

Septin-Mediated Plant Cell Invasion by the Rice Blast Fungus, *Magnaporthe oryzae*

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Abstract

To cause rice blast disease, the fungus *Magnaporthe oryzae* develops a pressurized dome-shaped cell called an appressorium, which physically ruptures the leaf cuticle to gain entry to plant tissue. Here, we report that a toroidal F-actin network assembles in the appressorium by means of four septin guanosine triphosphatases, which polymerize into a dynamic, hetero-oligomeric ring. Septins scaffold F-actin, via the ezrin-radixin-moesin protein Tea1, and phosphatidylinositide interactions at the appressorium plasma membrane. The septin ring assembles in a Cdc42- and Chm1-dependent manner and forms a diffusion barrier to localize the inverse-bin-amphiphysin-RVS-domain protein Rvs167 and the Wiskott-Aldrich syndrome protein Las17 at the point of penetration. Septins thereby provide the cortical rigidity and membrane curvature necessary for protrusion of a rigid penetration peg to breach the leaf surface.

Rice blast is the most devastating disease of cultivated rice and a constant threat to global food security. Each year up to 30% of the rice harvest is lost to blast disease—enough grain to feed 60 million people—and finding an effective way to control rice blast is therefore a priority. To infect rice leaves, *Magnaporthe oryzae* develops a special infection structure called an appressorium (Fig. 1), which generates turgor of up to 8.0 MPa (equivalent to 40 times that of a car tire) and translates this extreme pressure into physical force to break the leaf surface. Appressorium turgor is generated by rapid influx of water into the cell against a concentration gradient of glycerol, maintained in the appressorium by a specialized, melanin-rich cell wall (1). Turgor is translated into physical force and applied to the leaf surface by a narrow penetration peg that mechanically ruptures the tough leaf cuticle (2, 3). The cellular mechanism by which an appressorium breaches the cuticle is not known.

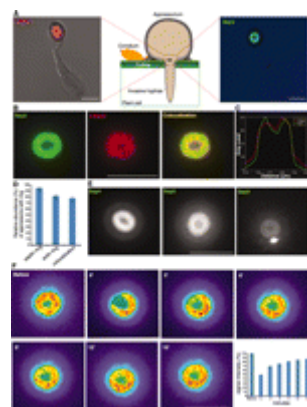


Fig. 1

A toroidal-shaped F-actin network and septin ring assemble at the appressorium pore in *M. oryzae*. (A) Cellular localization of LifeAct-RFP and Sep3-GFP visualized by laser confocal and laser excitation epifluorescence microscopy, respectively. ap indicates appressorium pore. (B) Sep3-GFP and LifeAct-RFP colocalization in the appressorium. (C) Linescan graph consistent with colocalization of the septin ring and F-actin network. (D) Bar charts showing septin ring and F-actin colocalization (mean \pm SD, three experiments, $n = 300$). (E) Sep3-GFP, Sep4-GFP, Sep5-GFP, and Sep6-GFP form a

ring at the appressorium pore. Sep6 also forms a distinct punctum. **(F)** Recovery of Sep3-GFP ring after partial photobleaching, with 87% recovery in fluorescence after 15 min (mean \pm SD, three experiments). Scale bars indicate 10 μ m.

We set out to investigate how the appressorium causes plant infection. We first carried out live-cell imaging of the actin cytoskeleton during appressorium maturation by expressing the actin-binding protein gene fusion, LifeAct-RFP (where RFP is red fluorescent protein) (4), in *M. oryzae*. This revealed an extensive toroidal F-actin network at the base of the infection cell surrounding the appressorium pore (Fig. 1A), a circular region that marks the point at which the penetration peg emerges to rupture the leaf cuticle (2). The appressorium pore initially lacks a cell wall, and the fungal plasma membrane makes direct contact with the rice leaf surface (2). Then, as the appressorium inflates to full turgor (with a mean diameter of 8.0 μ m), a pore wall overlay develops, and a narrow (780-nm mean diameter) penetration peg emerges. Assembly of an F-actin network during appressorium turgor generation, just before plant infection, suggests that specific reorientation of the F-actin cytoskeleton takes place at the base of the appressorium to facilitate plant infection (2). To investigate how actin is organized at this location, we decided to investigate the septin gene family in *M. oryzae*. Septins are small morphogenetic guanosine triphosphatases (GTPases); conserved from yeast to humans (5, 6); and involved in cytokinesis, polarity determination, and secretion (7, 8). Importantly, septins are thought to reorient and reorganize the cytoskeleton to determine cell shape (9–12) and act as partitioning diffusion barriers to recruit and maintain specific proteins at discrete subcellular locations (9, 10). We identified a family of five septin genes in *M. oryzae*, four of which showed similarity to core septins identified in the budding yeast *Saccharomyces cerevisiae* (Cdc3, Cdc10, Cdc11, and Cdc12) (9). Sep3 showed 47% amino acid identity to Cdc3, Sep4 showed 55% identity to Cdc10, Sep5 showed 45% identity to Cdc11, and Sep6 showed 57% identity to Cdc12. We expressed the *M. oryzae* genes in temperature-sensitive *S. cerevisiae* septin mutants that show defects in cell division and in all cases observed complementation of the cell separation defect consistent with the *M. oryzae* proteins acting as functional septins (fig. S1). Next, we expressed Sep3-GFP, Sep4-GFP, Sep5-GFP, and Sep6-GFP (where GFP is green fluorescent protein) gene fusions in *M. oryzae*, and each septin formed a 5.9- μ m ring that colocalized with the F-actin network at the appressorium pore (Fig. 1, A and D, and movie S1). In the case of Sep6-GFP, we also observed an additional bright punctate structure consistently associated with the appressorium septin ring (Fig. 1D). *M. oryzae* septins formed a wider range of structures in hyphae and during invasive growth, including bars, gauzes, collars, and rings (figs. S2 and S3). As expected, they also formed rings at sites of septation (10), such as the neck of nascent appressoria (fig. S2). To understand the nature of the septin ring, we investigated fluorescence recovery after partial photobleaching. We found 87% recovery of fluorescence after 15 min, consistent with the appressorium septin ring being a dynamic structure (Fig. 1F). We then carried out targeted gene deletions to produce isogenic mutants lacking *SEP3*, *SEP4*, *SEP5*, or *SEP6* (fig. S4). Septin null mutants showed mislocalization of the remaining septin-GFPs (fig. S5). Core *M. oryzae* septins therefore act cooperatively to form hetero-oligomers, which assemble into the large ring surrounding the appressorium pore. Consistent with this idea, coimmunoprecipitation experiments using Sep5-GFP identified Sep3, Sep4, and Sep6 interactions as well as physical interaction with actin, tubulins, and the Lte1 cell cycle control protein (11) (table S1). *M. oryzae* septin mutants showed a number of developmental phenotypes (fig. S6). Multiple rounds of nuclear division, for instance, took place during appressorium development in septin mutants (fig. S7, A and B) compared with a single round of mitosis and autophagy-associated cell death, which normally occurs in *M. oryzae* (12, 13). Septins took part in cytokinesis within hyphae (7–10), and localization of myosin II and myosin light chain to the septum, which separates the germ tube and appressorium (10), required septins (fig. S7, C and D).

We next decided to investigate the effect of deleting *M. oryzae* septin genes on actin organization and found that the appressorium F-actin network was disorganized in Δ sep3, Δ sep4, Δ sep5, and

$\Delta sep6$ mutants (Fig. 2A). We reasoned that F-actin, scaffolded by septins, might therefore provide cortical rigidity before peg emergence in a manner analogous to a yeast bud, where assembly of F-actin cables requires Cdc42 and the formins Bni1 and Bnr1 (14–16). To test this idea, we investigated F-actin and septin localization in a *M. oryzae* $\Delta cdc42$ mutant (Fig. 2). We observed many aberrant, $\sim 0.5\text{-}\mu\text{m}$ -diameter Sep3-GFP rings in a $\Delta cdc42$ mutant but no central septin ring in the appressorium (Fig. 2, B and C). Similarly, targeted deletion of *M. oryzae* *CHM1* (17, 18) prevented formation of either the septin or F-actin networks (Fig. 2, B and C). In yeast, Chm1 encodes a kinase that phosphorylates septins (17). Septin ring formation also required the cell integrity mitogen-activated protein kinase Mps1 (19) and the Mst12 transcription factor (20), which are necessary for appressorium function (fig. S8). Furthermore, we discovered that septin ring formation depends on cell cycle progression, which regulates appressorium development in *M. oryzae* (12) (fig. S9). Taken together, we conclude that septin ring assembly is necessary to scaffold F-actin as a toroidal network at the base of the appressorium before plant infection.

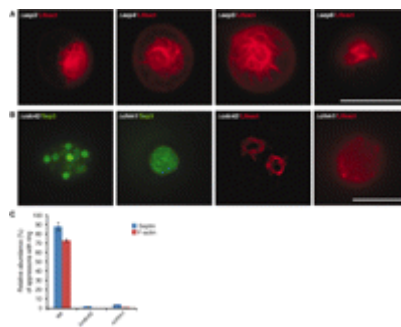


Fig. 2

Septin-dependent F-actin network organization in *M. oryzae* appressoria. (A) Micrographs of LifeAct-RFP in $\Delta sep3$, $\Delta sep4$, $\Delta sep5$, and $\Delta sep6$ mutants. (B) Live-cell imaging of Sep3-GFP and F-actin in $\Delta cdc42$ and $\Delta chm1$ mutants of *M. oryzae*. In a $\Delta cdc42$ mutant, Sep3-GFP localizes to $0.5\text{-}\mu\text{m}$ -diameter rings. In a $\Delta chm1$ mutant, the Sep3-GFP ring did not form at the appressorium pore. $\Delta cdc42$ and $\Delta chm1$ mutants do not form an F-actin network. (C) Bar charts showing percentage of appressoria in which septin and F-actin network formation was observed ($n = 300$, mean \pm SD, three experiments). Scale bars, $10\ \mu\text{m}$.

To test whether septin-dependent assembly of the F-actin network is essential for rice blast disease, we inoculated a susceptible rice cultivar with each septin null mutant and scored disease symptoms. Septin mutants were nonpathogenic, causing either no symptoms at all ($\Delta sep3$) or small necrotic flecks associated with abortive infection attempts that stimulate a rice defense response (Fig. 3, A and B). Transmission electron microscopy and live-cell imaging of rice leaf sheath infections by $\Delta sep3$ mutants confirmed the inability of appressoria from septin mutants to rupture plant cuticles efficiently (Fig. 3, C and D). All septin mutant phenotypes were complemented by reintroduction of either a wild-type allele or the corresponding septin-GFP fusion (fig. S10).

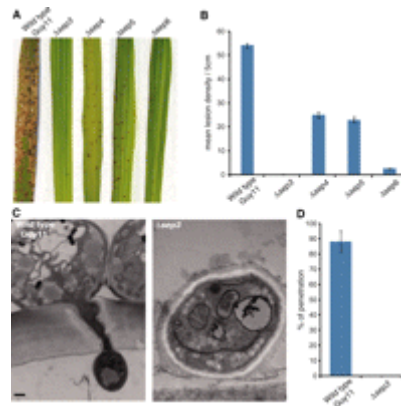


Fig. 3

M. oryzae septin mutants are unable to cause rice blast disease. (A) Targeted deletion of septin genes resulted in loss of pathogenicity on susceptible rice cultivar CO-39. The $\Delta sep3$ mutant caused no disease symptoms; $\Delta sep4$, $\Delta sep5$, and $\Delta sep6$ mutants elicited necrotic flecks because of abortive infection attempts. Septin mutants did not produce spreading disease lesions observed in the isogenic wild-type Guy11. (B) Bar chart shows frequency of disease lesions or necrotic flecks per 5 cm² of leaf surface ($n = 30$ plants) (mean \pm SD, three experiments), reflecting frequency of penetration attempts. (C) Transmission electron micrographs of transverse section of wild-type (24 hours) and $\Delta sep3$ mutant appressoria (36 hours) during leaf infection. No penetration pegs were observed in $\Delta sep3$ mutants. Scale bar, 2 μ m. (D) Bar chart showing frequency of rice epidermal cell rupture at 400 infection sites, 36 hours after conidial germination in wild type (Guy11) and $\Delta sep3$ mutants (mean \pm SD, three experiments).

To understand the nature of the cortical F-actin network in appressoria, we decided to investigate plasma membrane linkages at the appressorium base. It has been suggested that some F-actin–plasma membrane linkages may occur via ezrin, radixin, moesin (ERM) proteins, which contain a C-terminal actin-binding domain and an N-terminal ERM domain that binds transmembrane proteins, such as integrins (21). We identified a putative ERM protein-encoding gene in *M. oryzae*, *TEA1* (which showed 61% identity to *S. pombe* Tea1), and found that Tea1-GFP localized as a punctate ring in the appressorium, colocalized with the F-actin and septin networks (Fig. 4A and fig. S11B). By contrast, in a $\Delta sep5$ mutant, Tea1-GFP was mislocalized (Fig. 4A and fig. S11E). Phosphorylation of ERM proteins is potentiated by phosphatidylinositol (PtdIns)–4,5-bisphosphate binding (22). Yeast septins, for example, associate with PtdIns-4-phosphate and PtdIns-4,5-bisphosphate via an N-terminal polybasic domain (23, 24). We identified the *M. oryzae* PtdIns-4-kinase–encoding gene, *STT4* (showing 53% identity to *S. cerevisiae* *STT4*), and the PtdIns-4-phosphate-5-kinase–encoding gene, *MSS4* (64% identity), and found that Stt4-GFP and Mss4-GFP localized to the appressorium pore, bounded by F-actin and the septin ring (Fig. 4B). To test whether *M. oryzae* septins associated with membrane domains enriched in phosphoinositides, we deleted the N-terminal polybasic domain of *M. oryzae* Sep5 and expressed the resulting Sep5 Δ pb-GFP fusion protein in a $\Delta sep5$ mutant. The Sep5 Δ pb-GFP formed normal septin rings at hyphal septa or the neck of the nascent appressorium, demonstrating that stability of the septin was unaffected by deletion of the polybasic domain (Fig. 4C). However, the septin ring at the base of the appressorium did not form (Fig. 4C). We conclude that septin-PtdIns interactions and ERM protein-actin linkages occur at the appressorium pore. *M. oryzae* Chm1-GFP also localized to the appressorium pore (Fig. 4D and fig. S11, A and F), consistent with a role in septin phosphorylation (25).

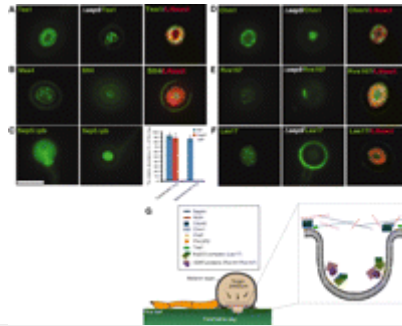


Fig. 4

Septin-dependent localization of specific proteins to the appressorium pore in *M. oryzae* and model of septin-mediated plant infection. (A) Micrographs show localization of Tea1-GFP in Guy11 and in a $\Delta sep5$ mutant and colocalization with LifeAct-RFP (mean \pm SD, three experiments, $n = 300$). (B) Micrographs show PtdIns 4-kinase (Stt4-GFP) and PtdIns-4-phosphate 5-kinase (Mss4-GFP) localization at center and periphery of the appressorium pore and colocalization with LifeAct-RFP. (C) Micrographs and bar chart show that the N-terminal polybasic-rich domain (pb) of Sep5 is dispensable for septin ring formation at site of cytokinesis at appressorium neck (left) but necessary for septin ring formation at the appressorium pore (right). (D) Chm1-GFP colocalizes with LifeAct-RFP, and this is impaired in a $\Delta sep5$ mutant. (E) The I-BAR protein (Rvs167-GFP) localizes to puncta in the appressorium pore, and its organization is affected in a $\Delta sep5$ mutant. (F) Localization of the N-WASP protein (Las17-GFP), a component of the actin-polymerizing Arp2/3 complex, to the center of the appressorium pore septin ring and impairment of its localization in a $\Delta sep5$ mutant. Scale bar, 10 μm . (G) Model to show septin-mediated rice leaf infection by *M. oryzae*.

We reasoned that, as well as scaffolding F-actin, the septin ring might also act as a diffusion barrier to constrain lateral diffusion of membrane-associated proteins involved in plant infection. Septin rings are known, for example, to act as diffusion barriers at the mother bud neck in *S. cerevisiae* (26, 27). We decided to test whether *M. oryzae* septins affect distribution of proteins potentially involved in penetration peg development. Bin-amphiphysin-Rvs (BAR) domain proteins have been shown to form oligomeric scaffolds involved in membrane curvature (28). Whereas F-BAR proteins are well known to play roles in membrane invagination during endocytosis, the inverse BAR (I-BAR) proteins are involved in negative membrane curvature leading to cellular protrusions (28). Because emergence of a penetration hypha from the appressorium requires extreme negative membrane curvature (2, 29), we decided to ask whether septins play a role in I-BAR protein localization. *M. oryzae* Rvs167 contains an I-BAR domain, showing 75% identity to *S. cerevisiae* Rvs167p. We found that Rvs167-GFP localized to the center of the appressorium pore, before penetration peg emergence. However, distribution of Rvs167-GFP was disrupted in a $\Delta sep5$ mutant (Fig. 4E and fig. S11, C and G). I-BAR proteins are proposed to link curved membrane structures to polymerization of cortical F-actin via nonspecific electrostatic interactions (30) mediated by Src homology 3 (SH3) domains of I-BAR proteins and components of the Wiskott-Aldrich syndrome protein (WASP)/WASP-family verprolin-homologous protein (WAVE) complex (31). We isolated a WASP/Arp2/3 complex component, Las17 (32), homologous to Las17p of *S. cerevisiae*. *M. oryzae* Las17-GFP localized to the base of the appressorium, bounded by the septin ring (Fig. 4F), whereas in a $\Delta sep5$ mutant Las17-GFP localization was disrupted, with the protein distributed diffusely in the cytoplasm and plasma membrane (Fig. 4F and fig. S11, D and H). On the basis of the evidence presented in this report, we propose that appressoria of the rice blast fungus infect plants by using a septin-dependent mechanism summarized in Fig. 4G. In this model, isotropic expansion of the pressurized appressorium is directed into mechanical force at the base of the infection cell. This is dependent on assembly of an extensive toroidal F-actin network at the appressorium pore to provide cortical rigidity at the initially wall-less region of the appressorium. Septins organize the actin network, making direct phosphoinositide linkages to the plasma membrane and facilitating the action of ERM proteins, such as Tea1, which link cortical F-actin to the

membrane. The septin ring also acts as a diffusion barrier to ensure localization of proteins, such as the Rvs167 I-BAR protein, and the WASP/WAVE complex involved in membrane curvature at the tip of the emerging penetration peg and F-actin polymerization (Fig. 4G). In this way, the rice blast fungus extends a rigid penetration peg that ruptures the leaf cuticle and invades the host plant tissue.

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