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Putative RhoGAP proteins orchestrate vegetative growth, conidiogenesis and pathogenicity of the rice blast fungus *Magnaporthe oryzae*

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ABSTRACT

Rho GTPases, acting as molecular switches, are involved in the regulation of diverse cellular functions. Rho GTPase activating proteins (Rho GAPs) function as negative regulators of Rho GTPases and are required for a variety of signaling processes in cell development. But the mechanisms underlying Rho GAPs in Rho-mediated signaling pathways in fungi are still elusive. There are eight RhoGAP domain-containing genes annotated in the *Magnaporthe oryzae* genome. To understand the function of these RhoGAP genes, we generated knockout mutants of each of the RhoGAP genes through a homologous recombination-based method. Phenotypic analysis showed that growth rate of aerial hyphae of the *Molrg1* deletion mutant decreased dramatically. The *ΔMolrg1* mutant showed significantly reduced conidiation and appressorium formation by germ tubes. Moreover, it lost pathogenicity completely. Deletion of another Rho GAP (MoRga1) resulted in high percentage of larger or gherkin-shaped conidia and slight decrease in conidiation. Appressorial formation of the *ΔMoRga1* mutant was delayed significantly on hydrophobic surface, while the development of mycelial growth and pathogenicity in plants was not affected. Confocal fluorescence microscopy imaging showed that MoRga1-GFP localizes to septal pore of the conidium, and this localization pattern requires both LIM and RhoGAP domains. Furthermore, either deleting the LIM or RhoGAP domain or introducing an inactivating R1032A mutation in the RhoGAP domain of MoRga1 caused similar defects as the *Morga1* deletion mutant in terms of conidial morphology and appressorial formation, suggesting that MoRga1 is a stage-specific regulator of conidial differentiation by regulating some specific Rho GTPases. In this regard, MoRga1 and MoLrg1 physically interacted with both *MoRac1-CA* and *MoCdc42-CA* in the yeast two-hybrid and pull-down assays, suggesting that the actions of these two GAPs are involved in MoRac1 and MoCdc42 pathways. On the other hand, six other putative Rho GAPs (MoRga2 to MoRga7) were dispensable for conidiation, vegetative growth, appressorial formation and pathogenicity, suggesting that these Rho GAPs function redundantly during fungal development. Taking together, Rho GAP genes play important roles in *M. oryzae* development and infectious processes through coordination and modulation of Rho GTPases.

1. Introduction

Cell polarity is a fundamental feature in eukaryotes and is essential for morphogenesis, cell division and migration (Orlando and Guo, 2009; Thompson, 2013). Polarized growth is coordinated by continuous synthesis of different materials of cell growth and asymmetrical delivery of these components to a specific cellular site, which is dynamically modulated by cytoskeleton reorganization and membrane trafficking (Bryant and Mostov, 2008). Rho GTPases belonging to the Ras superfamily have been proven to be vital polarity determinants (Hall, 2005; Perez and Rincón, 2010). Rho GTPases exert a large variety of cellular functions including actin cytoskeleton polarization, cell cycle control and cell morphogenesis (Iden and Collard, 2008; Perez and Rincón, 2010). These molecular switches alternate between a GDP-bound inactive state and a GTP-bound active state in a highly regulated manner (Bos et al., 2007). Emerging evidence indicates that Rho GTPase-activating proteins (Rho GAPs), which inactivate Rho by accelerating GTP hydrolysis, are required for a number of Rho-mediated signaling pathways in cell development and differentiation (Tcherkezian and Lamarche-Vane, 2007; Zygmunt and Spagnoli, 2013).

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Since the first Rho GAP was reported 24 years ago (Garrett et al., 1989), more than 70 members have been identified in eukaryotes from yeast to mammals (Tcherkezian and Lamarche-Vane, 2007). In all the species, the number of Rho GAPs is far more than that of the Rho GTPases, suggesting their functional diversity in regulating specific Rho GTPase signaling pathways in different tissues.

To date, most members of Rho GAPs in yeast have been identified, such as Rga1 (Stevenson et al., 1995), Rga2 (Smith et al., 2002), Lrg1 (Lorberg et al., 2001), Bem2 (Wang and Bretscher, 1995), Bem3 (Zheng et al., 1994), Sac7 (Schmidt et al., 2002), Bag7 (Roumanie et al., 2001), Rgd1 (Barthe et al., 1998) and Rgd2 (Roumanie et al., 2001). The functions of these Rho GAPs involve polarized growth of different yeast strains. In *Saccharomyces cerevisiae*, both Rga1 and Rga2 behave as GAPs for Cdc42 and facilitate the interaction between active Cdc42 and PAK kinase Ste20, leading to regulation of haploid invasive growth (Smith et al., 2002). In particular, Rga1 can direct the new polarity axis and establish concentric zone adjacent to but not within the old cell division site by inactivating Cdc42 (Tong et al., 2007). Rga1 is also required for GTP hydrolysis and spatiotemporal regulation of Cdc42-mediated diploid daughter cell polarization during the budding (Lo et al., 2013). Lrg1 is a GAP for Rho1, and its deletion is shown to cause reduced rate of cell fusion and diploid formation, suggesting its role in Rho1-dependent cell fusion and potential involvement in cell wall synthesis of mating cells (Fitch et al., 2004). In *Schizosaccharomyces pombe*, Rga2 functions as a critical substrate of cyclin-dependent kinase involved in G1 phase of cell cycle. Phosphorylation of Rga2 by CDK can inhibit Rga2 activity to modulate appropriate level of active Cdc42 crucial for establishment of cell polarity during G1-phase progression (Sopko et al., 2007).

In fission yeast *S. pombe*, Rga4, one of the nine Rho GAPs present in the genome, not only negatively regulates Cdc42 for cell polarity and morphogenesis, but also functions as a Rho2 GAP involved in regulating the activity of MAPK Pmk1 cell integrity pathway (Cansado et al., 2010; Soto et al., 2010). Furthermore, cell integrity maintenance also requires another GAP, Rga5, which is specific for Rho1 (Calonge et al., 2003). Altered expression of Rga5 and abnormal septation upon exposure to nitrosative stress suggests a novel mechanism of Rga5-mediated cell cycle regulation under nitrosative stress (Majumdar et al., 2012). In addition, Rho GAPs, such as Lrg1 and Rgd1 in higher fungi, also regulate cell morphogenesis like polar tip growth (Vogt and Seiler, 2008) and filamentous formation (Ness et al., 2010).

In general, as negative regulators of Rho GTPases, Rho GAPs are involved in the regulation of critical biological processes of polarized growth in fungal cells, ranging from cell cycle control to cellular morphogenesis. However, the specificity and functions of Rho GAPs in plant pathogenic fungi are not well understood.

Rice blast, caused by the ascomycete phytopathogenic fungus *Magnaporthe oryzae*, is a serious threat to rice production worldwide and has emerged as an important model to uncover molecular mechanisms of fungal pathogenesis in plants. Similar to other pathogenic fungi, every step of the infection cycle including mycellial development, conidiation, appressorial differentiation and infectious hyphae extension is required for the success of *M. oryzae* infection and expansion over a large area. All these processes are closely associated with cell polarity, which are regulated by Rho GTPases and their regulators and effectors. Previously, we have identified three Rho GTPases of *M. oryzae*, MoRac1, MoCdc42 and MoRho3, playing important roles in the regulation of fungal polarized growth, conidiation, appressorium differentiation and pathogenicity in either coordinated or independent manners (Chen et al., 2008; Zheng et al., 2007, 2009). In particular, real-time PCR indicates that MoCdc42 expression is increased upon deletion or dominant inactivation of MoRac1, while MoCdc42 expression is decreased if MoRac1 is under constitutively activation or overexpression (Chen et al., 2008). It raises the possibility that Cdc42 and Rac1 may function antagonistically in *M. oryzae*. Given that yeast two-hybrid assays reveal that both MoCdc42 and MoRac1 interact with a downstream effector Chm1 (Chen et al., 2008), they may share the same regulator(s) as well, such as Rho GAP(s). Since deleting either of the two Rho GTPases blocks conidiogenesis, appressorial formation and pathogenicity (Chen et al., 2008), it is important to uncover which Rho GAPs regulate MoCdc42 and MoRac1.

Here, we systematically characterized all eight putative Rho GAPs in *M. oryzae* and revealed that MoRga1 and MoLrg1 are both essential for conidiogenesis and appressorial formation, while MoRga2 to MoRga7 function redundantly in fungal development and pathogenesis. Deletion of MoLrg1, in particular, exhibited dramatic reduction of vegetative growth and complete loss of pathogenicity. Additionally, yeast two-hybrid and pull down assays indicated that both MoRga1 and MoLrg1 interact with Cdc42 and Rac1 in a GTP-dependent manner. Furthermore, either deletion of LIM or RhoGAP domain or introduction of an inactivating R1032A mutation in the RhoGAP domain causes similar defects as the MoRga1 null mutant, consistent with its role as Rho GAPs. These results illustrate the importance of Rho GAPs in the development of *M. oryzae* throughout the disease cycle via selective modulation of specific Rho GTPases.

2. Materials and methods

2.1. M. oryzae strains and culture conditions

M. oryzae strains *Δku70* and *Δku80* (Villalba et al., 2008) and fungal transformants generated in this study were maintained and cultured on complete medium plates (CM: 0.6% yeast extract, 0.6% casein hydrolysate, 1% sucrose, 1.5% agar) as described before (Chen et al., 2008). Genomic DNA, total RNA and the protoplast were isolated from mycelia collected from 3 to 4-day-old culture in liquid starch yeast medium (SYM: 0.2% yeast extract, 1% starch, 0.3% sucrose) under 150-rpm shaking at 25 °C. Congo red (200 μg/ml), sorbitol (1 M), or SDS (0.04%, 0.05%, 0.06% and 0.07%) was added to the CM agar plates to determine the effects on fungal growth. Conidia were harvested from 10-day-old culture grown on rice-polish agar medium (2% rice-polish, 1.5% agar, pH 6.0) at 25 °C under constant light to promote conidial development. The selective top agar medium for transformation was supplemented with either 400 μg/ml of hygromycin B (Roche Applied Science) or 600 μg/ml of G418 (Invitrogen Corp.) based on the selection marker on the plasmid vector.

2.2. Targeted gene deletion and complementation

Deletion mutant alleles for eight Rho GAP encoding genes were constructed through a homologous recombination-based method as described before (Chen et al., 2008). In brief, around 1 kb of the upstream and downstream flanking sequences of the target gene were amplified with respective gene specific primers (Table S1). The resulting PCR products were digested and ligated with the hygromycin phosphotransferase (*hph*) gene released from pCX62 (Zhao et al., 2004). After ligation, the resulting gene replacement construct was then transformed into protoplasts of the highly efficient gene targeting *Magnaporthe* strain *Δku70* or *Δku80* as described previously (Sweigard et al., 1998). The positive transformants were further verified by Southern blot analysis with the digoxigenin high prime DNA labeling and detection starter Kit I (Roche, Mannheim, Germany). The complement fragments for each Rho GAP, which contain the entire wild-type gene and native promoter region, were amplified by PCR with primers (Table S1).