***Magnaporthe oryzae* Abp1, a MoArk1 Kinase-Interacting Actin Binding Protein, Links Actin Cytoskeleton Regulation to Growth, Endocytosis, and Pathogenesis**

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The actin cytoskeleton and actin-coupled endocytosis are conserved cellular processes required for the normal growth and pathogenesis of the rice blast fungus *Magnaporthe oryzae*. We have previously shown that actin regulating kinase MoArk1 regulates actin dynamics and endocytosis to play a key role in virulence of the fungus. To understand the underlying mechanism, we have characterized the actin-binding protein MoAbp1 that interacts with MoArk1 from *M. oryzae*. The Δ Moabp1 mutant exhibited delayed endocytosis and defects in growth, host penetration, and invasive growth. Consistent with its putative function associated with actin-binding, MoAbp1 regulates the localization of actin patches and plays a role in MoArk1 phosphorylation. In addition, MoAbp1 interacts with MoCap (adenylyl cyclase-associated protein) affecting its normal patch localization pattern and the actin protein MoAct1 through its conserved domains. Taken together, our results support a notion that MoAbp1 functions as a protein scaffold linking MoArk1, MoCap1, and MoAct1 to regulate actin cytoskeleton dynamics critical in growth and pathogenicity of the blast fungus.

Keywords: actin cytoskeleton, endocytosis, *Magnaporthe oryzae*, virulence

Actin cytoskeleton dynamics are required for cellular functions that include the establishment of polarity, motility, cytokinesis, sensing of environmental forces, exocytosis, and endocytosis (Blanchoin et al. 2014; Pollard and Cooper 2009; Porat-Shliom et al. 2013). For example, dynamic actin cytoskeletal changes generate mechanical forces to induce membrane invagination and vesicle scission from the plasma

membrane (Goode et al. 2015; Qualmann et al. 2000; Schafer 2002). In the budding yeast *Saccharomyces cerevisiae*, actin filament assembly occurs at sites of plasma membrane internalization, which are characterized by cortical actin patches (Kaksonen et al. 2003, 2006). The actin cytoskeleton is a dynamic network that is precisely controlled by a number of related proteins, including the actin-regulating kinase (Ark1) (Cope et al. 1999), the Arp2/3 complex (Galletta et al. 2008), the cyclase-associated protein (Cap1) (Balcer et al. 2003), and actin-binding protein 1 (Abp1) (Goode et al. 2001).

Abp1 plays an important role in linking functions of the actin cytoskeleton to endocytosis (Dieckmann et al. 2010; Kessels et al. 2001; Quintero-Monzon et al. 2005). In mammalian cells, Abp1 (mAbp1) mediates the link between cortical actin cytoskeleton and endocytosis and serves as a hub in which several signaling pathways converge (Kessels et al. 2001). mAbp1 colocalizes with dynamin, a GTPase that functions in endocytosis at actin-rich sites proximal to the cell body during synaptogenesis (Kessels et al. 2001). mAbp1 binds to F-actin to have a role in receptor endocytosis (Kessels et al. 2001; Mise-Omata et al. 2003). mAbp1 is also involved in the dorsal ruffle formation and cell motility through interactions with various other proteins (Cortesio et al. 2010; Fenster et al. 2003; Han et al. 2003; Kessels et al. 2001). In *S. cerevisiae*, Abp1 binds to F-actin through the actin-depolymerizing factor homology (ADFH) domain and activates the Arp2/3 complex through two acidic motifs (Goode et al. 2001). In addition, Abp1 binds to other proteins involved in actin assembly and endocytosis, including Cap1, Scp1, Sjl2, and Ark1 (Fazi et al. 2002; Freeman et al. 1996; Haynes et al. 2007; Lila and Drubin 1997; Stefan et al. 2005; Stollar et al. 2009). The deletion of the *ABP1* gene causes a moderate defect in endocytosis (Tuo et al. 2013), but deletions of *ABP1* and *SAC6*, *SLA1*, or *SLA2* that encode actin-associated proteins are synthetic lethal (Holtzman et al. 1993).

Intracellular trafficking and actin cytoskeleton maintenance also play an important role in the rice blast fungus *Magnaporthe oryzae*, in particular, the function of appressoria that invade the plant tissue (Wilson and Talbot 2009). The development of appressoria is linked to remodeling of the actin cytoskeleton, mediated by a family of septin small GTPases (Dagdas et al. 2012). The actin cytoskeleton is remodeled into an F-actin ring surrounding the appressorium, a circular region that marks the point at which the penetration peg emerges to rupture the leaf cuticle (Dagdas et al. 2012). The appressorium pore initially lacks a cell wall, and the fungal plasma membrane makes direct contact with the rice leaf surface. Then, as

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the appressorium inflates to full turgor, a pore wall overlay develops and a narrow penetration peg emerges (Howard et al. 1991). Assembly of an F-actin network during appressorium turgor generation, just prior to infection, takes place at the base of the appressorium. The F-actin ring at the base of appressorium

provides cortical rigidity at the initially wall-less region of the appressorium and directs the isotropic expansion into a mechanical force at the base of the appressorium toward the host cell (Dagdas et al. 2012). In addition, MoTea4, a polarity determinant protein important in F-actin assembly, was shown to be required for growth and pathogenesis of the rice blast fungus (Patar et al. 2010). Finally, MoCap1, a protein associated with the function of adenylate cyclase MoMac1, was also implicated in actin cytoskeleton dynamics based on its localization pattern in appressoria (Zhou et al. 2012).

We have previously found that the actin-regulating kinase MoArk1 has a role in the actin cytoskeleton and endocytosis and it is important in growth, development, and pathogenicity of the fungus (Wang et al. 2013). We have also characterized the role of R-SNARE protein MoSec22 and the vesicle-associated membrane protein MoVam7 in endocytosis, growth, development, and pathogenicity (Dou et al. 2011; Song et al. 2010; Wang et al. 2013). As an important regulator protein, MoArk1 may interact with multiple proteins involved in actin cytoskeleton dynamics or endocytosis. Recently, we found that MoArk1 also mediates the phosphorylation of the F-actin capping protein MoCAP and the Eps15 domain-containing protein MoEnd3 to coordinate the actin cytoskeleton and endocytosis (Li et al. 2017a and b). Here, we report how the MoArk1-interacting actin-binding protein MoAbp1 plays a role in actin assembly and endocytosis to govern growth and pathogenicity of *M. oryzae*.

RESULTS

Identification of MoAbp1 through interaction with MoArk1.

MoArk1 is an actin-regulating kinase with important regulatory roles in the actin cytoskeleton dynamics, endocytosis, growth, and virulence of *M. oryzae* (Li et al. 2017a; Wang et al. 2013). To elucidate MoArk1 functional mechanisms, we have identified MoArk1-interacting proteins through affinity purification coupled with mass spectrometry (Li et al. 2017a). One of the proteins, encoded by the locus MGG_06358.6, showed high amino acid (aa) sequence homology to Abp1 characterized from other fungi (Supplementary Fig. S1). We designated it as MoAbp1 and obtained the $\Delta Moabp1$ mutants (Supplementary Fig. S2).

MoAbp1 consists of 799 aa residues that share 62-, 61-, and 63% aa sequence identity, respectively, to *Colletotrichum orbiculare* CoAbp1 (ENH88454.1), *Neurospora crassa* NcAbp1 (XP_957869.1), and *Ophiocordyceps sinensis* OsAbp1 (EQK97779.1). MoAbp1 is distant from *S. cerevisiae* Abp1 (NP_010012.1), sharing 23% aa sequence identity. Further sequence analysis indicated that MoAbp1 contains one conserved ADFH domain and two Src homology 3 (SH3) domains.

To validate the interaction between MoAbp1 and MoArk1, we performed yeast two-hybrid (Y2H) analysis by cotransformation of pGADT7-*MoABP1* and pGBKT7-*MoARK1* into the yeast host AH109 and the result validated the interaction (Fig. 1A). Additionally, fluorescence complementation was also performed. *MoARK1*-YFP^C and *MoABP1*-YFP^N fusion constructs were transformed into the wild-type (WT) strain Guy 11. The yellow fluorescent protein (YFP) signal in hyphae and conidia indicated that these two proteins also interacted in vivo (Fig. 1B).

MoAbp1 interacts with MoAct1 and MoCap1.

Abp1p was originally identified as an actin-binding protein by actin-affinity chromatography in *S. cerevisiae* (Drubin et al. 1988) and it has been shown to bind to actin filaments (Goode et al. 2001; Lappalainen et al. 1998). We therefore examined the interaction between MoAbp1 and actin by coimmunoprecipitation (co-IP) assays. The *MoABP1*-GFP (green fluorescent protein) and *MoACT1*-S constructs were generated and

Fig. 1. MoAbp1 interacts with MoArk1. **A**, Yeast two-hybrid assays for the interaction between MoAbp1 and MoArk1. The activating domain (AD) and DNA-binding domain (BD) plasmids were cotransformed into yeast AH109, and transformants were plated on SD-Leu-Trp for 3 days and on selective SD-Leu-Trp-His-Ade with 1 mM X- α -gal for 5 days. **B**, Bimolecular fluorescence complementation assays for the interaction between MoAbp1 and MoArk1. Vegetative hyphae and conidia of transformants were examined by differential interference contrast and epifluorescence microscopy. The strains expressing the MoAbp1-YFP^N and empty YFP^C, MoArk1-YFP^C and empty YFP^N, empty YFP^N and empty YFP^C constructs were used as negative controls. Bar = 5 μ m.

cotransformed into the WT strain. Transformants expressing the *MoACT1-S* and *MoABP1-GFP* constructs were confirmed by Western blot analysis with the anti-S and anti-GFP antibodies, respectively. A 40-kDa protein band of the expected size of MoAct1 and a 111-kDa protein band of MoAbp1 were found. In proteins eluted from GFP-Trap-A beads, the same protein band was detected with the anti-S antibody, indicating that MoAbp1 interacts with MoAct1 (Fig. 2A). This interaction was also verified by Y2H (Fig. 2B) and *MoABP1-YFP^N* and *MoACT1-YFP^C* fluorescence complementation in Guy 11 (Fig. 2C).

To investigate whether MoAbp1 colocalizes with F-actin, the *MoABP1-GFP* construct was cotransformed with the actin reporter LifeAct-RFP (red fluorescent protein) into the WT strain. Most of the MoAbp1-GFP colocalizes with apical cortical patches in hyphae (Fig. 3A and C). MoAbp1-GFP appears in the center of the appressoria as patches or puncta and surrounds the actin ring (Fig. 3B). When hyphae and appressoria were treated with latrunculin B (Lat B), an actin polymerization inhibitor, the subcellular localization pattern of MoAbp1-GFP

was completely disrupted. Instead of forming patches, the GFP signal was mainly observed as large cytoplasmic aggregates (Supplementary Fig. S3).

In addition to MoAbp1, MoCap1, encoded by MGG_01722.6, which shares aa sequence similarity to *S. cerevisiae* cyclase-associated protein (Cap1) Srv2, was also among those with which MoArk1 interacts (Li et al. 2017a). Cap1 is a multifunctional protein and its conserved C-terminal domain serves an important cytoskeletal regulatory function in vivo (Mattila et al. 2004; Zhou et al. 2012). We again verified the MoArk1-MoCap1 interaction through Y2H. However, an interaction cannot be reproduced (Supplementary Fig. S4), suggesting that the interaction may not be direct and additional proteins are needed.

In *S. cerevisiae*, Cap1 can bind to Abp1 through the SH3 domain (Lila and Drubin 1997). To detect this interaction between MoAbp1 and MoCap1, we again performed Y2H, co-IP, and bimolecular fluorescence complementation (BiFC) assays. All three methods successfully demonstrated this interaction (Fig. 4A, B, and C).

Fig. 2. MoAbp1 interacts with MoAct1. **A**, Coimmunoprecipitation assays. The *MoABP1-GFP/MoACT1-S* and *GFP/MoACT1-S* were coexpressed in the wild-type strain, respectively. Total proteins isolated from mycelium of transformants (Total) and proteins eluted from the green fluorescent protein (GFP)-Trap-A beads (Elution) were analyzed by Western blotting with anti-GFP and anti-S antibodies (Total 1 and Elution 1, *MoABP1-GFP/MoACT1-S*; Total 2 and Elution 2, *GFP/MoACT1-S*). **B**, Yeast two-hybrid assays for the interaction between MoAbp1 and MoAct1. The activating domain (AD) and DNA-binding domain (BD) plasmids were cotransformed into yeast AH109, and transformants were plated on SD-Leu-Trp for 3 days and on selective SD-Leu-Trp-His added with 1 mM 3AT (3-amino-1,2,4-triazole) and 1 mM X-gal. **C**, Bimolecular fluorescence complementation assays for the interaction between MoAbp1 and MoAct1. Vegetative hyphae and conidia of transformants were examined by differential interference contrast and epifluorescence microscopy. The strains expressing the MoAbp1-YFP^N and empty YFP^C, MoAct1-YFP^C and empty YFP^N, empty YFP^N and empty YFP^C constructs were used as negative controls. Bar = 5 μ m.

MoAbp1 contributes to the normal patch localization pattern of MoArk1 and MoCap1 and is crucial for the phosphorylation of MoArk1.

In *S. cerevisiae*, Abp1 is required for the localization of Ark1 and Cap1 (Fazi et al. 2002; Freeman et al. 1996). To examine whether MoAbp1 has a similar function, we constructed the *MoARK1-GFP* and *MoCAP1-GFP* plasmids and transformed them into the Δ *Moabp1* mutant and the WT strains. In the Δ *Moabp1* mutant, the localization of MoArk1-GFP in patches (hyphae, 64%; conidium, 55%; appressorium, 58%) was significantly reduced and the GFP signal was mainly observed in large cytoplasmic aggregates, in contrast to the WT strain (Fig. 5A, B, and C). In addition, in the Δ *Moabp1* mutant, the patch localization pattern of MoCap1-GFP (hyphae, 97%; conidium, 95%; appressorium, 96%) was also completely disrupted. Cortical patches of MoCap1-GFP were scarcely observed and, instead, dispersed and clumpy GFP signals were found (Fig. 5E, F, and G). These results indicated that MoAbp1 is required for the normal patch localization of MoArk1 and MoCap1.

The Ark/Prk serine/threonine kinases initiate phosphorylation cycles that control the endocytic machinery in mammalian cells and in yeast as well as the actin cytoskeleton in yeast (Smythe and Ayscough 2003). As kinases are commonly regulated by phosphorylation (Smith et al. 1993) and yeast Prk1 undergoes cortical localization-dependent autophosphorylation in vivo (Huang et al. 2009), we examined whether MoAbp1 regulates the level of MoArk1 phosphorylation through Phos-tag sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Guan et al. 2016; Kinoshita et al. 2006). The shifts in the mobility of MoArk1-GFP from the WT strain and Δ *Moabp1* mutant cells were examined by immunoblotting with the anti-GFP

antibody. The slower mobility form of MoArk1-GFP was present in WT cells but not in the Δ *Moabp1* mutant cells and the phosphatase-treated WT cells (Fig. 5D), suggesting that MoAbp1 regulates the phosphorylation of MoArk1.

We then observed the localization of MoAbp1-GFP in the Δ *Moark1* and Δ *Mocap1* mutant strains. In Δ *Moark1*, the MoAbp1-GFP patches were aggregated into large clumps (Supplementary Fig. S5), while these aggregates were reduced in Δ *Mocap1*. These results indicated that MoArk1 and MoCap1 also contribute to the normal patch localization pattern of MoAbp1.

MoAbp1 regulates actin architecture.

Actin-binding proteins are key regulators of actin organization and dynamics (Goode et al. 2001). To examine whether the loss of *MoABP1* affects actin organization in *M. oryzae*, we performed an actin assay in the Δ *Moabp1* mutant and the WT strain expressing the actin reporter LifeAct-RFP (Berepiki et al. 2011). The subcellular localization of LifeAct-RFP in vegetative and invasive hyphae was completely disrupted in the Δ *Moabp1* mutant. Of WT, 80% showed apical cortical patches distributed in the periphery of the hyphae. Although 20% of WT exhibited abnormal actin localization, the abnormal actin showed varied patterns. In the Δ *Moabp1* mutant, the mislocalized actin appeared homogeneous with actin patches aggregated as large clumps unevenly distributed inside the cell (vegetative hyphae, 38%; invasive hyphae, 39%) (Fig. 6A and C). In addition, whereas the WT strain showed 81% of appressoria containing an intact actin ring, the null mutant Δ *Moabp1* showed more than 58% of mislocalization of the actin ring (Fig. 6B), suggesting that MoAbp1 regulates the actin architecture.

MoAbp1 is required for normal endocytosis.

Abp1 proteins regulate the actin architecture that is directly related to endocytosis (Goode et al. 2001). To test if MoAbp1 is required for this cellular process, hyphae were stained with *N*-(3-triethylammoniumpropyl)-4-(β -diethylamino-phenyl-hexatrienyl) pyridinium dibromide (FM4-64) dye. FM4-64 was internalized within 1 min in WT hyphae (81%) and complemented null mutants (80%), with the signal appearing on the plasma membrane and endomembrane compartments, including vacuoles and endosomes. When Lat B was added 30 min prior to the addition of FM4-64 and was kept present during the course of the experiment, FM4-64 internalization did not occur until 40 min after staining in about 75% of the mutant hyphae. Even at 40 min, only little FM4-64 was internalized (Fig. 7A). Similarly, the internalization of FM4-64 was delayed in the hyphae of the Δ *Moabp1* mutant. The internalization of FM4-64 did not occur until 4 min after staining in about 76% of the mutant hyphae. At 5 min, vesicular fluorescence was observed in the hyphae of the Δ *Moabp1* mutant (Fig. 7B). We then observed FM4-64 uptake in Δ *Mocap1* and Δ *Moark1* mutants and found that both mutants exhibited a delay in endocytosis (Fig. 7C and D). We also observed a similar delay of FM4-64 uptake in appressoria (Supplementary Fig. S6). These results indicated that MoAbp1, MoArk1, and MoCap1 are all required for endocytosis during hyphal growth.

MoAbp1 is required for growth and full virulence.

To examine cellular functions of MoAbp1, we measured the expression of *MoABP1* at various developmental stages by quantitative real-time polymerase chain reaction (qRT-PCR). *MoABP1* was expressed at all stages examined, with the highest transcript level (4.42-fold) occurred at the 8-h infection stage when compared with the mycelial stage (Supplementary Fig. S7), suggesting a potential role in infection.

Fig. 3. Assays for colocalization of MoAbp1 and F-actin. **A**, MoAbp1 colocalizes with the F-actin in vegetative hyphae. Vegetative hyphae of the transformants expressing the MoAbp1-GFP (green fluorescent protein) fusion construct with the established F-actin marker LifeAct-RFP (red fluorescent protein) were observed after incubated in liquid complete medium for 48 h. **B**, Appressoria of the transformants expressing the MoAbp1-GFP fusion construct with the established F-actin marker LifeAct-RFP were observed after conidia incubated on hydrophobic glass plates for 24 h. **C**, MoAbp1 colocalizes with the F-actin in invasive hyphae. The observation was carried out at 24 h postinoculation. Bar = 5 μ m.

To assess the role of MoAbp1 in virulence, we inoculated conidia of the WT strain, $\Delta Moabp1$ mutants, and complemented null mutants onto rice seedlings at a concentration of 5×10^4 conidia per milliliter. $\Delta Moabp1$ showed significantly impaired virulence with few blast lesions developed on rice seedlings (Fig. 8C). To further observe the defect in virulence, we examined *M. oryzae* 28S rDNA, relative to rice Rubq1 DNA, by qRT-PCR. The results indicated that the expression level of rice blast fungus growth was markedly decreased in the $\Delta Moabp1$ mutant (Fig. 8D). To provide another perspective of virulence attenuation, we infected barley epidermal cells with the same set of strains. Penetration and invasive growth of the $\Delta Moabp1$ mutant were again significantly attenuated. More than 18% failed to show appressorium penetration (type 1) and less than 63% extended invasive hyphae (types 3 and 4) were

Since the appressorial turgor pressure is critical for host penetration, we measured the appressorial turgor pressure using the cytorrhysis assay. $\Delta Moabp1$ mutants showed an increased appressorial collapsing rate in 1 to 4 M glycerol solutions (Fig. 8G), suggesting that the turgor pressure is significantly lower than that in the WT strain.

MoAbp1 is a multidomain protein containing an N-terminal ADFH domain, a C-terminal E-SH3 domain, and a F-SH3 domain (Fig. 9A). To examine the interactions of each of these domains with MoAct1, MoArk1, and MoCap1, we performed

Fig. 4. MoAbp1 interacts with MoCap1. **A**, Coimmunoprecipitation assays. The *MoABP1*-GFP/*MoCAP1*-S and GFP/*MoCAP1*-S were coexpressed in the wild-type strain, respectively. Total proteins isolated from mycelium of transformants (Total) and proteins eluted from the green fluorescent protein (GFP)-Trap-A beads (Elution) were analyzed by Western blotting with anti-GFP and anti-S antibodies (Total 1 and Elution 1, *MoABP1*-GFP/*MoCAP1*-S; Total 2 and Elution 2, GFP/*MoCAP1*-S). **B**, Yeast two-hybrid assays for the interaction between MoAbp1 and MoCap1. The activating domain (AD) and DNA-binding domain (BD) plasmids were cotransformed into yeast AH109, and transformants were plated on SD-Leu-Trp for 3 days and on selective SD-Leu-Trp-His-Ade with 1 mM X-gal for 5 days. **C**, Bimolecular fluorescence complementation assays for the interaction between MoAbp1 and MoCap1. Vegetative hyphae and conidia of transformants were examined by differential interference contrast and epifluorescence microscopy. The strains expressing the MoAbp1-YFP^N and empty YFP^C, MoCap1-YFP^C and empty YFP^N, empty YFP^N and empty YFP^C constructs were used as negative controls. Bar = 5 μ m.

the Y2H assay that showed MoAbp1 interacts with MoAct1 via the ADFH domain (Fig. 9B; Supplementary Fig. S8), MoArk1 via F-SH3, and MoCap1 via E-SH3 (Fig. 9C and D). These results suggested that MoAbp1 may encode different functions through its different domains.

As an actin-binding protein, MoAbp1 interacts and colocalizes with actin (Figs. 2 and 3). Since MoAbp1 associated with MoAct1, MoArk1, and MoCap1 via its distinct domains, we examined the regulatory effects of these three domains on the subcellular localization of MoAbp1. Regardless of which of the MoAbp1 domains was deleted, the assembly of actin was abnormal. MoAbp1^{ΔE-SH3}-GFP and MoAbp1^{ΔF-SH3}-GFP colocalize with actin patches, while the localization of MoAbp1^{ΔADFH}-GFP to actin patches was markedly reduced (Fig. 9E). These observations indicated that the ADFH domain is required for correct localization of MoAbp1. These results also indicated that the SH3 domains of MoAbp1 could mediate, in part, the localization to actin patches, likely by interacting with other patch components in the absence of the ADFH domain.

All MoAbp1 domains are required for normal actin architecture and proper endocytosis, growth, and pathogenicity.

To further analyze individual domain function, we generated domain-deletion constructs, MoABP1^{ΔADFH}-GFP, MoABP1^{ΔE-SH3}-

GFP, and MoABP1^{ΔF-SH3}-GFP, and transformed them into the *ΔMoabp1* mutant. The resulting transformants were screened for the GFP signal. In addition, we transformed LifeAct-RFP into the transformants displaying the GFP signal. Microscopic observations indicated that the subcellular localization patterns of LifeAct-RFP in all domain-deletion mutants were disordered. In *ΔMoabp1/MoABP1^{ΔADFH}* and *ΔMoabp1/MoABP1^{ΔE-SH3}* mutants (39 and 41%, respectively), the localization of actin patches to the hyphae tip was significantly reduced compared with the WT. In the *ΔMoabp1/MoABP1^{ΔF-SH3}* mutant (40%), the actin patches were gathered in plaques instead of forming apical cortical patches, as seen in WT strains (Fig. 10A). However, during appressorium formation, the actin ring was formed at the base of the infected cell surrounding the appressorium pore in the WT strain (82%), whereas actin was gathered in two to four clumps in *ΔMoabp1/MoABP1^{ΔADFH}* (40%), *ΔMoabp1/MoABP1^{ΔE-SH3}* (39%), and *ΔMoabp1/MoABP1^{ΔF-SH3}* (41%) mutants, similar to the observations in *ΔMoabp1* (Fig. 10B). These data suggested that all domains of MoAbp1 are required for actin organization in both vegetative hyphae and in appressorium actin ring formation.

Next, we examined whether all MoAbp1 domains are also required for normal endocytosis. FM4-64 dye was internalized and the clear vesicular fluorescent signal was observed in the hyphae of WT strain within 1 min (80%), but, similar to *ΔMoabp1*, there was a delay of 5 min in *ΔMoabp1/MoABP1^{ΔADFH}* (77%),

Fig. 5. MoAbp1 is required for the normal patch localization pattern of MoArk1 and MoCap1 and is crucial for MoArk1 phosphorylation. **A** to **C**, Observation of subcellular localization of MoArk1 in hyphae, conidia, and appressorium of different indicated strains. **D**, Phos-tag assay analysis of MoArk1 phosphorylation. The green fluorescence MoArk1 fusion protein (MoArk1-GFP) from the *ΔMoabp1* mutant cell extracts treated with phosphatase inhibitors were subjected to Phos-tag sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and normal SDS-PAGE followed by immunoblotting with the anti-GFP antibody. The MoArk1-GFP from wild-type strain cell extracts treated with phosphatase inhibitors or calf intestinal alkaline phosphatase were used as controls. **E** to **G**, Subcellular localization of MoCap1 in hyphae, conidia, and appressorium of different indicated strains. Bar = 5 μm.

$\Delta Moabp1/MoABP1^{\Delta E-SH3}$ (75%), and $\Delta Moabp1/MoABP1^{\Delta F-SH3}$ (74%) mutants (Fig. 10C). These results suggested that all MoAbp1 domains are essential for proper endocytosis.

Finally, we attempted the testing of individual MoAbp1 domains in growth and pathogenicity. Compared with the WT and complemented strains, the growth rates of all individual domain-deletion mutants were significantly reduced, similar to the $\Delta Moabp1$ mutant. In addition, the domain-deletion mutants produced few and small lesions on rice leaves, similar to the $\Delta Moabp1$

mutant (Fig. 10D). These results indicated that all the ADFH, E-SH3, and F-SH3 domains are critical for the function of MoAbp1 in regulating the growth and pathogenicity of *M. oryzae*.

DISCUSSION

Intracellular trafficking and associated actin cytoskeleton dynamics are conserved cellular processes that are fundamental to the growth and development of living cells. In the rice blast

Fig. 6. MoAbp1 regulates actin architecture. **A**, Actin morphologies in hyphae of wild type (WT) and mutants. Micrographs of F-actin were observed by express LifeAct-RFP (red fluorescent protein) in WT and mutants. Hyphae of the WT and mutants expressing LifeAct-RFP were cultured in liquid complete medium for 48 h. The numbers indicate the percentage of the hyphae exhibiting the actin morphology as seen in the figure. The experiment was repeated three times ($n = 100$). **B**, Actin morphology in appressoria of WT and mutants. Conidial suspensions (3×10^4 conidia per milliliter) were inoculated onto glass coverslips and were incubated for 24 h. The numbers indicate the percentage of the appressoria exhibiting the actin morphology as seen in the figure. The experiment was repeated three times ($n = 100$). **C**, Actin morphologies in invasive hyphae of WT and mutants. Three-week-old rice cultivar CO-39 was inoculated with 100 μ l of conidial suspension (1×10^5 spores/ml) on the inner leaf sheath cuticle cells and was incubated under humid conditions at 28°C. These pictures were taken after incubation for 24 h. The numbers indicate the percentage of the invasive hyphae exhibiting the actin morphology as seen in the figure ($n = 100$). Bar = 5 μ m.

fungus *M. oryzae*, such processes not only govern fungal growth and development but also pathogenicity. One of our ongoing research efforts is to understand the mechanism by which membrane transport and actin cytoskeleton maintenance play in the formation and function of the infectious structure named appressorium, a fungal structure critical for fungal penetration of the host cell. One of these studies indicated that the actin-regulating kinase MoArk1 regulates intracellular trafficking and is required for conidial development, stress resistance, and pathogenicity (Wang et al. 2013). To explore targets of MoArk1, we employed affinity purification coupled with mass spectrometry to identify candidate proteins that associate with MoArk1. As a result, we identified the actin-binding protein MoAbp1 and characterized its function.

Consistent with findings in the budding yeast, we showed that MoAbp1 interacts with MoAct1, MoArk1, and MoCap1, and these interactions are mediated by the ADFH, F-SH3, and E-SH3 domains, respectively. Further individual domain deletion and functional characterization provided substantial evidence indicating that MoAbp1 is required for the proper localization and, thereby, functions of MoArk1 and MoCap1. The evidence strongly suggested that MoAbp1 functions as an adaptor protein linking MoArk1 and MoCap1 to MoAct1.

In yeast, endocytosis is mediated by actin patch assembly and movement (Huckaba et al. 2004; Kaksonen et al. 2003, 2005; Newpher et al. 2005; Walther et al. 2006). In this study, we found

that MoAbp1 mediates the localization of MoCap1 to cortical patches via E-SH3. In addition, a mutant with deletion of the E-SH3 domain of MoAbp1, $\Delta Moabp1/MoABP1^{\Delta E-SH3}$, still showed defects of actin assembly and endocytosis. The growth and virulence of $\Delta Moabp1/MoABP1^{\Delta E-SH3}$ were also similar to $\Delta Moabp1$. We also found that MoAbp1 mediates the localization of MoArk1 to cortical patches via F-SH3 that is crucial for the phosphorylation of MoArk1. A mutant with deletion of the F-SH3 domain of MoAbp1, $\Delta Moabp1/MoABP1^{\Delta F-SH3}$, showed defects in actin assembly and endocytosis. All evidence indicated that MoAbp1 modulate actin architecture and endocytosis by regulating the functions of MoArk1 and MoCap1 through linking them to actin patches.

In *M. oryzae*, MoArk1 and MoCap1 all contain a proline-rich (PR) motif that likely binds to the SH3 domain of MoAbp1 to control the localization of MoArk1 and MoCap1 to sites of actin assembly and this trait is essential for full virulence (Wang et al. 2013; Zhou et al. 2012). When the PR motif is deleted, the MoArk1^{ARPTAPPKP}-GFP protein was dispersed in the cytoplasm instead of showing the actin-like localization pattern and could not suppress the virulence defect of $\Delta Moark1$ (Wang et al. 2013). Similarly, in the $MoCAP1^{\Delta P2}$ transformants, the GFP signal was observed in the cytoplasm instead of the actin-like localization pattern seen in the WT strain, and the second PR motif deletion mutant, i.e., $\Delta Mocap1/MoCAP1^{\Delta P2}$, exhibited reduced pathogenicity (Zhou et al. 2012). Although

Fig. 7. MoAbp1, MoArk1, and MoCap1 are required for proper endocytosis. **A**, Latrunculin B (Lat B) blocked endocytosis. The wild-type (WT) strains were grown in liquid complete medium (CM) for 48 h and were pretreated with 0.08 μ g of Lat B per milliliter for 30 min or not, prior to the addition of FM4-64. Lat B was added and was kept present during the course of the FM4-64 dye. Photographs were taken under confocal fluorescence microscope after different times of exposure to FM4-64. **B** to **D**, MoAbp1, MoArk1, and MoCap1 are required for proper endocytosis. Strains were grown in liquid CM for 48 h before the addition of FM4-64, and photographs were taken under a confocal fluorescence microscope after different times of exposure to FM4-64. Camera exposure is indicated in seconds (800 ms). The experiment was repeated three times with the same results. The numbers indicate the percentage of the hyphae exhibiting different endocytosis as seen in the figure ($n = 100$). Bar = 5 μ m.

Fig. 8. MoAbp1 is important for growth and full virulence. **A** and **B**, Measure and statistical analysis of colony diameters following growth for 7 days. The indicated strains were inoculated on complete medium (CM), minimal medium (MM), straw decoction and corn agar (SDC), and oatmeal agar (OM) and were cultured at 28°C for 7 days in the dark. Error bars represent standard deviation (SD) and double asterisks indicate statistically significant differences (**, $P < 0.01$). **C**, Rice (*Oryza sativa* cv. CO39) seedlings were sprayed with conidial suspensions and were examined 7 days postinoculation. **D**, Severity of blast disease was evaluated by quantifying *Magnaporthe oryzae* genomic 28S rDNA relative to rice Rubq1 DNA. Mean values of three determinations with standard deviations are shown. Error bars represent the SD and double asterisks indicate statistically significant differences (**, $P < 0.01$). **E** and **F**, Close observation and statistical analysis of infectious growth on barley. Excised barley leaves from 7-day-old barley seedlings were inoculated with conidial suspension (5×10^4 spores/ml of each strain). Infectious growth was observed at 24 h postinoculation. Four types of invasive hyphae were observed in barley tissues. Statistical analysis was made for each type of infectious hyphal shape. For each tested strain, 100 infecting hyphae ($n = 100$) were counted per replicate and the experiment was repeated three times. Bar = 10 μ m. **G**, Statistical analysis of collapsed appressorium after 24 h of incubation in 1, 2, 3, and 4 M glycerol solution for 5 min. For each glycerol concentration, at least 100 appressoria were observed and the number of collapsed appressoria was counted. Error bars represent the SD and double asterisks indicate statistically significant differences (**, $P < 0.01$).

Fig. 9. MoAbp1 interacts with MoAct1, MoArk1, and MoCap1, respectively, through the actin-depolymerizing factor homology (ADFH), free SH3 (F-SH3), and extended SH3 (E-SH3) domains and the ADFH domain is crucial for MoAbp1 normal localization. **A**, Schematic representation of MoAbp1. **B** to **D**, Yeast two-hybrid assays for the interaction between domains of MoAbp1 and MoAct1, MoArk1, MoCap1. The activating domain (AD) and DNA-binding domain (BD) plasmids were cotransformed into yeast AH109, and transformants were plated on SD-Leu-Trp for 3 days and on selective SD-Leu-Trp-His added with 1 mM 3AT (3-amino-1,2,4-triazole) and 1 mM X-gal or SD-Leu-Trp-His-Ade with 1 mM X-gal for 5 days. **E**, The complement construct *MoABP1*-GFP (green fluorescent protein) and domain-deletion constructs *MoABP1*^{ΔADFH}-GFP, *MoABP1*^{ΔE-SH3}-GFP, and *MoABP1*^{ΔF-SH3}-GFP were cotransformed with LifeAct-RFP into $\Delta Moabp1$ mutant. The resulting transformants were cultured in liquid complete medium for 48 h and were examined under a confocal fluorescence microscope. Bar = 5 μ m.

Fig. 10. All MoAbp1 domains are required for normal actin architecture and proper endocytosis and growth and pathogenicity. **A**, Actin morphology of hyphae from wild type (WT) and domain-deletion mutants. The domain-deletion constructs $MoABP1^{\Delta ADFH}$, $MoABP1^{\Delta E-SH3}$, and $MoABP1^{\Delta F-SH3}$ were cotransformed with LifeAct-RFP (red fluorescent protein) into the $\Delta Moabp1$ mutant. Hyphae of indicated strains were cultured in liquid complete medium (CM) for 48 h. **B**, Actin morphology in appressoria of WT and domain-deletion mutants. Conidial suspensions at 3×10^4 conidia/ml were inoculated onto glass coverslips for 24 h and were examined under a confocal fluorescence microscope. Bar = 5 μ m. **C**, All domains of MoAbp1 are required for proper endocytosis. Hyphae of indicated strains were cultured in liquid CM for 48 h, were stained with FM4-64, and were examined under a confocal fluorescence microscope. Bar = 5 μ m. **D**, All domains of MoAbp1 are required for growth and pathogenicity. Seven-day-old cultures of different strains on CM plates; $\pm$ standard deviation was calculated from three repeated experiments and upper case letters indicate statistically significant differences ($P < 0.01$). Disease symptoms on rice seedlings sprayed with conidial suspensions at 7 days postinoculation.

Cap1 is able to interact with G-actin, the localization of Cap1 to sites of actin rearrangement still needs the P2 region to bind to the SH3 domain of Abp1 (Freeman et al. 1996; Mattila et al. 2004; Zhou et al. 2012). In this study, MoAbp1 was shown to link MoArk1 and MoCap1 to actin patches via the F-SH3 and E-SH3 domains, respectively. $\Delta Moabp1/MoABP1^{\Delta E-SH3}$ and $\Delta Moabp1/MoABP1^{\Delta F-SH3}$ mutants also exhibited abnormal actin assembly, delayed endocytosis, slow growth, and reduced virulence. These observations also confirmed that MoAbp1 regulates the functions of MoArk1 and MoCap1 to modulate the architecture of actin and endocytosis that govern the growth and pathogenicity of *M. oryzae*.

Our study showed that MoAbp1 is also required for proper endocytosis. Endocytosis is a process involved in transporting and sorting membrane proteins and lipids, absorption of nutrients, regulation of membrane receptors, and maintenance of cell polarity in eukaryotic cells (Higuchi et al. 2009; Peñalva 2010). Previous studies have shown that endocytosis directly or indirectly contributes to the growth and virulence of many pathogenic fungi. In the corn pathogen *Ustilago maydis*, the tSNARE protein Yup1 was found to mediate endocytosis to govern pathogenicity (Fuchs et al. 2006; Wedlich-Söldner et al. 2000). The vacuolar protein sorting protein Vps27 is essential for proper endocytosis and contributes to hyphal growth and full virulence of *Fusarium graminearum* (Xie et al. 2016). Our previous studies showed that the Eps15 domain-containing protein MoEnd3 regulates appressorium formation and virulence through mediating endocytosis (Li et al. 2017b). The F-actin capping proteins MoCAP coordinate endocytosis and actin dynamics to govern the development and virulence (Li et al. 2017a). MoEnd3 and MoCAP proteins are regulated by MoArk1 through phosphorylation (Li et al. 2017a and b). In addition, we have shown that MoArk1 mediates endocytosis to regulate the growth and pathogenicity of *M. oryzae* (Wang et al. 2013). Moreover, we have also found that SNARE proteins MoSec22 and MoVam7 are both required for the normal patch localization pattern of MoArk1, proper endocytosis, growth, and pathogenicity (Dou et al. 2011; Song et al. 2010; Wang et al. 2013). Here, we further our studies by demonstrating that

the actin-binding protein MoAbp1 is also required for the normal patch localization pattern of MoArk1, proper endocytosis and F-actin assembly, and for growth and pathogenicity. Taken together, evidence is overwhelming that any disturbance to normal endocytosis and associated F-actin organization would result in defects in growth and development of the fungus, even pathogenicity.

We thus propose a model that MoAbp1 is a protein chaperon in association with at least MoArk1 and MoCap1 in *M. oryzae* (Fig. 11). MoAbp1 colocalizes with actin patches through the ADFH domain and recruits MoArk1 and MoCap1 to actin patches through F-SH3 and E-SH3 domains. In this way, MoAbp1 mediates F-actin assembly and endocytosis to regulate growth and pathogenicity of *M. oryzae*.

MATERIALS AND METHODS

Strains and culture conditions.

The *M. oryzae* Guy11 strain was used as the WT strain for transformation and all strains were maintained on CM plates at 28°C. For vegetative growth, small hyphae blocks cut from the edge of 3-day-old cultures were placed onto fresh media plates (CM, MM, OM, and SDC) and were cultured in the dark at 28°C for 7 days (Zhang et al. 2011a). For conidiation, mycelium blocks were maintained on SDC (100 g of straw, 40 g of corn powder, 15 g of agar in 1 liter of distilled water) agar media at 28°C for 7 days in the dark, followed by 3 days continuous illumination under fluorescent light (Zhang et al. 2011a).

Gene disruption, domain deletion, and complementation.

MoABP1 targeted gene replacement was performed using one-step replacement strategy. Two 1.0-kb sequences flanking the targeted gene *MoABP1* were amplified with primer pairs (Supplementary Table S1). The resulting PCR product of *MoABP1* was digested with restriction endonucleases and was ligated with the *HPH* cassette amplified from pCX62. The 3.4-kb completed fragments containing the flanking sequences and hygromycin resistance cassette were amplified and were

Fig. 11. A working model of MoAbp1 function. During growth, appressorium formation, and infection of *Magnaporthe oryzae*, the actin network is a very dynamic structure with continuous polymerization and disassembly. MoAbp1 is localized to actin patches through the actin-depolymerizing factor homology (ADFH) domain. It recruits MoArk1 and MoCap1 to actin patches through the free SH3 (F-SH3) and extended SH3 (E-SH3) domains, respectively. In this way, MoAbp1 mediates endocytosis and F-actin assembly to regulate growth and pathogenicity in *M. oryzae*. Solid arrows indicate conclusions based on our studies.

transformed into protoplasts of Guy11. Putative mutants were screened by PCR and were confirmed by Southern blot analysis. The complement fragments, which contain the *MoABP1*, *MoABP1*^{ΔADF_H}, *MoABP1*^{ΔE-SH₃}, and *MoABP1*^{ΔF-SH₃} with its native promoter, were amplified with primers, were cloned into pYF11 (bleomycin resistance) by the yeast gap repair approach (Bruno et al. 2004), and were transformed into protoplasts of the *ΔMoabp1* mutant.

Virulence assays.

Two-week-old rice seedlings (*Oryza sativa* cv. CO-39) were sprayed with 5 ml of conidial suspension of 5×10^4 conidia per milliliter and were incubated as described previously (Zhang et al. 2011b). Lesion formation was examined at 7 days after inoculation. Conidial suspension with a concentration of 5×10^4 conidia per milliliter in a 0.2% (wt/vol) gelatin solution were spotted on the lower epidermis of barley leaves (*H. vulgare* cv. Four-arris) and were then incubated in a moist and dark chamber at 28°C. Microscopy observations were performed at 24 h after inoculation, respectively. For the 'relative fungal' assay, total DNA was extracted from 1.5 g of diseased leaves and was tested by qRT-PCR with 28S/Rubq1-specific primer sets (Guo et al. 2010).

Endocytosis assays.

FM4-64 staining was conducted following the procedure previously described (Dou et al. 2011). Hyphae of all mutant strains were cultured in liquid CM for 48 h, were stained with FM4-64 followed by double-distilled H₂O washing, and were then examined under a confocal fluorescence microscope. Hyphae of WT strains were grown in liquid CM for 48 h and were pretreated or not with Lat B for 30 min prior to the addition of FM4-64. Lat B was added and kept present during the course of the FM4-64 dye. Photographs were taken under confocal fluorescence microscope (Zeiss LSM710, 63× oil) after a different time of exposure to FM4-64.

Co-IP.

To test the interactions between MoAbp1 and MoAct1, MoCap1 in vivo, the *MoABP1* was cloned into pYF11 and *MoACT1* and *MoCAP1* were cloned into pXY203, respectively, by the yeast gap repair approach (Fischer-Parton et al. 2000). The pYF11-*MoABP1* was cotransformed with pXY203-*MoACT1* or pXY203-*MoCAP1* into Guy11 protoplasts. Total proteins were isolated from transformants expressing both MoAbp1-GFP and MoAct1-S or MoCap1-S and were incubated with GFP-Trap-A beads (Chromo Tek). Proteins bound to GFP-Trap-A beads were eluted after incubation. Total proteins and eluted proteins were analyzed by Western blotting with anti-GFP and anti-S antibodies (Abmart).

Y2H assays.

The cDNA of *MoARK1*, *MoACT1*, and *MoCAP1* were cloned into pGBTK7 as the bait constructs, respectively. The prey constructs were generated by cloning *MoABP1* full-length cDNAs into pGADT7. The resulting bait and prey constructs were confirmed by sequencing analysis and were transformed in pairs into yeast strain AH109 and transformants were identified as described (Clontech).

Glutathione-S-transferase (GST) pull-down assays.

To construct GST-fusion plasmids, *MoARK1* was inserted into the vector pGEX4T-2. To construct the His-fusion plasmid, *MoABP1* was inserted into the vector pET-32a. The resultant plasmid was expressed in *Escherichia coli* BL21 (DE3). Pull-down assay was conducted following the procedure previously described (Zhong et al. 2016). Briefly, soluble proteins were

incubated with 50 μl of glutathione agarose beads for 1 h at 4°C. The beads were washed five times and were then incubated with an equal amount of bacterial lysates containing His-MoAbp1 for another hour at 4°C. The beads were washed five times, again, and the presence of His-MoAbp1 was detected by immunoblotting, using anti-His antibody (Abmart), following separation by electrophoresis.

BiFC assays.

MoABP1-YFP^N was generated by cloning *MoABP1* fragments into pHZ65. *MoARK1*, *MoACT1*, and *MoCAP1* were cloned into pHZ68 to generate, respectively, the *MoARK1*-YFP^C, *MoACT1*-YFP^C, and *MoCAP1*-YFP^C fusion constructs. Plasmids *MoABP1*-YFP^N and *MoARK1*-YFP^C, *MoACT1*-YFP^C, or *MoCAP1*-YFP^C were cotransformed into protoplasts of Guy11. Transformants resistant to both hygromycin and zeocin were isolated and examined under a confocal fluorescence microscope (Zeiss LSM710, 63× oil).

Phos-tag analysis.

The *MoARK1*-GFP fusion construct was transferred into the WT strain and the *ΔMoabp1* mutant. The positive transformants were cultured in liquid CM for 48 h. For protein isolation, about 150 to 200 mg of mycelia were ground into powder in liquid nitrogen and were resuspended in 1 ml of extraction buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5 mM EDTA, 0.5% NP40, 1 mM phenylmethylsulfonyl fluoride, 10 μl of protease inhibitor cocktail [Sigma], and 10 μl of phosphatase inhibitor cocktail 3 [Sigma]). For the preparation of the phosphatase-treated cell lysates, the phosphatase inhibitor cocktail was omitted for alkaline phosphatase (2.5 U/ml final concentration) (P6774, Sigma), and the sample was incubated for 1 h with the addition of 1 mM MgCl₂ (37°C). The samples were then resolved on an 8% sodium dodecyl sulfate-polyacrylamide gel prepared with 50 μM acrylamide-pendant Phos-tag ligand (Wako) and 100 μM MnCl₂, according to instructions provided by the Phos-tag Consortium.

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