Endocytic protein Pal1 regulates appressorium formation and is required for full virulence of *Magnaporthe oryzae*

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Abstract
Endocytosis plays key roles during infection of plant-pathogenic fungi, but its regulatory mechanisms are still largely unknown. Here, we identified a putative endocytosis-related gene, *PAL1*, which was highly expressed in appressorium of *Magnaporthe oryzae*, and was found to be important for appressorium formation and maturation. Deletion of *PAL1* significantly reduced the virulence of *M. oryzae* due to defects in appressorial penetration and invasive growth in host cells. The Pal1 protein interacted and colocalized with the endocytosis protein Sla1, suggesting it is involved in endocytosis. The *Δpal1* mutant was significantly reduced in appressorium formation, which was recovered by adding exogenous cAMP and 3-isobutyl-1-methylxanthine (IBMX). Moreover, the phosphorylation level of Pmk1 in *Δpal1* was also reduced, suggesting Pal1 functions upstream of both the cAMP and Pmk1 signalling pathways. As a consequence, the utilization of glycogen and lipid, appressorial autophagy, actin ring formation, localization of septin proteins, as well as turgor accumulation were all affected in the *Δpal1* mutant. Taken together, Pal1 regulates cAMP and the Pmk1 signalling pathway for appressorium formation and maturation to facilitate infection of *M. oryzae*.

KEYWORDS
actin ring, appressorium formation, autophagy, cAMP pathway, endocytosis, Pmk1 pathway

1 | INTRODUCTION

The ascomycete fungus *Magnaporthe oryzae* is the causal agent of rice blast, which has become a model organism for studying plant-pathogen interactions (Dean et al., 2012; Ebbole, 2007). During infection, *M. oryzae* forms a melanized appressorium on the host surface, breaches the host cuticle with a penetration peg, and spreads across host cells through invasive hyphae (Ebbole, 2007; Wilson & Talbot, 2009). The formation and maturation of the appressorium is a key and complex process that is determined by spore storage components (such as glycogen and lipid) and is regulated by a variety of signalling pathways (Rho et al., 2009; Talbot, 2003).

The cAMP signalling pathway is one of the most important signalling pathways in the formation of the appressorium in *M. oryzae* (Adachi & Hamer, 1998; Mitchell & Dean, 1995; Xu & Hamer, 1996). Previous studies have confirmed that intracellular cAMP...
concentration gradually increases during appressorium differentiation of *M. oryzae* (Lee & Dean, 1993). cAMP acts as a second messenger in cells, transmitting intracellular and extracellular signals to regulate the formation and differentiation of the appressorium (Lee & Dean, 1993; Xu & Hamer, 1996). The Pmk1-MAPK signalling pathway is another pathway that participates in the late stage of appressorium formation, called appressorium maturation. This pathway consists of the Mst11–Mst7–Pmk1 protein kinase cascade (Wilson & Talbot, 2009; Xu & Hamer, 1996). Although the key components of cAMP and the Pmk1–MAPK signalling pathway have been identified, their upstream regulator mechanism in *M. oryzae* is still unclear. Mucin protein MoMsb2 is responsible for cell surface recognition, is required for Pmk1 phosphorylation, and may also play a role upstream of the cAMP signalling pathway (Liu et al., 2011; Wang et al., 2015).

In *M. oryzae*, during the formation and maturation of the appressorium, a series of biological processes are activated, so that the appressorium has all the functions needed to ensure successful invasion and colonization (Foster et al., 2017; Rebollar & López-García, 2013). These processes include glycoll, lipid, and trehalose utilization (Foster et al., 2017; Jong et al., 1997; Thines et al., 2000), peroxisome biogenesis (Chen et al., 2017), the autophagy process (Kershaw & Talbot, 2009; Veneault-Fourrey et al., 2006), endocytosis, actin ring formation, and melanin synthesis (Li, Chen et al., 2017), as well as turgor accumulation (Jong et al., 1997). In addition to the strong turgor effect, the appressorium also needs enough mechanical pressure to produce the penetration peg, which is provided by the appressorium actin ring formed in the middle of the contact between the mature appressorium and the host (Li, Gao et al., 2017). The appressorium actin ring is assembled by septin proteins, which are composed of Sep3, Sep4, Sep5, and Sep6 (Dagdas et al., 2012; Ryder et al., 2013).

Endocytosis is a complex cellular process that mainly includes cargo sorting, membrane invagination, vesicle scission, and targeting (Kaksonen et al., 2005). The first and most interesting step of endocytosis is site formation or establishment (Carroll et al., 2012; Toret & Drubin, 2006). In budding yeast, dozens of proteins participate in this step, including early coat, WASP/Myo, and amphiphysin (Carroll et al., 2012; Kaksonen et al., 2005; Munn, 2000). The recruitment of proteins to endocytic sites is carried out in a highly precise order. In *Saccharomyces cerevisiae*, Ede1p, Syp1p, clathrin, Pal1p (Pal1 domain-containing protein), and the AP2 complex are the first proteins to arrive at nascent endocytic sites (Carroll et al., 2012; Ge et al., 2005; Newpher et al., 2005). Proteins containing clathrin, AP2 complex, yap1801p, yap1802p, and Pal1p work as a new early coat module and internalize with vesicles before disassembly (Carroll et al., 2012; Sun et al., 2006). In *M. oryzae*, several endocytosis genes, such as MoEND3, MoABP1, and MoARK1, have been identified to play important roles in receptor internalization, signalling pathway activation, cytoskeleton remodelling, and cellular morphogenesis (Li, Chen et al., 2017).

Recently, there have been two reports on the endocytosis-related protein Pal1 in *Fusarium graminearum* and *Ustilaginoidea virens* (Chen et al., 2020; Yin et al., 2020). In *F. graminearum*, disruption of FgPAL1 leads to defects in vegetative growth, conidial morphology, and pathogenicity (Yin et al., 2020). In *U. virens*, in addition to participating in asexual development and full virulence, *UvPal1* also interacts with other endocytic proteins as well as the septin protein Cdc11 (Chen et al., 2020). However, the regulatory mechanism of Pal1 in plant-pathogenic fungi is still not clear. Interestingly, Pal1 is also predicted to be a mucin protein, so we speculated whether the function of Pal1 related to endocytosis may be similar to MoMsb2, which play roles upstream of both the cAMP and Pmk1 signalling pathways in *M. oryzae*. Consistent with our speculation, in this study we demonstrate that Pal1 affects appressorium formation and virulence through regulating cAMP signalling and the Pmk1–MAPK signalling pathway. A series of biological processes, including utilization of glycogen and lipid, vesicle transport, assembly of actin ring, and localization of septin proteins, were blocked or defective in the PAL1 deletion mutants. Our study elucidated the mechanism of the endocytosis protein Pal1 regulating appressorium formation and maturation in rice blast fungus.

## RESULTS

### 2.1 Identification of an appressorium highly expressed gene PAL1 in *M. oryzae*

Through whole transcriptome data (authors’ unpublished data), we found the gene MGG_02779 was highly expressed at the appressorium development stage of *M. oryzae*. This gene encodes a 526 amino acid protein with a Pal1 domain and eight exons (Figure S1a). We named this gene as PAL1 in *M. oryzae*. Phylogenetic analysis showed that Pal1 is highly conserved in fungi, and the Pal1 protein of *M. oryzae* has the highest homology with that of *Gaumannomyces graminis* and *Neurospora crassa*. In addition, *M. oryzae* Pal1 is also closely related to the related proteins of *Fusarium graminearum* and other fungi (Figure S1b).

To confirm the expression pattern and determine the function of PAL1 in *M. oryzae*, we examined the expression of PAL1 at different developmental stages of *M. oryzae*. Total RNA extracted from the hyphae (HY), conidia (CO), appressoria at 3 h postinoculation (hpi, AP-3h) and 12 hpi (AP-12h), and infectious hyphae at 18 hpi (IH-18h), 24 hpi (IH-24h), and 48 hpi (IH-48h) of *M. oryzae* were used to perform reverse transcription quantitative PCR (RT-qPCR) analysis. We found the expression level of *PAL1* at AP-12h was four to five times as much as that of other developmental stages (Figure S2), suggesting that PAL1 may play an important role in the appressorium of *M. oryzae*.

To determine the function of PAL1 in *M. oryzae*, we disrupted this gene in the wild-type strain P131 using a split-PCR strategy (Goswami, 2012). The transformants were screened by PCR-based methods (Figure S3a), and the RT-qPCR result indicated that the candidate deletion mutants lacked PAL1 (Figure S3b). Two independent PAL1 deletion mutants (PAL1KO1 and PAL1KO2) were obtained and...
used for the subsequent analyses. The complementary strains were also generated by introducing the 1.5 kb native promoter-driven PAL1 coding region into the deletion mutant PAL1KO1. Most of the transformants were recovered in all tested phenotypes, therefore one of the complementation transformants, cPAL1, was used for further experiments.

2.2 | PAL1 is involved in endocytosis in *M. oryzae*

Pal1 was first reported to be gathered in the initial stage of endocytosis in budding yeast (Carroll et al., 2012). We tested whether Pal1 plays similar roles in the rice blast fungus. In the process of cell active transport, the dye FM4-64 can enter cells and specifically bind to vesicle membranes and other structures (Penalva, 2005). To examine whether Pal1 is required for endocytosis, we used FM4-64 to stain the hyphae of different strains, and then observed the endocytosis process. The FM4-64 dye quickly appeared in the cytoplasm at the hyphal tip of the wild-type strain P131 but stayed on the cytoplasmic membrane of the Δpal1 mutant after 1 min of staining. Five minutes later, stronger fluorescence signal showed in the cytoplasm of the wild type stained with FM4-64 dye, which still showed in the plasma membrane of the Δpal1 mutant. After 15 min of staining, most wild-type mycelia were stained internally but there was still only weak internal fluorescence in the cytoplasm of the Δpal1 mutant (Figure 1a,b). This experiment indicated that Pal1 is indeed involved in endocytosis in *M. oryzae*.

To further determine whether Pal1 is required for endosome-mediated signal compartmentalization and transduction, we fused Rab5, an endosome marker protein, with green fluorescent protein (GFP). The Rab5-GFP construct was transformed into the wild-type and Δpal1 mutant strains. When the localization of Rab5-GFP in the

![Figure 1](image-url)

**Figure 1** Pal1 plays a role in the endocytosis process. (a) Endocytosis process observation of the hyphal tips at different time points after staining with FM4-64. Bar = 10 μm. (b) Bar chart showing the fluorescence intensity at the indicated regions of the hyphal tip in (a). At least 10 hyphae of each strain were used for measurement using ImageJ software. Error bars represent standard deviation and asterisks represent significant differences (p < 0.01). a.u., arbitrary units. (c) Subcellular localization of the endosome protein Rab5-GFP in hyphae, conidia, and germ tubes of the wild type and the Δpal1 mutant. White arrows indicate the endosomes. Bar = 10 μm. (d) Yeast two-hybrid (Y2H) assay was performed to detect interaction between Pal1 and Sla1. The blue interaction between pGBK7T7-53 (BD-53) and pGADT7-T (AD-T) was used as the positive control, and interaction between pGBK7T7-Lam (BD-Lam) and pGADT7-T was used as the negative control. Positive colonies on SD/-Leu-Trp-His were stained with X-α-gal. (e) Colocalization of Pal1 and Sla1. White arrows indicate the puncta. Bar = 10 μm.
hyphal tips was observed, we found that bright puncta were distributed in the cytoplasm adjacent to the cell membrane in the wild type but not in the Δpal1 mutant (Figure 1c). A similar phenomenon was also clearly observed in the conidia and germ tubes (Figure 1c). This suggests that endosomal trafficking is involved in Pal1-mediated endocytosis.

2.3 | Pal1 interacts and colocalizes with the endocytic protein Sla1

Previous studies reported that in yeast, Pal1p reaches the endocytosis site at the earliest stage of the endocytosis process, then works together with other proteins such AP2 complex proteins, Sla1p, and Ent1p (Carroll et al., 2012). Therefore, we conducted yeast two-hybrid (Y2H) assay to test whether Pal1 interacted with those proteins present at the earliest stage of endocytosis in M. oryzae. As expected, the Y2H experiment showed that Pal1 can interact with Sla1 in M. oryzae (Figure 1d).

To further determine whether Pal1 is colocalized with Sla1 in M. oryzae, we generated GFP-Pal1 and red fluorescent protein (RFP)-Sla1 constructs driven by the PAL1 and the SLA1 native promoters, respectively. The GFP-Pal1 fusion vector was transformed into the Δpal1 mutant strain. Phenotypes of the positive transformants, including vegetative growth, conidiation, and virulence, were all recovered to the wild type state (data not shown), indicating that the GFP-Pal1 construct functioned fully. Subsequently, the GFP-Pal1 and RFP-Sla1 constructs were transformed into the wild-type strain. Green and red fluorescence signals were both observed in the cytoplasm with some puncta in the hyphae, conidia, and appressoria (Figure S4). To further confirm the relationship of localization of the two proteins, we cotransformed the GFP-Pal1 and RFP-Sla1 constructs into the Δpal1 mutant to observe their localization at different developmental stages. The results showed that Pal1 and Sla1 colocalized in the cytoplasm as well as in puncta structures in all tested samples, including the mycelium, conidium, and appressorium (Figure S4). Thus, we concluded that Pal1 interacted and was colocalized with the early-stage endocytic protein Sla1 in M. oryzae, suggesting that it also plays a key role in the initial stage of endocytosis.

2.4 | Pal1 is important for cell wall integrity

We then tested whether Pal1 is involved in resistance to environmental stress during fungal development. Various stresses, including cell wall inhibitors (0.1 mg/ml Calcofluor white [CFW], 0.2 mg/ml Congo red [CR]), high osmotic pressure (0.5 M NaCl, 1 M sorbitol), and oxidative stress (H₂O₂) were added into a complete medium (CM) plate for testing. Acidic or alkaline pH was also used for testing, by adjusting the pH of the CM medium to different values (pH 5.8, 6.8, and 7.8). Compared with the wild-type and complementary strains, the Δpal1 mutant was more sensitive to CFW and CR, indicating that PAL1 is involved in cell wall integrity. On the other hand, the Δpal1 mutant was more resistant to 1 M sorbitol, 10 mM H₂O₂, and acidic/alkaline pH conditions (Figure 2a,b), suggesting that deletion of PAL1 may lead to compensation effects in the fungal response to these stresses. Together, our results show that deletion of PAL1 affects the fungal response to different stresses.

2.5 | PAL1 regulates asexual growth and conidial formation of M. oryzae

To investigate the role of PAL1 in fungal growth and development, we cultured the Δpal1 mutants, the wild type, and the complementary strain on oatmeal tomato agar (OTA) medium for 5 days at 28°C. The colony diameters of the Δpal1 mutants were significantly smaller than those of the wild type and complementary strains (Figure 3a,b). The CFW staining assay showed the mycelial cell length of the mutants was obviously shorter than that of the wild type (Figure S5). This result accounts for the decreased growth rate of the mutants compared with that of the wild type. Therefore, PAL1 plays an important role in fungal vegetative growth.

We also examined the effect of PAL1 deletion on spore formation and development. Although the deletion mutant could form normal conidia, the number of spores attached to conidia was significantly less than that of wild-type and complementary strains (Figure 3c). In comparison, the number of conidia of the two deletion mutants was 36% less than that of the wild-type and complementary strains (Figure 3d). This result is consistent with the observation of conidiophores, and demonstrates that PAL1 affects spore formation of M. oryzae. Moreover, the spore morphology of Δpal1 was evidently different from that of the wild type. Around 60% of Δpal1 conidia did not show the normal two/septa/three-cells morphology. By contrast, 90% of P131 and cPAL1 spores formed two-septa conidia, and only 10% of their spores formed one or no septum (Figure 3e,f). These results suggest that PAL1 is involved in maintaining the spore morphology of M. oryzae.

2.6 | PAL1 is responsible for full virulence of M. oryzae

To detect the effect of PAL1 deletion on the pathogenicity of M. oryzae, we sprayed rice and barley leaves with a conidial suspension of Δpal1, P131, and cPAL1. After 4 days, only a small number of disease spots formed on the plant leaves inoculated by Δpal1. However, more and bigger lesions appeared on the leaves inoculated with P131 and cPAL1 (Figure 4a,b). These results indicate that PAL1 plays an important role in the full virulence of M. oryzae.

To determine whether the expansion of fungal growth in Δpal1 was affected, rice seedlings were scratched with needles, and mycelial blocks of different strains were inoculated to the inoculation site. Four days later, we observed that the leaves of rice inoculated with P131 and cPAL1 showed extended disease lesions, while lesions of
leaves inoculated with Δpal1 could not extend beyond the inoculation sites (Figure 4c). This evidence suggests that the expansion of Δpal1 in host cells is blocked.

To further confirm if the expansion of the infectious hyphae was blocked in Δpal1, the formation of invasive hyphae was observed in barley epidermis cells. Statistical analysis showed that at 24 hpi more than 80% of P131 or cPAL1 attachment cells formed infectious hyphae with one or more branches, while about 70% of Δpal1 conidia could only form appressoria (Figure 4d,e). At 30 hpi, more than 70% of appressoria of P131 or cPAL1 had formed expanded hyphae, while only 10% of appressoria of Δpal1 formed secondary infectious hyphae and nearly 40% of fungal cells still stayed at the appressorium stage (Figure 4d,e). Obviously, the infection rate of Δpal1 was significantly lower than that of the wild-type and complementary strains. These results indicate that PAL1 is responsible for both appressorium-mediated penetration and infectious hyphae expansion.

2.7 | Pal1 is involved in the inhibiting host defence response

On fungal infection, plant cells often produce reactive oxygen species (ROS) for resistance. Because Δpal1 was deficient in colonization in host cells, we detected ROS accumulation in host cells infected with the PAL1 deletion mutant. The 3,3’-diaminobenzidine (DAB) staining assay was conducted on barley epidermis inoculated with a conidial suspension of Δpal1, P131, and cPAL1 at 30 hpi. We observed and photographed the epidermal cells under the microscope after decolourization. More than 60% of the plant cells inoculated with Δpal1 showed a reddish brown colour, representing massive ROS accumulation, while less than 20% of the cells infected by P131 or cPAL1 were stained, suggesting less ROS accumulation (Figure S6a,b). These data indicate more ROS accumulated in plant cells infected by Δpal1 than by P131 or cPAL1, which suggests that PAL1 is involved in inhibiting host defence response.
2.8 | Pal1 regulates appressorium formation

Considering that Pal1 is highly expressed in appressoria, we wondered whether deletion of PAL1 might affect appressorium formation. Conidia of different strains were inoculated on hydrophobic glass slides and were used to observe appressorium formation at different time points (6, 12, and 24 h). At 6 hpi, many immature appressoria formed at the tip of the germ tube in Δpal1, while half of the wild-type conidia had formed mature appressoria. At 12 hpi, around 70% of P131 or cPAL1 conidia produced mature appressoria, much higher than in the deletion mutant. At 24 hpi, more than 80% of the wild-type conidia formed mature appressoria, while it was only about 50% in Δpal1 (Figure 5a,b). In summary, the appressorium formation rate of Δpal1 was markedly slower than that of the wild-type and complementary strains, indicating that Pal1 regulates appressorium formation in M. oryzae.

2.9 | Pal1 plays a role upstream of the cAMP signalling pathway

In M. oryzae, the cAMP signalling pathway is one of the main pathways regulating appressorium formation. The cellular cAMP concentration is closely determined by adenylate cyclase and phosphodiesterase, which are responsible for synthesis and degradation of cAMP, respectively (Sassone-Corsi, 2012). Due to the defect of Δpal1 in appressorium formation, we speculated whether Pal1 acts upstream of the cAMP signalling pathway. We exogenously added a membrane-permeable cAMP variant, 8-Na-cAMP, or a cyclophosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), to increase the cAMP level during the formation of appressoria. At 6 hpi, the percentage of appressorium formation of Δpal1 was only 40% in water. When the Δpal1 conidial suspension was supplemented with 1 mM cAMP or 2.5 mM IBMX, the percentage of appressorium formation was restored to around 60%, comparable to that of the wild type. At 12 and 24 hpi, adding cAMP or IBMX increased the appressorium formation ratio of Δpal1 to the wild-type level (Figure 5c,d). These results indicate that Pal1 plays a role upstream of the cAMP signalling pathway in M. oryzae. Consistent with this, we found that the content of endogenous cAMP in the mycelium of Δpal1 was reduced nearly twice as much as that of the wild-type strain (Figure 5e). This suggests that Pal1 plays an important role in cAMP signalling pathway-mediated appressorium formation in M. oryzae.
Pal1 is require for Pmk1 phosphorylation

The observation of the infection process showed that Pal1 is also important for appressorium-mediated penetration. Considering that the Pmk1 signalling pathway is required for appressorium maturation and penetration, we speculated whether Pal1 also regulates the Pmk1 signalling pathway. We detected the phosphorylation level of the Pmk1 protein in different strains. Proteins of different stages of the wild type and Δpal1 mutant were extracted and analysed by western blot. No visible difference was found in the protein level of Pmk1 (42 kDa) between wild type (P131) and Δpal1 (Figure 5f, bottom panel). However, when using the phospho-MAPK antibody for testing, Pmk1 phosphorylation was clearly detected at the mycelium, 8 h appressorium, and 18 h appressorium stages, while only a weak signal was detected in the conidium. In contrast, reduced and delayed Pmk1 phosphorylation were detected in the Δpal1 appressoria (Figure 5f, upper panel). This finding suggests that Pal1 also contributes to Pmk1 phosphorylation and plays a role upstream of the Pmk1 signalling pathway during appressorium development.
2.11 | Pal1 affects utilization of glycogen and lipid during appressorium formation

Glycogen and lipids stored in conidia are nutrients required for appressorium formation and maturation in M. oryzae. Utilization of glycogen and lipids directly determines the function of the appressorium. Because Pal1 plays roles upstream of both the cAMP and Pmk1 signalling pathways, we inferred that utilization of glycogen and lipid should be blocked in the \( \Delta \text{pal1} \) mutant. Different time points during appressorium formation of the wild type and \( \Delta \text{pal1} \) were stained with \( \text{I}_2 / \text{KI} \) to detect glycogen utilization and with Nile red to detect lipid utilization. The glycogen staining assay showed that in the wild-type strain, glycogen began to transfer from spore to appressorium at 8 h and was totally utilized at 12 h. In the \( \Delta \text{pal1} \) mutant, glycogen could still be observed by staining at 18–24 h (Figure 6a), indicating that the utilization of glycogen in \( \Delta \text{pal1} \) was severely blocked. Similar results were found in lipid utilization. In the wild type, lipid droplets began to be degraded at 8 h, and could not be detected at 12 h. In \( \Delta \text{pal1} \), lipid droplets could be still detected at 12 h (Figure 6b), indicating that the utilization of lipid in \( \Delta \text{pal1} \) was delayed. These results suggest that Pal1 affects the metabolism of glycogen and lipid during transition from the conidium to appressorium.

2.12 | Pal1 is important for autophagy

Nutrition transition from the conidia to appressoria is a consequence of autophagy followed by mitosis and nuclear migration (Veneault-Fourrey et al., 2006). Because Pal1 influences the transition of glycogen and lipid, we determined whether or not disruption of \( \text{PAL1} \) affects the autophagy process. Mycelia of the wild-type and \( \Delta \text{pal1} \) strains were cultured in liquid minimal medium (MM) with reduced nitrogen (MM−N). We used \( \text{GFP}:\text{MoATG8} \) as a functional marker to detect delivery of vesicles to vacuoles and the breakdown of autophagosomes. Free GFP cannot be hydrolysed from \( \text{GFP}:\text{MoAtg8} \) in normal autophagy (Kim et al., 2001; Mizushima et al., 2004). Therefore, we examined autophagy by detecting \( \text{GFP}:\text{MoAtg8} \) in mycelia under nitrogen starvation. A GFP signal was detected in 63% of hyphal cells of wild type (P131) and 59% of cells of \( \Delta \text{pal1} \)
after 2 h of nitrogen starvation treatment. However, after 5 h of nitrogen starvation, a GFP signal was observed in 88% of wild type mycelial cells, significantly higher than the 68% in Δpal1 cells. This phenomenon was confirmed by a staining assay with the vacuole dye 7-amino-4-chloromethylcoumarin (CMAC) (Figure 7a,b). Furthermore, western blotting was conducted to detect proteolysis of GFP:MoAtg8. Total proteins were extracted from wild type and Δpal1 expressing GFP:MoAtg8 followed by 0, 2, and 5 h of culture in MM−N medium. Free GFP and the fusion protein GFP:MoAtg8 (41 kDa) were then detected using the anti-GFP antibody. The ratio of GFP intensity to the sum of GFP:MoAtg8 and GFP (GFP+GFP:MoAtg8) were measured. In normal conditions and MM−N exposure for 2 h, the ratio was comparable in wild type and Δpal1. After 5 h of nitrogen starvation, the ratio increased to 0.95 in wild type, significantly higher than the 0.78 in Δpal1 (Figure 7c,d), indicating autophagy was blocked in Δpal1. Based on this, we conclude that disruption of PAL1 influences autophagy.

2.13 | Turgor accumulation is reduced in the Δpal1 mutant

Because the utilization of glycogen and lipid droplets was severely reduced in the Δpal1 mutant, and the autophagy process was also blocked in the mutant, we inferred that the appressorial turgor should be also reduced in the mutant. Therefore, turgor accumulation was examined in appressoria of the Δpal1 mutant by a cytorrhysis assay. When treated with different concentrations of polyethylene glycol (PEG) 8000, the mature appressoria formed by the Δpal1 mutant were more easily collapsed than those of the wild
Type (Figure 5g), suggesting the turgor pressure in the Δpal1 mutant is significantly reduced.

2.14 | Pal1 regulates the assembly of actin rings in appressorium

During the development of appressorium, the actin ring, which is located at the base of the infected cell surrounding the appressorium pore, provides cortical rigidity and promotes the emergence of the penetration peg to invade the host plant. Because Δpal1 was defective in appressorium maturation and plant infection, we examined whether the deletion of PAL1 affected actin ring formation in the appressorium. The F-actin marker Lifeact-RFP was transformed into the wild-type and Δpal1 mutant strains. At 24 hpi, 85% of wild-type appressoria formed an actin ring, while 70% of Δpal1 appressoria exhibited dispersed actin localization that failed to form a ring (Figure 8a). This result suggests that Pal1 is required for assembly of the actin ring in the appressorium.

2.15 | Pal1 determines localization of septin proteins

The actin ring is organized with the assistance of septin proteins (Dagdas et al., 2012). We investigated whether localization of septin proteins was affected by Pal1. GFP-Sep5 and GFP-Sep6 constructs were transformed into the wild-type strain or the Δpal1 mutant. Positive transformants were used to observe the localization of GFP-Sep5 and GFP-Sep6. In the wild-type strain, GFP signals gathered as a ring in the centre of the appressorium, while in the Δpal1 mutant, GFP-Sep5 and GFP-Sep6 were mainly distributed in granular form throughout the appressorium and did not form a ring (Figure 8b,c). These results indicate that Pal1 is responsible for accurate localization of septins.

3 | DISCUSSION

As previously reported, Pal1p is very important for maintaining the cylindrical cell morphology in Schizosaccharomyces pombe (Ge et al.,
2005) and S. cerevisiae (Carroll et al., 2012). Deletion of S. cerevisiae Pal1p results in spherical or pear-shaped cell morphology (Carroll et al., 2012). Recently, two studies in the plant-pathogenic fungi F. graminearum and U. virens have demonstrated roles for Pal1 in vegetative growth, conidial morphology, pathogenicity, polarized tip growth, and developments of ascospores (Chen et al., 2020; Yin et al., 2020). However, the Pal1 regulatory mechanisms in plant-pathogenic fungi are still not well understood. In this study, we revealed that Pal1-mediated endocytosis plays key roles in appressorium formation and maturation, which function upstream of both cAMP and Pmk1–MAPK signalling pathways in M. oryzae.

For many filamentous fungi, the appressorium is a specialized structure required to penetrate host tissue for colonization. The expression level of PAL1 in appressoria was obviously higher than that in vegetative hyphae, conidia, and infectious hyphae. We speculated that Pal1 may play a key role during appressorium formation and maturation. Several important signalling pathways, including the cAMP signalling pathway (Lee & Dean, 1993; Xu & Hamer, 1996), the Pmk1–MAPK signalling pathway (Xu & Hamer, 1996; Zhao et al., 2005), the Ca2+ signalling pathway (Lee & Lee, 1998), and the TOR signalling pathway (Marroquin-Guzman & Wilson, 2015; Marroquin-Guzman et al., 2017), are involved in regulating the formation and maturation of appressoria. In this study, we detected that the content of cAMP was less in Δpal1 than in the wild type. Exogenous addition of cAMP content restored the appressorium formation ratio of Δpal1 to the wild-type level. This evidence suggests that PAL1 affects appressorium formation by regulating the cAMP signalling pathway. We also found the Pmk1 phosphorylation level was significantly reduced in the Δpal1 mutant, suggesting that Pal1 may regulate the Pmk1–MAPK signalling pathway. All these suggest Pal1 is a key upstream signalling regulator for appressorium formation.

MoMsb2 is a mucin protein responsible for cell surface recognition. The phosphorylation level of Pmk1 was reduced in MoMSB2 deletion mutants, indicating that MoMsb2 may function upstream of the Pmk1 MAPK cascade (Liu et al., 2011). The G protein coupled receptor (GPCR) Pth11 is a receptor protein involved in surface sensing. Disruption of PTH11 leads to defects in appressorium formation, which can be recovered by exogenous cAMP (DeZwaan et al., 1999). MSB2 and PTH11 may be functionally related in sensing signals of the leaf surface, but the detailed mechanism is still unknown. Interestingly, Pal1 is also a predicted mucin protein, suggesting endocytosis could play a role in surface signal transduction. Whether or not MoMsb2 is also involved in endocytosis is fascinating. Previous study for MoEnd3, another endocytic protein, indicated that MoEnd3 mediates the transport of Pth11 (DeZwaan et al., 1999; Li et al., 2010) and membrane sensor MoSho1, triggers the downstream phosphorylation of Pmk1, and promotes autophagy, appressorium formation, and penetration (Li, Gao et al., 2017). Deletion of MoEND3 delays the endocytosis of Pth11 and MoSho1 and affects appressorium formation, which proves that endocytosis plays an important role in the signal transduction and pathogenesis of M. oryzae (Li, Gao et al., 2017). Together with these studies, our study shows that Pal1-mediated endocytosis is closely connected with cAMP and Pmk1 signalling pathways by transducing upstream signals to downstream signalling pathway.

![FIGURE 8 Pal1 regulates actin ring assembly and localization of septin proteins. (a) Observation of the actin ring in appressoria of wild type (P131) and Δpal1 expressing RFP-Lifeact. (b) Localization of Sep5-GFP in wild type and ΔPal1. Bars = 10 μm. (c) Localization of Sep6-GFP in wild type and ΔPal1. Bars = 10 μm. For (a) to (c), linescan graphs show fluorescence intensity in a transverse section of individual appressoria as the arrow-indicated direction.](image-url)
pathways for appressorium formation and maturation. However, considering that Pal1 is an endocytosis factor, it could also be possible that Pal1 affected all the prerequisite processes required for appressorium formation, not only required for appressorium formation, by acting upstream of important signaling pathways.

The actin ring is a ring structure of the hetero-oligomer from mature appressorium, supporting enough mechanical pressure to form the penetration peg to infect the plant epidermis. During the development of the appressorium, an actin ring will form in the center of the cell to assist penetration peg formation (Dagdas et al., 2012). The septin proteins Sep3, Sep4, Sep5, and Sep6 are involved in the assembly of the actin ring and support F-actin to form cytoskeleton in the appressorium (Dagdas et al., 2012). In this study, using the actin marker Lifeact-RFP, we found standard actin ring formation was impaired in Δpal1 by affecting localization of the septins. This suggests Pal1 is involved in the assembly of the actin ring for appressorium penetration. Interestingly, in U. virens, Pal1 was also proved to interact with a septin protein Cdc11 (Chen et al., 2020), suggesting that this regulatory mechanism may be conserved in fungi.

In yeast, another Pal protein, Pal2, is also known to participate in clathrin-mediated endocytosis. Double deletion of PAL1 and PAL2 causes a slightly slower growth defect than the wild type (Ghosh et al., 2021), while in M. oryzae, Pal2 homologous protein was found. However, one protein (MGG_00684) with low similarity to yeast Pal2 was found and also annotated as a Pal1-like protein. As many proteins are involved in endocytosis, the viability of the strain lacking PAL1 in M. oryzae may be a result of functional redundancy of Pal1 and Pal2, as well as other unknown endocytotic proteins that remain to be discovered.

Altogether, Pal1 regulates CAMP and Pmk1 signalling pathways, and as a consequence many important processes in appressorium differentiation and maturation are regulated by Pal1, including glycogen and lipid droplet utilization, appressorial autophagic process, actin ring formation, and turgor accumulation. Considering that Pal1 is very important for functional appressorium formation, and it is well-conserved and specific in fungi, this membrane-associated protein may be a valid target for developing broad-spectrum fungicides.

4 | EXPERIMENTAL PROCEDURES

4.1 | Strains and culture conditions

P131 was used as the wild-type strain. All strains listed in Table S1 were cultured on OTA plates at 28°C. For genomic DNA and RNA extraction, all strains were cultured in liquid CM at 28°C. Colony diameters on the OTA plates were measured at 120 hpi. Conidia from 7-day-old colonies cultured on OTA plates were harvested for experiments.

To test fungal response to environmental stresses, strains were inoculated onto the CM plates supplemented with 0.2 mg/ml CR (Sigma-Aldrich), 0.1 mg/ml CFW (Sigma-Aldrich), 0.5 M NaCl, 1 M sorbitol, and 10 mM H2O2. The colony diameters were measured after 5 days postinoculation (dpi).

4.2 | RT-qPCR

To evaluate the PAL1 expression profile, tissues harvested from different developmental stages (Liu et al., 2018) were used to extract total RNA for preparing the cDNA templates. The qPCR was performed by using an SYBR Green PCR Master Mix kit (Takara) on an ABI 7500 real-time PCR system (Applied Biosystems).

4.3 | Gene disruption and complementation

A split-PCR strategy was used for gene disruption as previously described (Goswami, 2012). Left boarder and right boarder were confirmed by PCR using the Pal1LBCK/ HYG-LBCK and Pal1RBCK/ HYG-RBCK primer pairs. Gene deletion was further confirmed by RT-PCR of a c.900 bp internal fragment of PAL1. For complementation, a complementation vector containing 1.5 kb promoter region, the PAL1 gene-coding region, and the adjacent 0.5 kb downstream region was transformed into the Δpal1 mutant. CM plates supplemented with 250 μg/ml hygromycin B (Roche Diagnostics) were used to select the deletion transformants, and CM plates supplemented with 400 μg/ml neomycin (Amresco) were used to select the complementation transformants.

4.4 | Staining assays

The fungal mycelia incubated in liquid CM were harvested and stained with 10 μg/ml FM4-64 (Sigma-Aldrich) at room temperature for 1, 5, 10, or 15 min. For CFW staining, the hyphae and conidia were stained with 10 μg/ml CFW (Sigma-Aldrich) solution for 10 min. The samples were washed twice with phosphate-buffered saline (PBS), and observed and photographed under a microscope (Nikon). For glycogen and lipid droplet staining, the spore suspension (105 spores/ml) was incubated on the hydrophobic plastic cover glass and stained with KI/ I2 solution (60 mg/ml KI, 10 mg/ml I2) for glycogen staining or Nile red solution (50 mM Tris/maleate buffer, pH 7.5) for lipid droplet staining. For vacuole staining, the mycelia were treated with 10 μM prewarmed CMAC (Thermo Fisher Scientific) for 30 min and observed under a microscope.

4.5 | Infection assay

The rice cultivar Oryza sativa ‘CO-39’ grown for 1 month and the barley cultivar Hordeum vulgare ‘E9’ grown for 1 week were used for the virulence test. Conidial suspension (5 × 106 conidia/ml in 0.025% Tween 20) was used to spray plants, which were then incubated under full humidity conditions at 28°C. The disease lesions were photographed at 5 dpi.
4.6 Subcellular localization

The PAL1, SEP5, SEP6, and Rab5 genes with native promoters were amplified and ligated into the C-terminal end of the GFP gene in the vector pKNRG (Tables S2 and S3; Liu et al., 2018). The subsequent vector pKNRG-PAL1 was transformed into the Δpal1 mutant. The constructed vectors pKNRG-SEP5, pKNRG-SEP6, and pKNRG-Rab5 were transformed into wild-type strain P131 or the Δpal1 mutant. Subsequent transformants were used to observe subcellular localization at different developmental stages under a confocal microscope TCS SP8 (Leica Microsystems). To observe the actin ring in appressorium, the pKNRR-LifeAct vector was constructed and transformed into wild-type strain P131 and the Δpal1 mutant. Transformants with fluorescence were used for observation under the confocal microscope.

4.7 Yeast two-hybrid experiment

The full-length cDNA of PAL1 was inserted into vector pGADT7, and the full-length cDNA of SLA1 was inserted into vector pGBKKT7. Both vectors were cotransformed into yeast strain AH109. The transformants were selected on plates of SD−Leu−Trp−His (Clontech), followed by culture at 30°C for 3–4 days to observe the colony growth. The colonies were then transferred to a new SD−Leu−Trp−His plate for staining by 5 μl X-α-Gal, followed by incubation at 30°C in darkness for 2 h. The interaction between pGBKKT7-53 and pGADT7-T was used as the positive control, and the interaction between pGBKKT7-Lam and pGADT7-T was used as the negative control.

4.8 cAMP and IBMX treatment

For determination of cAMP content, the mycelia of different strains were cultured in CM medium for 48 h. The mycelia were collected and treated with liquid nitrogen then freeze-dried for 16 h. Then cAMP levels were quantified using cAMP Biotrak Enzyme Immunoassay system (Biotrak).

To test the effect of cAMP and IBMX on appressorium formation of the Δpal1 deletion mutant, spore suspension (10^5 spores/ml) was inoculated on a hydrophobic glass slide and then 1 mM cAMP (Macklin) and 2.5 mM IBMX (Solarbio) were added to observe the appressorium formation ratio at different time points.

4.9 Pmk1 phosphorylation detection assay by western blotting

Around 200 mg of mycelia was harvested and ground into powder with liquid nitrogen, and resuspended in 1 ml of extraction buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% Triton X-100) with 1 mM phenylmethanesulfonyl fluoride (PMSF) and 10 μl of protease inhibitor cocktail (Sigma). Total proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. The Pmk1 protein level was assayed by using anti-p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technology), and the phosphorylated Pmk1 was assayed by anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell Signaling Technology).

4.10 Autophagy assay

The MoAtg8-GFP construct was transformed into P131 and Δpal1. Subsequent transformants were cultured in liquid minimal medium with nitrogen starvation (MM−N). After 0, 2, or 5 h incubation, the mycelia were harvested for extracting total proteins. A western blot was then performed using total proteins, and the GFP:MoAtg8 fusion protein and free GFP were detected using an anti-GFP antibody. Protein levels were normalized using an anti-actin antibody.

4.11 Cytorrhysis assay

Appressorium turgor was assessed by dropping conidial suspension (10^5 conidia/ml) onto a hydrophobic coverslip and incubating it in a moistened chamber at 28°C for 24 h to form mature appressoria. The coverslip was then placed into solution containing different concentrations of PEG 8000 (25%, 30%, 35%, and 40% wt/vol). After 10 min, the percentage of collapsed appressoria was calculated under a microscope. For each experiment, at least 100 conidia were examined and three replicates were performed.

4.12 Statistical analysis

Data were subjected to analyses of variance using SPSS v. 19.0 software (IBM). Means were separated using the test of least significant difference (α = 0.05).

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CONFLICT OF INTEREST

The authors declare no conflict of interest exists.

AUTHOR CONTRIBUTIONS

J.X. and X.L.C. conceived the study. J.X. and X.L.C. designed the experiments. D.C., H.H., W.H., S.Z., M.T., S.X., C.L., A.H., and M.K. performed the experiments. J.X., X.L.C., D.C., and H.H. analysed data and wrote the manuscript. J.X., X.L.C., J.H., L.Z., and H.L. supervised the project. All authors discussed the results and contributed to the final manuscript.
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