INTRODUCTION

Rice blast disease, which is caused by the filamentous ascomycete fungus *Magnaporthe oryzae* (syn. *Pyricularia oryzae*), is a destructive disease that threatens crop production annually worldwide (Dean et al., 2012; Yan & Talbot, 2016). Infection by *M. oryzae* is established by the development of a specialized infection structure, the appressorium, which is differentiated from the tip of the conidial germ tube.
and regulated by the cyclic-3',5'-adenosine monophosphate/protein kinase A (cAMP/PKA) signalling pathway (Li et al., 2012). Then, high turgour pressure is generated in the appressorium due to the accumulation of osmolytes, such as glycero, which forces the penetration peg into host cells, and primary invasive hyphae grow with sequential colonization (Foster et al., 2017; Wilson & Talbot, 2009). Recent studies have shown that the formation of a functional appressorium per conidium is precisely regulated to ensure the pathogenicity of M. oryzae (Galhano et al., 2017; Zhou et al., 2014). The dominant active GTPase Ras2 causes conidia to form two appressoria and lose pathogenicity (Zhou et al., 2014). In addition, a small proportion of conidia can also form two appressoria in the deletion mutant of MoRGS1, which is a regulator of G protein signalling and plays many roles in development and pathogenicity in M. oryzae (Zhang et al., 2011). Recently, disruption of the transcription factor MoTPC1 has been shown to lead to the dysregulation of cell growth polarity and the production of two dysfunctional appressoria on one conidium (Galhano et al., 2017). To date, great progress has been made in understanding the mechanism of appressorium formation, but the regulation of the number of appressoria remains unclear.

In yeast, the WHI2 gene was initially found to play a crucial role in inhibiting cell proliferation after perceiving extracellular nutrient unavailability (Saul & Sudbery, 1985). Further studies showed that the potential functions of ScWhi2 mainly include three aspects. First, the Whi2 protein forms a complex with Psr1 and participates in the regulation of the expression of a series of stress-responsive genes by dephosphorylating the downstream translational factor Msn2 (Kaida et al., 2002). Second, ScWhi2 is involved in cAMP/PKA signalling by targeting Ras2 to vacuoles for inactivation of this signalling pathway (Leadsham et al., 2002). Second, ScWhi2 is involved in cAMP/PKA signalling by targeting Ras2 to vacuoles for inactivation of this signalling pathway (Leadsham et al., 2002). Additionally, Whi2 negatively regulates the activity of the target of rapamycin (TOR) signalling complex 1 (TORC1) under limited amino acid conditions (Chen et al., 2018). In the plant pathogen Collectotrichum orbiculare, CoWhi2 has been reported to play key roles in the regulation of the transition from biotrophic infection to necrotrophic infection via regulation of TOR signalling (Harata et al., 2016). The functions of the ScWhi2 homolog in M. oryzae, which is a model rice–pathogen interaction system, remain unclear (Valent, 1990).

The TOR kinase complex is an evolutionarily conserved protein kinase, coordinating nutrients and energy to regulate cell proliferation, the cell cycle, cell wall integrity, and cell metabolic activities by interacting with different subunits (Gonzalez & Hall, 2017; Qian et al., 2018, 2020). Recently, it has been confirmed that TOR signalling functions in controlling appressorium formation in M. oryzae. The GATA-type transcription factor Asd4, which is required for glutamine hydrolysis, promotes appressorium formation by relieving the suppression on the cAMP/PKA signalling pathway by TOR (Marroquin-Guzman & Wilson, 2015). In addition, deletion of ABL1, which encodes a subunit of AMP-activated protein kinase, inhibits TOR signalling in response to low glucose levels, resulting in cell cycle arrest at the G2/M phase, initiation of autophagy, and appressorium formation. On hydrophilic surfaces, germ tubes undergo multiple rounds of the cell cycle and cannot efficiently form appressoria due to failure in G2/M arrest and autophagic cell death (Marroquin-Guzman et al., 2017). Furthermore, a feed-forward regulatory mechanism has been reported to exist between TOR and the cAMP/PKA signalling pathway, controlling appressorium formation in M. oryzae (Sun et al., 2019). In addition, TOR has been revealed to function in maintaining the integrity of the biotrophic interface membrane and promoting invasive hyphal growth via the vacular protein Imp1 (Sun et al., 2018). Whether TOR signalling also regulates the formation of multiple appressoria is still unknown.

In this study, we characterized the function of MoWhi2 and its partner MoPsr1 in M. oryzae. Disruption of MoWhi2 and MoPsr1 displayed similar phenotypic changes, such as reduced growth, decreased conidiation, and reduced virulence. Interestingly, we found that the ΔMowhi2 and ΔMopsr1 mutants formed appressoria on a hydrophilic surface, which was probably caused by increased intracellular cAMP contents. Our results showed that inappropriate activation of TOR signalling in the ∆Mowhi2 or ∆Mopsr1 mutants caused delayed conidial autophagic cell death, which was one of the main reasons for the formation of multiple appressoria per conidium and the low infection efficiency of the rice sheath infection. Our results indicate that MoWhi2, in collaboration with MoPsr1, participates in infection-related morphogenesis and plant infection in M. oryzae.

2 | RESULTS

2.1 | MoWHI2 is required for growth, conidiation, and virulence

In our previous research, we screened the T-DNA insertion mutant library of M. oryzae and obtained a mutant that formed multiple appressoria per conidium with pathogenicity defects. By analysing the flanking sequence of the T-DNA insertion site in the mutant, it was found that T-DNA was inserted into the MGG_11241 gene, encoding a protein with a SKP1/BTB/POZ domain. Using the BLASTP program and the NCBI database, we found that the amino acid sequence encoded by MGG_11241 displayed high similarity (43%) with Saccharomyces cerevisiae Whi2, thus MGG_11241 was named MoWhi2. To elucidate the functions of MoWhi2 in M. oryzae during appressorium formation, the disruptant ΔMowhi2 mutant was generated by targeted replacement based on the principle of homologous recombination. A Southern blot assay was conducted to confirm the correct knockout and exclude random insertion (Figure S1). To ensure that the visible defects of the ΔMowhi2 mutant were due to the deletion of MoWhi2, a genomic allele of MoWhi2 was reintroduced into the null mutant ΔMowhi2 to obtain the complemented strain Mowhi2c.

To investigate the biological roles of MoWHI2 in M. oryzae, vegetative growth, conidiation, and pathogenicity were assayed by comparing wild type (WT), the ΔMowhi2 mutant, and the complementation strain Mowhi2c. As shown in Figure 1a,b, the ΔMowhi2 strain grew more slowly than the WT and Mowhi2c strains on prune agar (PA) plates after 6 days of incubation. In addition, the ΔMowhi2 mutant produced eight times fewer conidia than the WT and Mowhi2c strains (Figure 1c). To examine the effect of MoWHI2 on virulence, conidial suspensions collected from the WT, ΔMowhi2, and Mowhi2c strains were sprayed on 3-week-old susceptible rice
CO39 (Oryza sativa) seedlings. At 7 days postinoculation (dpi), a few small lesions were observed on the rice leaves inoculated with the \( \Delta Mowhi2 \) strain in comparison with those of the WT and complemented strains (Figure 1d). These data demonstrate that MoWHI2 plays vital roles in conidiation and pathogenicity in \( M. oryzae \).

### 2.2 | MoWhi2 interacts with MoPsr1, a homolog of \( S. cerevisiae \) phosphatase Psr1

To unpick the underlying mechanism of the function of MoWhi2, a yeast two-hybrid screening assay was conducted with an \( M. oryzae \) cDNA library using pGBK7-MoWhi2 as bait. A homolog protein of \( S. cerevisiae \) phosphatase Psr1 (MGG_03646) was identified as a candidate that interacted with MoWhi2. Then, a prey vector carrying the pGADT7-MoPSR1 construct was prepared to perform the yeast two-hybrid assay. The results showed that there was a positive interaction between MoPsr1 and MoWhi2 in yeast (Figure 2a). The binding of MoPsr1 to MoWhi2 was further confirmed by an in vivo coimmunoprecipitation (Co-IP) assay. In the Co-IP assay, the fusion protein MoWhi2-FLAG was detected in the protein solutions eluted from green fluorescent protein (GFP)-trap beads incubated with total protein extracts of the strain coexpressing MoWhi2-FLAG and MoPsr1-GFP but not in the protein solutions eluted from GFP-trap beads of the strain expressing MoWhi2-FLAG, further confirming that MoWhi2 interacts with MoPsr1 in vivo (Figure 2b).

To observe the site of interaction of MoWhi2 and MoPsr1 in \( M. oryzae \), a GFP-MoPsr1 fusion construct driven by its native promoter was introduced into the \( \Delta Mopsr1 \) mutant to obtain the GFP-MoPSR1 strain. Then, a MoWhi2-mCherry fusion construct was transformed into the resultant GFP-MoPSR1 strain. The localization of MoWhi2-mCherry and GFP-MoPsr1 was monitored during the mycelial growth, appressorium formation, and in planta growth stages. In the vegetative and invasive hyphae, the GFP and mCherry fluorescence signals overlapped in the cell periphery, indicating that they were located on the plasma membrane (Figure 3a). During the appressorium development...
stage, except for the cell periphery, both the GFP and mCherry fluorescent signals were also visible in the vacuole, which was confirmed by 7-amino-4-chloromethylcoumarin (CMAC) staining (Figure 3b,c). These results suggest that MoWhi2 predominantly colocalizes with MoPsr1 at the cell plasma membrane in different developmental stages. In conclusion, MoWhi2 interacts with MoPsr1 on the plasma membrane in M. oryzae during the hyphal growth and infection stages.

2.3 | MoPSR1 is important for growth, conidiation, and virulence

To investigate the biological roles of MoPSR1 in M. oryzae, the ΔMopsr1 mutant was generated and verified by Southern blot analysis (Figure S1). As shown in Figure S2, deletion of MoPSR1 caused slower vegetative growth on the PA plate, a 5-fold reduction in conidiation, and fewer lesions on the rice leaves compared with the WT strain. Reintroduction of MoPSR1 into the null mutant ΔMopsr1 restored the defects in growth, conidiation, and pathogenicity, suggesting that MoPsr1 is also crucial for conidiation and pathogenicity in M. oryzae.

2.4 | MoWhi2 and MoPsr1 are involved in appressorium formation

The rice blast fungus uses a specialized structural appressorium to infect host cells. Because of the defective pathogenicity of both the ΔMowhi2 and ΔMopsr1 strains, we assumed that appressorium formation and penetration might be affected. To verify this hypothesis, the conidia of the WT, ΔMowhi2, and ΔMopsr1 strains were inoculated on hydrophobic coverslips that mimicked the inductive surface characteristics during the appressorial assays in vitro. In the WT strain, the conidium usually formed one appressorium on the hydrophobic surface (Figure 4a). In contrast to the WT strain, more than one appressorium was formed on the hydrophobic surface in a large number of conidia from the ΔMowhi2 (56.2 ± 3.5%) and ΔMopsr1 (50.6 ± 1.8%) mutants (Figure 4b). In addition, appressoria formation on a hydrophilic surface was also examined. Conidia of the ΔMowhi2 or ΔMopsr1 mutants formed appressoria at higher proportions of nearly 20% and 47%, respectively, while a few (approximately 1.3%) conidia of the WT strain formed appressoria on the hydrophilic surface (Figure 4c,d). Abnormal appressorium formation was restored in the complemented strains Mowhi2c and Mopsr1c on either the hydrophobic or hydrophilic surface, suggesting that MoWhi2 and MoPsr1 are involved in regulating appressorium formation in M. oryzae.

The cAMP/PKA signalling pathway regulates surface recognition and appressorium morphogenesis in M. oryzae (Mitchell & Dean, 1995). It has been reported that hyperactivation of the cAMP/PKA signalling pathway results in appressorium formation on a hydrophilic surface (Liu et al., 2007; Ramanujam & Naqvi, 2010). To determine whether MoWhi2 and MoPsr1 were involved in cAMP/PKA signalling, the cAMP content was measured in the WT, ΔMowhi2, ΔMopsr1, Mowhi2c, and Mopsr1c strains. In comparison to the WT strain, the ΔMowhi2 and ΔMopsr1 strains had increased cAMP levels. cAMP accumulation was restored to the WT level in the complemented strains Mowhi2c and Mopsr1c on either the hydrophobic or hydrophilic surface, suggesting that MoWhi2 and MoPsr1 are also crucial in the negative regulation of cAMP accumulation.

2.5 | Disruption of MoWhi2 and MoPsr1 results in defects in appressorium-mediated penetration and invasive growth

To further explore whether MoWhi2 and MoPsr1 are involved in appressorium-mediated penetration, the infection process was observed by inoculating conidia of the WT, ΔMowhi2, and ΔMopsr1 strains on rice sheaths. At 24 hr postinoculation (hpi), similar to the artificial hydrophobic surface, conidia of the ΔMowhi2 and ΔMopsr1
strains differentiated more than one appressorium, while the WT conidia formed only one appressorium (Figure 5a). Beneath the appressorium, callose deposition was induced by pathogen-associated molecular patterns, such as chitosan, in the invaded cell, which could be used to quantify appressorium-mediated penetration (Sun et al., 2006). By aniline blue staining we found that fewer callose deposits formed in the ΔMowhi2 or ΔMopsr1 mutants than in the WT strain and the complemented strains Mowhi2c and Mopsr1c (Figure 5b,c).
FIGURE 4 MoWhi2 and MoPsr1 regulate appressorium formation in *Magnaporthe oryzae*. (a) Conidial suspensions of the wild-type (WT), ΔMowhi2, ΔMopsr1, and complemented strains Mowhi2c and Mopsr1c were inoculated on hydrophobic coverslips. Micrographs displayed appressorium formation at 24 hr postinoculation (hpi). Scale bar = 10 μm. (b) The percentage of conidia forming appressorium in the WT and derivative strains. (c) Conidial suspensions of the WT, ΔMowhi2, ΔMopsr1, Mowhi2c, and Mopsr1c strains were inoculated on the hydrophilic surface of GelBond coverslips and photographed at 24 hpi. Scale bar = 12 μm. (d) Bar charts show the percentage of conidia forming appressorium on the hydrophilic surface. **Significant at p < 0.005 and *p < 0.01. (e) cAMP content was increased in the ΔMopsr1 and ΔMowhi2 strains. Mean and SD were calculated from three biological replicates. Significant at **p < 0.005 and *p < 0.01
MoWhi2 and Mo Psr1 are required for appressorium-mediated penetration and invasive growth. (a) Monitoring of appressorium formation on the rice sheath. Conidial suspensions were dropped on the rice sheath surface and appressorium formation was observed at 24 hr postinoculation (hpi). Bar = 5 µm. (b) Papillary callose deposits were visualized by staining with aniline blue at 36 hpi. (c) Numbers of papillary callose deposits formed underneath appressoria were counted and presented with mean ± SD. (d) Rice sheath infection assay. Conidial suspensions were inoculated on the excised rice sheath and invasive growth in rice cells was observed at 36 hpi. Infectious hyphae were divided into four types: no penetration (Type 1), primary infectious hyphae (Type 2), secondary infectious hyphae (Type 3), and infectious hyphae extending to neighbouring cells (Type 4). Statistical analysis was performed and the results are presented as means and SD. Bar = 10 µm.
indicating that MoWhi2 and MoPsr1 play important roles in forming functional appressoria to penetrate host cells. The invasive hyphae were also examined at 36 hpi. In contrast to approximately 20% of WT cells showing type 4 infectious hyphal growth in which infection expanded into neighbouring cells, most invasive cells of the ΔMowhi2 or ΔMopsr1 strains (approximately 45% and 45%) were type 2 and
strains were degraded, similar to those in the RFP-ATG8 Mowhi2Δtophagosomes in the conidia from the above three strains (Figure 7). At 24 hpi, the number of fluorescent dots decreased and almost completely degraded in the RFP-ATG8 strain. However, certain autophagosomes remained in the conidia of the ∆Mowhi2(RFP-ATG8) and ∆Mopsr1(RFP-ATG8) strains at 24 hpi, even extending to 30 hpi (Figure 7), indicating that the autophagic process during appressorium formation was prolonged when MoWHI2 and MoPSR1 were disrupted.

2.6 | Conidial nuclear degradation is retarded in the ∆Mowhi2 and ∆Mopsr1 strains

During appressorium formation of M. oryzae, the three-celled conidium undergoes one round of mitosis, one newly formed nucleus is transferred into the appressorium, and three nuclei are degraded in the conidium (Oses-Ruiz & Talbot, 2017). To determine whether the abnormalities in appressorium formation in the ∆Mowhi2 and ∆Mopsr1 strains were associated with the behaviour of nuclei, green fluorescent protein (GFP) was fused with histone H1 to visualize nuclei in M. oryzae. In the WT strain, most conidia (approximately 83%) contained three nuclei and underwent one round of mitosis to form the fourth nucleus, which was then transferred into the appressorium. At 24 hpi, nuclei in the WT conidia were degraded and only one nucleus was left in the appressorium (Figure 6a,b). In contrast, most conidia (approximately 79%) in the ∆Mowhi2 or ∆Mopsr1 mutants initially had two nuclei due to defects in conidal morphogenesis (Figure S3). In the ∆Mowhi2 or ∆Mopsr1 mutants, conidial nuclear degradation was significantly impaired after one round of mitosis in the apical conidial cells and one round of mitosis occurred, resulting in approximately 60% of conidia containing four nuclei. Even at 48 hpi, more than 20% of conidia contained two or three nuclei in the ∆Mowhi2 or ∆Mopsr1 mutants (Figure 6c,d). These data suggest that MoWHI2 and MoPSR1 are involved in regulating conidial nuclear degradation during appressorium formation.

2.7 | Autophagic cell death in conidia is prolonged in the ∆Mowhi2 and ∆Mopsr1 mutants

Autophagic conidial cell death is crucial for appressorium development in M. oryzae (Liu et al., 2012; Veneault-Fourrey et al., 2006). To determine whether the delay in conidial nuclear degradation in the ∆Mowhi2 and ∆Mopsr1 strains was caused by defects in autophagic conidial cell death, the strains ∆Mowhi2(RFP-ATG8) and ∆Mopsr1(RFP-ATG8) were constructed by disrupting MoWHI2 and MoPSR1, respectively, in the RFP-ATG8 strain that was produced previously (Deng et al., 2009). Conidial suspensions of the RFP-ATG8, ∆Mowhi2(RFP-ATG8), and ∆Mopsr1(RFP-ATG8) strains were inoculated on artificial hydrophobic surfaces to observe autophagosomes under a fluorescence microscope. The numbers of fluorescent dots representing autophagosomes in the conidia from the RFP-ATG8, ∆Mowhi2(RFP-ATG8), and ∆Mopsr1(RFP-ATG8) strains were not obviously different at 0 and 8 hpi. Additionally, RFP-Atg8-labelled autophagosomes in the ∆Mowhi2(RFP-ATG8) and ∆Mopsr1(RFP-ATG8) strains were degraded, similar to those in the RFP-ATG8 strain, suggesting that the autophagy initiation process was also normal in the above three strains (Figure 7). At 24 hpi, the number of fluorescent dots decreased and almost completely degraded in the RFP-ATG8 strain. However, certain autophagosomes remained in the conidia of the ∆Mowhi2(RFP-ATG8) and ∆Mopsr1(RFP-ATG8) strains at 24 hpi, even extending to 30 hpi (Figure 7), indicating that the autophagic process during appressorium formation was prolonged when MoWHI2 and MoPSR1 were disrupted.

2.8 | Disruption of MoWHI2 and MoPSR1 causes inappropriate activation of MoTor

Because the conidial autophagy process in the ∆Mowhi2 and ∆Mopsr1 was prolonged and TOR signalling plays important roles in the negative regulation of autophagy (He et al., 2018; Marroquin-Guzman et al., 2017), we postulated that MoTor was inappropriatel activated in the ∆Mowhi2 and ∆Mopsr1 strains. Initially, the rapamycin (an inhibitor of TOR kinase) sensitivity assay showed that both the ∆Mowhi2 and ∆Mopsr1 strains were more sensitive to rapamycin than WT, indicating that MoWHI2-MoPSR1 is related to MoTOR signalling (Figure S4). Then, to determine whether the defects in appressorium formation in the ∆Mowhi2 and ∆Mopsr1 strains were associated with MoTOR signalling, appressorium formation was observed by adding rapamycin to conidial drops of the WT, ∆Mowhi2, and ∆Mopsr1 strains. It was found that most conidia of the ∆Mowhi2 and ∆Mopsr1 strains formed one appressorium, similar to the WT strain after rapamycin treatment, indicating that suppressing MoTor activity could partially restore the defects in the ∆Mowhi2 and ∆Mopsr1 strains (Figure 8a,b). Therefore, we postulated that rapamycin could inhibit MoTor activity, leading to the suppression of autophagy, and consequently, the suppression of conidial nuclear degradation. Furthermore, upstream of MoTor, the expression levels of MoRS3 were examined by quantitative reverse transcription PCR (RT-qPCR) (Qian et al., 2020). The results showed that MoRS3 expression levels were up-regulated in the ∆Mowhi2 and ∆Mopsr1 strains compared with those in the WT strain (Figure 8c,d), suggesting that the transcriptional level and enzyme activity were up-regulated after knockout of MoWHI2 and MoPSR1. In conclusion, these data suggest that inappropriate activation of MoTOR signalling is one of the main reasons for the formation of multiple appressoria per conidium in the ∆Mowhi2 or ∆Mopsr1 mutants.

3 | DISCUSSION

The appressorium is a specialized infection structure of many plant-pathogenic fungi (Ryder & Talbot, 2015). In this study, we identified a gene MoWHI2 that regulates appressorium differentiation in M. oryzae. Conidia of the ∆Mowhi2 strain formed multiple appressoria but the pathogenicity was significantly reduced. Further assays revealed that MoWHI2 interacts with MoPSR1, participating in the regulation of appressorium formation and the pathogenic process by regulation of the cAMP levels and the TOR signalling pathway.
Previous studies have demonstrated that the Whi2–Psr1 complex is an important regulator of growth and development. In yeast, the ScWhi2 protein forms a complex with plasma membrane-localized phosphatase Psr1 and participates in growth and stress responses (Kaida et al., 2002). In Colletotrichum orbiculare, CoWhi2 interacts with CoPsr1 and is involved in the biotrophic infection stage (Harata et al., 2016). Systematic analysis of phosphatases in the wheat head blight pathogen Fusarium graminearum and human opportunistic fungal pathogen Cryptococcus neoformans revealed that Psr1 homologs are involved in infection-related development and virulence (Jin et al., 2020; Yun et al., 2015). In our study, we found that MoWhi2 colocalized with MoPsr1 on the plasma membrane and functioned together during appressorium development and invasive growth. In addition to the above conserved roles among Whi2 homologs, there exists functional divergence between ScWhi2 and MoWhi2. Although ScWHI2 showed a similar sequence to MoWHI2, ScWHI2 did not fully complement the defects of ΔMowhi2 except for increasing mycelial pigmentation (Figure S5). In addition, ScWHI2 is required for responding to general stresses, while the correlation between MoWHI2 and stress is not obvious (Figure S6), which also indicates that there are differences in the functions of these two genes. Similarly, in C. orbiculare, CoWHI2 is also not involved in the cell wall stress response (Harata et al., 2016). These results suggest that Whi2 and Psr1 homologs are important factors required for development and virulence in pathogenic filamentous fungi and have functional divergences from yeast homologs.

Under suitable conditions, one cell of the three-celled rice blast fungus conidium germinates and undergoes one round of mitosis to activate the formation of a single appressorium on an inductive surface rather than on a hydrophilic surface (Oses-Ruiz & Talbot, 2017). Here, we found that the conidium of the ΔMowhi2 and ΔMopsr1 strains formed an appressorium on a hydrophilic surface, which was
probably related to the increased cAMP level (Zhou et al., 2014). In addition, nearly half of the conidia of the ΔMowhi2 and ΔMopsr1 strains formed multiple appressoria on the artificial hydrophobic surface and host cell surface. Although the ΔMowhi2 and ΔMopsr1 strains formed more appressoria after inoculation on the surface of rice, the ability of appressoria to penetrate rice cells was significantly reduced, suggesting that MoWhi2 and MoPsr1 are critical for appressorium function during plant infection. These malformations of multiple appressoria might not be caused directly by the increased cAMP level in the ΔMowhi2 and ΔMopsr1 mutants because exogenous cAMP did not induce the formation of multiple appressoria in the WT strain (Choi & Dean, 1997; Zhou et al., 2012).

The TOR signalling is a well-known negative regulator of the autophagy process, which is inactivated during appressorium formation in M. oryzae (Marroquin-Guzman & Wilson, 2015). Recent reports have further revealed that G2 arrest is necessary for autophagy induction and appressorium formation in M. oryzae (Marroquin-Guzman et al., 2017). In S. cerevisiae and Podospora
anopheles. WHI2 and PSR1 orthologs act as conserved negative regulators of TOR and play important roles in regulating the entrance into the stationary phase when external nutrients are exhausted (Timpano et al., 2016). Deletion of Whi2 or Psr1 homologs will cause a failure to halt cell growth and lead to continued cell division (Radiciflffe et al., 1997). According to our further study, some evidence showed that MoWhi2- and MoPsr1-mediated suppression of Tor activity is mainly responsible for the formation of multiple appressoria per conidium. First, the conidium of the ΔMowhi2 and ΔMopsr1 strains formed only a single appressorium after treatment with the Tor inhibitor rapamycin. Second, the expression of MoTOR and MoRS3, a marker gene of MoTor activity, increased in the ΔMowhi2 and ΔMopsr1 strains, indicating that MoTor activity increased after deletion of MoWHI2 or MoPSR1. Third, autophagy-mediated nuclear degradation in the ΔMowhi2 and ΔMopsr1 strains was delayed. It is possible that, similar to CoWhi2 and ScWhi2, MoWhi2 and MoPsr1 might be negative regulators of the MoTor signalling pathway in M. oryzae.

Activated MoTor signalling plays an important role in the suppression of cAMP/PKA signalling, which leads to germ tube growth, multiple rounds of mitosis, and no appressorium formation on a hydrophilic surface in M. oryzae (Sun et al., 2019). Consistently, inappropriate TOR activation disrupts the progression of cell division and results in multiple rounds of mitosis in the ΔMowhi2 or ΔMopsr1 strains. In addition, deletion of MoWHI2 or MoPSR1 also leads to an increase in cAMP content, indicating that other signalling pathways may be involved in controlling the cAMP/PKA signalling pathway to induce appressorium formation, alleviating suppression from the activated MoTor signalling pathway. How MoTor signalling, cAMP/PKA signalling, and the conidial cell cycle are tightly intertwined during appressorium formation needs to be investigated further. Overall, this evidence implicates the pivotal roles of Whi2 and Psr1 in controlling fungal cell proliferation and differentiation by integrating the MoTor and cAMP/PKA signalling pathways.

4 | EXPERIMENTAL PROCEDURES

4.1 | Fungal strains and culture conditions

The M. oryzae strain B157 was used as WT and genetic background for deletion of MoWHI2 and MoPSR1. Both the WT strain and derivative strains were cultured on prune agar (PA) for quantitative analysis of growth and conidiation. Mycelial plugs were inoculated at the centre of PA plates for 2 days in darkness and then treated with constant light for 5 days before counting the conidia of each colony (Kou et al., 2017). To test the sensitivity to stresses, mycelial plugs of WT and ΔMowhi2 were inoculated on complete medium (CM) plates amended with 0.005% sodium dodecyl sulphate (SDS), 1 M sorbitol, 2 mM H2O2, and 250 μM menadione. Genomic DNA and total RNA were extracted from mycelia cultured in liquid CM.

4.2 | Yeast two-hybridization assay

A bait construct MoPsr1BD was generated by cloning the MoPSR1 coding sequence with primers Psr1-pGBKTK7F/Psr1-pGBKTK7R (Table S1) and ligated into EcoRI/BamHI-digested pGBKTK7. The MoWHI2 coding region was cloned and ligated into EcoRI/BamHI-digested pGADT7 as a prey construct. After sequencing, a pair of bait and prey vectors were cotransformed into the yeast AH109 strain according to the Matchmaker Gold Yeast Two-Hybrid System User Manual (Clontech). Yeast colonies growing on SD–Trp/Leu plate were diluted to SD–Trp/Leu/Ade/His plate to further determine the binding ability. The pair of pGBKTK7-p53 and pGADT7-T was used as a positive control, and the pair of pGBKTK7-lam and pGADT7-T was a negative control.

4.3 | Coimmunoprecipitation assay

A construct expressing MoWhi2 labelled with 3×FLAG was introduced into the GFP-MoPSR1 expressing strains and the WT strain, respectively. Total proteins were extracted from mycelia of the resultant coexpressing GFP-MoPSR1 and MoWHI2-3×FLAG strains and the MoWHI2-3×FLAG-expressing strain with protein extraction buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail). After centrifugation at 10,000 g for 10 min, the supernatant solutions of the lysates were incubated with GFP-trap magnetic beads (Chromtek, gta-20) at 4 °C for 5 hr. Total proteins and eluted proteins were resolved in 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylpyrrolidone (PVDF) membrane, which was detected by western blot with primary antibodies including anti-GFP antibody (HUABIO) and anti-FLAG antibody (Sigma), and secondary antibodies (A0208; Beyotime). The pictures were collected with a Bio-Rad imaging system using a Clarity western chemiluminescence kit (Bio-Rad).

4.4 | Knockout and complementation of MoWHI2 and MoPSR1

To construct MoWHI2 knockout vector, the 5′ untranslated region (UTR) and the 3′ UTR of MoWHI2 were cloned with primers Whi2-5F/5R and Whi2-3F/3R (Table S1), then ligated into PstI/HindIII and EcoRI/KpnI sites of pFGL821 (Addgene 58223) with a hygromycin resistance gene, respectively. The resultant knockout vector pFGL821-koMoWHI2 was transformed into the WT strain by Agrobacterium-mediated transformation (ATMT). The candidate disruptants ΔMowhi2 were verified with PCR assay and further Southern blot assay with a DIG-High Prime DNA Labeling and Detection Starter Kit (Roche). To generate the complemented strains Mowhi2c, a genomic gene sequence driven by the MoWHI2 native promoter was amplified and ligated into Xmal/HindIII-digested pFGL822 (Addgene 58225) with a glufosinate ammonium resistance gene. The complemented construct was transformed into the ΔMowhi2 strain. To complement the ΔMowhi2 strain with ScWHI2,
ScWHI2 was cloned with primers Whi2-γf/r driven by the ScWHI2 native promoter amplified with primers Whi2-γSf/Sr and introduced into the ΔMowhi2 strain. We used the same principle to construct a knockout vector of MoPSR1 pFGL820 (Addgene 58221)-koMoPSR1 and obtained the corresponding knockout mutant ΔMopsr1 and complemented strains Mopsr1c. The primers used in this study are listed in Table S1.

4.5 | Fluorescent microscopic observation

To construct the GFP-MoPSR1 fusion expression vector, its native promoter and open reading frame (ORF) was cloned with primers and ligated into the EcoRI/Kpn1 site and the BamHI/XbaI site of pFGL820-GFP, respectively. The resulting GFP-MoPSR1 vector was introduced into the WT strain via ATMT. To observe colocalization of MoWhi2 with MoPSR1, the genomic ORF of MoWHI2 driven by its native promoter was amplified with primers MoWhi2-mcF/mcR and ligated into PsiI/HindIII-digested pFGL822-mCherry. The resultant MoWhi2-mCherry construct was transformed into the GFP-MoPSR1 expressing strain to produce coexpression transformants. Conidia suspension (5 × 10^4/ml) was inoculated on the hydrophobic plastic coverslip to induce appressorium formation.

To observe the conidial autophagic process, the knockout vector of MoWHI2 or MoPSR1 was introduced into an RFP-ATG8 strain and a H1-GFP strain to produce ΔMowhi2 (RFP-ATG8), ΔMopsr1 (RFP-ATG8) strains, ΔMowhi2 (H1-GFP), and ΔMopsr1 (H1-GFP) strains, respectively. GFP and red fluorescent protein (RFP) signals were monitored during appressorium development at indicated time points of incubation on hydrophobic coverslips.

The fluorescence was captured by confocal fluorescent LSM700 microscope (Zeiss) as previously described. GFP, RFP, and CMAC were imaged with 488, 543, and 405 nm laser excitation, respectively. Images were processed with ImageJ and Adobe Illustrator CS6 software. To observe conidial morphology, conidia collected from WT were imaged with 488, 543, and 405 nm laser excitation, respectively.

4.6 | Appressorium formation

Conidial suspensions (5 × 10^4/ml) collected from 7-day-old culture plates of the WT strain, ΔMowhi2, ΔMopsr1, and the complemented strains Mowhi2c and Mopsr1c were applied on hydrophobic coverslips or hydrophilic coverslips (Gelbond) and incubated under humid and dark condition at 25 °C. Ratios of appressorium formation were counted at 24 hpi. Rapamycin (1 μg/ml) was supplemented into conidial suspensions of the WT strain, ΔMowhi2, and ΔMopsr1 to test effects of MoTor suppression on appressorium formation. The state of appressorium formation was pictured under a bright field using a BX53 microscope (Olympus).

4.7 | cAMP content measurement

Mycelia cultured in CM for 3 days were collected for cAMP content measurement as previously described (Shi et al., 2016) with an anti-cAMP antibody based direct cAMP ELISA kit (NewEast Biosciences).

4.8 | Rice seedling and rice sheath infection assay

To test pathogenicity changes, 2 ml of conidial suspension (10^5/ml) harvested from the WT, ΔMowhi2, ΔMopsr1, and the relevant complemented strains Mowhi2c and Mopsr1c was sprayed on 2-week-old rice seedlings (CO39). To observe appressorium formation and penetration on the host surface, conidial drops were inoculated on the rice sheath. Penetration pegs were observed by staining papillary callose deposits with aniline blue (Sigma) at 36 hpi. Four types of infection were counted at 36 hpi with a BX53 microscope (Olympus).

4.9 | RT-qPCR assay

Total RNA was extracted by RNAiso (TaKaRa) from mycelia of WT, ΔMowhi2, and ΔMopsr1 cultured in liquid CM. cDNA was synthesized with a PrimeScript RT reagent kit (TaKaRa). qPCR was performed with a CFX connect real-time system (Bio-Rad) using TB Green Premix Ex Taq (TaKaRa). The β-tubulin gene was used as the reference gene and the expression levels of MoTOR and MoRS3 were calculated by the 2^{-ΔΔCt} method (Livak & Schmittgen, 2001). The primers used are listed in Table S1.

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CONFLICT OF INTEREST

There is no conflict of interest.

AUTHOR CONTRIBUTIONS

Y.K. planned and designed the research. H.S., S.M., J.Q., C.W., and Y.S. performed experiments and analysed data. Y.K. and H.S. wrote the manuscript.

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 online in the Supporting Information section.

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