INTRODUCTION

Rice blast disease caused by the fungus *Magnaporthe oryzae* is a devastating rice (*Oryza sativa*) disease that causes severe losses (Dean et al., 2012; Li et al., 2012; Wilson & Talbot, 2009). *M. oryzae* builds elaborate infection structures and secretes effector proteins into plant tissues to suppress immunity and support pathogen growth (Giraldo et al., 2013). However, the molecular mechanisms in these processes are not totally understood.

The actin cytoskeleton is an important component of eukaryotic cells and is mainly composed of polymeric filamentous actin (F-actin), motor proteins, actin-binding proteins, and actin-scaffolding septins; actin also occurs as monomeric globular actin (G-actin) (Berepiki et al., 2011; Moseley & Goode, 2006; Schmidt & Hall, 1998). In fungi, the actin cytoskeleton is crucial for cell polarity and growth, cellular division, motility, and host interactions (Giraldo et al., 2013; Li et al., 2012; Wilson & Talbot, 2009). The actin cytoskeleton is also involved in the formation of infection structures, such as appressoria and haustoria, which are essential for pathogen entry and survival inside the host plant (Dean et al., 2012; Li et al., 2012; Wilson & Talbot, 2009).

Abstract

Actin assembly at the hyphal tip is key for polar growth and pathogenesis of the rice blast fungus *Magnaporthe oryzae*. The mechanism of its precise assemblies and biological functions is not understood. Here, we characterized the role of *M. oryzae* twinfilin (MoTwf) in the *M. oryzae* infection through organizing the actin cables that connect to Spitzenkörper (Spk) at the hyphal tip. MoTwf could bind and bundle the actin filaments. It formed a complex with Myosin2 (MoMyo2) and the Woronin body protein Hexagonal peroxisome 1 (MoHex1). Enrichment of MoMyo2 and MoHex1 in the hyphal apical region was disrupted in a *ΔMotwf* loss-of-function mutant, which also showed a decrease in the number and width of actin cables. These findings indicate that MoTwf participates in the virulence of *M. oryzae* by organizing Spk-connected actin filaments and regulating MoHex1 distribution at the hyphal tip.

KEYWORDS

actin cytoskeleton, *Magnaporthe oryzae*, plant fungal disease
the actin cytoskeleton is usually organized as actin patches; F-actin also accumulates at the Spitenkörper (Spk) and forms actin cables (Berepiki et al., 2011; Riquelme, 2013). In Neurospora crassa, Aspergillus nidulans, and Colletotrichum graminicola, actin filaments form a sub-apical collar of actin patches, a concentration of actin at the Spk, or actin cables and patches in the hyphae (Bartnicki-Garcia et al., 2018; Berepiki et al., 2010; Delgado-Alvarez et al., 2010; Echauri-Espinosa et al., 2012; Upadhyay & Shaw, 2008). In M. oryzae, a toroidal F-actin ring assembles in the appressorium to facilitate breaching of the leaf surface by the rigid penetration peg (Dagdas et al., 2012; Dulal et al., 2021) and a bowl-like actin structure capping the hyphal tip was observed during M. oryzae hyphal polar growth (Li et al., 2020). These observations of the diverse organization of the actin cytoskeleton in fungi provide crucial information for understanding the functions of the actin cytoskeleton in fungal development and pathogenesis.

The actin cytoskeleton has been implicated in various crucial fungal processes: polar growth, cytokinesis, endocytosis, exocytosis, and cell shape determination (Echauri-Espinosa et al., 2012; Fischer et al., 2008; Harris, 2006; Jin et al., 2011; Papadopoulos, 2017; Tang et al., 2016). Much progress in recent decades has been achieved in elucidating the crucial roles of the actin cytoskeleton in fungal morphogenesis and pathogenesis, especially in M. oryzae. The M. oryzae actin motor proteins, Myosin 2 (MoMyo2) and MoMyo5, and the actin-binding proteins, MoCap, the endocytic proteins, MoEnd3, and Coronin were reported to be key factors in M. oryzae development and virulence (Dulal et al., 2021; Guo et al., 2017; Li et al., 2017a, 2017b; Motaung & Tsilo, 2017; Tang et al., 2018). In addition, septin proteins, which scaffold cortical F-actin at the point of plant infection in the M. oryzae appressorium, provide cortical rigidity for physically rupturing the leaf cuticle (Dagdas et al., 2012). The actin crosslinking protein α-actinin is reported to function in M. oryzae mitogen-activated protein kinase, Pmk1-mediated fungal penetration from a rice cell to its neighbour (Sakulkoo et al., 2018). The actin cytoskeleton is implicated in appressorium turgor, which facilitates the invasion of host cells (Ryder et al., 2019).

Twinfilin is a member of the actin depolymerization factor-homology (ADF-H) domain protein family and usually consists of two ADF-H domains. Twinfilin binds ADP-bound G-actin to inhibit the assembly of the actin filament (Vartiainen et al., 2000) or directly interacts with the barbed ends of actin filaments to accelerate actin depolymerization (Johnston et al., 2015). A mouse (Mus musculus) Twinfilin protein, mTwf1, has been shown to bind CARMIL, which protects the capping protein from barbed-end displacement (Johnston et al., 2018). These findings indicate that Twinfilin is a key actin-associated protein that functions in actin assembly through various regulations. Currently, we do not know the roles of Twinfilin in actin organization and in the development or pathogenesis of M. oryzae.

The Woronin body (WB) is a peroxisome-derived organelle that is unique to filamentous ascomycete fungi and functions to maintain cellular integrity after hyphal damage by sealing septal pores, thereby preventing cytoplasmic leakage. The WB major protein Hexagonal peroxisome 1 (Hex1) self-assembles into extremely large protein complexes that form the dense core of the WB (Jedd & Chua, 2000). Deletion of Hex1 in N. crassa results in bleeding of cytoplasm through septal pores after cellular damage (Jedd & Chua, 2000; Tenney et al., 2000). WBs are also important for pathogenesis of fungal pathogens. In M. oryzae and Fusarium graminearum, lack of WBs results in defects in appressorium formation and host plant infection (Son et al., 2013; Soundararajan et al., 2004).

Recently, we revealed that M. oryzae forms a unique actin structure at the hyphal tip that governs hyphal polar growth and infection (Li et al., 2020). In this study, we further characterized the role of an actin-related protein, MoTwf, in M. oryzae hyphal tip actin organization. We demonstrate that MoTwf localizes at the Spk-connected actin cables at the subapical region of the growing hypha. MoTwf possesses actin binding and bundling activity, and M. oryzae lacking MoTwf was compromised to assemble the Spk-connected actin cables in endocytosis and protein secretion. Furthermore, we show that MoTwf can form a complex with the actin motor protein MoMyo2 and the WB major protein MoHex1. Distribution of MoHex1 at the hyphal tip region depends on MoTwf-mediated organization of the actin–myosin system. The misdistribution of MoHex1 in the MoTwf loss-of-function mutant led to low levels of reactive oxygen species (ROS) at the hyphal tip region. These results revealed the physiological functions of MoTwf, which plays crucial roles in hyphal tip actin cable assembly and hyphal tip distribution of MoHex1. Our results may provide new insight for understanding the actin organization mechanism in M. oryzae growth and infection.

2 | RESULTS

2.1 | Deletion of the Twinfilin gene decreases the virulence of M. oryzae

Twinfilins are conserved actin-associated proteins and are present in all eukaryotes, except plants (Nevalainen et al., 2009). The functions of Twinfilin in M. oryzae have not been studied. Through sequence BLAST searches and Southern blot analysis, we found that one Twinfilin (MoTwf) gene is present in the M. oryzae Y34 genome (Figure S1a). The phylogenetic alignment shows that Twinfilin proteins from fungi, yeast, and mammals formed different subbranches (Figure S1b). We knocked out MoTwf in M. oryzae strain Y34 (Figure S2) and found that the ΔMoTwf mutant grew moderately slower than the wild-type (WT) and complemented (Com) strains when cultured on complete medium (CM) or straw rice bran (SRB) medium (Figure 1a,b). Next, we found that the conidiation, the morphogenesis of the conidia, and the mycelium of the mutant were not changed significantly (Figure S3). However, when the same number of conidia were used for punch- or spray-inoculation assays for rice leaf infection, the virulence of the ΔMoTwf mutant was significantly decreased compared with the WT and the complemented strain (Figure 1c–g). These results indicated that MoTwf is required for the virulence of M. oryzae.
FIGURE 1  Defects of the ΔMotwf mutant in growth and plant infection. (a, b) Seven-day-old cultures of wild-type (WT), ΔMotwf, and complemented (Com) strains on complete medium (CM) (a) and straw rice bran (SRB) medium (b). The graphs indicate the hyphal growth rate of the WT, ΔMotwf, and complemented strains. The colony diameters were measured 7 days after inoculation. Error bars indicate SD calculated for three replicates. Pathogenicity assay of the WT, ΔMotwf, and complemented strains in punch-inoculation (c) and whole-plant spray inoculation assays (e). The same amount of conidia (10^5 spores/ml) from the WT, ΔMotwf, and complemented strain was used to infect punctured rice leaves or spray to rice leaves (Oryza sativa ‘Nipponbare’). Photographs were taken 6 days after infection. Quantification of the lesion area (d) in punch inoculation, and lesion numbers per cm² (f) or lesion area (g) in spray inoculation assay of the rice leaves are calculated from (c) and (e), respectively. Error bars represent SD (n = 20) and asterisks (***) represent a significant difference (p < .001).
2.2 | MoTwf contributes to the organization of Spk-connected actin cables

Given that Twinfilin is associated with actin, we next investigated the actin cytoskeleton in the WT and the ΔMotwf mutant by using the Lifeact-green fluorescent protein (GFP) labelling system (a marker used for visualizing F-actin) (Dagdas et al., 2012; Qu et al., 2017). A cluster of actin was observed to accumulate at the growing hyphal tip in the WT hyphae. This included the actin at the Spk, Spk-connected actin cables, and small actin patches distributed at the collar region and cytoplasm (Figures S4 and 2a, and Movie S1a) as we reported previously. However, compared with the WT and the complemented strain, we found that the number and width of Spk-connected actin cables were decreased in the ΔMotwf mutant (Figure 2 and Movie S1). In addition, we also noticed that the area of actin at Spk between the WT and the mutant was not significantly changed (the mean number was 1.35 µm² for the WT and 1.28 µm² for the ΔMotwf mutant), and the growth rate of the mutant was slightly decreased (the mean growth rate was 0.78 µm/min for the WT and 0.71 µm/min for the ΔMotwf mutant; Figure S2d).

2.3 | MoTwf functions in vesicle transport and protein secretion

The actin cables in the ΔMotwf mutant were much less organized compared to the WT, which indicated delivery of vesicles or proteins to the hyphal tip may be compromised. To verify this, we stained the hyphae with FM4-64, a widely used marker of endocytosis. The results showed that in the WT, FM4-64 was quickly internalized into the cytoplasm and transported to the hyphal tip where it colocalized with the actin at the Spk at 0.5 min (Figure S5a). After that, more FM4-64 gradually accumulated at the hyphal tip at 5–15 min (Figure S5a). However, compared with the WT, the internalization and transport of FM4-64 in the ΔMotwf mutant, which had less organized actin cables, were compromised. Even at 15 min after incubation with FM4-64, a considerable amount of the FM4-64 in the cytoplasm could not be delivered to the hyphal tip (Figure S5b). We also examined protein secretion in the WT and ΔMotwf strains. We found that the ΔMotwf mutant exhibited a 50% reduction in protein secretion compared to the WT (Figure S6).

2.4 | MoTwf localizes at the Spk-connected actin cables

Localization of MoTwf was investigated in M. oryzae by a live-cell imaging system. MoTwf-mCherry driven by the MoTwf native promoter and Lifeact-GFP were coexpressed in the ΔMotwf mutant, and the colocalization of GFP and mCherry was observed in growing hypha. Time-lapse imaging of the hypha revealed that MoTwf formed dense patches at the subapical region of the hypha and some of the patches in the cytoplasm localized at the Spk-connected actin cables (Figure 3a and Movie S2). The line-scan analysis further showed that MoTwf could localize to these actin cables, and rare MoTwf spots were detected at the actin at Spk (Figure 3b–d). We drew a simple model of the MoTwf distribution pattern according to our observations (Figure 3e). Localization of MoTwf in invasive hypha was similar to that in the vegetative hypha, in that MoTwf-mCherry was distributed at the hyphal tip area (Figure 3f). Furthermore, we found that MoTwf-mCherry colocalized with the actin cytoskeleton in the M. oryzae conidia, the germ tube, and the appressorium (Figure S7).

2.5 | MoTwf binds to F-actin and facilitates the formation of actin cables

We next examined the biochemical functions of MoTwf in vitro. As shown in Figure 4a (left panel), the F-actin was observed as fine actin filaments after phalloidin staining. However, when F-actin was incubated with 1 µM purified MoTwf, actin cables appeared (Figure 4a, middle panel). Furthermore, 3 µM MoTwf induced larger actin cables (Figure 4a, right panel).

MoTwf is composed of an N-terminal ADF (N-ADF) motif and a C-terminal ADF (C-ADF) motif (Figure S8a). We produced His-tagged recombinant MoTwf and GFP-labelled MoTwf proteins including GFP-MoTwf, MoTwf-GFP, N-ADF-GFP, and C-ADF-GFP (Figure S8b). To further elucidate the molecular mechanism of the actin bundling activity of MoTwf, we incubated the polymerized F-actin with the labelled MoTwf proteins listed above. When we incubated the polymerized F-actin with GFP, it could be observed that GFP did not bind to F-actin (Figure 4b) or affect the polymerization of F-actin (Figure 4a, left panel). However, when GFP-MoTwf was incubated with F-actin, it showed that GFP-MoTwf colocalized with the bundled F-actin, indicating that MoTwf binds directly to F-actin and induces actin cable formation (Figure 4c). Similar results were obtained with MoTwf-GFP (Figure 4d). We then analysed the biochemical properties of the two domains of MoTwf. Comparing with GFP alone (Figure 4e), the N-ADF domain only possessed the actin binding property but the C-ADF domain exhibited both actin binding and bundling activities (Figure 4f, g). We also introduced an M. oryzae actin depolymerization factor, cofilin (MoCof), as a parallel control. We found that F-actin was depolymerized to small fragments in the presence of MoCof (Figure 4h).

We further performed yeast two-hybrid (Y2H) assays to examine the properties of the two MoTwf domains. The results showed that N-ADF but not C-ADF could interact with itself, which indicated that N-ADF could form homodimers (Figure 4i).

2.6 | MoTwf associates with Myosin and MoHex1

To further explore the molecular mechanism of MoTwf in pathogenesis, we performed immunoprecipitation in combination with mass spectrometry (IP-MS) to identify MoTwf-interacting proteins. We then expressed GFP and MoTwf-GFP driven under the promoter of
FIGURE 2  Defects of the ΔMotwf mutant in the organization of Spk-connected actin cables. (a–c) Time-lapse images showing actin dynamics in growing wild-type (WT) (a), ΔMotwf (b), and complemented (c) hyphae. The corresponding movie is provided as Movie S1. The red arrows indicate the presence of the Spk-connected actin cables. The numbers at the top right corner indicate the timestamps (mins:s). Bars = 2 μm. (d) Quantitative analysis of the Spk-connected actin cables. The numbers at the top right corner indicate the timestamps (mins:s). Bars = 2 μm. (d) Quantitative analysis of the mean width of the actin cables, mean number of F-actin, mean area of actin at Spk, and mean growth rate in the WT, ΔMotwf, and complemented strains (100 hyphae each of the WT, ΔMotwf, and complemented strains in three experimental repeats)
MoTwf (termed pMoTwf::GFP and pMoTwf::MoTwf-GFP, respectively) in M. oryzae for IP-MS. GFP and MoTwf-GFP were well expressed and their expressed amount was comparable when examined by fluorescence microscopy and western-blot analysis (Figure S9a,b). Figure 5a lists the most abundant proteins identified from MoTwf-GFP IP-MS samples. The results show that a M. oryzae WB major protein, MoHex1, and an actin motor protein, MoMyo2, were the most abundant. Thus, we speculated that MoTwf may associate with MoHex1 and MoMyo2. The bimolecular fluorescence complementation (BiFC) assays showed that both MoTwf and MoHex1 could interact with MoMyo2 (Figure S9a,b), but no interactions were detected between MoTwf and MoHex1 (not shown). We also noticed that the interactions took place at the hyphal tip area. We then analysed the composition of the domains of the three proteins (Figure 5b) and used Y2H assays to further examine the interactions between their varied domains. We found that while the N-ADF motif of MoTwf could interact with the SH3 motif of MoMyo2, the interaction between MoMyo2 and MoHex1 occurred at the Tail and Hex domain (Figure 5b,e–h). In all, these results indicate that MoTwf may associate with MoMyo2 and MoHex1 to execute its functions.

2.7 MoTwf is involved in distribution of MoMyo2 and MoHex1 at the hyphal tip

The protein interaction analysis indicated that MoTwf may associate with MoMyo2 and MoHex1. To clarify their relationships, we first examined the expression of MoMyo2 and MoHex1 in the WT and ΔMotwf strains. The results showed that the expressions of the two genes were not significantly changed between the two strains (Figure S10). We then investigated their protein distributions in the WT and the ΔMotwf mutant. MoMyo2 accumulated as a bright spot at the WT hyphal tip.
However, in the ΔMotwf mutant, accumulation of MoMyo2 at the hyphal tip was seriously affected. MoMyo2 was uniformly distributed in the ΔMotwf hypha (Figure 6b and Movie S3b). We next investigated the distribution of MoMyo2 in the invasive hypha of the WT and ΔMotwf strains. The results revealed a decreased accumulation of MoMyo2 in the ΔMotwf mutant at the hyphal tip compared with that in the WT invasive hypha (Figure 6c,d). These results indicate that MoTwf is required for the localization of MoMyo2 to the hyphal tip.

2.8 | ΔMotwf mutant has lower level of ROS at the hyphal tip, which is not beneficial for formation of the actin cables

Hex1 protein self-assembles into large protein complexes and forms the dense core of the WB, which is a kind of peroxisome (Jedd & Chua, 2000). Distribution of Hex1 at the hyphal tip was affected in ΔMotwf mutant (Figure 7). We examined the reactive oxygen species (ROS) level in the WT and ΔMotwf strains using the ROS staining dye 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) (Han et al., 2013). We found more ROS accumulated at the hyphal tip region than at the hyphal tip in the WT (Figure 7g,h). These results indicate that MoTwf is required for the localization of MoMyo2 and MoHex1 to the hyphal tip.
other region in the WT. However, in the ΔMotwf mutant, the hyphal tip ROS accumulation pattern was abolished (Figure S11a,b).

Next, we treated the WT hyphae with diphenylene-iodonium chloride (DPI, 0.5 µM) to inhibit ROS production. We found that DPI significantly affected hyphal growth (Figures S11c,d and S12). We also found that DPI-treated WT hyphae were defective in the organization of hyphal tip actin cables when compared with the mock (Figure S11c–e and Movie S5). In all, these results indicate that distribution of MoHex1 at the hyphal tip region may be associated with the high ROS level at the hyphal tip region, and an impaired ROS homeostasis may affect the assembly of actin cables at the hyphal tip.

2.9 | MoTwf functions in organizing actin during invasive hyphal expansion in host plant cells

Penetration assays using rice sheath tissues were conducted to investigate how MoTwf functions in M. oryzae pathogenesis. Four types of invasive hyphae (IH) occur during host infection: type 1, no hyphal penetration; type 2, IH with fewer than two branches; type 3, IH with at least two branches; and type 4, IH that fully occupies a plant cell and moves into neighbouring cells (Li et al., 2017b). Accordingly, we calculated these infection types for the ΔMotwf mutant at 36 h after infection. We found that while most of the WT IH were type 4, those in the ΔMotwf mutant were type 2 and type 3 (Figure 8a,b). This indicates that infection of ΔMotwf was compromised. We next observed the actin organization for the IH of the ΔMotwf mutant. In the newly infected hyphal cells (but not in bulbous invasive hyphae, Figure S13, in the bulbous invasive hyphae, actin usually organized as actin patches), the actin usually accumulated at the hyphal tip in the WT cells (Figure 8c). However, in the ΔMotwf mutant, the accumulation of actin at the hyphal tip was usually abolished (Figure 8d). This may indicate that the polar growth or extension of ΔMotwf mutant in host cells is compromised.

3 | DISCUSSION

Twinfilin is a member of the actin depolymerization factor-homology (ADF-H) domain family of proteins, which is conserved...
across distant species of animals and fungi (Hilton et al., 2018). Mammalian Twinfilin isoforms have been shown to play important roles in regulating various cell functions such as cell migration, stereocilia formation, cancer progression, and platelet activation (Bockhorn et al., 2013; Peng et al., 2009; Rzadzinska et al., 2009; Wang et al., 2010). However, until now, the physiological functions of Twinfilin in filamentous fungi have not been elucidated. In this study, we investigated the roles of the actin-binding protein MoTwf in the model fungus *M. oryzae*. Our results showed that the ΔMotwf mutant exhibited differences in both hyphal development and virulence (Figure 1). These results broaden our knowledge of the crucial roles of the Twinfilin proteins in the pathogenesis of filamentous fungi.

### 3.1 MoTwf binds and bundles actin filaments

Twinfilin is one of five proteins in the ADF-H domain family, of which ADF/Cofilin is the founding member (Poukkula et al., 2011). Twinfilin is unique among the members of this family in that it contains two ADF-H domains that are joined by a linker region. Previously, Twinfilin proteins have been shown to interact with the actin monomer and the barbed ends of actin filaments to inhibit actin subunit addition (Vartiainen et al., 2000; Wahlström et al., 2001), and with F-actin or actin end binding proteins to accelerate the depolymerization at actin filament ends (Hilton et al., 2018; Johnston et al., 2015). Unlike the reported Twinfilin proteins, in this study we found that MoTwf directly bound F-actin to facilitate the formation of actin cables (Figure 4a–d). Further studies showed that the two ADF domains of MoTwf directly bound F-actin, but they possess different actin bundling properties. While the C-ADF domain strongly accelerated the formation of actin cables (Figure 4g), the N-ADF domain may form a homodimer in this process (Figure 4f,i). These results indicate new functions of Twinfilin family proteins in actin polymerization.

Plants do not contain *Twinfilin* genes, but multiple ADF domain-containing genes are found in their genomes. For example, in *Arabidopsis thaliana*, 11 ADF genes have been identified (Ruzicka et al., 2007) and most of these *Arabidopsis* ADFs can sever or depolymerize actin filaments (Maciver & Hussey, 2002; Ren & Xiang, 2007; Zheng et al., 2013). Interestingly, *Arabidopsis* ADF5 and ADF9 show actin-bundling activities, which were distinct from the activities of other conserved ADF family proteins (Nan et al., 2017; Tholl et al., 2011). Like ADF5 and ADF9, in our study the C-ADF motif of MoTwf also exhibited actin bundling activity. These findings indicate that the ADF family proteins show...
broad diversity in their biochemical properties, which also suggests that ADF genes have evolved in a divergent manner in their regulation of the actin cytoskeleton over the course of species diversification.

3.2 | MoTwf functions in organizing the Spk-connected actin cables for hyphal polarized exocytosis and protein secretion

*M. oryzae* dynamically builds elaborate actin structures during its development and host infection process (Fernandez & Orth, 2018; Zhang & Xu, 2014) and the actin cytoskeleton has been recognized as playing a major role in *M. oryzae* development and pathogenesis. In *M. oryzae*, the actin-binding proteins MoCap, MoEnd3, and Septins, the actin motor proteins MoMyo2 and MoMyo5, and the actin-regulating kinase, MoArk1 and Pmk1, which function in actin-associated protein modification, have been shown to have critical functions in the formation of infection structures, hyphal polar growth, endocytosis, and hyphal proliferation inside the host cells (Dagdas et al., 2012; Guo et al., 2017; Li et al., 2017a, 2017b; Sakulkoo et al., 2018; Tang et al., 2018).

*M. oryzae* assembles and relies on a unique actin distribution pattern at the hyphal tip: actin patches, actin at the Spk, and FIGURE 7  MoTwf functions in the distribution of MoHex1 at the hyphal tip. (a, b) MoHex1-mCherry driven by the MoHex1 native promoter was expressed in Lifeact-green fluorescent protein (GFP)-labelled wild-type (WT) (a) or ΔMotwf (b) vegetative hyphae. Time-lapse imaging was conducted to record the dynamic distribution of MoHex1 in the growing hypha. The asterisks indicate distribution of MoHex1 at the cell tip of WT or ΔMotwf hyphae, respectively. The numbers at the top right corner indicate the timestamps (mins). Bars = 5 μm. (c–f) Line-scan analysis of the distribution of MoHex1 related to the actin cytoskeleton. Representative images showing MoHex1-mCherry in Lifeact-GFP-labelled WT (g) or ΔMotwf (h) invasive hyphae, respectively. The asterisks indicate distribution of MoHex1 at the cell tip of the WT or ΔMotwf hyphae, respectively. Bars = 10 μm.
Spk-connected actin cables (Li et al., 2020). Currently, the organization mechanism and their functions in fungal growth and pathogenesis are not known. In this study, we characterized the role of MoTwf in actin assembly. We found that MoTwf localized at the Spk-connected actin cables at the hyphal apex (Figure 3). The ΔMotwf mutant exhibited a decrease in the width and number of actin filaments (Figure 2), coinciding with severely affected exocytosis and protein secretion (Figures S5 and S6). Considering that MoTwf can bind and bundle actin filaments, we speculate that MoTwf plays a role in organizing the Spk-connected actin cables and functions in vesicle delivery and protein secretion during pathogenesis.

3.3 | MoTwf is required for the distribution of MoHex1 at the hyphal tip

The major WB protein Hex1 self-assembles to form the vesicle’s dense core. The WB functions to maintain cellular integrity after hyphal damage. Deletion of Hex1 in N. crassa results in cells that lack WBs, and mutant hyphae bleed cytoplasm through septal pores after cellular damage (Jedd & Chua, 2000; Tenney et al., 2000). WBs have also been suggested to have crucial functions in fungal development and pathogenesis. In M. oryzae, deletion of Hex1 showed that WBs are required not only for appressorial morphogenesis but also for invasive growth in plants by enhancing resistance to nutritional stress (Soundararajan et al., 2004). WBs are distributed at the hyphal tips and septa (Kubo et al., 2015). Currently, we do not know the significance of the hyphal tip-localized WBs and the mechanism of how WBs are distributed at the hyphal tip.

In this study, we found that in the ΔMotwf mutant, accumulation of MoHex1 at the hyphal tip was severely affected (Figure 7). In particular, we found that MoHex1 interacted with the actin motor protein MoMyo2 (Figure 5). Considering that MoTwf is involved in the organization of hyphal tip actin cables and that an interaction occurs between MoTwf and MoMyo2, we speculate that distribution of MoHex1 at the hyphal tip region is dependent on the MoTwf-MoMyo2-organized actin system.
EXPERIMENTAL PROCEDURES

4.1 | Fungal strains and plant material

M. oryzae strain Y34 was used as the WT strain (kindly provided by Professor Lihuang Zhu, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). All strains were cultured on solid CM for growth and SRB medium for conidia generation at 28°C in the dark. Mycelia were harvested from liquid CM and used for genomic DNA and RNA extractions. Y34-susceptible rice (Oryza sativa subsp. japonica ‘Nipponbare’) was used for fungal infection analyses.

4.2 | Targeted gene deletion and plasmid constructions

The deletion of MoTwf in M. oryzae was according to the method reported previously (Tang et al., 2018). All the primers used in the gene deletion experiment are listed in Table S1. The obtained mutants were screened by PCR and followed with one single spore isolation. The complement DNA fragment that contains the entire MoTwf gene driven by the native promoter was amplified by PCR and ligated to mCherry and inserted into pKNTG binary vector.

To construct plasmids expressing MoMyo2-mCherry, MoHex1-mCherry, the related DNA fragments from the M. oryzae Y34 genome, and the 1.5-kb native promoter region were amplified and cloned into the pKNTG binary vector. To construct the Lifeact-GFP plasmid, the Lifeact DNA sequence was ligated to mCherry, the related DNA fragments from the PsulPH vector (Li et al., 2016; Zhou et al., 2017). To construct the plasmids in the BiFC experiments, the open reading frame (ORF) of MoTwf and MoHex1 fusion with N-YFP fragment was cloned into the pKNTG vector. The ORF of MoMyo2, MoHex1 fusion with C-YFP was cloned into the PBHT2 vector. All constructs were generated via homologous recombination cloning (ClonExpress MultiS One Step Cloning Kit; Vazyme Biotech); all primers with restriction enzyme sites are listed in Table S1. The recombinant plasmids were transformed into M. oryzae protoplasts as described previously (Tang et al., 2015).

4.3 | Pathogenicity assay

For determination of the pathogenicity of the ΔMotwf mutant, punch-inoculation assays were performed according to the method described previously (Zhou et al., 2020). The conidia from the WT, ΔMotwf, and complemented strains were harvested from SRB medium and were diluted to the same concentration (10^5 spores/ml). Then the same volume (5 µl) of the conidial suspension was used to infect punched rice leaves (the third leaf of each seedling, cv. Nipponbare). The disease phenotypes of the leaves were observed and imaged at 5 days postinfection (dpi).

For microscopic observation of penetration and expansion of infectious hyphae in rice sheath leaves, 100 µl of the conidial suspension (10^5 spores/ml in 0.2% wt/vol gelatin) was used to infect the inner leaf sheath cuticle cells and incubation under humid conditions at 28°C. The leaf sheaths were observed using confocal microscopy (LSM880; Zeiss).

4.4 | Yeast two-hybrid assay

To validate homodimer formation of the ADF domains and the interactions between MoTwf, MoMyo2, and MoHex1, Y2H assays were performed using the Matchmaker Yeast Two-Hybrid System (Clontech) following the manufacturer’s instructions. The cDNA encoding the ADFs and various indicated domains of MoTwf, MoMyo2, and MoHex1 were cloned into AD and BD vectors. The recombinant plasmids were cotransformed into AH109 cells and their growth examined on double-dropout (DDO) and quadruple-dropout (QDO) media. The pGADT7-pGBKTT7-53 (AD-T/BD-53) plasmid was used as a positive control, and pGADT7/pGBKTT7(AD/BD) was used as a negative control. All the primers are listed in Table S1.

4.5 | In vitro protein purification and F-actin binding/bundling assay

The cDNAs of MoTwf, its two ADF domains, and MoCof were fused to GFP and cloned into the pET28a vector to produce His-tagged MoTwf, MoTwf-GFP, GFP-MoTwf, N-ADF-GFP, C-ADF-GFP, and MoCof-GFP fusion proteins. All primers and restriction enzyme sites are listed in Table S1. All constructs were transformed into Escherichia coli BL21 (DE3). The expression and purification of these recombinant proteins were performed according to the previously reported method (Gao et al., 2021) and the manufacturer’s (TransGen) instructions.

Visualization of actin filaments in the presence of the recombinant proteins was performed by fluorescence microscopy as reported previously (Han et al., 2013). Briefly, prepolymerized F-actin (1 µM) was incubated with MoTwf and its domain proteins (1 µM) at room temperature for 30 min and labelled with Alexa561-phalloidin (Thermo Fisher). The images were obtained under a confocal microscope (LSM880; Zeiss) at 488 or 561 nm.

4.6 | Hyphal FM4-64 and ROS staining and drug treatment

FM4-64 and H2DCFDA (Thermo Fisher) solutions were prepared to examine the vesicle distribution and ROS level in M. oryzae. The M. oryzae hyphae were cultured in liquid CM for 24 h. Then they were stained with FM4-64 (10 µM) or H2DCFDA (5 µM) before being viewed by fluorescence microscopy.

For evaluating hyphal growth in the presence of H2O2 or DPI (ROS generation inhibitor), a conidial suspension (10^5 spores/ml) of the WT, ΔMotwf, and the complemented strains was cultured on solid CM with 3 mM H2O2 or 0.5 µM DPI at 28°C.
4.7 | RNA extraction and quantitative reverse transcription PCR analysis

Total RNA was extracted using a Total RNA Purification kit (TransGen). The extracted RNA was reverse transcribed using the oligo(dT) primer and M-MLV reverse transcriptase (Invitrogen). Quantitative reverse transcription PCR analysis of MoHex1 in the WT, ΔMotwf, and complemented strains was performed using SYBR Green real-time PCR Master Mix (Toyobo), with M. oryzae tubulin used as the internal control (Que et al., 2020). The experiment was conducted twice with three independent biological replicates.

4.8 | Extraction of secreted proteins from M. oryzae mycelia

Extraction of the secreted proteins from the WT, ΔMotwf, and complemented strains was mainly according to the method reported previously (Gupta et al., 2015). Equal weight of WT, ΔMotwf, and the complemented mycelia that were cultured in CM liquid medium were harvested and transferred to liquid glucose minimal medium (GMM) for 24 h. The secreted proteins in the medium were collected, condensed using an ultrafiltration tube (3 kDa; Millipore), and quantified using the Bradford Protein Assay Kit (Beyotime) (Yin et al., 2018).

4.9 | Observation of fluorescent signals by live-cell imaging and actin analysis

For observation of the fluorescence from the vegetative hyphae, mycelia were cultured in liquid CM for 24 h. The actively growing hyphae were observed under a confocal microscope (LSM880; Zeiss) equipped with an Airyscan detector. The maximum projection and time-lapse of the images were used to record the organization of the actin cytoskeleton in fast-growing M. oryzae hyphal cells. The laser lines included GFP (argon laser excitation at 488 nm, emission spectra 500–540 nm) and mCherry (neon laser excitation at 561 nm, emission spectra 600–640 nm).

Image processing and measurements were performed using ImageJ software (v. 1.51 s, http://imagej.nih.gov/ij) as described previously (Tian et al., 2015). Briefly, to analyse the actin area at Spk, images were selected using the “rectangle” tool and the mean grey value of the selected region was calculated using the “Analyze-Measure” plugin. To calculate the width of the F-actin, images were selected using the “straight line” and “Zoom in” tools. The line length of the F-actin width could be calculated according to the scale bar value. To calculate the number of F-actin, images were thresholded to create a binary image, followed by a skeletonization procedure (Process-Binary-Skeletonize), so all actin filaments were shown as black. The total number of black pixels in the images was counted as actin microfilaments.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Sequence data for the genes described in this study can be found in the GenBank/EMBL database at https://www.ncbi.nlm.nih.gov/genbank/ under the accession numbers ELQ35272 (MoTwf), ELQ43604 (MoMyo2), and ELQ36235 (MoHex1).

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Supporting Information

Additional supporting information may be found online in the Supporting Information section.

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