Homeostasis of cell wall integrity pathway phosphorylation is required for the growth and pathogenicity of *Magnaporthe oryzae*

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**Abstract**  
The cell wall provides a crucial barrier to stress imposed by the external environment. In the rice blast fungus *Magnaporthe oryzae*, this stress response is mediated by the cell wall integrity (CWI) pathway, consisting of a well-characterized protein phosphorylation cascade. However, other regulators that maintain CWI phosphorylation homeostasis, such as protein phosphatases (PPases), remain unclear. Here, we identified two PPases, MoPtc1 and MoPtc2, that function as negative regulators of the CWI pathway. MoPtc1 and MoPtc2 interact with MoMkk1, one of the key components of the CWI pathway, and are crucial for the vegetative growth, conidial formation, and virulence of *M. oryzae*. We also demonstrate that both MoPtc1 and MoPtc2 dephosphorylate MoMkk1 in vivo and in vitro, and that CWI stress leads to enhanced interaction between MoPtc1 and MoMkk1. CWI stress abolishes the interaction between MoPtc2 and MoMkk1, providing a means of deactivation for CWI signalling. Our studies reveal that CWI signalling in *M. oryzae* is a highly coordinated regulatory mechanism vital for stress response and pathogenicity.

**KEYWORDS**  
CWI, protein phosphatases, protein phosphorylation homeostasis, rice blast fungus

1 | INTRODUCTION

*Magnaporthe oryzae* is the causal agent of rice blast and also an established model organism to study plant-pathogen interactions (Deng & Naqvi, 2019; Meng et al., 2019; Nasir et al., 2018; Yan & Talbot, 2016; Zhang et al., 2016). In *M. oryzae*, the cell wall integrity (CWI) pathway consists of the MAP kinase kinase kinase (MoMck1), the MAP kinase kinase (MoMkk1), and the MAP kinase (MoMps1).
Similar to other model organisms, the CWI pathway amplifies cues of environmental fluxes and/or host immunity, allowing the activation of cell wall-related genes in response to cell wall stress (Heinisch, 2020; Yin et al., 2020).

Previous studies have demonstrated that protein kinase C (MoPkc1) phosphorylates MoMck1 to active the CWI pathway (Jeon et al., 2008; Penn et al., 2015) and that the MoMck1–MoMkk1–MoMps1 CWI pathway is important for the growth and virulence of M. oryzae (Yin et al., 2016, 2020). A recent study also showed that MoAtg1, an autophagy-related protein serine/threonine-protein kinase, phosphorylates MoMkk1 to also activate the CWI pathway in response to endoplasmic reticulum (ER) stress (Yin et al., 2016, 2020). Despite this well-elaborated phosphorylation mechanism of CWI signalling in M. oryzae, other mechanisms, including the initiation and termination of the pathway or additional regulation, remain unclear.

Protein dephosphorylation is regulated by protein phosphatases (PPases), with corresponding phosphorylation mediated by phosphokinases. By hydrolysing the conserved serine/threonine or tyrosine, PPases also significantly affect signalling pathways (Furukawa et al., 2018; Shi, 2009). In Saccharomyces cerevisiae, there are four specific PPase catalytic subunits in the cytoplasm. PPases PP1, PP2A, and PP2B are found complexed with other protein subunits, whereas PP2C is a monomeric protein (Barford, 1995; Cohen, 1989; Luan, 2003; Sajid et al., 2015; Shi, 2009). Studies have shown that PP2C is involved in stress response through a unique mechanism. In mammals, PP2C regulates stress signalling by modulating p38 and the JNK MAPK pathway (Takekawa et al., 1998). In Schizosaccharomyces pombe, PP2C regulates stress response independent of the stress-activated kinase cascade (Gaits et al., 1997). In Populus euphratica, the regulation of PP2C in abscisic acid signalling enhances drought tolerance (Chen et al., 2015). Interestingly, PP2C plays a role in avoiding the excessive activation of phosphorylation in fungi (Warmka et al., 2001). Lack of Ptc1 in yeast results in hyphal growth, conidiation, and virulence (Yin et al., 2016). To explore the molecular mechanism by which MoMkk1 maintains phosphorylation homeostasis of CWI in a spatiotemporal manner, we screened MoMkk1-interacting proteins and identified PP2C protein phosphatases MoPtc1 and MoPtc2. We demonstrated that MoPtc1 and MoPtc2 dephosphorylate MoMkk1 in vivo and in vitro. Deletions of MoPtc1 and MoPtc2 cause continuous activation of MoMkk1 phosphorylation and attenuate the virulence of the fungus. Moreover, CWI stress increases the affinity between MoPtc1 and MoMkk1 while disrupting the interaction between MoPtc2 and MoMkk1.

## RESULTS

### MoMkk1 interacts with MoPtc1 and MoPtc2 in vivo and in vitro

MoMkk1 is involved in cellular development and pathogenicity, and it is important to the CWI of M. oryzae (Yin et al., 2016). To elucidate the function of MoMkk1, we used a yeast two-hybrid approach to screen a M. oryzae cDNA library constructed with RNA pooled from various developmental stages, including conidia and infection (0, 2, 4, 8, 12, and 24 h), using MoMkk1 as a bait. Two protein phosphatases, MoPtc1 and MoPtc2, were identified. We then verified these interactions by yeast two-hybrid assay. Yeast cells transformed with both MoPtc1, MoPtc2 and MoMkk1 grew on synthetic dropout (SD) medium lacking leucine (Leu), tryptophan (Trp), histidine (His), and adenine (Ade), and exhibited β-galactosidase (LacZ) activities, indicating that MoMkk1 specifically interacts with MoPtc1 and MoPtc2 (Figure 1a). To further confirm these interactions, co-immunoprecipitation (co-IP) assays were performed. MoPtc1-GFP, MoPtc2-GFP, RP27-GFP, and MoMkk1-Stag were co-transformed into the wild-type strain Guy11. Total proteins were extracted from the mycelia of the putative transformants and immunodetected using anti-Stag and anti-GFP antibodies (Figure 1b). These tests confirmed the positive interactions, suggesting that MoPtc1 and MoPtc2 might have a potential role in MoMkk1-dependent signalling processes.

### MoPtc1 and MoPtc2 complementation in S. cerevisiae and expression patterns

MoPtc1 and MoPtc2 were found to share homology with yeast Ptc1 and Ptc2/Ptc3 proteins, respectively (Figure S1a). Amino acid sequence alignment showed that MoPtc1 and MoPtc2 also share high similarity with other fungal Ptc proteins, including phytopathogens Gaumannomyces tritici and Magnaporthe grisea, particularly at the conserved PP2C domain (Figure S1b). We then expressed MoPtc1 and MoPtc2 in the corresponding yeast mutants, and the results showed that MoPtc1 rescued the growth defect of ptc1 at 37°C and MoPtc2 rescued the growth defect of ptc2 at 37°C and ptc3 at 13°C (Figure S2).

We also examined the expression patterns at various developmental stages by reverse transcription-quantitative PCR (RT-qPCR) and found that MoPtc2 transcripts decreased 12 h postinoculation (hpi), 24 hpi, and 48 hpi, relative to that of the hyphal stage. However, the level of MoPtc1 remained constant (Figure S3), implying that MoPtc1 and MoPtc2 may have distinct roles in host interactions.

### MoPtc1 and MoPtc2 are important in vegetative growth and conidiation

To further examine MoPtc1 and MoPtc2 function, we generated ΔMoPtc1, ΔMoPtc2, and ΔMoPtc1ΔMoPtc2 mutant strains (Figure S4).
and complemented strains. When infected using mycelial pellets, the lesion areas of Moptc1, Moptc2, and Moptc1ΔMoptc2 mutants were all reduced after 4 days when compared with Guy11 and complemented strains (Figure 2c). These results indicated that both Mopc1 and Mopc2 are important for the full virulence of M. oryzae.

Appressorial turgor pressure is critical for infection. To test the role of Mopc1 and Mopc2 in turgor generation, we examined the turgor pressure and found that no appressorial turgor could be measured in either of the Moptc1 and Moptc2 mutants in the presence of 1, 2, 3, and 4 M glycerol (Figure 3a). We then examined the penetration and invasive hyphal extension in rice sheath cells. After incubation with conidial suspensions for 48 h, fewer than 35% invasive hyphae from Guy11 were found to be restricted in primary infected cells, while restrictions of 68% and 77% were found in Moptc1 and Moptc2 infected cells, respectively (Figure 3b,c). These results suggest that Mopc1 and Mopc2 play an important function in turgor pressure generation and invasive hyphal growth.

2.5  |  Mopc1 and Mopc2 are important for CWI

Because Mopc1 and Mopc2 interact with Mokkk1, we speculated that Mopc1 and Mopc2 might also play a role in CWI. Thus, we compared the effects of cell wall-degrading enzymes on the mycelia of mutants and the wild-type strain. Hyphae of the Moptc1, Moptc2, and Moptc1ΔMoptc2 mutants were well digested and released more protoplasts than did Guy11 and the complemented strains (Figure 4a,b). The Moptc1, Moptc2, and Moptc1ΔMoptc2 mutants also showed increased sensitivity to the cell wall agent Congo red (Figure 4c). These results collectively indicated that both Mopc1 and Mopc2 are important for the maintenance of CWI.

The phosphorylation levels of Mops1 were increased in the Moptc1, Moptc2, and Moptc1Moptc2 mutants (Figure 4d), indicating that Mopc1 and Mopc2 are involved in Mops1 phosphorylation. These results are consistent with the proposition that Mopc1 and Mopc2 play an important role in CWI.

2.6  |  Mopc1 and Mopc2 dephosphorylate Mokkk1 in vivo and in vitro

Given that Mopc1 and Mopc2 are PPases and they interact with Mokkk1 in vitro, we tested if they play a role in CWI by dephosphorylating Mokkk1. We found that levels of phosphorylated Mokkk1 were significantly increased in the Moptc1 and Moptc2 mutants (Figure 5a). We further expressed glutathione S-transferase (GST)-fused Mokkk1 protein in Escherichia coli, purified the protein by glutathione agarose beads, and co-incubated with purified His-Mopc1 and His-Mopc2 by Ni-NTA agarose. We detected apparent pro-Q magnitude increases, in comparison to the control group without Mopc1. The fluorescence intensity of the experimental group also decreased, similar to that of the Mopc2 group (Figure 5b). These results suggest Mopc1 and Mopc2 function to dephosphorylate Mokkk1.
Continued activation of MoMkk1 by phosphorylation attenuates virulence

Previous studies have found that MKK1^T369D, T375D results in constitutive activation of MoMps1 and the intensity of MoMps1 phosphorylation slightly increased in the ΔMomck1/MoMKK1^T369D, T375D strain compared to Guy11 (Fujikawa et al., 2009; Yin et al., 2016). Based on the finding that MoPtc1 and MoPtc2 dephosphorylate MoMkk1, we speculated that ΔMoptc1 and ΔMoptc2 mutants might exhibit a continuous activation of MoMkk1 phosphorylation that consequently results in the pathogenicity defect. Mycelial pellets of the ΔMomkk1/MoMKK1^T369D, T375D mutant were inoculated on wounded rice leaves for 4 days. Limited lesions, similar to those produced by ΔMoptc1 and ΔMoptc2 mutants, were observed, in comparison to the control strains (Figure 6a,b). This finding suggests that continued activation of MoMkk1 phosphorylation causes a pathogenicity defect and proper phosphokinase and PPase balance is critical for the full virulence of the fungus.
2.8 | CWI stress regulates MoPtc1- and MoPtc2-mediated MoMkk1 dephosphorylation

We have shown that MoPtc1 and MoPtc2 dephosphorylate MoMkk1 to regulate CWI and the pathogenicity of *M. oryzae*. During the interaction between the blast fungus and rice, host-derived immunity also inflicts higher CWI stress on *M. oryzae* (Bacete et al., 2018). We therefore assessed relationships between MoPtc1/MoPtc2 and MoMkk1 under sodium dodecyl sulphate (SDS)-induced cell wall stress. SDS is a detergent that disrupts cell...
membranes, activates CWI signalling, and restricts cell growth in *S. cerevisiae* (Schroeder & Ikui, 2019). We first treated the mycelia of Guy11, ΔMoptc1, ΔMoptc2, and ΔMoptc1 ΔMoptc2 with 0.005% SDS for 2 h and detected the phosphorylation levels of MoMps1. The results showed that SDS enhanced phosphorylation levels of MoMps1 in Guy11 and ΔMoptc1 ΔMoptc2 (Figure S6). We then examined the effect of SDS on interactions between MoPtc1/2 and MoMkk1 by co-IP. The results showed that SDS completely blocked the interaction between MoPtc2 and MoMkk1 (Figure 7b). However, the interaction between MoPtc1 and MoMkk1 was enhanced under the same conditions (Figure 7a). This finding raised an interesting proposition that MoPtc2 might regulate MoMkk1 distinctively from MoPtc1.

As the MoPtc1/MoPtc2–MoMkk1 interaction intensity may be correlated with levels of phosphorylation of MoMkk1, we used microscale thermophoresis (MST) (Baaske et al., 2010) to assess the affinity of MoPtc2 with MoMkk1, MoMkk1T369A, T375A, and MoMkk1T369D, T375D in vitro, based on the finding that MoPtc1/MoPtc2 dephosphorylation of MoMkk1 is dependent on Thr369 and Thr375. The results showed that MoPtc1 bound with MoMkk1T369D, T375D, with the highest affinity with MoMkk1 and the lowest with MoMkk1T369A, T375A. Meanwhile, MoPtc2 bound with MoMkk1T369A, T375A, with the highest affinity with MoMkk1 and the lowest with MoMkk1T369D, T375D (Figure 7c,d). These findings suggest that the affinity of MoPtc2 and MoMkk1 is negatively correlated with the degree of dephosphorylation of MoMkk1 and that both MoPtc1 and MoPtc2 reduce phosphorylation levels of MoMkk1 under CWI stress.

3 | DISCUSSION

The CWI signalling pathway plays an important role in responding to external stress and pathogenicity (Fuchs & Mylonakis, 2009; Herlaar
In *M. oryzae*, the CWI pathway consists of MoMkk1, MoMck1, and MoMps1, with MoMkk1 acting as a key element that mediates not only CWI but also various signalling pathways, such as autophagy (Yin et al., 2020), mitotic exit network (Feng et al., 2021), and the target of rapamycin pathway (Qian et al., 2018). However, the negative regulation of the CWI pathway and MoMkk1 in phosphorylation homeostasis has remained unexplored until now. We found that PPases MoPtc1 and MoPtc2 dephosphorylate MoMkk1 to ensure phosphorylation homeostasis of the CWI pathway. In addition, we demonstrated that CWI stress interrupted the interaction between MoPtc2 and MoMkk1 but enhanced the association between MoPtc1 and MoMkk1, which provides a means for negative regulation of CWI signalling (Figure 8).

Phosphorylation controls various events during the development of eukaryotic cells, including DNA transcription, cell-cycle regulation, energy metabolism, and signal transduction (Anand et al., 2020; Brehove et al., 2015; Wang et al., 2018; Yang et al., 2017). During the interaction between *M. oryzae* and rice (*Oryza sativa*), rice uses various strategies to subvert the infection. However, these defence strategies cause extracellular stress including CWI, osmotic, and oxidative pressures to the pathogen (Feng et al., 2012; Kadota et al., 2014). In return, *M. oryzae* activates the CWI signalling pathway through a phosphorylation cascade, including MoMck1 and MoAtg1 phosphorylating MoMkk1, which activates MoMps1 phosphorylation to counter the stress and promote infection (Yang et al., 2018; Yin et al., 2016, 2020). As phosphorylation homeostasis is important for protein kinase function, we hypothesize that continuous phosphorylation may be harmful to the fungi, and there might exist dephosphorylation regulatory mechanisms. To test this hypothesis, continuous activation of MoMkk1 was evaluated, and it was found that the continuous activation of MoMkk1 attenuated the pathogenicity in *M. oryzae* (Figure 6a,b). This defect is similar to that in the ΔMoptc1 and ΔMoptc2 mutants (Figure 6a,b), indicating that PP2C may function as the negative regulator of the CWI pathway.

Previous studies showed that the MoMkk1-mediated MAPK (MoMck1-MoMkk1-MoMps1) signal pathway is important for the pathogenicity of *M. oryzae*: deletion of *MoMCK1*, *MoMKK1* or *MoMPS1* caused defects in development, CWI, and pathogenicity (Yin et al., 2016). Given that MoPtc1/2 dephosphorylate MoMkk1 and disruption of MoPTC1/2 resulted in CWI defects, it is plausible that dysregulation of MoMkk1 phosphorylation also results in CWI defects. Previous studies showed that continuous activation of MoRgs1 attenuates the virulence of *M. oryzae*. Constitutively activated MoRgs1 causes protein mislocalization, leading to its

![Figure 6](image-url)

**Figure 6** Continuous activation of MoMkk1 phosphorylation attenuates pathogenicity. (a) Pathogenicity test on rice. Detached rice leaves, wounded by abrasion, were inoculated with the wild type (Guy11), ΔMmkk1, ΔMmkk1/MoMKK1T369D, T375D, ΔMoptc1, ΔMoptc2, and ΔMoptc1ΔMoptc2 strains. Photographs were taken 4 days postinoculation (dpi). (b) Pathogenicity test on barley. Detached barley leaves, wounded by abrasion, were inoculated with Guy11, ΔMmkk1, ΔMmkk1/MoMKK1T369D, T375D, ΔMoptc1, ΔMoptc2, and ΔMoptc1ΔMoptc2 strains. Diseased leaves were photographed 4 dpi. These experiments were performed three times with similar results.
functional defects (Yu et al., 2021). Nevertheless, the mechanisms by which MAPK kinase overphosphorylation affects its function remain unknown. As an important component in the MAPK signal pathway, MoMkk1 becomes activated on CWI stress. Following a series of cascade phosphorylations, the MAPK kinase transmits signals to downstream transcription factors whose function may also be adversely affected. In S. cerevisiae, Mpk1/Slt2 phosphorylates the transcription factors Rlm1 and the SBF complex, including Swi4 and Swi6, to regulate the nuclear expression of genes involved in cell wall biosynthesis and remodelling and cell cycle progression (Jendretzki et al., 2011). In M. oryzae, previous studies found that PPhases MoPtps dephosphorylate MoOsm1 to regulate MoOsm1/MoAtf1-mediated transcription of the antioxidant genes (Liu et al., 2020). Overexpression of these transcription factors is hypothesized to adversely affect cellular growth and development, even pathogenicity.
Also in *S. cerevisiae*, the deletion of Ptc1 causes hyperphosphorylated Tip41 that is unable to properly bind with Tap42, leading to a defect in response to rapamycin (Du et al., 2006; González et al., 2009; Warmka et al., 2001). In *M. oryzae*, phosphorylated levels of MoMps1 increase significantly in response to CWI stress (Yin et al., 2020). Here, we found that MoPtc1 and MoPtc2 negatively regulated MoMkk1 under CWI stress (Figure 5a,b). MoPtc1 and MoPtc2 act on the same signalling pathway, but with different regulating mechanisms, resulting in different outcomes (Figure 7a,b). MoPtc2 completely dissociated from MoMkk1 under CWI stress (Figure 7b).

Previous reports suggested that changes in protein interactions could be caused by external cues. In rice, CWI signals influence the structures of phosphatases to attenuate their catalytic activities. Abscisic acid-induced oxidation of Cys 350 and Cys 428 in PPase PP45 forms PP45 intermolecular dimers that block the interaction between PP45 and DMI3 and prevent the PP45-mediated inhibition of DMI3 activities (Ni et al., 2019). Also, the PP2C38 residue can be phosphorylated by Bik1 to dissociate from FLS2/EFR-BIK1 complexes and enable full Bik1 activation (Couto et al., 2016). Another scenario is that the CWI signal affects the positioning of MoMkk1 and MoPtc2, similar to PsAvh240-M3 interacting with GmAP1 described in *Phytophthora sojae* (Guo et al., 2019). In *S. pombe*, cleavage of the Ptc4 mitochondrial targeting sequence is greatly reduced by H₂O₂, in a specific manner, resulting in full-length forms of the phosphatase that leads to a stronger interaction with Sty1 (Di et al., 2012).

In summary, our studies demonstrated that PP2C PPases MoPtc1 and MoPtc2 play critical roles in the growth, conidiation, and pathogenicity of *M. oryzae*. MoPtc1 and MoPtc2 function on MoMkk1 through protein dephosphorylation. In addition, cell wall stress results in the disassociation of MoPtc2 from MoMkk1 while enhancing the MoPtc1–MoMkk1 interaction. This proper phosphorylation homeostasis allows CWI signalling to counter cell wall stresses (Figure 8). Our studies reveal a novel mechanism of MoPtc1 and MoPtc2 in regulating MoMkk1 and CWI signalling to counter cell wall stress and provide new insights into pathogenicity mechanisms of the blast fungus.

4 | EXPERIMENTAL PROCEDURES

4.1 | Phylogenetic tree construction and sequence alignment

The genes from this article can be found in the GenBank database under the following accession numbers: MoMck1 (MGG_00883), MoMps1 (MGG_04943), MoMkk1 (MGG_06482), MoPtc1 (MGG_05207), MoPtc2 (MGG_01351), MoActin (MGG_03982). The PP2C proteins (MGG_04943), MoMkk1 (MGG_06482), MoPtc1 (MGG_05207), MoPtc2 (MGG_01351), MoActin (MGG_03982). The PP2C proteins and MoMkk1 increase significantly in response to CWI stress (Yin et al., 2020). Here, we found that MoPtc1 and MoPtc2 negatively regulated MoMkk1 under CWI stress (Figure 5a,b). MoPtc1 and MoPtc2 act on the same signalling pathway, but with different regulating mechanisms, resulting in different outcomes (Figure 7a,b). MoPtc2 completely dissociated from MoMkk1 under CWI stress (Figure 7b).

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In summary, our studies demonstrated that PP2C PPases MoPtc1 and MoPtc2 play critical roles in the growth, conidiation, and pathogenicity of *M. oryzae*. MoPtc1 and MoPtc2 function on MoMkk1 through protein dephosphorylation. In addition, cell wall stress results in the disassociation of MoPtc2 from MoMkk1 while enhancing the MoPtc1–MoMkk1 interaction. This proper phosphorylation homeostasis allows CWI signalling to counter cell wall stresses (Figure 8). Our studies reveal a novel mechanism of MoPtc1 and MoPtc2 in regulating MoMkk1 and CWI signalling to counter cell wall stress and provide new insights into pathogenicity mechanisms of the blast fungus.

4.2 | Yeast two-hybrid assay

We cloned the cDNA of MoMkk1 in vector pGBKT7 by yeast transformation according to the producer (BD Biosciences Clontech). We screened interacting proteins in a yeast cDNA library using BD-MoMkk1. Next, we constructed AD-MoPtc1 and AD-MoPtc2 for one-to-one verification; this needed co-transformation expression on SD–Leu–Trp. Finally, we replicated individual colonies to SD–Leu–Trp–His–Ade. After 3 days, we observed their growth as independent colonies (AD-Rect and BD-53 as positive, AD-Rect and BD-Lamb as negative).

4.3 | Targeted gene deletion and complementation

The MoPTC1 and MoPTC2 gene deletion mutants were generated using the standard one-step gene replacement strategy. First, two fragments with 1.0 kb of sequences flanking the targeted gene were PCR amplified with primer pairs. The resulting PCR products were digested with restriction endonucleases and ligated with the hygromycin resistance cassette (HPH) released from pCX62. Finally, the recombinant insert was sequenced. The 3.4 kb fragment, which includes the flanking sequences and the HPH cassette, was amplified and transformed into Guy11 protoplasts. Putative mutants were first screened by PCR and later confirmed by Southern blotting analysis. The complement fragment, which contains the entire target gene-coding region and its native promoter region, was amplified by PCR with primers and inserted into pYF11 (bleomycin resistance) to complement the mutant strains. The MoPtc1 and MoPtc2 double mutants were the 3.4 kb fragment that includes the flanking sequences of MoPtc1 and the bleomycin resistance cassette, which was amplified transformed into ΔMoPtc1 protoplasts.

4.4 | RT-qPCR analysis

Total RNA was reverse transcribed into first-strand cDNA using oligo(dT) primer and HiScript II Q Select RT SuperMix for qPCR (Vazyme). The qPCR was run on the Applied Biosystems 7500 Real-Time PCR System with ChamQ SYBR qPCR Master Mix (Vazyme). Normalization and comparison of mean Cᵥ values were performed as previously described (Liu et al., 2020).

4.5 | Protein extraction and western blot analysis

The mutants and Guy11 strains were cultured in liquid CM for 2 days and then the total proteins were isolated from vegetative hyphae as described by Bruno et al. (2004). For protein phosphorylation analysis, the proteinase inhibitor cocktail (Complete; Sigma-Aldrich) was added. The intensity of the signal corresponding to phosphorylated MoMps1 was detected by binding of an antiphospho-p44/42MAP kinase antibody (Cell Signaling Technology), and the anti-p44/42
MAP kinase antibody (Cell Signaling Technology) was used as control. For green fluorescent protein (GFP)-tagged protein detection, samples were analysed by 8% SDS-PAGE followed by western blotting with the anti-GFP antibody (Abmart) and the anti-mouse antibody (Li-COR, IRDye), followed by detection using the ODYSSEY infrared imaging system (application software v. 2.1).

4.6 | In vitro phosphorylation assay

The Pro-Q Diamond Phosphorylation gel stain (Thermo Fisher Scientific), a phosphor-protein gel-staining fluorescence dye, was used in this assay. For protein kinase reaction, 2 μg of MoPtc1 or MoPtc2 was mixed with MoMkk1, MoMck1 in a kinase reaction buffer (100 mM phosphate-buffered saline, pH 7.5, 10 mM MgCl₂, 1 mM ascorbic acid; Sigma-Aldrich), with the addition of 50 mM ATP (Sigma Aldrich). The subsequent experiments were performed according to the protocol (Feng et al., 2021; Jin & Gou, 2016; Yin et al., 2020; Yu et al., 2021).

4.7 | Co-immunoprecipitation assay

MoMkk1 was amplified and cloned into pXY203 by the yeast gap repair approach to generate the Stag fusion constructs. A similar approach was employed to generate the GFP fusion construct pYF11 (bleomycin resistance) for MoPtc1 and MoPtc2. The resulting fusion constructs were transformed into the Guy11 strain. Transformants expressing the fusion constructs were confirmed by western blot analysis. For co-IP assays, total proteins were isolated and incubated with the anti-GFP agarose. Proteins eluted from agarose were analysed by western blot detection with anti-Stag and anti-GFP antibodies (Abmart).

4.8 | Assays for vegetative growth, cuticle penetration, and pathogenicity

Small squares of mycelia were picked from the edge of a 4-day-old colony and placed onto a variety of media (CM, minimal medium, OM, and SDC), supplemented with or without different compounds, and cultured in the dark at 28°C. The colony diameter was measured after incubation for 7 days. All the experiments were repeated three times with three replicates each time. Mycelia cultured in liquid CM for 2 days were harvested, used to inoculate barley leaf epidermis, and incubated under humid conditions at 28°C in the dark. After incubation for 30 h, appressorium formation and the development of invasive hyphae were examined under a microscope. The pathogenicity assay was performed as described by Zhang et al. (2021). To examine the pathogenicity of the mutants without conidia, mycelia cultured in liquid CM for 2 days were harvested and inoculated on wounded rice and barley leaves and kept in the same conditions as described above for 4 days.

4.9 | Microscale thermophoresis assay

The binding of His-MoPtc2 to GST-MoMkk1, GST-MoMkk1T369A, T375A, and GST-MoMkk1T369D, T375D was determined by MST using Monolith NT.115 (Nano Temper Technologies) according to the protocol (Xu et al., 2018). The GST-MoMkk1, GST-MoMkk1T369A, T375A, and GST-MoMkk1T369D, T375D were labelled with 5-carboxy-fluorescein (FAM). A constant concentration (10 μM) of the labelled promoter in MST buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.05% Tween 20) was titrated against increasing concentrations of MoMkk1, GST-MoMkk1T369A, T375A, and GST-MoMkk1T369D, T375D dissolved in double-distilled water. A constant concentration (45 μM) of the labelled promoter in the MST buffer was titrated against His-MoPtc1 and His-MoPtc2. MST premium-coated capillaries (Monolith NT.115 MO-K005) were used to load the samples into the MST instrument at 25°C using medium MST power. Laser on and off times were set at 30 and 5 s, respectively. All experiments were performed in triplicate. Data were analysed using Nano Temper Analysis software v. 1.2.101 (Nano Temper Technologies).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher’s website.

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