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MoMip11, a MoRgs7-interacting protein, functions as a scaffolding protein to regulate cAMP signaling and pathogenicity in the rice blast fungus *Magnaporthe oryzae*

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Abstract

The rice blast fungus *Magnaporthe oryzae* has eight regulators of G-protein signaling (RGS) and RGS-like proteins (MoRgs1 to MoRgs8) that exhibit both distinct and shared regulatory functions in the growth, differentiation, and pathogenicity of the fungus. We found MoRgs7 with a unique RGS-seven transmembrane (7-TM) domain motif is localized to the highly dynamic tubule-vesicular compartments during early appressorium differentiation followed by gradually degradation. To explore whether this involves an active signal perception of MoRgs7, we identified a Gbeta-like/RACK1 protein homolog in *M. oryzae* MoMip11 that interacts with MoRgs7. Interestingly, MoMip11 selectively interacted with several components of the cAMP regulatory pathway, including Gα, MoMagA and the high-affinity phosphodiesterase MoPdeH. We further showed that MoMip11 promotes MoMagA activation and suppresses MoPdeH activity thereby up-regulating intracellular cAMP levels. Moreover, MoMip11 is required for the response to multiple stresses, a role also shared by Gbeta-like/RACK1 adaptor proteins. In summary, we revealed a unique mechanism by which MoMip11 links MoRgs7 and G-proteins to reulate cAMP signaling, stress responses, and pathogenicity of *M. oryzae*. Our studies revealed the multitude of regulatory networks that govern growth, development, and pathogenicity in this important causal agent of rice blast.

Keywords

RGS proteins; Gbeta-like/RACK1 scaffold protein; cAMP regulation; stress response; pathogenicity

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Introduction

Heterotrimeric guanine-nucleotide binding protein (G-protein) signaling is one of the most essential mechanisms by which eukaryotic cells sense extracellular signals and integrate them into intrinsic signal transduction pathways, such as the cyclic AMP (cAMP)-dependent signaling pathway, allowing proper growth and development. The G-protein is heterotrimeric typically composed of α , β , and γ subunits. Activated $G\alpha$ and $G\beta\gamma$ moieties subsequently target downstream effectors, including adenylyl cyclases, ion channels, protein kinases, phosphodiesterases and phospholipases (Simon et al., 1991; Neer, 1995; Hamm, 1998; Neves et al., 2002). Activated G-proteins are later desensitized by the intrinsic GTPase activity of the $G\alpha$ subunit, which is regulated by the RGS (Regulators of G-protein Signaling) protein (Dohlman et al., 1998; Siderovski and Willard, 2005). In fungi, G-protein, upstream G-protein coupled receptors (GPCRs), and RGS proteins regulate multiple cellular functions and developmental pathways including vegetative growth, conidiation, appressorium formation and function, and pathogenicity (reviewed by (Bolker, 1998; Lengeler et al., 2000)).

G-protein and cAMP signaling have been shown to play important roles in the rice blast fungus *Magnaporthe oryzae* during the initial recognition of surface signals and formation of the appressorium, because losing the $G\alpha$ MoMagB subunit and/or adenylyl cyclase MoMac1 resulted in defects in appressorium formation and pathogenicity (Choi and Dean, 1997; Liu and Dean, 1997). In this fungus, three distinct $G\alpha$ proteins (MoMagA, MoMagB, and MoMagC), one $G\beta$ protein (MoMgb1) and one $G\gamma$ subunit (MoMgg1) were described (Liu and Dean, 1997; Nishimura et al., 2003; Li et al., 2015). MoMgb1 contains a distinct seven-bladed propeller structure with each blade composed of a conserved core of 40 amino acids flanked by Trp-Asp (WD) and is involved in regulating multiple steps of infection-related morphogenesis (Nishimura et al., 2003). With the seven WD-40 repeat motif significant homologous to $G\beta$, the $G\beta$ -like/receptor for activated protein kinase C1 (RACK1) is a highly conserved multifunctional adaptor protein (Ron et al., 1994; Omosigho et al., 2014).

In mammals, RACK1 is a scaffold protein that integrates many different processes through interacting with various proteins including the activated protein kinase C (PKC), integrin, and Src tyrosine kinase (Guillemot et al., 1989; Ron et al., 1994; Chang et al., 1998; Liliental and Chang, 1998; Ron et al., 1999). RACK1 has been shown to interact with several subunits of G protein (Dell et al., 2002; Chen et al., 2004; Chen et al., 2005). Interaction with Pde4D5, an isoform of Pde4D, was shown to suppress cAMP degradation (Yarwood et al., 1999). Moreover, RACK1 was also shown to be the major component of the ribosomes regulating protein translation and post-translational modification (Ceci et al., 2003; Nilsson et al., 2004). A RACK1 homolog was found to bind to $G\beta$ and all three tiers of the MAPK cascade in *Arabidopsis thaliana* (Cheng et al., 2015).

In the budding yeast *Saccharomyces cerevisiae*, Asc1, a Gbeta/RACK1 homolog, functions as guanine nucleotide dissociation inhibitor for $G\alpha$ Gpa2 (Zeller et al., 2007). Asc1 also regulates stress resistance and adhesion-dependent growth and is also characterized as a core component of the 40S ribosome (Gerbaso et al., 2004; Valerius et al., 2007). In the corn

pathogen *Ustilago maydis*, deletion of RAK1 (a homolog of RACK1/Asc1) affected cell growth and attenuated cell fusion, cell wall integrity, and pathogenicity (Wang et al., 2011). In the human fungal pathogen *Cryptococcus neoformans*, Gib2 not only interacts with G protein subunits Gpa1 and Gpg1/Gpg2, but also positively regulates cAMP levels in conjunction with Gpa1 (Palmer et al., 2006). Additional evidence suggested that Gib2 functions as a scaffold protein integrating a wide array of cellular functions (Wang et al., 2014; Ero et al., 2015; Bruni et al., 2017). Finally, a recent study has also found that Mip11, a RACK1 homolog in *M. oryzae*, interacted with Mst50 and that the *mip11* mutant had a defect in cell wall integrity and plant infection (Li et al., 2017a).

We previously found that *M. oryzae* have eight RGS family proteins (MoRgs1-MoRgs8) that orchestrate surface recognition, appressorium formation, intracellular cAMP levels, and pathogenicity of the fungus and that MoRgs7, specifically, has a role in cAMP signaling, appressorium formation, and pathogenicity (Zhang et al., 2011c). Interestingly, MoRgs7 contains a seven-transmembrane (7-TM) domain motif typical of G-protein coupled receptors (GPCRs) in addition to the RGS domain. We discovered that MoRgs7 is localized to the dynamic tubule-vesicular compartments during the early appressorium development stage. To further examine the significance of this observation, we searched MoRgs7 interacting proteins and identified MoMip11 (*Magnaporthe oryzae* Mip11). Given the established cellular functions of RGS/RGS-like and Gbeta-like/RACK1/Asc1/Rak1/Gib2 proteins, we thought to investigate functions of MoRgs7, MoMip11, and their interaction in the growth, development, and pathogenicity of the rice blast fungus.

Results

MoRgs7 is highly expressed and localized to the late endosome during early stages of appressorium formation

Previous studies suggested that MoRgs7 is involved in the regulation of appressorium function, cAMP levels, and virulence and that MoRgs7 also contains a 7-TM domain in addition to the RGS domain (Zhang et al., 2011c). To dissect whether this domain has additional roles in MoRgs7, we examined its subcellular localization and protein dynamic that are often associated with GPCRs.

To investigate the spatial and temporal distribution of MoRgs7 during the appressorium germinating process, we constructed a *MoRgs7/MoRGS7-RFP* strain in which the expression of the C-terminal RFP fusion protein is under the control of the native MoRgs7 promoter. Imaging was performed on germinating conidia at 2 and 4 hours on an inductive surface, with un-germinated conidia as control. We found that MoRgs7-RFP could be localized to the tubule-vesicular structure, a morphologically distinct and highly dynamic membrane vesicle, during early stages of germination and that MoRgs7-RFP was actively transported towards the incipient appressorium (Fig. 1A; Movie S1). This is in sharp contrast to MoRgs7 being limited to the less dynamic vesicles of non-germinated conidia (Fig. 1A; Supporting Information Movie S1). In order to rule out the possibility that the complementation strain (*MoRgs7/MoRGS7-RFP*) may not perfectly restored all defects to the wild-type status, we also observed the intracellular localization of *MoRGS7-RFP* in a

transformant derived from the wild type strain Guy11 and observed a similar result (Supporting Information Fig. S1).

MoRGS7 was highly expressed during the entire germination progress with the expression peaked at 8 h following germination (Fig. 1E). To further explore the tubule-vesicular/endosomal localization of MoRgs7, we used the GFP-tagged Rab5 and Rab7 proteins that are markers of early and late endosomes, respectively (Lachmann et al., 2011). Indeed, MoRgs7-RFP co-localized with Rab7 to the late endosomes, but not Rab5 (Fig. 2). Interestingly, as the appressorium matures, the MoRgs7-RFP signal became less apparent at 8 and 24 h after germination (Fig. 1A-1C), which is consistent with the decreased protein content detected by Western blot analysis (Fig. 1D). Moreover, this degradation was further linked to the ubiquitin-mediated protein degradation pathway (Supporting Information Fig. S2 and Fig. S3).

While still being preliminary, these data suggested that MoRgs7 might have a role in surface signal perception, particularly during the early stage of appressorium formation.

The seven-transmembrane (7-TM) domain is important for MoRgs7 function

To examine the possible function of the 7-TM domain in MoRgs7, we generated a 7-TM domain deletion allele (MoRgs7TM) and expressed it in the *Morgs7* mutant. No effect on tubulo-vesicular structure localization of MoRgs7 was found but MoRgs7TM affected the degradation progress in later stages of germination (Supporting Information Fig. S4A-S4C). Intracellular cAMP levels and pathogenicity of the MoRgs7TM were found to be less severe than the *Morgs7* null mutant but not to the levels of the wild type, suggesting that the 7-TM domain has a role in full functions of MoRgs7 (Supporting Information Fig. S4D-S4F).

MoRgs7 interacts with MoMip11

To further characterize MoRgs7 functions, we used co-immunoprecipitation (co-IP) assay to identify putative MoRgs7-interacting proteins. We generated the *MoRGS7*-3xFLAG construct and introduced it into the wild-type strain. After verifying MoRgs7 expression, we immobilized it to the anti-FLAG M2 beads and analyzed proteins co-purified with FLAG tagged MoRgs7 by mass spectrometry (MS). Among the proteins that MoRgs7 interacts (Supporting Information Table S1), we identified a protein gi|351650345 (MGG_04719.7) with an initial annotation of a G-protein associated protein and also has high matching unique peptides. We thus selected MGG_04719.7 for further characterized. MGG_04719.7 encodes a protein with a 7-WD40 repeat domain and shares highly amino acid sequence identity with *S. cerevisiae* Asc1 (58%), *C. neoformans* Gib2 (72%), and human RACK1 (71%) (Fig. 3A and 3B). A recent study have reported this protein as a Mst50-interacting protein which thus named it Mip11, we further stick to the name of MoMip11. Based on its high amino acid sequence conservation with *C. neoformans* Gib2 whose crystal structure is available (PDB: 4D6V) (Ero et al., 2015), we determined the structure of MoMip11 (Fig. 3C and 3D). The interaction between MoMip11 and MoRgs7 was verified by yeast two-hybrid (Y2H) and, again co-IP assays (Fig 3E and 3F).

MoMip11 is important for growth, asexual and sexual development

To characterize the functions of MoMip11, a *Momip11* deletion mutant was generated by replacing the coding region with a hygromycin-resistance cassette (*HPH*) (Supporting Information Fig. S5). A complemented mutant strain was also generated. The *Momip11* mutant showed significantly growth reduction on CM, MM, SDC, and OM media (Fig. 4A and 4B). Nearly no aerial hyphae was found following continuously incubation for more than 10 days (Fig. 4C). In comparison to Guy 11, only 6.7% conidiation rate was estimated after 10 day's growth on decoction and corn agar (SDC) cultures (Fig. 4D and 4E).

To determine whether MoMip11 plays a role in mating, the Guy11, *Momip11* and *Momip11/MoMIP11* strains were crossed to a standard tester strain, TH3 (*MAT1-1*). After 3 weeks, abundant perithecia were observed at the junctions of the cross between the Guy11 and TH3, complement transformants (*Momip11/MoMIP11*) and TH3, but not *Momip11* and TH3 (Supporting Information Fig. S6). These results suggested that MoMip11 has a major regulatory role in hyphal growth, conidiation, and mating.

MoMip11 is required for infection and virulence

To test the virulence of the *Momip11* mutant, 2-week-old rice seedlings of cultivar CO-39 were sprayed with conidia suspensions. Only few small brown spots (not typical blast lesions) were observed on leaves infected with the *Momip11* mutant, in contrast to controls (Fig. 5A). Similar results were obtained from injection inoculation assay on rice (Fig. 5B).

To explore why the *Momip11* mutant exhibits reduced virulence, we performed penetration and invasive hyphal growth assays on rice sheath and found that there was no successful appressorium penetration by the *Momip11* mutant at 24 h, whereas more than 95% of penetration was successfully by Guy 11 (Fig. 5D). Even if the observation time was extended to 48 h, only few penetrations were found with infectious hyphae seriously restricted into one plant cell (Fig. 5E and 5F). These results suggested that MoMip11 is important for appressorium mediated infection, invasive hyphal growth, and virulence.

MoMip11 is required for appressorium turgor pressure

Appressorium-mediated penetration requires enormous internal turgor pressure to generate sufficient mechanical force to breach the rice leaf cuticle (Thines et al., 2000; Talbot and Foster, 2001; Wang et al., 2007). Since appressorium formation appears to be normal in the *Momip11* mutant, we tested whether the turgor pressure is the factor for the penetration defect. In an incipient cytorrhysis (cell collapse) assay using a 1–4 molar concentration of glycerol solution, appressoria of the *Momip11* mutant showed increased collapse rates in comparison to the wild type Guy11 (Fig. 5C), consistent with that the reduced pathogenicity of the *Momip11* mutant was caused by abnormal turgor pressure. Additionally, examination of cellular distributions of glycogen essential for appressorium-mediated host penetration revealed that glycogen mobilization was significantly delayed in the *Momip11* mutant (Supporting Information Fig. S7).

MoMip11 is involved in the regulation of intracellular cAMP levels

To test whether MoMip11 has a role in cAMP signaling, we measured intracellular cAMP levels in hyphae stage of the wild type Guy11, *Momip11*, and *Momip11/MoMIP11* strains. We also included *Morgs7*, *MopdeH*, and *Momgb1* ($G\beta$) mutant strains that are known to have aberrant cAMP levels (Nishimura et al., 2003; Zhang et al., 2011b; Zhang et al., 2011c). The cAMP level was significantly decreased in the *MoMip11* mutant, in comparison with the wild type Guy11 and complemented *MoMip11/MoMIP11* strains (Supporting Information Fig. S8).

Considering the cAMP defect of *Momip11*, we further tested if exogenously adding 1, 2, 5, and 10 mM cAMP (8-Bromoadenosine 3', 5'-cyclic monophosphate, Sigma, B5386) to the conidia suspension could suppress the *Momip11* infection defect. Observation and statistical analysis of invasive growth in rice sheath cells found that at 10 mM 8-Bromoadenosine cAMP could partially suppress the defect (Supporting Information Fig. S9). At the same time, we also introduced a constitutively activated MoMagA (*MoMagA^{G187S}*) construct into the *Momip11* mutant and found that *Momip11/MoMAG^{G187S}* transformants have normal or relatively higher levels of cAMP compared to the wild type (Fig. 6B). Sparing assays on rice seedlings and further infection assays on barley leaf epidermis both showed that the constitutively activated MoMagA (*MoMagA^{G187S}*) partially restored virulence (Fig. 6A and 6C and Supporting Information Fig. S10). These results indicated that MoMip11 has a positive role in the regulation of intracellular cAMP levels that are essential for pathogenicity.

MoMip11 interacts with $G\alpha$ MoMagA but not activated $G\alpha$ MoMagA^{G187S}

Since both exogenous cAMP and endogenous constitutively activated $G\alpha$ MoMagA^{G187S} (*MoMagA^{GS}*) partially restored the virulence defect of the *Momip11* mutant, we tested whether MoMip11 directly interacts with any $G\alpha$ proteins through Y2H screening. We found that MoMip11 interacts with MoMagA but not the activated $G\alpha$ MoMagA^{GS} allele, in contrast to MoRgs7 that interacts with both MoMagA and MoMagA^{GS} (Fig. 7A). The interactions were verified by an *in vitro* GST-pull down assay using GST-MoMip11 and His-MoMagA/MoMagA^{GS} constructs (Fig. 7B and 7C) and co-IP assay (Fig. 7D and 7E).

Considering that Gbeta-like/RACK1/Asc1/Gib2 are scaffold proteins interacting with a wide variety of proteins, we were curious about if MoMip11 also has a similar function in *M. oryzae*. We found that MoMip11 interacted with the high-affinity phosphodiesterase MoPdeH (Supporting Information Fig. S11), but not $G\beta$ and $G\gamma$ subunits or any components downstream of the MAPK cascade (Fig. 7A; Supporting Information Fig. S11).

MoMip11 promotes the intracellular transport of MoMagA

Both MoRgs7 and MoMip11 interacted with MoMagA (Fig. 7A) and the interaction between MoRgs7 and MoMagA occurs at the distinct tubulo-vesicular structures (Supporting Information Fig. S12). To further examine the significance of the MoMip11-MoMagA interaction, we introduced a MoMagA-RFP construct into Guy11 and the *Momip11* mutant, respectively. Two hours following germination, MoMagA-RFP was found in the distinct tubulo-vesicular structures in Guy11 (Fig. 6D), but it remained mostly

on the membrane in the *Momip11* mutant (Fig. 6E). These results suggested that MoMip11 could facilitate the transport MoMagA (G α) from the plasma membrane into the tubulo-vesicular structure, a process associated with MoMagA function.

MoMip11 interferes with MoRgs7-MoMagA^{GS} interaction

Since MoRgs7 interacts with both MoMagA^{GS} and MoMip11, we wondered if the interactions are mutually competitive. To test this, we conducted an *in vitro* pull-down assay of GST-MoRgs7 and His-MoMagA^{GS} with or without His-MoMip11. We found that MoRgs7 interacted with MoMagA^{GS} *in vitro* and the addition of His-MoMip11 could weaken this interaction (Fig. 8A). To confirming this result, we conducted the pull-down assay in the presence of different concentrations of His-MoMip11. Interestingly, as the concentration of MoMip11 increases, the enrichment of MoMagA^{GS} in the MoRgs7-bound resin decreased gradually (Fig. 8B).

MoMip11 interacts with MoPdeH to suppress its phosphodiesterase activity

MoMip11 interacts with MoPdeH, a high-affinity cAMP phosphodiesterase important for cAMP signaling of the rice blast fungus (Ramanujam and Naqvi, 2010; Zhang et al., 2011b) through Y2H. This interaction was also validated using both co-IP and GST-pull down assays (Fig. 9A and 9B).

Phosphodiesterase inhibitors such as IBMX could suppress the infection defect of the *Momip11* mutant on barely leaves (Supporting Information Fig. S9), we further confirmed this infection suppression using rice sheath and found that IBMX could indeed restore the infection of the *Momip11* mutant at both 24 hpi and 48 hpi (Fig. 9C). We then tested whether MoMip11 suppresses the phosphodiesterase activity of MoPdeH by measuring phosphohydrolase activities with or without MoMip11 using purified GST tagged proteins and a fluorescence based assay method for free phosphates (Brune et al., 1994). Results showed that the samples treated with MoPdeH alone has a strong fluorescence intensity, whereas the samples treated with various concentrations of MoMip11 have comparatively less intense fluorescence (Fig. 9D), support that MoMip11 could suppress the phosphohydrolase activity of MoPdeH through the interaction.

MoMip11 partially complements the function of MoMgb1 (G β) during appressorium formation

Previous studies showed that G β MoMgb1 protein is essential for appressorium formation (Nishimura et al., 2003). Since MoMip11 also had a G β -like 7-WD repeats motif similar to MoMgb1, we wondered if MoMip11 might functionally compensate for a lack of MoMgb1 in appressorium formation. We thus overexpressed *MoMIP11* in the *Momgb1* mutant and found that the *Momgb1/MoMIP11* strain produced long germ tubes and appressoria although abnormal in comparison to the controls (Supporting Information Fig. S13). Meanwhile, intracellular cAMP levels in the *Momgb1/MoMIP11* strain were also higher than those in the *Momgb1* mutant (Supporting Information Fig. S8). While a definitive mechanism(s) remains to be further studied, the results suggested that MoMip11 could partially compensate for functions of MoMgb1 during appressorium formation.

MoMip11 is involved in multiple responses to the environment

Considering that Gbeta-like/RACK1/Asc1/Gib2 are scaffolding proteins regulating wide arrays of cellular functions including stress responses, we tested whether MoMip11 may also associate with the response to environmental stress in *M. oryzae*. As shown in Fig. 10A, the *Momip11* mutant revealed an increased sensitivity to the phenylpyrrole fungicide fludioxonil. Simultaneously, the *Momip11* mutant exhibited equally increased sensitivity to oxidative stress (H₂O₂), high temperature stress, and endoplasmic reticulum stress (DTT) (Fig. 10A and 10B). To further investigate how MoMip11 might participate in this stress response, we measured the gene expression of several heat shock genes (*MoSSB1*, *MoSSQ1*, *MoSSZ1*, and *MoLTH*), ribosomal gene (*MoRPP0*), UPR target genes (*MoSCJ1*, *MoSIL1*, and *MoKAR2*), as well as the catalase gene (*MoCAT1*) using qRT-PCR. We found that all of these genes were up-regulated upon the stress in the wild type strain Guy 11 (Fig. 10C-10E). However, substantially reduced amplitude of genes up-regulation or even no differences were found in the *Momip11* mutant (Fig. 10C-10E).

Discussion

RGS proteins function as negative regulators of G-protein signaling to also govern growth, development, and/or pathogenicity. Our previous studies have characterized eight RGS and RGS-like proteins from *M. oryzae* and we provided evidence showing all have certain roles in conidiation, appressorium formation, cAMP levels, and pathogenicity (Zhang et al., 2011c). Among MoRgs1, MoRgs3, MoRgs4, and MoRgs7 that are directly involved in pathogenicity, MoRgs7 stood out as it also contains a 7-TM domain motif typical of GPCRs, in addition to the conserved RGS domain. Here, we focused on this RGS-like protein and characterized its subcellular localization during conidia germination and maturation of appressorium. Importantly, we have provided initial evidence that MoRgs7 has a cellular dynamic similar to that of GPCR. In addition, we have identified MoRgs7-interacting MoMip11 as a homolog of Gbeta-like/RACK1/Asc1/Gib2 and that MoMip11 has multiple functions in *M. oryzae*. Whereas the 7-TM motif mediated function of MoRgs7 remains to be further defined, our extensive characterization of MoMip11 provides additional insights into the mechanisms that govern fungal growth, differentiation, and pathogenicity. Studies of MoMip11 will also help to further address functions of MoRgs7.

MoMip11 is a WD40 repeat protein containing a seven-bladed β -propeller that shares similarities with G-protein β subunit. The modelled structure of MoMip11 features the conserved seven β -propeller architect with each propeller blade, made up of four-stranded antiparallel β -sheets, arranged in sequential order. Although differences exist, MoMip11 shows great agreements in amino acid sequence and structure with those of human Gbeta-like/RACK1 (PDB ID 4AOW), *S. cerevisiae* Asc1 (PDB ID 3FRX) and *C. neoformans* Gib2 (PDB ID 4D6V) (Coyle et al., 2009; Ruiz Carrillo et al., 2012; Ero et al., 2015). Gbeta-like/RACK1 orthologues are scaffold proteins that integrate many different intracellular processes through interacting with various proteins including the activated protein kinase C (PKC), MAPK cascade, G-protein subunits (α , β , γ), phosphodiesterase Pde4D5 (Ron et al., 1999; Yarwood et al., 1999; Dell et al., 2002; Chen et al., 2004; Chen et al., 2005; Omosigho et al., 2014; Cheng et al., 2015). In *M. oryzae*, we found that MoMip11 interacts with

MoMagA and MoPdeH, in addition to MoRgs7. To our knowledge, it is the first report for an interaction between Gbeta-like/RACK1/Asc1/Gib2 homolog and RGS or RGS-like proteins.

Intriguingly, unlike other Gbeta-like/RACK1 homologs, interactions between MoMip11 and G β or G γ subunit, MAPK cascade components (MoMkk1, MoMps1 and MoOsm1), or MoPkc1, could not be established for *M. oryzae* (Supporting Information Fig. S11). Instead, MoMip11 was found to associated with several major regulatory components of the cAMP signaling pathway including G α subunit MoMagA and phosphodiesterase MoPdeH, in addition to MoRgs7 (Choi and Dean, 1997; Zhang et al., 2011b; Zhang et al., 2011c; Zhou et al., 2012; Zhang et al., 2016), which lead us to speculate that MoMip11 might be one of the primary regulators for cAMP signaling. Despite that MoPdeH was linked to cAMP regulation in *M. oryzae* (Ramanujam and Naqvi, 2010; Zhang et al., 2011b; Zhang et al., 2011c), reports on the specific mechanism and the related regulation network remain limited in fungus. In this study, we provided evidence to demonstrate that MoMip11 has positive role in regulating intracellular cAMP levels through interacting with MoMagA and MoPdeH.

In humans, Gbeta-like/RACK1 regulates intracellular cAMP levels through the inhibition of the Pde4D5 PDE activity (Yarwood et al., 1999). Analogous to this, supplementing cAMP both exogenously and endogenously (constitutive expression of activated MoMagA) also restored, albeit partially, the defect in infection. We also discovered that endosome-localized MoMagA during germination decreased remarkably in the *Momip11* mutant that supports an important role of MoMip11 in promoting MoMagA function during infection. In addition, we found MoMip11 could interfere with the interaction of activated MoMagA and MoRgs7 that provides additional means in the regulation of cAMP signaling (Fig. 11). Interestingly, we also found that MoMip11 could interact with a negative regulator of cAMP signaling MoPdeH, in order to suppress its phosphodiesterase activity which also have a positive role on final intracellular cAMP level. Taken together, our study is therefore highly significant.

During our studies, we found that MoMip11 not only interact with MoRgs7 but also additional RGS proteins (Supporting Information Fig. S14), providing the possibility that MoMip11 might has a common regulatory role on RGS and RGS-like proteins. We found the ability of MoMip11 to interact with multitude of proteins to impact their pathways in the hallmark of scaffold adaptor proteins. Our studies of MoMip11 provided convincing evidence indicating its adaptor roles and forecast that additional important regulatory functions may also be found for MoMip11. Indeed, a recent study identified a Mst50-interacting protein Mip11 from *M. oryzae* is involved in cell wall integrity pathway and Mps1 activation (Li et al., 2017a). Since Mip11 is indeed MoMip11, this study further corroborates with that of ours that the function MoMip11 is not limited to cAMP signaling and stress, but also other functions during the growth and development of *M. oryzae*.

MoRgs7 has a unique domain structure by also containing a 7-TM motif typical of GPCR proteins. To address any activities associated with the unique motif, we explored whether MoRgs7 could be translocated into the late endosome, especially during the early stage of

germination when active signaling mediated by G-proteins is anticipated. Recent studies showed that the spatio-temporal dynamics of the G-protein cascade and intracellular signal transduction are necessary for proper appressorium differentiation and the late endosomal HOPS complex anchors active G-protein signaling is essential for the pathogenesis of *M. oryzae* (Ramanujam et al., 2013; Kou and Naqvi, 2016). Similarly, the ability of the late endosomes to traverse in the germ tube and navigate in both directions possibly facilitates the recycling and the transport of signaling components to or away from the sites of active signaling on both ancillary sites and plasma membrane (Ramanujam et al., 2013). Co-localization with late endosomes suggested that MoRgs7 could respond to the inductive host cues and assist with the regulation of the G-protein signaling pathway in the early stage of germination. As an interacting protein, MoMip11 does not affect the localization of MoRgs7 but interferes with the interaction between MoRgs7 and activated G α to maintain balanced cAMP signaling. Degradation of MoRgs7 assisted by ubiquitin-26s proteasomes accompanied the maturation of appressorium also supports a role of MoRgs7 and its 7-TM domains in direct perception of developmental cues.

Intriguingly, a recent study showed that MoRgs1 that does not have any transmembrane (TM) domains can also be localized to the late endosomes (Ramanujam et al., 2013). This finding may not be inconsistent with our findings employing the MoRgs7TM mutant. Nevertheless, MoRgs7TM mutant showed obviously defect in intracellular cAMP levels and pathogenicity, indicating that the 7-TM domain has essential role in full functions of MoRgs7, thus additional studies to further dissect MoRgs7 and its 7-TM motif functions are necessary and are currently underway.

In eukaryotic cells, various signaling networks converge to regulate growth, differentiation, and pathogenicity. Here, we provided evidence that MoRgs7 interacting MoMip11 functions to regulate G-protein-cAMP signaling and stress responses affecting pathogenicity. Our studies of MoMip11 further highlight the multifaceted roles of adaptor proteins in growth, differentiation, and/or pathogenicity. Considering the crucial roles of MoMip11 in sustaining intracellular cAMP levels and pathogenicity of *M. oryzae*, and the urgency of developing novel fungicides for rice blast control, further studies on MoMip11 and its regulatory network are warranted.

Methods

Strains and culture conditions

The *M. oryzae* Guy11 strain was used as wild-type in this study. All strains were cultured on CM agar plates for 3–15 days in the dark at 28°C (Talbot et al., 1993). Mycelia were harvested from liquid CM medium and used for DNA and RNA extractions. For conidiation, culture blocks were inoculated on SDC (100 g of straw, 40 g of corn powder, 15 g of agar in 1 l of distilled water) agar media (Zhang et al., 2011b) at 28°C for 7 days in the dark followed by 3 days continuous illumination under fluorescent light. For medium containing stress agents, the final concentrations were 2.5 mM DTT (Dithiothreitol), 2.5 mM H₂O₂, 25 ppm Fludioxonil. 30°C was used for high temperature stress.

Targeted gene deletion and complementation

To generate the *MoMIP11* gene deletion vector, two fragments with 1.0 kb of sequences flanking the targeted gene were PCR amplified with primer pairs (Supporting Information Table S2). The resulting PCR products were ligated with the hygromycin resistance cassette (*HPH*) released from pCX62, as previously described (Yin et al., 2016). Putative mutants were first screened by PCR and confirmed by Southern blotting analysis (Supporting Information Fig. S5). To complement the *Momip11* mutant strain, a fragment containing the entire *MoMIP11* gene coding region and its native promoter region, was amplified by PCR with primers (Supporting Information Table S2) and inserted into the pYF11 (bleomycin resistance) vector prior to transferring into the *Momip11* mutant as previously described (Dong et al., 2015).

Observation of the localization of MoRgs7-RFP and MoMagA-RFP in germinating process

To investigate the cellular localization of MoRgs7, the *MoRGS7* gene was tagged with RFP at the C-terminus and was transformed into the wild-type Guy11 strain. Considering that tagging at the C- or N-terminus with a fluorescent marker is known to compromise functions of G-alpha subunit, we introduced the RFP coding sequence in frame into the alphaB-alphaC loop of *M. oryzae* MoMagA (between amino acids 116 and 117) (Gibson and Gilman, 2006; Li et al., 2017c). The MoMagA-RFP construct was then transformed into the *Momip11* mutant and the wild-type Guy11 strains, respectively. Conidia harvested from 10-day-old SDC medium plates and germinated on an inductive surface for 0 to 24 h and observed under a confocal fluorescence microscope (Zeiss LSM710, 63x oil).

Appressorium formation and turgor estimation

For appressorium formation and germinating, conidia were harvested from 10-day-old SDC cultures and adjusted to 5×10^4 conidia/mL in sterile water. Aliquots (30 μ l) were applied on the cover glass (Fisher Scientific, St Louis, MO, USA) and incubated under humid conditions at 28°C. Conidial germination and appressorium formation were examined at 0, 2, 4, 8 and 24 h post incubation. Appressorium turgor was measured using an incipient cytorrhysis (cell collapse) assay with a 1.0 to 4.0 M glycerol solution as described (Zhong et al., 2016). The percentages of appressoria that had collapsed after 10 min was obtained and at least 100 appressoria were counted per replicate at 24 h after germination.

Evaluation of pathogenicity and plant infection

For pathogenicity assays, conidia were harvested from 10-day-old SDC cultures and filtered through three layers of lens paper, and were resuspended by a 0.2% (wt/vol) gelatin solution to a concentration of 5×10^4 spores per milliliter. Two-week-old seedlings of rice (*O. sativa* cv. CO39) were used for spray inoculation assays as described (Zhang et al., 2011a). Inoculated plants were kept in a growth chamber at 25°C with 90% humidity and kept dark for the first 24 h, followed by a 12 h-light and 12 h-dark cycle. Lesion formation was daily detected and photographed 7 days after inoculation. For plant infection assays, conidial suspension (1×10^5 spores/ml) was inoculated into the leaf sheath and incubated for 24h and 48h at 28°C with 90% humidity in dark before microscope observation under Zeiss Axio Observer A1 inverted microscope.

cAMP extraction and HPLC (high-performance liquid chromatography) analysis

Mycelia cultured in liquid CM for 2 days were harvested, frozen in liquid nitrogen and lyophilized for 24 h. Every 1 mg of lyophilized mycelia filled with 20 μ l of 6% TCA solution and then turbulence. Samples were centrifuged at $13,000 \times g$ for 15 min. The supernatant was collected and washed twice with five times the volume of anhydrous ether for HPLC analysis. HPLC analysis was performed as described previously (Liu et al., 2016).

Detection of MoPdeH phosphodiesterase activity *in vitro*

Glutathione-S-transferase (GST), GST-MoPdeH, His-MoMip11 were expressed in *Escherichia coli* BL21-CodonPlus (DE3) cells (Stratagene, Cedar Creek, Texas, USA). Samples were induced with 0.1 mM IPTG (isopropyl β -D-1-thiogalactopyranoside) at 18°C for 4 h. Centrifuged at 4000 rpm for 10 min, and the precipitated cells were resuspended in lysis buffer (1 $\times$ PBS, 10% glycerol). The target proteins were collected by sonication and further purified with glutathione Sepharose beads and His-tag agarose beads, respectively, and then incubated at 4°C for 3 h. Centrifuged at 500 rcf for 5 min and washed with washing buffer {for GST-beads: 20 mM Tris (pH 7.5), 0.25 mM NaCl, 2 mM EDTA, 2 mM EGTA; for His-beads: 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM Imidazole, pH 8.0}, then eluted with elution buffer (for GST-beads: 15 mM glutathione with 5% glycerol, for His-beads: 50mM NaH₂PO₄, 300 mM NaCl, 250mM Imidazole, pH 8.0) and stored at -70 °C.

The phosphodiesterase activity was detected through coupling it with a phosphatase. The phosphate sensor is a phosphate-binding protein modified with a MDCC fluorophore (Brune et al., 1994). For the positive control, 2 $\times$ Amp (Sigma A2252) and 2 $\times$ alkaline phosphatase (Calbiochem P/N 524545) were incubated in PDE enzymatic reaction buffer for 60 min at room temperature, and 2 $\times$ 1 μ M phosphate sensor (Invitrogen P/N PV4406) was added to each well. The plate was mixed and reading was immediately taken at excitation 420 nm and emission 450 nm. For the negative control, 2 $\times$ cAMP (Meilune P/N MB3159), 2 $\times$ alkaline phosphatase and GST protein were incubated in PDE enzymatic reaction buffer for 60 min at room temperature, before adding 2 $\times$ 1 μ M Phosphate Sensor. The protein concentration was measured using the Bradford Protein Assay Kit (Beyotime P0006). All experiments were repeated three times, each with three replicates.

Yeast two-hybrid assays and GST pull down assays

The full length cDNAs of *MoMIP11* (MGG_04719) and *MoRGS7* (MGG_11693) were cloned into pGADT7 vector. Full length cDNAs of *MoRGS7*, *MoMAGA* (MGG_01818), *MoMAGA^{GS}*, *MoMAGB* (MGG_00365), *MoMAGC*, *G β* (MGG_05201), *G γ* (MGG_10193), *MoPKC1* (MGG_08689), *MoMKK1* (MGG_06482), *MoMPS1* (MGG_04943), *MoPDEH* (MGG_05664), *MoOSM1* (MGG_01822) were amplified using HiScript II Q RT SuperMix for qPCR (R233-01, Vazyme Biotech Co., Nanjing, China) cloned into pGBKT7 vector (BD Biosciences Clontech, Oxford, UK), respectively. The resulting prey and bait constructs were confirmed by sequencing analysis and transformed in pairs into yeast strain AH109 following the manufacturer's recommended protocol (BD Biosciences Clontech). Transformants grown on synthetic medium lacking leucine and tryptophan (SD-Leu-Trp) were further transferred to synthetic medium lacking leucine,

tryptophan, adenine and histidine (SD–Leu–Trp–Ade–His). Yeast stains for positive and negative controls were provided by the BD library construction & screening kit.

For protein production in *Escherichia coli*, the full length of *MoMIP11*, *MoMAGA* and *MoMAGA^{GS}* cDNA were inserted into pGEX4T-2 and pET32a vectors, respectively. The resultant plasmid DNA was transformed into BL21-CodonPlus (DE3) cells (Stratagene). And the pull down expression and elution assays were performed as described previously (Yin et al., 2016; Li et al., 2017b).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Dynamic subcellular localization of MoRgs7 during early and late stages of spore germination

(A and B) MoRgs7-RFP conidia were inoculated on plastic cover slips and incubated in a moist chamber before microscopic observations. DIC and epifluorescence images were captured at the indicated time points. The arrows highlight the localization pattern of MoRgs7-RFP fusion protein at 2 hpi and 4 hpi (tubulo-vesicular structures). Part of appressorium at 8 hpi and 24 hpi showed weak RFP intensity. Scale bar, 10 μ m. (C) One hundred appressorium at 8 hpi and 24 hpi were quantified for fluorescence observation, respectively. Error bars represent the standard deviations, asterisks denote statistical

significances ($P < 0.01$). (D) Cells from different stages were analyzed by Western blot for MoRgs7-RFP detecting. (E) Expression level screening of *MoRGS7* at different stages during germination by qRT-PCR. Asterisks denote statistical significances ($P < 0.01$).

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Fig. 2. Identification of tubule-vesicular compartments that harbor MoRgs7 during the early stage of germination

(A) GFP-Rab7 (a late endosome marker) co-localizes with MoRgs7-RFP during early stages of germination. The arrowhead depicts the region magnified in the inset. Scale bar, 10 μm .

(B) GFP-Rab5 (an early endosome marker) does not co-localize with MoRgs7-RFP during the early stages of germination. Scale bar, 10 μm . (C) The linescan graph is consistent with the co-localization of Rab7 and MoRgs7. (D) A linescan graph is also consistent with non-co-localization of Rab5 and MoRgs7.

Fig. 3. Identification of MoRgs7-interacting MoMip11

(A) Multiple sequence alignment was made by ClustalW program. (B) Sequence alignment of MoMip11 orthologues. All conserved residues are shown in black and similar residues in gray. The positions of the conserved WD and GH repeats, as well as structurally conserved S and D residues in WD proteins were shown below the sequences. MoMip11 shares high homologous to Gib2/RACK1/Asc1 proteins sharing 72%, 71%, 58% in amino acid sequence similarities, respectively. (C and D) The crystal structure of MoMip11. Ribbon diagram of *C. neoformans* Gib2 (PDB: 4D6V) was used as the template for MoMip11 model.

Molecules were colored using the chainbow scheme from blue (N-terminus) to red (C-terminus) and visualized using Cn3D macromolecular structure viewer. The seven β -propeller blades were numbered, and individual β -sheets for the second blade were also labelled. The extended loop between blades six and seven is also indicated. (E) Yeast two-hybrid analysis. The pair of plasmids pGBKT7-53 and pGADT7-T was used as the positive control. The pair of plasmids pGBKT7-Lam and pGADT7-T was used as the negative control. Columns in each panel represent serial decimal dilution. Plates were incubated at 30°C for 3 days before being photographed. (F) co-immunoprecipitation assay. Western blot analysis with total proteins (Total) isolated from transformants co-expressing the *MoMIP11*-GFP and *MoRGS7*-3xFLAG constructs and proteins eluted from anti-GFP beads (Elution). The presence of MoMip11 and MoRgs7 was detected with an anti-GFP and an anti-FLAG antibody, respectively.

Fig. 4. MoMip11 is important for vegetative hyphal growth and conidium formation

(A) The *Momip11* mutant displays significantly reduced mycelial growth on complete media (CM), minimal media (MM), oatmeal media (OM) and straw decoction and corn media (SDC) after incubation at 28°C for 7 days in darkness. (B) Statistical analysis of the diameters of hypha from the wild-type Guy11, the *Momip11* mutant, and the complemented strains on different medium. Error bars represent standard deviations, asterisks denote statistical significances ($P < 0.01$). (C) Aerial hyphae growth is reduced in the *Momip11* mutant after incubation for more than 10 days. Strains were grown under the same conditions as above and colony side views are displayed. “a” stands for Guy11 and “b” stands for the *Momip11* mutant. (D) Conidia formation was observed under a light microscope 24 h at room temperature after induction of conidiation under cover slips. Scale bar, 30 μm . (E) Statistical analysis of conidia production. Asterisks denote statistical significances ($P < 0.01$).

Fig. 5. MoMip11 is required for infection and pathogenicity

(A and B) Two-week-old and four-week-old rice seedlings were sprayed or injected with conidial suspensions (5×10^4 spores/ml) of the Guy11, the $\Delta Momip11$ mutant and the complemented strains, respectively. Diseased leaves were photographed 7 days after inoculation. The arrow points to the injection site. (C) Different concentrations of glycerol (1 to 4 M) were used for cytorrhysis analysis. For each concentration, at least 100 appressoria were counted and the experiment was repeated three times with the same result. Asterisks denote statistical significances ($P < 0.01$). (D) The conidial suspension (1×10^5 spores/ml) was inoculated in the excised rice sheath of 4-week-old rice seedlings. The invasive hyphae growth was observed at 24 and 48 hpi. Asterisks denote the sites that the infectious hyphae stretched to the next cell. (E and F) The infectious hyphae were divided into four types. For each strain tested, 100 hyphae were counted per replicate and the experiment was repeated three times. Scale bar, 10 μ m.

Fig. 6. MoMip11 promotes intracellular trafficking of MoMagA

(A) Pathogenicity test on rice seedlings. Four milliliters of conidial suspension (5×10^4 spores/ml) of Guy11, *Momip11*, *Momip11/MAGA^{GS(G187S)}*, and complemented strains were used for spraying and symptoms examined 7 dpi. (B) Quantification of intracellular cAMP levels. Bar charts showing the quantifications of intracellular cAMP levels in the mycelia stage following 2 days of culture in CM. Two biological repetitions with three replicates were assayed. The error bars represent SD of three replicates. The different small letters indicate statistical significance of difference at $P = 0.05$. (C) Excised barley leaves

from 7-day-old barley seedlings were inoculated with mycelia and infectious growth was observed at 24 hpi. For each tested strain, 100 infecting hyphae were counted per replicate and the experiment was repeated three times. Scale bar, 10 μ m. (D) MoMagA-RFP was found in the distinct tubule-vesicular structures in Guy11 after germinating for 2 h. The arrowhead depicts the magnified region in the inset. The linescan graph is consistent with the localization observation. Scale bar, 10 μ m. (E) MoMagA-RFP is observed mostly on the plasma membrane in the *Momip11* mutant. The linescan graph consistent with the localization observation. Scale bar, 10 μ m.

Fig. 7. MoMip11 interacts with G-protein G α MoMagA but not activated G α , G β , or G γ
 (A) Yeast two-hybrid analysis. MoMip11 and MoRgs7 were co-introduced with G α (MoMagA, MoMagB, MoMagC), activated G α (MoMagA^{GS}), G β , and G γ into AH109 strain, respectively. Yeast transformants growth on SD-His-Leu-Trp-Ade plates. Plasmids pGBKT7-53 and pGADT7-T were used as the positive control and pGBKT7-Lam and pGADT7-T were used as the negative control. (B, C) *In vitro* pull down assays to confirm the interaction between MoMip11 and G α (MoMagA). The recombinant GST-MoMip11 or GST- bound to glutathione Sepharose beads was incubated with *E. coli* cell lysate containing

His-MoMagA and MoMagA^{GS}, respectively. Eluted proteins were analyzed by immunoblot (IB) with the monoclonal anti-His and anti-GST antibodies. (D and E) co- IP assay. Western blots of total proteins, suspensions and proteins eluted from anti-GFP agarose from transformants co-transformed with MoMip11-GFP and MoMagA-FLAG and MoMip11-GFP and MoMagA^{GS}-FLAG were detected with the anti-FLAG or anti-GFP antibodies.

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Fig. 8. MoMip11 interferes with the interaction between MoRgs7 and MoMagA^{GS}

(A) MoMip11 weakens the interaction between MoRgs7 and MoMagA^{GS}. The recombinant GST-MoRgs7 or GST- bound to glutathione Sepharose beads was incubated with *E. coli* cell lysate containing His-MoMagA^{GS} and His-MoMip11. Eluted proteins were analyzed by immunoblot (IB) with the monoclonal anti-His and anti-GST antibodies. (B) MoMip11 interferes with the interaction between MoRgs7 and MoMagA^{GS} *in vitro* in a dose-dependent manner. Co-precipitation of MoRgs7 and MoMagA^{GS} in the presence of MoMip11 was examined by Western blotting before (input) and after affinity purification (GST pull-down). Different gradient dilutions of MoMip11 were added into the mix in the form of total protein extracts prepared from *E. coli*.

Fig.9. MoMip11 interacts with MoPdeH to suppress its phosphodiesterase activity

(A) co-IP assay. Western blot analysis with total proteins (Total) isolated from transformants co-expressing *MoMIP11*-GFP and *MoPDEH*-3xFLAG and proteins eluted from anti-GFP beads (Elution). The presence of MoMip11 and MoPdeH was detected with the anti-GFP and anti-FLAG antibody, respectively. (B) *In vitro* pull down assay showing the interaction between MoMip11 and MoPdeH. Recombinant GST-MoPdeH or GST- bound to glutathione Sepharose beads was incubated with *E. coli* cell lysate containing His-MoMip11, respectively. Eluted proteins were analyzed by immunoblotting (IB) with the anti-His and anti-GST antibodies. (C) The invasive hyphae growth was observed at 24 and 48 hpi. Asterisks denote the sites where the infectious hyphae expanded to the next host cells. CK represents the conidia suspension without IBMX. (D) Detection of protein phosphodiesterase activities *in vitro*. Target proteins were expressed in *E. coli* BL21-CodonPlus (DE3) cells. The phosphate sensor was used to bind free inorganic phosphate (Pi), and fluorescence was read immediately at excitation 420 nm and emission 450 nm. The error bars indicate standard deviations of three replicates. Different letters indicate statistically significant differences ($p < 0.01$). T represents the same total protein concentrations of MoPdeH and MoMip11. 0.1T and 0.2T represent the concentrations of MoMip11 following dilution.

Fig. 10. MoMip11 is involved in multiple environmental responses

(A) Sensitivity test of strains to high temperature (30°C), ER stress (DTT), oxidative stress (H₂O₂), and Fludioxonil. Strains were incubated on CM supplemented with 2.5mM DTT, 2.5mM H₂O₂, or 25ppm Fludioxonil at 28°C or 30°C for 7 days. (B) The inhibition rate was determined by plotting the percentage of colonies in the presence of various stresses against the control. Asterisks denote statistical significances ($P < 0.01$). (C) Expression analysis of four heat shock genes (*MoSSB1*, *MoSSQ1*, *MoSSZ1*, and *MoLTH*) and a ribosomal gene (*MoRPP0*) at 28°C (CK) and 30°C (high temperature stress) by qRT-PCR. (D) Expression analysis of three putative UPR target genes (*MoSCJ1*, *MoKAR2*, and *MoSIL1*) following DTT treatment of 4 hours. (E) Expression analysis for the catalase gene (*MoCAT1*) following 2.5 mM H₂O₂ treatment of 2 hours. Asterisks denote statistical significances ($P < 0.01$).

Fig. 11. A proposed model of MoMip11 in the regulation of cAMP signaling

MoRgs7 responds to the inductive surface cues in the early stage of germination and degrades gradually accompanied with appressorium maturation. MoMip11 promotes MoMagA activation while suppresses MoPdeH activities thereby up-regulating intracellular cAMP levels. MoMip11 also interferes with the interaction between MoRgs7 and activated Gα resulting in a positive role in cAMP signaling. In addition, MoMip11 could partially compensate for lacking of Gβ in appressorium formation.