

## ORIGINAL ARTICLE

# The MoPah1 phosphatidate phosphatase is involved in lipid metabolism, development, and pathogenesis in *Magnaporthe oryzae*

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## Funding information

National Natural Science Foundation of China, Grant/Award Number: 32172373 and 31801723; Fundamental Research Funds for the Central Universities, Grant/Award Number: 2662020ZKPY006

## Abstract

As with the majority of the hemibiotrophic fungal pathogens, the rice blast fungus *Magnaporthe oryzae* uses highly specialized infection structures called appressoria for plant penetration. Appressoria differentiated from germ tubes rely on enormous turgor pressure to directly penetrate the plant cell, in which process lipid metabolism plays a critical role. In this study, we characterized the *MoPAH1* gene in *M. oryzae*, encoding a putative highly conserved phosphatidate phosphatase. The expression of *MoPAH1* was up-regulated during plant infection. The MoPah1 protein is expressed at all developmental and infection stages, and is localized to the cytoplasm. Disruption of *MoPAH1* causes pleiotropic defects in vegetative growth, sporulation, and heat tolerance. The lipid profile is significantly altered in the *Mopah1* mutant. Lipidomics assays showed that the level of phosphatidic acid (PA) was increased in the mutant, which had reduced levels of diacylglycerol and triacylglycerol. Using a PA biosensor, we showed that the increased level of PA in the *Mopah1* mutant was primarily accumulated in the vacuole. The *Mopah1* mutant was blocked in both conidiation and the formation of appressorium-like structures at hyphal tips. It was nonpathogenic and failed to cause any blast lesions on rice and barley seedlings. RNA sequencing analysis revealed that MoPah1 regulates the expression of transcription factors critical for various developmental and infection-related processes. The *Mopah1* mutant was reduced in the expression and phosphorylation of Pmk1 MAP kinase and delayed in autophagy. Our study demonstrates that MoPah1 is necessary for lipid metabolism, fungal development, and pathogenicity in *M. oryzae*.

## KEYWORDS

appressorium formation, autophagy, lipid metabolism, *Pyricularia oryzae*, virulence

Juan Zhao and Peng Sun contributed equally to this work.

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## 1 | INTRODUCTION

*Magnaporthe oryzae* is the causative agent of the rice blast disease that causes annual losses that are sufficient to feed more than 60 million people worldwide, seriously threatening global food security (Dean et al., 2012; Fernandez & Orth, 2018). As a typical filamentous ascomycete, *M. oryzae* uses infection-specific structures called appressoria for plant infection. Because of the critical role of appressoria in plant penetration, appressorium formation has been extensively studied (Kong et al., 2013; Li et al., 2012). Similar structures called appressorium-like structures (ALSs), which differentiate from hyphal tips, can also penetrate cell walls (Kong et al., 2013). The process of appressorium or ALS formation is coordinately regulated by multiple signalling pathways, including the cyclic AMP-protein kinase A (cAMP-PKA), and the Pmk1 and Mps1 mitogen-activated protein kinase (MAPK) pathways, with Pmk1 playing the most critical role (Kronstad et al., 1998; Zhang et al., 2021). Additionally, autophagy regulates nutrition recycling and is involved in virulence in *M. oryzae*. Multiple mutants that lack key autophagy components are nonpathogenic (He et al., 2013; Kershaw & Talbot, 2009).

Enormous turgor pressure is generated in the appressorium from accumulating glycerol and facilitates the penetration peg to directly puncture the plant cell wall (Howard et al., 1991; Wang et al., 2007). The above process is energy-consuming, and glycogen stored in conidia is rapidly metabolized to generate sufficient energy during germination (Thines et al., 2000). Mutants lacking glycogen metabolic genes *AGL1* or *GPH1* are able to form appressoria but are reduced in virulence on rice (Badaruddin et al., 2013). Additionally, many enzymes, including Gpd1, Dak1, Had1, Gut1, Gpp1, and Gfd1, are involved in glycerol metabolism. Gpd1, a cytosolic form of glycerol-3-phosphate dehydrogenase, is important for glycerol accumulation in the appressorium (Foster et al., 2017). Disruption of *GPD1* or *GPD2* leads to reduced fungal virulence on rice (Shi et al., 2018). Metabolites related to glycerol metabolism including phospholipids could be important for fungal infection.

Phospholipids, as amphipathic molecules, are major components of the cell membrane and play a critical role in various biological processes (Xie, 2019). In *M. oryzae*, sphingolipid is a type of phospholipid required for appressorium formation. MoLag1 is involved in the biosynthesis of the sphingolipid of ceramide, and deletion of *MoLAG1* causes a severe reduction in conidiation and virulence. This shows that ceramide is important for fungal pathogenicity (Liu, Yun, et al., 2019a; Liu, Liang, et al., 2019b). *MoLPPs* also encode a type of phosphatidate phosphatases (PAPs), and *Molpp3* and *Molpp5* mutants display reductions in appressorium formation and virulence that can be restored by exogenous supplementation of diacylglycerol (Skamnioti & Gurr, 2007). Because of the critical role of phospholipids in *M. oryzae*, key enzymes involved in phospholipid biosynthesis are used as targets of rice blast fungicides. The widely used fungicides phosphorothiolates and isoprothiolane inhibit the transmethylation step of choline biosynthesis (Uesugi, 2001). The impact of phospholipid biosynthesis on virulence has also been reported in the human pathogen *Candida albicans*, and phosphatidylserine (PS)

and phosphatidylethanolamine (PE) are important for pathogenesis (Mu et al., 2019). Deletion of *CHO1* causes the loss of PE biosynthesis from PS and in turn attenuates virulence on mice in *C. albicans* (Cassilly & Reynolds, 2018). Therefore, investigating the genes involved in phospholipid biosynthesis is necessary for understanding the mechanism of fungal virulence and providing potential targets for novel fungicides.

PAPs catalyse the  $Mg^{2+}$ -dependent dephosphorylation of phosphatidic acid (PA) to synthesize diacylglycerol (DAG) (Adeyo et al., 2011). Four PAPs, Dpp1, Lpp1, App1, and Pah1, have been elucidated in *Saccharomyces cerevisiae* (Chae & Carman, 2013; Han et al., 2008; Pascual & Carman, 2013). Furthermore, only four out of eight PAPs have been characterized in *M. oryzae*, and disruption of *MoLPP3* or *MoLPP5* results in reduced virulence on rice (Sadat et al., 2014). In *S. cerevisiae*, the *pah1* mutant grows slowly and is hypersensitive to high temperature (Park et al., 2015). Pah1 is involved in the regulation of autophagy via the Nem1/Spa7-Pah1 complex (Rahman et al., 2018). The phosphorylation of Pah1 is regulated by multiple protein kinases in *S. cerevisiae* (Karanasios et al., 2010).

In this study, we characterized *MoPAH1* in *M. oryzae*. A *MoPAH1* deletion mutant was severely defective in vegetative growth, ALS formation, and pathogenicity. RNA sequencing (RNA-Seq) analysis supported this impact of *MoPAH1* deletion on fungal development and pathogenicity. The mutant was hypersensitive to the cell wall stressor Congo red and heat stress, and lipid profiles were widely altered. The PA biosensor indicated that PA is distributed in the hyphae and showed that PA accumulated in the vacuoles of the mutant. Additionally, Pmk1 activation and autophagy were attenuated in the *Mopah1* mutant. Overall, the evidence indicates that *MoPah1* plays pivotal roles in hyphal growth, conidiation, ALS formation, and pathogenesis in *M. oryzae*.

## 2 | RESULTS

### 2.1 | Characterization and analysis of MoPah1 in *M. oryzae*

To characterize the *MoPah1* in *M. oryzae*, the yeast Pah1 protein sequence was used to search the *M. oryzae* genome database (Howe et al., 2020) and only one homolog was obtained, named *MoPah1* (MGG\_01311). *MoPah1* contains two protein domains, LNS2 and Lipin\_N (Figure S1) (Irie et al., 1993), whereas Pah1 homologs from rice and *Arabidopsis* contain an extra protein domain, Lipin\_mid (Han et al., 2012). Phylogenetic analysis shows that Pah1 is highly conserved, and *MoPah1* has a close relationship with Pah1 homologs from filamentous fungi *Neurospora crassa* and *Fusarium graminearum*. The *MoPah1* protein shows a 70% identity with those of *N. crassa* and *F. graminearum*.

High-quality transcriptomes of *M. oryzae* during the infection process (Jeon et al., 2020) indicated that expression of PAP genes increases in infected rice tissues, with *MoPAH1* the highest (Figure S2), suggesting that *MoPAH1* is involved in plant infection.

## 2.2 | The *Mopah1* mutant is defective in hyphal growth and conidiation

To investigate the role of the *MoPAH1* gene in *M. oryzae*, we deleted *MoPAH1* (Figure S3a). A screen of 120 transformants by PCR identified two *Mopah1* mutants, M21 and M22. The *Mopah1* mutant M21 was used for further analyses (Table 1 and Figure S3b).

Strains were grown on complete medium (CM), oatmeal medium (OMA), and minimal medium (MM) plates. The growth rate of the *Mopah1* mutant was reduced up to 83% on OMA compared to the wild type (WT) and complementation strains (Figure 1a,b). A cross-section of colonies on CM plates revealed that mycelia of the *Mopah1* mutant grew into the solid medium under the inoculation plug (Figure 1g). Measurements of Calcofluor white (CFW)-stained hyphae indicated that the average cell length of apical hyphal cells of the *Mopah1* mutant was 33% shorter compared to the WT and complementation strains (Figure 1d,e). The aerial hyphae of the *Mopah1* mutant also appeared compact compared to the WT (Figure 1h). These results indicate that *MoPAH1* is involved in vegetative growth.

Microscopic observations showed that conidiophores of the *Mopah1* mutant failed to produce conidia (Figure 1c,f). Conidiation in the complemented strain was similar to the WT. An analysis of the expression of conidiation-related genes *COM1*, *CON1*, *CON2*, *CON6*, *CON7*, *COS1*, and *HTF1* indicated a significant reduction in mutant M21 compared to WT (Figure 1i) (Liu et al., 2010; Shi et al., 1998; Shi & Leung, 1995; Yang et al., 2010). These results indicated that *MoPAH1* is required for conidiation in *M. oryzae*.

## 2.3 | Deletion of *MoPAH1* alters the lipid profile

*MoPAH1* encodes a putative PAP. To assay the function of *MoPAH1* in vivo, we examined the lipid profile of the *Mopah1* mutant by two-dimensional thin layer chromatography (2D-TLC) and high-performance liquid chromatography/mass spectrometry (HPLC-MS). The 2D-TLC analysis showed that the lipid profile was altered in the *Mopah1* mutant compared with the WT strain (Figure 2a). The lipidomics results using HPLC-MS showed that the PA level in the *Mopah1* mutant was two-fold higher than in the WT strain. In addition, phosphatidylcholine (PC), phosphatidylethanolamine (PE),

phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS) were increased 234%, 93%, 18%, 71%, and 150%, respectively, in the *Mopah1* mutant. In contrast, the DAG and triacylglycerol (TAG) content were reduced by 64% and 57%, respectively, in the *Mopah1* mutant in comparison to the WT strain (Figure 2b). These results demonstrate that deletion of *MoPAH1* widely alters the lipid metabolism in *M. oryzae*.

## 2.4 | Disruption of *MoPAH1* alters PA distribution

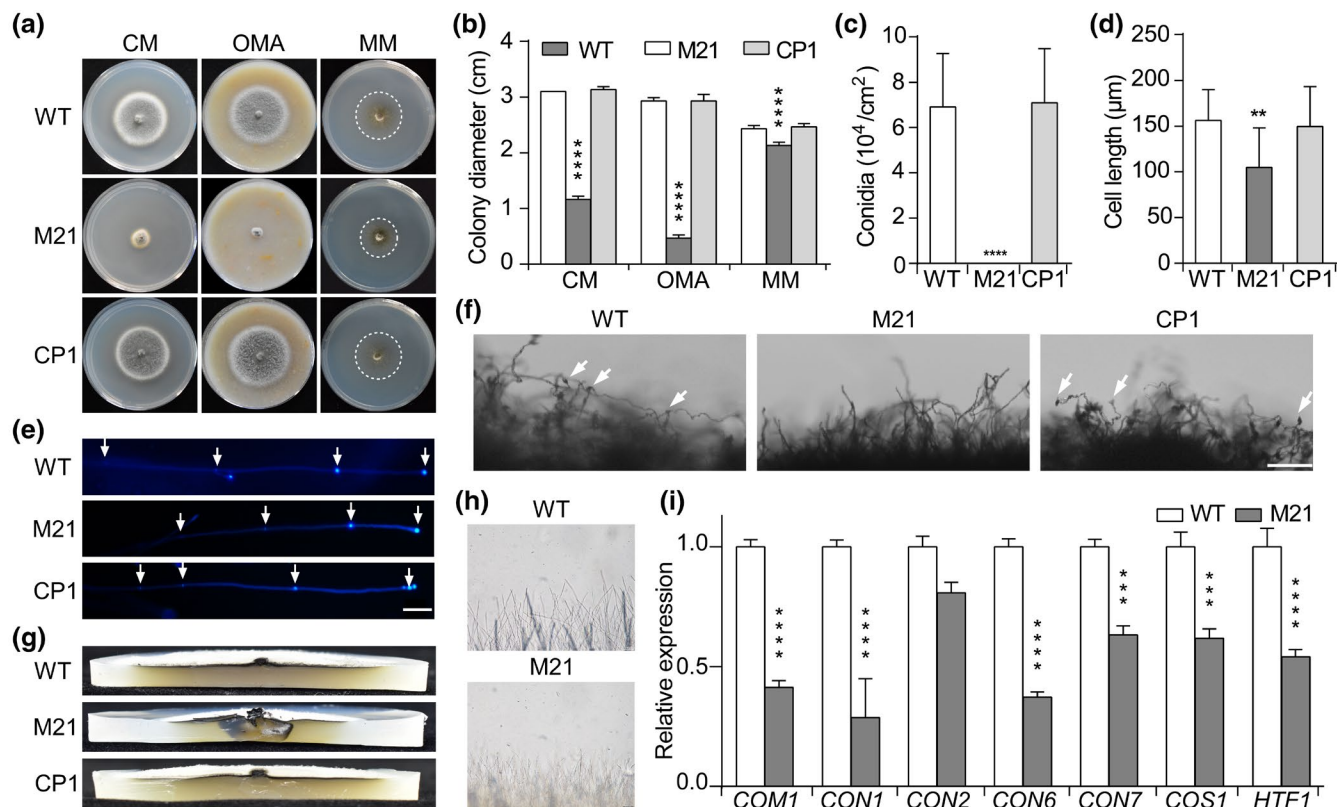
The yeast Opi1 protein (ScOpi1) binds to PA, and its binding domain has been extensively used as a marker for cellular PA (Ganesan et al., 2015). To study the subcellular PA, we fused the PA-binding domain of ScOpi1 with red fluorescent protein (RFP) and transformed the construct into the WT and *Mopah1* mutant strains. The ScOpi1-RFP protein was detected by western blotting in both the transformed WT (OPW3) and *Mopah1* mutant (OPM5) strains (Figure 3b). The protein was not detected in the nontransformed WT control. Under confocal microscopy, red fluorescence was observed in appressoria and invasive hyphae of OPW3 (Figure 3a). In hyphae, fluorescent signals were distributed in the cytoplasm and vacuole (Figure 3c). These results indicate that PA is required for the infection process and accumulated in the vacuole of OPM5. These results suggest that deletion of *MoPAH1* affects PA distribution in hyphae, which probably results from the accumulation of PA in the *Mopah1* mutant.

## 2.5 | *MoPAH1* regulates responses to multiple stressors

To investigate whether *MoPAH1* is involved in stress responses, WT, *Mopah1* mutant (M21), and complementation (CP1) strains were grown on CM and treated with stress conditions. These results showed that growth of the *Mopah1* mutant was completely inhibited by the high temperature of 34°C. The cell wall-disturbing reagent Congo red (CR, 0.2 mg/ml) inhibited growth by 33% compared to the WT and CP1 strains. With osmotic stressors NaCl (0.7 M) and sorbitol (1 M), the *Mopah1* mutant grew slightly faster than on the CM medium control (Figure 4a,b). In contrast, growth of the WT and

| Strain   | Genotype description                              | Reference             |
|----------|---------------------------------------------------|-----------------------|
| WT       | Wild-type strain (P131)                           | Xue et al. (2012)     |
| M21      | <i>MoPAH1</i> mutant of WT                        | This study            |
| M22      | <i>MoPAH1</i> mutant of WT                        | This study            |
| CP1      | <i>MoPAH1</i> complemented transformant           | This study            |
| OPW3     | Transformant of WT expressing ScOpi1-RFP          | Han and Carman (2017) |
| OPM5     | Transformant of M21 expressing ScOpi1-RFP         | Han and Carman (2017) |
| EP3      | Transformant of WT expressing <i>MoPAH1</i> -GFP  | This study            |
| ATG8-WT5 | Transformant of WT expressing <i>MoAtg8</i> -GFP  | This study            |
| ATG8-M1  | Transformant of M21 expressing <i>MoAtg8</i> -GFP | This study            |

TABLE 1 Wild-type and mutant *Magnaporthe oryzae* strains used in this study



**FIGURE 1** MoPah1 is involved in vegetative growth and conidiation. (a) Five-day-old cultures on complete medium (CM), oatmeal medium (OMA), and minimal medium (MM) plates. The wild-type (WT), *Mopah1* mutant (M21), and *Mopah1* complementation transformant (CP1) cultured at 28°C for 5 days (a–c). (b) The colony diameter was measured on CM, OMA, and MM plates. Means and standard deviations were calculated with data from three replicates. (c) Conidiation on OMA plates. Means and standard deviations were calculated from four independent repeats. (d) Hyphal cell length was measured for at least 20 cells for each strain. (e) The septa stained with Calcofluor white (CFW). White arrows indicate cell septa. Bar, 20 μm. (f) The conidiophores were examined under light microscopy. Bar, 50 μm. White arrows indicate the conidiophores. (g) Cross-section of colonies grown on CM plates for 7 days. (h) The branching of vegetative hyphae in WT and the *Mopah1* mutant. Bar, 100 μm. (i) Relative expression levels of conidiation-related genes in the WT and M21 strains. The expression level of each gene in the WT was set to 1. Means and standard deviations were calculated from three independent replicates. Data were analysed with Student's *t* test. Asterisks represent significant differences (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ )

complementation strains was reduced by these two stressors. These results indicate that MoPAH1 plays different roles in the response to various stress conditions.

## 2.6 | MoPah1 is required for ALS formation and pathogenicity

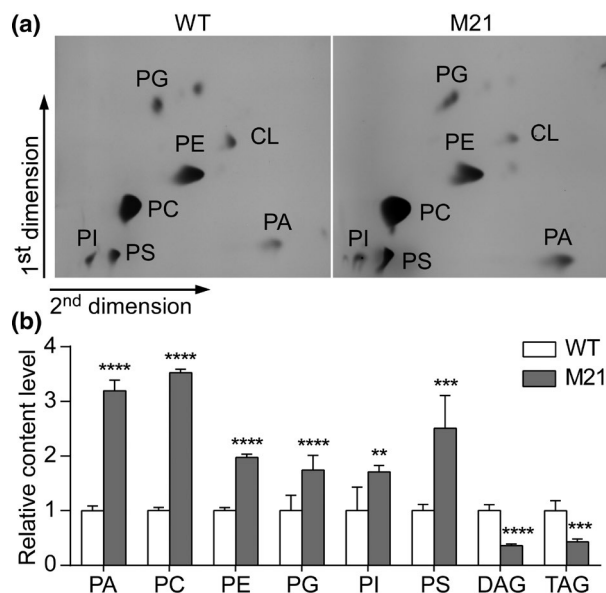
ALSs formed at hyphal tips are critical in plant infection for *M. oryzae* (Kong et al., 2013). Because the *Mopah1* mutant does not produce conidia, fresh mycelial blocks were used to test the ability to produce ALSs. When placed on hydrophobic cover slides and barley leaves, the *Mopah1* mutant failed to form any ALS after 48 h of incubation (Figure 5a,b). In contrast, the WT and complementation strains formed abundant melanized, dome-shaped ALSs. Therefore, MoPAH1 is required for the formation of ALSs.

To investigate whether deletion of MoPAH1 affects the plant infection capacity of the fungus, we tested the virulence of the WT, M21, and CP1 strains on 4-week-old rice seedlings of cultivar

Lijiangxintuanheigu and 6-day-old barley seedlings of cultivar E9. At 14 days postinoculation (dpi) with WT and CP1 strains, plants developed typical large blast lesions on rice leaves. In contrast, M21 caused no visible lesions. On barley leaves, the *Mopah1* mutant was completely nonpathogenic and did not produce lesions at 4 dpi, whereas WT and CP1 strains formed typical lesions (Figure 5c). These results indicate that MoPah1 is required for plant infection in *M. oryzae*.

## 2.7 | Distribution of MoPah1 during developmental and infection processes

The subcellular localization of MoPah1 at different developmental stages was investigated by transforming a MoPah1-green fluorescent protein (GFP) construct into the WT strain. The expression and integrity of the MoPah1-GFP fusion protein in transformant EP3 was verified by western blot analysis (Figure S4). Confocal microscopy revealed that the MoPah1-GFP protein was expressed at all tested

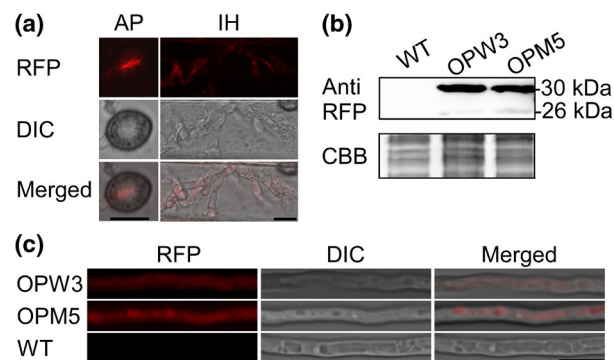


**FIGURE 2** Disruption of *MoPAH1* widely alters the lipid profile in *Magnaporthe oryzae*. (a) Two-dimensional thin layer chromatography (TLC) analysis of total lipids. Total lipids were isolated from the wild-type (WT) strain and the *Mopah1* mutant (M21). Equal amounts of lipids of each sample were loaded on the TLC plate. CL, cardiolipin; DAG, diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglycerol. (b) High-performance liquid chromatography/mass spectrometry (HPLC-MS) analysis of each lipid in the WT strain and the M21 mutant. Means and standard deviations were calculated from five independent replicates. Data were analysed with Student's *t* test. Asterisks represent significant differences (\*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001)

developmental stages, including conidia, appressoria, hyphae, and invasive hyphae (Figure S5). The GFP signals appeared mostly in the cytoplasm at different stages. These results suggest that *MoPah1* is important during all developmental and plant infection processes for *M. oryzae*.

## 2.8 | Deletion of *MoPAH1* alters the expression of genes involved in development and pathogenesis

To investigate the genome-wide effect of the *MoPAH1* disruption on gene transcriptional expression, we performed RNA-Seq analysis of the WT and *Mopah1* mutant strains. RNA was extracted from 4-day-old MM cultures of the WT and M21 strains. RNA samples were sequenced and analysed using an established bioinformatic pipeline (Wang et al., 2009). Approximately 40 million reads were generated from each RNA-Seq library. Most of the reads mapped (96%) to the reference *M. oryzae* genome. The expression profile analysis identified 2459 differentially expressed genes (DEGs) with 1035 up-regulated and 1424 down-regulated genes from the *Mopah1* mutant in comparison with the WT strain (*p* ≤ 0.05, log<sub>2</sub>



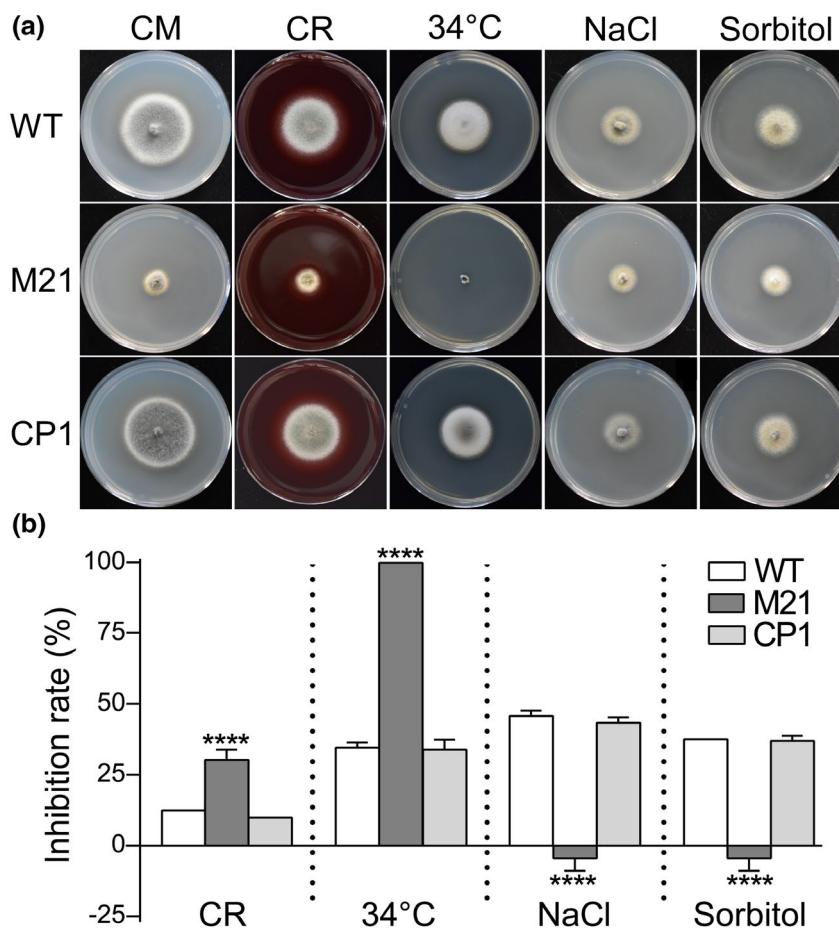
**FIGURE 3** ScOpi1-RFP fluorescence signals in the *Mopah1* mutant accumulated in the vacuole. (a) Subcellular localization of ScOpi1-RFP in appressoria (AP) and invasive hyphae (IH) of the wild type (WT; OPW3). Bar, 10 μm. (b) The ScOpi1 protein level was assessed using western blot analysis in the OPW3 and OPM5 strains. Proteins from the WT strain were used as the negative control. CBB, Coomassie Brilliant blue staining. (c) Subcellular localization of ScOpi1-RFP in hyphae of the WT (OPW3) and *Mopah1* mutant (OPM5) strains. Bar, 10 μm

FC ≥ 1; Figure 7a). The analysis of the KEGG pathway and GO enrichment showed that autophagy and the metabolic pathways of lipid, glycerolipid, and glycolysis/gluconeogenesis were altered in the *Mopah1* mutant (Figures S6 and S7). Based on the functionally characterized genes in the PHI database (Urban et al., 2020), 104 DEGs were categorized into groups representing growth, conidiation, appressorium, and pathogenicity functions (Figure 6b). Genes involved in conidiation and pathogenicity account for the most in the Venn diagram. Analysis by reverse transcription quantitative PCR (RT-qPCR) confirmed the reduced expression in the *Mopah1* mutant of DEGs involved in fungal growth (*FLB3*, *HOX1*, *HOX4*, *Dac*, *CRZ1*, *RGA4*, *COD1*, *CRF1*, *MSN2*), conidiation (*TDG1*, *TDG7*, *FAR2*, *CYP51B*, *CRZ1*, *RGA4*, *CHS1*, *COD1*, *CRF1*, *MSN2*), appressorium formation (*CBP1*, *CHS7*, *PTH11*, *RGA4*, *CHS1*, *COD1*, *CRF1*, *MSN2*), and pathogenicity (*RSY1*, *Dac*, *CYP51B*, *PTH11*, *CHS7*, *CRZ1*, *CHS1*, *COD1*, *CRF1*, *MSN2*) (Figure 6c). Similar analysis of the strains grown on CM indicated a significant reduction of the expression of conidiation genes in the *Mopah1* mutant (Figure 1i). Based on these results, we conclude that *MoPah1* is associated with proper expression of genes involved in fungal growth, conidiation, appressorium formation, and pathogenicity in *M. oryzae*.

## 2.9 | Pmk1 phosphorylation was reduced in the *Mopah1* mutant

The MAPK pathway is required for appressorium formation in *M. oryzae* (Li et al., 2012). To determine whether the deletion of *MoPAH1* altered the MAPK signalling pathway in the *Mopah1* mutant, assays for Pmk1 and Mps1 MAP kinase were conducted using western blotting. The protein level of Pmk1, but not Mps1, was reduced dramatically in the *Mopah1* mutant. The Pmk1 phosphorylation level was reduced but the Mps1 phosphorylation level

**FIGURE 4** Pleiotropic defects of the *Mopah1* mutant in response to stressors. (a) Colony morphology of the wild type (WT), *Mopah1* mutant (M21), and *Mopah1* complementation (CP1) strains supplemented with different stressors on CM plates. CM, complete medium; CR, Congo red. (b) The inhibition rate was calculated relative to the growth rate of each untreated control. The inhibition extent (%) = [(diameter of untreated colony – diameter of treated colony)/diameter of untreated colony] × 100. Means and standard deviations were calculated from three independent replicates. Data were analysed with Student's *t* test. Asterisks represent significant differences (\*\*\*\**p* < 0.0001)



increased in the *Mopah1* mutant compared to the WT strain. In contrast, the ratio of phosphorylated Pmk1 to Pmk1 was much higher in the *Mopah1* mutant than that in the WT, while the same ratio for Mps1 was slightly changed in *Mopah1* (Figure 7a,b). Taken together, these results indicate that MoPah1 is mainly involved in the Pmk1 MAPK signalling pathway in *M. oryzae*, in comparison to the Mps1 MAPK pathway.

## 2.10 | Autophagy is delayed in the *Mopah1* mutant

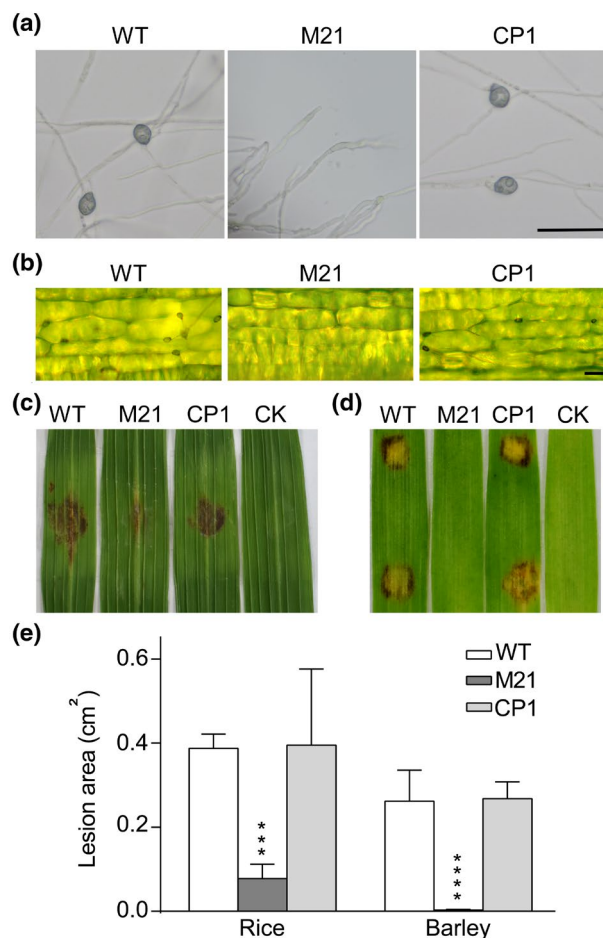
To determine whether MoPah1 is involved in autophagy, MoAtg8, a widely used autophagy marker (Klionsky, 2011), was fused to GFP and transformed into the WT and *Mopah1* mutant strains to yield the transformant strains ATG8-WT5 and ATG8-M5, respectively. Growth on nitrogen starvation medium-induced autophagy in both the WT and mutant strains was measured and observed by the increase of free GFP. Surprisingly, autophagy was strikingly delayed in the *Mopah1* mutant compared to the WT strain. After 5 h, the fluorescent signals were translocated from the cytoplasm into the vacuole completely in the WT strain (Figure 8a). In contrast, only a portion of fluorescent signals were translocated into the vacuole in the *Mopah1* mutant. In immunoblotting assays, ratios of free GFP to the total amount of GFP-MoAtg8 and free GFP were 90% in the WT strain and only 60% in the *Mopah1* mutant (Figure 8b). These

results demonstrate that MoPAH1 is required for proper nonspecific autophagy in *M. oryzae* under conditions of nitrogen starvation.

## 3 | DISCUSSION

Phospholipids are important for various biological processes but direct evidence of a role for phospholipids in *M. oryzae* pathogenesis was lacking. Recent studies have shown the versatile roles of PA, the simplest phospholipid molecule, in mammalian cells and plants (Dubots et al., 2012; Kim & Wang, 2020). In this study, we set out to evaluate the role of PA in fungal pathogenesis by focusing on the major PAP, MoPah1, an enzyme for PA metabolism. Our results showed the critical role of MoPAH1 in lipid metabolism, fungal development, and pathogenicity.

Among all PAP knockout mutants, only the *Mopah1* mutant showed obvious and pleiotropic phenotypes, suggesting an indispensable role of MoPah1 in *M. oryzae* (Sadat et al., 2014). Deletion of MoPAH1 resulted in an increased level of PA, which has also been shown in the *pah1* mutant of *S. cerevisiae* (Han et al., 2006). Decreased levels of DAG and TAG were observed in the *Mopah1* mutant, which has also been observed in *pah1* mutants of *S. cerevisiae*, *F. graminearum*, and *Yarrowia lipolytica* (Karanasios et al., 2010; Oh et al., 2017; Ukey et al., 2020). The reduction of TAG levels leads to a reduced level of lipid droplets in *pah1* mutants of *Tetrahymena*



**FIGURE 5** MoPah1 is required for formation of appressorium-like structures (ALSs) and pathogenicity. (a, b) ALSs formed from hyphal tips on glass coverslips and barley leaves of the wild type (WT) and *Mopah1* complementation (CP1) strains, but not the *Mopah1* mutant (M21). Bar, 50  $\mu$ m. (c) Inoculation of rice leaves with mycelial plugs for 14 days. CK, inoculation with agar blocks. (d) Infection assays on detached barley leaves with mycelial plugs for 4 days. CK, inoculation with agar blocks. (e) Statistical analysis of lesion area on rice and barley leaves. Means and standard deviations were calculated from three and five independent replicates in rice and barley leaves, respectively. Data were analysed with Student's *t* test and asterisks represent significant differences (\*\* $p < 0.001$ , \*\*\* $p < 0.0001$ )

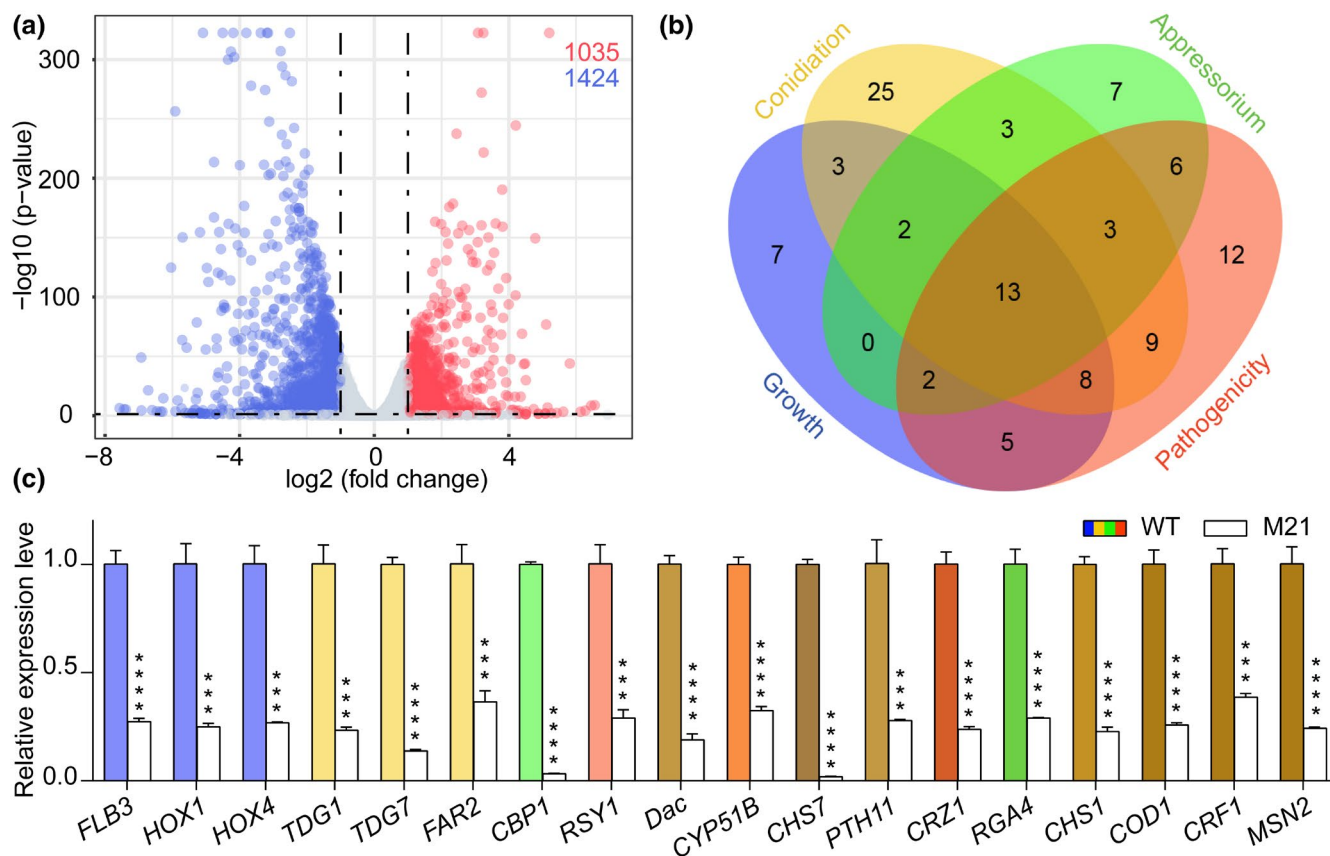
*thermophila* and *F. graminearum* (Liu, Yun, et al., 2019a; Liu, Liang, et al., 2019b; Pillai et al., 2017; Shi et al., 2018). The KEGG pathway and GO term analyses of the *Mopah1* mutant showed that many DEGs are related to lipid and glycolysis/gluconeogenesis metabolism. These results indicate a conserved function for Pah1 in lipid metabolism in fungi.

The *Mopah1* mutant was severely defective in mycelial growth, hypersensitive to cell wall stressors, and completely inhibited by elevated temperatures. These phenotypes are similar to the *pah1* mutants of *S. cerevisiae*, which is hypersensitive to oxidative stress and elevated temperature (Carman & Han, 2019), and to the *Fgpah1* mutant of *F. graminearum*, which is defective in mycelial growth and response to cell wall stressors (Liu, Yun, et al., 2019a; Liu, Liang, et al.,

2019b). Furthermore, the down-regulation of the growth-related genes *MoMSN2* and *MoFLB3* suggests their involvement in reduced growth phenotypes of the *Mopah1* mutant (Figure 6). As a pathogen adapted to paddy field conditions, heat tolerance is especially important for *M. oryzae*. We found that *HSP60* and *HSP10*, related to heat tolerance, are up-regulated in the thermosensitive *Mopah1* mutant, indicating that the up-regulation of these *HSP* genes is insufficient to compensate the phenotype or dysregulated genes, rather than other commonly known *HSPs* that may be involved in reduced heat tolerance of the *Mopah1* mutant (Li et al., 2011). Conidia are important for plant infection and rice blast spread in the field (Matheis et al., 2017). Unlike the *Fgpah1* mutant that produces conidia, the *Mopah1* mutant is completely defective in conidiation (Kong et al., 2012; Liu, Yun, et al., 2019a; Liu, Liang, et al., 2019b; Xue et al., 2012). The largest category of DEGs was related to conidiation in the *Mopah1* mutant, including transcription factors *COM1*, *COS1*, and *CON7*, confirming the critical role of MoPah1 in conidiation (Figures 1 and 8). Mutants lacking *COM1*, *COS1*, or *CON7* are also reduced in virulence (Shi et al., 1998; Yang et al., 2010). Taken together, down-regulated genes involved in development and stress responses in *Mopah1* could be also important for fungal virulence.

The *Mopah1* mutant is nonpathogenic on plant leaves, similar to the corresponding mutants in *C. albicans* (*Capah1*) and *F. graminearum* (*Fgpah1*), which are reduced in virulence or are completely avirulent, respectively (Liu, Yun, et al., 2019a; Liu, Liang, et al., 2019b; Mu et al., 2019). The nonpathogenicity of the *Mopah1* mutant is probably caused by its defects in ALS formation and the dysregulation of the Pmk1 MAPK pathway, which is responsible for ALS formation (Kong et al., 2013). The ratio of phosphorylated Pmk1 to total Pmk1 was increased in the *Mopah1* mutant, though the level of phosphorylated Pmk1 was reduced (Figure 7). Similarly, an increased phosphorylated MAPK relative to corresponding total proteins has been observed in the *Lpin2*<sup>-/-</sup> (*pah1*) mutant of human macrophages (Lordén et al., 2017). In mice, an increased level of PA activates the MEK-Erk MAPK pathway (Nadra et al., 2008). The two-fold increase of PA content in the *Mopah1* mutant could induce hyperphosphorylation of Pmk1, given the dramatically reduced protein level of Pmk1. Delayed autophagy could result in delayed glycogen metabolism, which is possibly another reason for loss of virulence in the *Mopah1* mutant (Figure 5). The delayed autophagy could be due to the down-regulation of the genes *MoATG1* and *MoATG9* in the *Mopah1* mutant (Lv et al., 2017). Additionally, PA is an important component of the cellular membrane system including the vacuole. The *S. cerevisiae* *pah1* mutant lacks vacuolar fusion (Sherr et al., 2017), which plays a critical role in autophagy. Thus, delayed autophagy in the *Mopah1* mutant might result from the altered vacuolar membrane due to membrane phospholipid composition changes. Taken together, the altered lipid metabolism in the *Mopah1* mutant probably affects Pmk1 phosphorylation activation and autophagy, which subsequently results in complete loss of virulence.

In conclusion, this study has demonstrated that MoPah1, the major PAP, is involved in several developmental and infection-related processes in *M. oryzae*. In particular, the MoPah1 PAP is



**FIGURE 6** MoPah1 regulates genes involved in development and pathogenesis in *Magnaporthe oryzae*. (a) Differentially expressed genes (DEGs) are shown in the volcano plot. The x axis shows the fold change in gene expression between wild type (WT) and *Mopah1* mutant M21, and the y axis shows the statistical significance of the difference. Red dots represent up-regulated genes; blue dots represent down-regulated genes. (b) Venn diagram of DEGs classified by biological function. The coloured circles represent genes involved in fungal growth, conidiation, appressorium formation, and pathogenicity. The number at the overlapping area stands for DEGs with two or more functions. (c) Reverse transcription quantitative PCR assays of DEGs between the WT and the *Mopah1* mutant. The column colour of the WT strain indicates the biological functions shown in (b). Means and standard deviations were calculated from three independent replicates. Data were analysed with Student's *t* test. Asterisks represent significant differences (\*\*\**p* < 0.001, \*\*\*\**p* < 0.0001)

essential for conidiation and ALS formation, indicating that MoPah1 plays a broader role in *M. oryzae* development than in *C. albicans* and *F. graminearum* (Liu, Yun, et al., 2019a; Liu, Liang, et al., 2019b; Mu et al., 2019). *MoPAH1* is involved in protein expression and phosphorylation of Pmk1, the most critical MAP kinase in *M. oryzae*, which advances our understanding of the role of MoPah1 and PA in *M. oryzae* pathogenicity. However, the detailed mechanism on how *MoPAH1* functions is still unknown. The increased level of plant PA negatively modulates relevant transcription factors in their binding to downstream genes (Kim et al., 2019), and similar mechanisms might apply to the *Mopah1* mutant, which shows increased PA content and many down-regulated infection-related genes (Figures 2 and 8). Notably, down-regulated genes in the *Mopah1* mutant include *MoMSN2*, *COS1*, *HTF1/HOX2* and *CHS7*, and 22 of them overlap 62 down-regulated genes in the mutant lacking *MoMSN2*, which regulates lipid metabolism, appressorium formation, and invasive growth (Zhang et al., 2014), indicating that down-regulated *MoMSN2* could be related to many phenotypes of the *Mopah1* mutant. Additional studies

are needed to investigate whether MoPah1 phosphorylation/de-phosphorylation regulates the enzymatic activity of the versatile protein, like reports in yeast and other filamentous fungi (Carman & Han, 2019). These studies will provide a clear role of MoPah1 in *M. oryzae* pathogenicity, particularly on Pmk1 phosphorylation and regulation of important infection-related transcription factors.

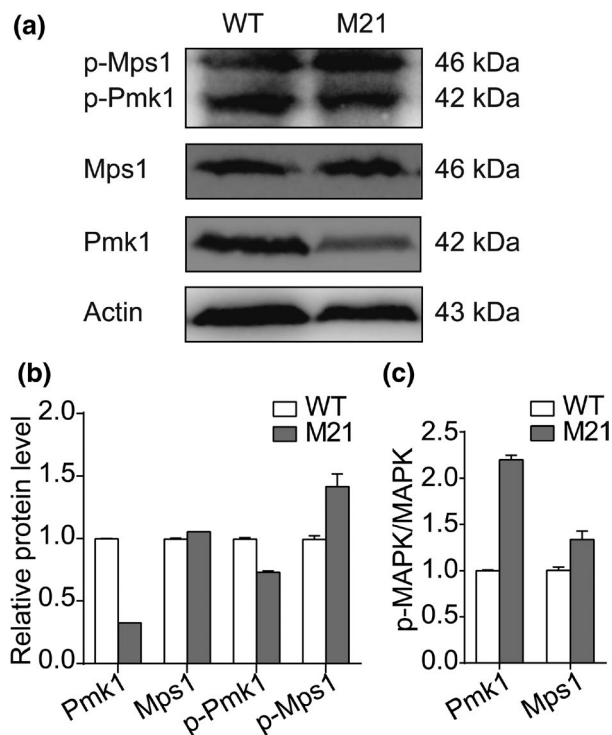
## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Strains and culture conditions

*M. oryzae* P131 was used as the WT in this study (Xue et al., 2012). The WT strain and transformants were routinely cultured on OMA or CM agar plates at 28°C. The growth rate was measured on CM, MM, and OMA at 28°C for 5 days (Li et al., 2017; Zhong et al., 2016). Conidiation was assayed on OMA plates and conidiophores were observed as previously described (Dubey et al., 2019). For

tolerance tests, fungal strains were cultured at 28°C on CM plates supplemented with 0.2 mg/ml CR, 0.7 M NaCl, or 1 M sorbitol. For heat tolerance assays, the temperature was set at 34 °C. For

transformation, all transformants were selected on TB3 medium with 250 µg/ml hygromycin B (Genview) or 400 µg/ml geneticin (GLP BIO).



**FIGURE 7** Defects of the MAPK signalling pathways in the *Mopah1* mutant. (a) Assays of the protein level of Pmk1 and Mps1, phosphorylated Pmk1, and Mps1 were detected using anti-Phospho-p44/42 MAPK antibody. Mps1 antibody, Pmk1 antibody and anti-actin antibody. (b) Analysis of the Pmk1 and Mps1 protein level, phosphorylated Pmk1, and Mps1 relative to that of actin. (c) Relative expression of phosphoproteins against total proteins. The protein contents of phospho-Mps1, Mps1, phospho-Pmk1, and Pmk1 relative to that of actin

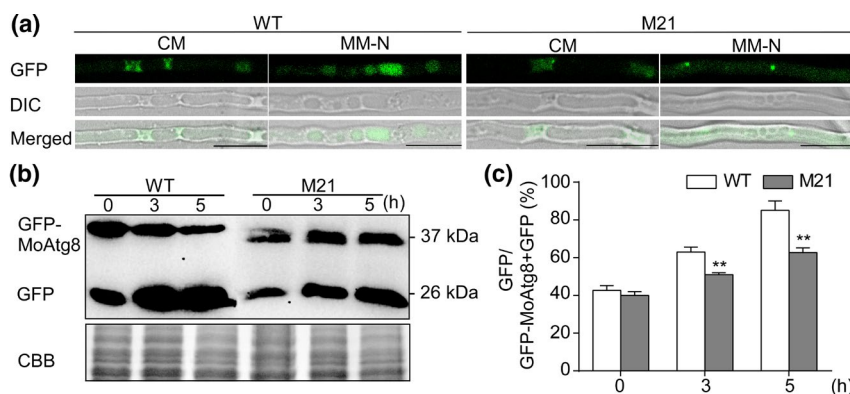
## 4.2 Targeted gene deletion and complementation of *MoPAH1*

A split-marker strategy was used to delete the *MoPAH1* gene (Catlett et al., 2003). The 1.0 kb upstream and downstream flanking fragments were amplified using primer pairs 1F/2R and 5F/6R, respectively. Primers HYG-F/R were used to amplify the *hph* cassette from plasmid pCB1003 (Carroll et al., 1994). The *hph* cassette was ligated with flanking fragments using primers 1F/HYG-UR and HYG-DF/6R. PCR products were directly used to transform into protoplasts of P131 using the polyethylene glycol (PEG)-mediated approach as described (Yang et al., 2010). Putative deletion transformants were verified by PCR.

For complementation assays, we used primers *MoPAH1*-CF/CR to amplify the full length of the *MoPAH1* gene, including its 1.5-kb native promoter, and clone into the pKNTG plasmid (Chen et al., 2014). The constructs were sequenced and transformed into protoplasts of M21. Putative complementation transformants were verified by PCR (Table S1).

## 4.3 Phylogenetic analysis

We downloaded Pah1 protein sequences (*M. oryzae*, *C. albicans*, *S. cerevisiae*, *Oryza sativa*, *Arabidopsis thaliana*, *Schizosaccharomyces pombe*, *N. crassa*, *F. graminearum*, *Aspergillus nidulans*, *Sclerotinia sclerotiorum*, and *Botrytis cinerea*) from the NCBI database to construct a multispecies neighbour-joining phylogenetic tree. Multiple amino acid sequences of Pah1 proteins from different species were aligned



**FIGURE 8** Autophagy is delayed in mutant *Mopah1*. (a) The fluorescence signal of GFP-MoATG8 in the wild-type strain (WT) and *Mopah1* mutant (M21) when cultured in complete medium (CM) or nitrogen starvation medium (MM-N). Bar, 10 µm. (b) Total proteins were extracted from ATG8-WT5 and ATG8-M5 strains cultured in MM-N for 0, 3, and 5 h. The protein level of GFP-Atg8 was detected using immunoblotting. Coomassie Brilliant blue (CBB) staining indicates the protein loading for each lane. (c) Autophagy was delayed in the *Mopah1* mutant. The extent of autophagy was analysed by calculating the amount of free GFP compared with the total amount of GFP-MoAtg8 and free GFP. Quantitative analysis was conducted using ImageJ. Means and standard deviations were calculated from three independent replicates. Data were analysed with Student's *t* test. Asterisks represent significant differences (\*\**p* < 0.01)

using ClustalW. The neighbour-joining phylogenetic tree was constructed using MEGA 7.0 in the p-distance model with 1000 bootstrap replicates.

#### 4.4 | Calcofluor white staining assay

For CFW staining, mycelia from liquid CM culture with shaking (120 rpm) at 28°C for 24 h were harvested and stained with 10 mg/ml CFW (Sigma-Aldrich) in the dark for 5 min (Guo et al., 2017). Stained mycelia were observed by microscopy (Ni90; Nikon). The cell length was measured using ImageJ software.

#### 4.5 | RNA-Seq analysis

The WT and *Mopah1* mutant (M21) strains were grown in liquid MM with shaking (120 rpm) at 28°C for 4 days. Total RNA was extracted using the RNeasy Pure Plant Kit (Qiagen). RNA quantity and quality were assayed on a NanoDrop spectrophotometer ND-1000 (Thermo Scientific) and 2100 Bioanalyzer (Agilent). Samples were sequenced on a BGISEQ-500 sequencer (BGI) following the manufacturer's instructions. Quality control of sequencing data was performed using FastQC (Brown et al., 2017). We used HISAT2 v. 2.0.4 to map the clean reads to the *M. oryzae* MG8 genome (<http://fungi.ensembl.org/index.html>) (Kim et al., 2015). Quantification of gene expression was performed using featureCounts (Liao et al., 2014). DESeq2 was used for differential expression analysis between WT and M21 (Love et al., 2014). DEGs were identified with criteria  $p \leq 0.05$  and  $\log_2 FC \geq 1$  (FC, fold-change) (Oh et al., 2017). The volcano plot was produced from R analysis (Zhou et al., 2021). The genes used in the Venn diagram were retrieved from PHI-base (Urban et al., 2020) and drawn using Evenn (Chen et al., 2021).

For heatmap analysis, the transcriptional expression levels of PAP-encoding genes in *M. oryzae* were obtained from the previous study (Jeon et al., 2020). The heatmap was drawn using TBtools, as previously described (Chen et al., 2020).

#### 4.6 | RT-qPCR

Total RNA was isolated from mycelia grown on CM plates with cellophane overlays and used for both RT-qPCR assays and RNA-Seq analysis. For RT-qPCR assays, 1st Strand cDNA Synthesis Kit (Vazyme) was used to generate complementary DNA (cDNA). The qPCR assay was performed on the CFX Connect Real-time PCR System (Bio-Rad) using TransStart Tip Green qPCR SuperMix (TransGen Biotech) (Li et al., 2011). The  $\beta$ -tubulin gene (MGG\_00604) was used as the internal control, and the expression level of target genes was calculated using the  $2^{-\Delta\Delta Ct}$  method (Livak & Schmittgen, 2001). The RT-qPCR assay was repeated three times with independent samples.

Data were analysed with GraphPad software. The primers used are listed in Table S1.

#### 4.7 | ALS formation and plant infection assay

To observe ALSs at hyphal tips, fresh mycelial blocks (2 × 2 mm) were incubated on hydrophobic glass coverslips at 28°C for 48 h. Formation of ALSs was examined under a microscope (Ni90; Nikon) (Liu et al., 2010).

Six-day-old barley (*Hordeum vulgare*) seedlings of cultivar E9 and 4-week-old rice (*O. sativa*) seedlings of cultivar Lijiangxintuanheigu were used for infection assays. Fungal culture blocks were inoculated on barley and rice leaves in a humid chamber at 28°C. Symptoms on rice and barley were examined at 14 and 4 dpi, respectively. Plant inoculation, incubation, and lesion examination were performed as previously described (Guo et al., 2019).

#### 4.8 | Lipid analysis

Mycelia were harvested from liquid MM cultured at 28°C with shaking (120 rpm) for 4 days and freeze-dried. The sample was incubated in hot (75°C) isopropanol containing 0.05% (vol/vol) butylated hydroxytoluene (Sigma-Aldrich) for 15 min. Total lipid was extracted using chloroform/methanol (2:1) with 0.01% butylated hydroxytoluene, and this extraction procedure was repeated five times. Then 1 M KCl and water were added to the sample for centrifugation, and the upper phase was discarded. The solvent was evaporated under an nitrogen gas stream and redissolved in chloroform (5 mg/ml) (Bligh & Dyer, 1959). TLC plates were incubated at 110°C for 90 min and samples were spotted at one corner of the plates (Macherey Nagel). Chloroform/ethanol/ammonium hydroxide (65:25:2, vol/vol/vol) and chloroform/ethanol/acetic acid/water (85:15:10:3, vol/vol/vol/vol) were used for first- and second-dimension separation, respectively. After drying, the plates were exposed to iodine vapour for 90 s in the tank. The lipid contents were measured as described using an Agilent HPLC system coupled with a triple quadrupole/ion trap 4000 QTrap mass spectrometer (Applied Biosystems) (Nakamura et al., 2014).

#### 4.9 | Subcellular localization assays

The MoPah1-GFP and ScOpi1-RFP constructs were generated using a One Step Cloning Kit (Vazyme) and verified with sequencing. The constructs were transformed into the WT and *Mopah1* mutant strains (Yang et al., 2010). The transformant strains were used for observing GFP and RFP fluorescence signals at different developmental stages, including hyphae, conidia, appressoria, and invasive hyphae, under a confocal microscope (TCS SP8; Leica). Confocal images were collected with 40× objective lenses. Excitation and

emission spectra of eGFP and mRFP were 498/550 nm and 571/620 nm, respectively. The images were acquired and processed using LAS X software.

#### 4.10 | Western blotting

Total proteins were extracted from mycelia cultured in liquid CM for 48 h as previously described (Li et al., 2011). Proteins were separated with 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PDVF) membranes. The target proteins on the PDVF membrane were detected with anti-GFP and anti-RFP antibodies (Abbkine), along with horseradish peroxidase (HRP)-conjugated secondary antibodies (Abbkine). Signals were visualized with a Western Blotting Detection Kit (Advansta) and the membrane was photographed using the ChemiDoc Touch imaging system (Bio-Rad). The protein loading was monitored by staining proteins in acrylamide gels with Coomassie Brilliant blue (CBB) for 2 h and destaining the gel overnight before imaging (Wang et al., 2018).

#### 4.11 | Autophagy analysis

For autophagy analysis, the construct of MoAtg8 fused with a GFP tag at its N-terminus was transformed into the WT and *Mopah1* mutant (Veneault-Fourrey et al., 2006). The GFP-Atg8-tagged WT and mutant strains were cultured in CM for 2 days. Mycelia were rinsed with water and transferred to nitrogen starvation medium for 2–5 h to induce nonselective autophagy. The fluorescence signals were observed under a confocal microscope (TCS SP8; Leica). Proteins were extracted and analysed by western blotting with anti-GFP antibodies as described (Li et al., 2017). The amount of free GFP and GFP-Atg8 was quantified by densitometric analysis with ImageJ (Zhang et al., 2018; Zhong et al., 2016).

#### 4.12 | Assays for the nonphosphorylated and phosphorylated MAP kinases

Mycelia were harvested from liquid CM for 48 h and used for protein extraction as described (Li et al., 2011). Expression and phosphorylation of Pmk1 and Mps1 were detected with Phospho-p44/42 MAP kinase antibody kits, and anti-Mps1 and anti-Pmk1 antibodies (Cell Signaling Technology; ABclonal Biotechnology) following the manufacturer's instructions. The amount of protein was quantified by densitometric analysis with ImageJ (Qu et al., 2021).

#### ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (32172373, 31801723) and Fundamental Research Funds for the Central Universities (2662020ZKPY006) to G. L. We thank Drs Charles P. Woloshuk and Jin-Rong Xu at Purdue University for critical reading of this manuscript and also thank Dongqin Li from the

National Key Laboratory of Crop Genetic Improvement for technical assistance in lipidomics analyses.

#### CONFLICT OF INTEREST

There is no conflict of interest.

#### DATA AVAILABILITY STATEMENT

The RNA-Seq data used in this study can be accessed at the National Center for Biotechnology Information (NCBI) at <http://www.ncbi.nlm.nih.gov/> with the accession number PRJNA777402.

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## SUPPORTING INFORMATION

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**How to cite this article:** Zhao, J., Sun, P., Sun, Q., Li, R., Qin, Z., Sha, G., et al (2022) The *MoPah1* phosphatidate phosphatase is involved in lipid metabolism, development, and pathogenesis in *Magnaporthe oryzae*. *Molecular Plant Pathology*, 23, 720–732. <https://doi.org/10.1111/mpp.13193>