C**, Wild-type Guy11, Δ MoYvh1 mutant, and the complemented strain were incubated on complete medium (CM) plates containing different concentrations of Congo Red (CR) at 28°C for 7 days. The growth rate was determined by plotting the percentage of colonies in the presence of various concentrations of CR against regular CM; asterisks denote statistical significances ($P < 0.01$). **D**, GlcNa determination shows significantly decreased chitin contents in the Δ MoYvh1 mutant. Data comprise three independent experiments with triple replications each time that yielded similar results. Asterisks indicate significant differences at $P < 0.01$. **E**, Proteins were prepared from Guy11, Δ MoYvh1, Δ MoYvh1/MoYVH1^{ΔN}, and Δ MoYvh1/MoYVH1^{ΔC} mutant mycelia cultured in liquid CM media; signal intensity of phosphorylated Mps1 decreased in the Δ MoYvh1 and Δ MoYvh1/MoYVH1^{ΔC} mutant.

MATERIALS AND METHODS

Strains and culture condition.

M. oryzae Guy11 strain was used as the wild type in this study. All strains were cultured on CM agar plates for 3 to 15 days at 28°C (Talbot et al. 1993). Fungal mycelia were harvested from liquid CM and were used for genomic DNA and RNA extractions. Protoplasts were prepared and transformed as described (Sweigard et al. 1992). Transformants were selected on TB3 medium (3 g of yeast extract, 3 g of casamino acids, 200 g of sucrose, and 7.5 g of agar in 1 liter of distilled water) with 300 µg of hygromycin B per milliliter (Roche) or 200 g of zeocin per milliliter (Invitrogen).

qRT-PCR analysis.

For qRT-PCR, total RNA was reverse transcribed into first-strand cDNA using the oligo (dT) primer and M-MLV reverse transcription (Vazyme Biotech). qRT-PCR reactions were performed following previously established procedures and each experiment was repeated three times (Guo et al. 2010). qRT-PCR was performed with the ABI 7500 Fast real-time system (ABI Co.) and transcripts were analyzed using the 7500 System SDS software (ABI Co.). To compare the relative abundances of target gene transcripts, the average threshold cycle (Ct) was normalized to that of Actin (qRT-actinF/qRT-actinR) for each of the treated samples.

MoYVH1 gene disruption complementation.

To generate the *MoYVH1* gene replacement vector pCX62, approximately 1-kb upstream and 1-kb downstream fragments

were amplified with primer pairs (Supplementary Table S2). The resulting PCR products were ligated to the hygromycin-resistance cassette released from pCX62, as previously described (Dong et al. 2015). Putative mutants were screened by

Fig. 9. A proposed model for the cross-talk between the cAMP pathway and the cell-wall integrity (CWI) pathway regulated by MoYvh1 in *Magnaporthe oryzae*. Putative cross-talks between the CWI pathway and cAMP pathway mediated by MoPdeH and regulated by the MoYvh1.

Fig. 8. Function of phosphatase domain and zinc-binding domain of MoYvh1. Vegetative growth, conidia formation, and pathogenicity in four different mutants (Δ MoYvh1/MoYvh1^{ΔC}, Δ MoYvh1/MoYvh1^{ΔN}, Δ MoYvh1/MoYvh1^{C123S}, and Δ MoYvh1).

PCR and were confirmed by Southern blotting analysis. To complement the $\Delta Moryh1$ mutant, a DNA fragment including the putative promoter and the coding sequence was amplified and inserted into pYF11 (bleomycin resistance) by homologous recombination in *Saccharomyces cerevisiae*. The plasmids were extracted and were transformed into *Escherichia coli*-competent cells, and then, the plasmids with correct inserts were transferred into protoplasts, as previously described (Dong et al. 2015).

Conidiation, appressorium formation, and turgor assays.

After 7 days of cultivation, conidia were collected with 5 ml of distilled water, were filtered through three layers of lens paper, and were counted with a hemacytometer under a microscope. Conidial germination and appressorium formation were measured on a hydrophobic surface (Qi et al. 2012). Conidial suspensions of $30 \mu\text{l}$ (1×10^5 spores per milliliter) were dropped onto a hydrophobic surface and were placed in a moistened box at 28°C . Appressorium formation rate was then calculated under the microscope at 12 hpi. More than 200 appressoria were counted for each strain and the experiment was repeated three times (Zhang et al. 2011a). Photographs were taken at 24 hpi. The appressorium turgor was measured using an incipient cytorrhysis (cell collapse) assay and a 1.0 to 4.0 M glycerol solution (Zhang et al. 2009b). Conidial suspension the ($20 \mu\text{l}$, 5×10^4 spores per milliliter) were placed on plastic coverslips and were incubated for 24 h at 28°C . The water surrounding the conidia was removed carefully and was replaced with an equal volume ($20 \mu\text{l}$) of glycerol (1.0 to 4.0 M). The number of appressoria that had collapsed after 10 min was recorded and at least 100 appressoria were counted for each strain. The experiments were repeated three times.

Pathogenicity, plant infection, and rice-sheath penetration assays.

Conidia were harvested from 10-day-old SDC agar cultures, were filtered through three layers of lens paper, and were resuspended to a concentration of 5×10^4 spores per milliliter in a 0.2% (wt/vol) gelatin solution. For the leaf assay, leaves from two-week-old seedlings of rice (*Oryza sativa* cv. CO39) were used for spray inoculation and 7-day-old seedlings of barley were used for infection assays. Spray inoculation on the rice leaves, 5 ml of a conidial suspension of each treatment were sprayed onto rice with a sprayer. Inoculated plants were kept in a growth chamber at 25°C with 90% humidity and in the dark for the first 24 h, followed by a 12-h-light and 12-h-dark cycle. Lesion formation was observed daily and was photographed 7 days after inoculation (Guo et al. 2011).

cAMP extraction and high-performance liquid chromatography (HPLC) analysis.

All of the strains were cultured on CM medium at 28°C , were cut into 1×1 -mm squares, and were cultured in liquid CM for another 2 days. Filtering to collect mycelium and quickly ground into powder. Every 1 mg of mycelium mixed with $20 \mu\text{l}$ of 6% TCA solution was turbulenced. Samples were centrifuged ($1,377 \times g$, 15 min), the top layers were collected and were washed twice with five times the volume of anhydrous ether. The pellet was collected for HPLC. HPLC analysis was done with a programmable Agilent Technology Zorbax 1200 series liquid chromatograph. The solvent system consisted of methanol (90%) and water (10%), at a flow rate of 1 ml per minute; 0.1 mg of cAMP solution per milliliter was eluted through the column (SB-C₁₈, $5 \mu\text{l}$, 4.6×250 mm) and was detected at 259 nm UV. Each sample was eluted through the column in turn and peak values were detected with the same time as the standard.

Measurement of the chitin content.

Chitin (N-acetylglucosamine [GlcNAc]) content was analyzed as described (Bulik et al. 2003; Song et al. 2010). First,

mycelial samples were freeze-dried, and then, 5 mg of dried mycelia were resuspended in 1 ml of 6% KOH and were heated at 80°C for 90 min. Samples were centrifuged ($16,000 \times g$, 10 min) and pellets were washed with phosphate buffered saline in three cycles of centrifugation and suspension ($16,000 \times g$, 10 min) before final suspension in 0.5 ml of McIlvaine's buffer (pH 6). An aliquot of 100 μl (13 units) of *Streptomyces plicatus* chitinase (Sigma) was added and was incubated for 16 h at 37°C with gentle mixing. Samples (100 μl) were then combined with 100 μl of 0.27 M sodium borate (pH 9), were heated for 10 min at 100°C , and finally, 1 ml of freshly diluted (1:10) Ehrlich's reagent (10 g p-dimethylaminobenzaldehyde in 1.25 ml of HCl and 8.75 ml of glacial acetic acid) was added. After incubating at 37°C for 20 min, 1 ml of the sample was transferred to a 2.5-ml plastic cuvette (Greiner) and the absorbance at 585 nm was recorded. Standard curves were prepared with GlcNAc (Sigma). The experiment was repeated three times.

Western blot analysis of protein phosphorylation.

The mutant and Guy11 strains were cultured in liquid CM media for 2 days. The intensity of the signal corresponding to phosphorylated Mps1 was detected by binding of an antiphosphop44/42 MAPK antibody (Cell Signaling Technology) and the Mpk1 antibody (N-terminal anti-Mpk1) from Santa Cruz Biotechnology was used as a control. Each experiment was repeated three times.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Broad Institute *Magnaporthe grisea* database:
http://www.broad.mit.edu/annotation/genome/magnaporthe_grisea/Home.html