# A Rho3 Homolog Is Essential for Appressorium Development and Pathogenicity of *Magnaporthe grisea*<sup>∇</sup>

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The small GTPase Rho3 is conserved in fungi and plays a key role in the control of cell polarity and exocytosis in yeast. In this report, we show that a Rho3 homolog, MgRho3, is dispensable for polarized hyphal growth in the rice blast fungus *Magnaporthe grisea*. However, MgRho3 is required for plant infection. Appressoria formed by the Mgrho3 deletion mutants are morphologically abnormal and defective in plant penetration. Conidia of the Mgrho3 deletion mutants are narrower than those of the wild-type strain and delayed in germination. Transformants expressing a dominant negative Mgrho3 allele exhibit similar phenotypes as the Mgrho3 deletion mutant, while transformants expressing a constitutively active allele of MgRho3 can produce normal conidia but remain defective in appressorium formation and plant infection. In contrast, overexpression of wild-type MgRho3 increases the infectivity of *M. grisea*. Our results reveal a new role for the conserved Rho3 as a critical regulator of developmental processes and pathogenicity of *M. grisea*.

Magnaporthe grisea is an ascomycete pathogen of important cereal crops, such as rice, barley, and wheat. It causes rice blast, one of the most severe fungal diseases of rice throughout the world (33, 35). The fungus infects rice plants in a sophisticated manner, like many other foliar pathogens. Germ tubes produced from conidia differentiate into specialized infection structures called appressoria and then use the enormous turgor pressure generated in appressoria for plant penetration (8). Mature appressoria develop thin penetration pegs to physically pierce the host surface and enter plant epidermal cells. At early stages, the penetration peg contains high concentrations of actin filaments (3) and recruits the rice cell membrane to form the extrainvasive hyphal membrane, while infection hyphal growth and its co-opt plasmodesmata for cell-to-cell movement (18). It has been hypothesized that actin and cytoskeleton elements may be involved in the reestablishment of polarized growth by determining the penetration site and in stabilizing the tip of the penetration peg (15). After penetration, the peg differentiates into infectious hyphae that grow inter- and intracellularly and result in development of blast lesions.

Signal transduction pathways that regulate infection-related morphogenesis have been extensively studied in *M. grisea* during the past few years (34, 41). In general, cyclic AMP (cAMP) signaling is involved in surface recognition and initiation of appressorium formation (1, 20, 22, 44). However, appresso-

rium formation is regulated by the *Pmk1* mitogen-activated protein kinase pathway (25, 42, 46). The *pmk1* deletion mutant fails to form appressoria and is nonpathogenic. One putative transcription factor regulated by PMK1 is MST12, which is homologous to Saccharomyces cerevisiae Ste12 and essential for pathogenesis (27). The mst12 deletion mutant forms melanized appressoria that have normal appressorium turgor but fail to develop penetration pegs, probably due to cytoskeleton defects in mature appressoria (26). MST12 may function downstream of PMK1 to regulate genes involved in appressorial penetration and infectious growth, but other factors must exist in M. grisea to regulate appressorium formation. Other M. grisea mutants that are known to form melanized appressoria but that fail to penetrate plant cells include PLS1 (encoding a tetraspanin-like protein) (6) and MPS1 (encoding a mitogen-activated protein kinase) mutants (43). The mps1 mutant appears to have weaker cell walls, but the penetration defect of the pls1 mutant may be related to cytoskeleton changes associated with appressorial penetration.

Besides the importance of infection structure differentiation, it is also known that secretion of elicitor proteins plays a key role in the pathogenesis of most pathogenic fungi. For example, genome analysis has revealed that the plant fungal pathogen *M. grisea* has 739 secreted proteins, nearly twice the proteins found in the nonpathogenic fungus *Neurospora crassa* (7). It is very important to understand how these proteins are secreted into the host cells. However, the mechanisms that regulate protein secretion in pathogenic fungi are still unknown.

The Rho family of small GTPases regulate a wide spectrum of cellular functions, especially those involving the actin cytoskeleton (36). However, different members in the Rho family have specific functions. Rho3, one of the Rho family members, first isolated in *S. cerevisiae* (21), has been shown to act as a key regulator of cell polarity and exocytosis through modulating vesicle delivery function, mediated by the unconventional myosin Myo2, and vesicle docking and fusion functions, which are

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mediated by the exocytosis component Exo70 (2, 16, 23, 29). In *Rho3* promoter shutdown experiments, a strong cell polarity defect and a partially depolarized actin cytoskeleton were observed in *Candida albicans* (10). Rho3-regulated exocytosis also is critical for cell division, cell separation, and polarized cell growth in *Schizosaccharomyces pombe* (38). In filamentous fungi, both growth and protein secretion of *Trichoderma reesei* in cellulose cultures are remarkably decreased in *rho3* disruptant strains (37). Deletion of AgRho3 caused early lysis of emerging germ tubes and characteristic swellings at the hyphal tips of *Ashbya gossypii* (40). Recently, it has been shown that AgRho3p is able to directly activate formin-driven actin cable nucleation through its interaction with the SH3/PH domain-containing protein AgBoi1/2p (19).

In this study, we identified and characterized the Rho3 homolog in *M. grisea* (named MgRho3) and determined its role in pathogenesis. The Mgrho3 deletion mutant was viable and had a normal growth rate. Slender conidia from the Mgrho3 mutant were delayed in germination and defective in appressorium formation. Appressoria formed by the Mgrho3 deletion mutant were morphologically abnormal and defective in plant penetration. Expression of a dominant active or dominant negative Mgrho3 also resulted in defects in appressorium formation and plant penetration, but overexpression of wild-type MgRho3 resulted in more efficient plant infection. These results indicate that MgRho3 is dispensable for polarized hyphal growth but required for pathogenesis in *M. grisea*.

### MATERIALS AND METHODS

Fungal strains, culture, and transformation. Magnaporthe grisea (Herbert) Barr strain 70-15 (7) and its derivative strains described in this paper were maintained on sterile filter paper and cultured in complete medium. For sporulation, oatmeal agar or rice polish agar medium was used. Fungal transformations were performed as described by Sweigard et al. (32), using hygromycin B (at a final concentration of 400  $\mu$ g/ml; Roche Applied Science, Mannheim, Germany) or glufosinate ammonium (at a final concentration of 300  $\mu$ g/ml; Sigma-Aldrich Co., St. Louis, MO) as a selective marker, depending on the selection marker gene on the plasmid vector to be transformed.

**Isolation of MgRho3.** Primers P6f and P6r (Table 1) were used to amplify MgRho3 from 70-15 genomic DNA by PCR consisting of 30 cycles of 45 s at 94°C, 45 s at 58°C, and 2 min at 72°C, followed by 7 min at 72°C. PCR products were then cloned in the pGEM-T Easy vector (Promega Corp., Madison, WI) and sequenced. Corresponding cDNA was isolated by reverse transcription-PCR (RT-PCR) with primers P6f and P6r, cloned in the pGEM-T Easy vector, and sequenced. Total RNA and cDNA used in the study were all isolated with the SuperScript first-strand synthesis system for RT-PCR (Invitrogen Corp., Carlsbad, CA).

**MgRho3 replacement and mutants.** To generate the MgRho3 gene replacement vector, a 2.0-kb fragment of MgRho3 was amplified with primers P1f and P1r and cloned between KpnI and SacI sites on pCSN43 as pRHO3. The *hph* gene was released from pCSN43 with XhoI and EcoRV sites and cloned into the XhoI and SmaI sites of pRHO3 as the MgRho3 gene replacement vector pKOR3. To construct the complementation vector pCOR3, a 3.03-kb fragment including a native promoter and open reading frame (ORF) of MgRho3 was amplified with primers P2f and P2r and then cloned between SacI and BamHI sites of pBARKS1 containing the Basta resistance gene.

Site-directed mutagenesis was used to generate dominant MgRho3 mutants (MgRho3-CA [constitutively active] and MgRho3-DN [dominant negative]) by PCR-mediated amplification from cDNA of strain 70-15. Two primers including the forward primer P3f and reverse primer P3r were used to generate MgRho3-CA by replacing the glycine (G22) of MgRho3 with valine, and a dominant negative MgRho3 mutant (MgRho3-DN) was generated by replacement of the aspartic acid (D128) with alanine by recombinant PCR with primers P4f, P5r, P5f, and P4r. All mutated DNA fragments were amplified with Pfu polymerase (Stratagene) and sequenced. Expression of MgRho3-CN and MgRho3-DN was driven by the constitutive RP27 promoter built within pTE11.

TABLE 1. Primers used for this study

Name	Sequence $(5' \rightarrow 3')$								
P1f	CGGGGTACCTGGTTGAGCAGTTG								
P1r	CGAGCTCACGGGTCTCAGAAGTT								
P2f	CGAGCTCAGCATTCACGACACAG								
P2r	GGGGATCCCTACATGACGGTGC								
P3f	ATGCCTTTATCGCTTTGCGGAGGAAGCAA								
	AACGGTCCAGCGCAAGCTGGTATTGCTT								
	GGCGACGTTGCTT								
P3r	CTACATGACGGTGCACTTGCTCTCCTCG								
P4f	CCGCTCGAGCTATGCCTTTATCGCTT								
P4r	CCGCTCGAGCTACATGACGGTGC								
P5f	TGCGCTCAAGTGCGCCTTGCGCCAGGAGC								
P5r	GCTCCTGGCGCAAGGCGCACTTGAGCGCA								
P6f	CGGGATCCGTATGCCTTTATCGCTTTGCG								
P6r	GCCTGCAGCTACATGACGGTGCACTTG								
P7f	GCTGTCCTCGTCGATCTCGA								
P7r	CAGAGCAGGTCAGGTAACGA								
P8f	TTGGCGACGGTGCTTGT								
P8r	GTTCTCGAATACTGTTGGCTCGT								
P9f	TCCGTGGAAAGGTTTCCATG								
P9r	ATCCACTCGACGAAGTACGA								
Phf	ATGCGATCGCTGCGGCCGATCTTAG								
S1	GATCGACGCGAACCCCCATA								
S2	AGAAAGCCCGGAAGTCTGTGTAA								
S3	TGTCAAGTTGGTGCTGGTTGC								

The MgRho3 genomic DNA fragment amplified from 70-15 with primers P4F and P4R was also cloned into the site of XhoI in pTE4 and driven by the promoter RP27 to generate the overexpression vector pROE3.

Reverse transcription-PCR and real-time PCR analysis. Total RNA samples were prepared using an SV total RNA isolation system (Promega Corp.) from growing hyphae of *M. grisea*. cDNAs were synthesized as described above. For reverse transcription-PCR, a 0.76-kb PCR fragment for the  $\beta$ -tubulin gene Mg-*TUB* (MGG\_00604.5) was amplified as an internal control using primers P7f and P7r. Mg*Rho3* was amplified through RT-PCR with the primers P6f and P6r.

In a real-time PCR, primers P8f and P8r were used to amplify a 92-bp amplicon of Mg*Rho3*. As an endogenous control, an 85-bp amplicon of the  $\beta$ -tubulin gene was amplified with primers P9f and P9r. Quantitative real-time RT-PCR was performed with the MJ Research Opticon real-time detection system using TaKaRa SYBR Premix Ex *Taq* (Perfect Real Time; Takara, Japan). To verify that the amplification efficiencies of Mg*Rho3* and Mg*TUB1* were approximately equal, PCRs were performed with serial dilutions of cDNA templates. The relative quantification of the Mg*Rho3* transcript was calculated by the  $2 - \Delta\Delta Ct$  method (20a).

Analysis of hyphal morphology, conidium germination, appressorium formation, and penetration. Conidial suspensions (5  $\times$  10<sup>4</sup> conidia/ml) were prepared from 10-day-old oatmeal agar cultures, and aliquots (50 µl) were applied to either the hydrophobic or hydrophilic side of a GelBond film (Cambrex Bio-Science, Rockland, ME). The conidial droplets were incubated in a moist petri dish at room temperature. Conidial germination and appressorium formation were examined at 1, 2, 4, 8, and 24 h postincubation. Appressorium penetration on onion epidermal cells was assayed as described elsewhere (44). Photographs were taken with an Olympus BX51 universal research microscope. Cell wall and septum of hyphae were visualized by calcofluor white staining as described previously (13). To observe the Spitzenkörper localization, FM4-64 staining of hyphal tips was conducted following the procedure described by Fischer-Parton et al. (11). Mycelia of MgRho3 mutants were stained with a 7.5 µM aqueous working solution of FM4-64 and observed immediately. Confocal images were acquired using a Zeiss Axioskop 2 microscope equipped with a Zeiss LSM 510 Meta system. Spectral data were collected with 514-nm excitation using 1-mW helium-neon lasers. The appressorium turgor was examined with the incipient cytorrhysis technique using 2 M glycerol (14). For intracellular cAMP measurements, mycelia were collected from 3-day-old liquid complete medium cultures by filtering through Miracloth and frozen in liquid nitrogen immediately. All fungal samples were lyophilized for 16 h and weighed. Dry fungal tissues (100 µg) were ground to a powder in liquid nitrogen and resuspended in 1 ml ice-cold 6% (wt/vol) trichloroacetic acid (24) and then centrifuged in Eppendorf tubes at 12,000 rpm for 5 min at 4°C. The supernatant (200 µl) was transferred to a new tube and extracted five times with an equal volume of chloroform. The concen-

				G	1				G2		G3	
A	*. *		* ::	***	* ***	.**		** *	**:*:**		* ******	*::*:**.**
MgRho3	MPLSLCGGS-	KTV	QRKLVL	LGDG	ACGKT	SLLNV	FTRO	SYFPTVY	EPTVFENYVH	DIFVDN-VHI	ELSLWDTAGQ	EEFDRLRSLS
CgRho3	MA-FLCGSSS	SQSSGSKKPV	ERKIVI	LGDG	ACGKT	SLLNV	FTRO	SYFPEVY	EPTVFENYIH	DIFVDN-RHI	TLSLWDTAGQ	EEFDRLRSLS
ScRho3	MS-FLCGSAS	TSNKPI	ERKIVI	LGDG	ACGKT	SLLNV	FTRO	SYFPEVY	EPTVFENYIH	DIFVDS-KHI	TLSLWDTAGQ	EEFDRLRSLS
Y1Rho3	MA-ICGGAP-	KAI	SRKIVI	LGDG	ASGKT	SLLNV	FTRO	SYFPHVY	EPTVFENYVH	DIFIDG-QPV	QLSLWDTAGQ	EEFDRLRSLS
SpRho3	MS-SCFGS	KKKPI	YRKIVI	LGDG	AAGKT	SLLNV	FTKO	SYFPQVY	EPTIFENYIH	DIFVDG-NSI	ELSLWDTAGQ	EEYDQLRSLS
GzRho3	MP-ALCGGS-	KTV	QRKLVL	LGDG	ACGKT	SLLNV	FTRO	SYFPTVY	EPTVFENYVH	DIFVDN-VHI	ELSLWDTAGQ	EEFDRLRSLS
NcRho3	MP-CGLGGS-	KTV	QRKLVL	LGDG	ACGKT	SLLNV	FTRO	SYFPTVY	EPTVFENYVH	DIFVDN-VHI	ELSLWDTAGQ	EEFDRLRSLS
HjRho3	MPLCGGS-	KTV	QRKLVL	LGDG	ASGKT	SLLNV	FTRO	SYFPTVY	EPTVFENYVH	DIFVDN-VHI	ELSLWDTAGQ	EEFDRLRSLS
UmRho3	MTLCGLVH	DAQR-ESHRI	ARKVLV	LGDG	ASGKT	SLLFV	LVR	<b>IEFPQTY</b>	EPTVFENYTH	IQHTHTGLAV	ELTLWDTAGQ	EEFDKLRSLS
AfRho3	MASGPA-	TQ	SLKCVV	TGDG	AVGKT	CLLIS	YTT	NAFPGEY	IPTVFDNYTA	SVMVDG-RPI	SLGLWDTAGQ	EDYDRLRPLS
								G4				
	* :*::*	*::	** :*	*:	1.000		**.	: ***				:
MgRho3	YDDTDLIMLC	YSVDSKDSLE	NVESKW	VGEI	ADNCP	GVKLV	LVA	LKCDLRO	EOADDAA	E	NGNATDPS00	REKKPMITYD
CgRho3	YSDTHTIMLC	FSIDSRDSLE	NVKNKW	VGEI	TDHCE	GVNLV	LVA	LKCDLRN	NÊNNVITP	NNIQN	NRNNNNN	NLITYE
ScRho3	YSDTQCIMLC	FSIDSRDSLE	NVQNKW	VGEI	TDHCE	GVKLV	LVA	LECDLRN	NENESNAITP	NNIQQDNSVS	NDNGNNINST	SNGKNLISYE
Y1Rho3	YSDTHTIMLC	FSVDSRDSLE	NVQTKW	VGEI	ADHCE	GVKLV	LVA	LKCDLRN	NEE		GGEGAKKT	YVTYE
SpRho3	YSDTHVIMIC	FAVDSRDSLE	NVITKW	LPEV	SSNCP	GVKLV	LVA	LKCDLRG	ADE		-EQVDHSK	IIDYE
GzRho3	YDDTDLIMLC	YSVDSKDSLE	NVESKW	VGEI	ADNCP	GTKLV	LVA	LKCDLR-	EQGEDDE	N	AAAGGESEQP	REKKPMINYD
NcRho3	YDDTDLIMLC	YSVDSKDSLE	NVESKW	QGEI	AENCP	GVKIV	LVA	LKCDLR-	EATEDEE	D	GGANEDGAQ-	REKKPMISYD
HjRho3	YDDTDLIVLC	YSVDSKDSLE	NVESKW	VGEI	ADNCP	GVKLV	LVA	LKCDLR-	QQEDDEP	E	DQAAADGNAQ	REKPPTISYD
UmRho3	YADTDVVLLC	FSVDNPVSLQ	NVESRW	IAEI	RHHCP	AVKIM	LVA	LKCDLR-			EPRNPR	TLSYN
AfRho3	YPOTDVFLIC	FSIVSPPSFD	NVKAKW	YPEI	EHHAP	NVPII	LVG	FKLDLR-			-DDPATAESL	ROKKMDLSRI
	G5					CAAX			C box			
	. : . *	* :*****		. *	*				*			
MaRho3	TOLEVARRIN	AL RYL ECSAM	RNRGVNI	FAFT	FAARV	AT SVK	KDRE	FSK	CT	VM		
CaRho3	FOLAMAKOTO	AL RYL FCSAK	INKGVN	FAFT	FAARV	ALTAK	NATI	NINSNS	SSNEKDSECT	TC		
ScRho3	FGLAMAKKIG	ALRYLECSAK	LNKGVN	FAFT	FAARV	ALTAG	PVAT	FVKSDS	GSSCT	TM		
VIRho3	OGLEVAKKIG	ALRYLECSAK	MNRGVN	FAFT	FAARC	ALGAK	PKG	AADDKE	ESSKCV	TM		
SpRho3	EGLAAAKKIN	AVRYLECSAK	LNRGVN	EAFT	FAARV	ALAAO	PRG	CKDGADE	SHGTGCT	TA		
GzRho3	OGLEVARRIG	AORYLECSAM	KNRGVN	FAFT	FAARV	ALSVK	KDR	FGK	CT	VM		
NcRho3	OGLEVARRIK	AL RYLECSAM	RNRGVN	FAFT	FAARV	ALSVK	KDR	FSK	CV	VM		
HiDho2	FOLEWAYDIG	ACOVI FOCAM	VNDCUAL	EXET	FAADU	AT CUV	VEDI	DNIV	OT	TM		



tration of intracellular cAMP was determined with the enzyme immunoassay system (Amersham-Pharmacia Biotech) according to the manufacturer's instructions.

**Infection assays.** Rice (*Oryza sativa* L.), cultivar CO39, was grown under greenhouse conditions. Fifteen-day-old seedlings were used for infection assays. Conidial suspensions  $(1 \times 10^5$  conidia/ml in 0.02% Tween solution) were prepared from rice polish agar cultures and spray inoculated onto plant seedlings as described elsewhere (44). Root infection assays were carried out as described previously (9).

# RESULTS

**MgRho3 encodes a homolog of Rho GTPases.** In the *M. grisea* genome sequence (7), there is one Cdc42 gene homolog, one Rac1 gene homolog, and five other Rho-GTPase-encoding genes. The predicted gene MGG\_10323.5 encodes a protein that shares 88%, 87%, 66%, and 65% identity with Rho3 homologs from *N. crassa, Hypocrea jecorina, S. cerevisiae*, and *S. pombe*, respectively. It contains all the consensus domains characteristic of Rho GTPases, including a GTP/GDP binding domain, an effector binding site, a Rho insert domain, and a membrane localization domain CAAX box (Fig. 1A). Thus, we named the gene product MgRho3 (for *M. grisea* Rho3). Phylogenetic analysis revealed that MgRho3 is closely related to Rho3 homologs from other fungi (Fig. 1B).

**MgRho3** is dispensable for vegetative growth in *M. grisea*. To study the function of Mg*Rho3*, the 728-bp fragment of the Mg*Rho3* ORF was replaced with the hygromycin phosphotransferase (*hph*) gene in strain 70-15. Three putative Mg*rho3* deletion mutants,  $\Delta$ Mg*rho3-5*,  $\Delta$ Mg*rho3-9*, and  $\Delta$ Mg*rho3-22*, were identified by PCR with primers Phf and P2r and confirmed by Southern blot analysis (Fig. 2A and B). No Mg*Rho3* transcripts could be detected by RT-PCR in the deletion mutant  $\Delta$ Mg*rho3-22* (Fig. 2C). Two additional Mg*rho3* deletion mutants,  $\Delta$ Mg*rho3-5* and  $\Delta$ Mg*rho3-9*, had similar hybridization patterns as  $\Delta$ Mg*rho3-22* in a Southern blot analysis (data not shown).

The growth rate of the Mgrho3 deletion mutant was similar to that of the wild-type strain, but conidiation was significantly reduced in the mutant  $\Delta Mgrho3-22$  (see Table 2). Conidia formed by the Mgrho3 deletion mutant were three celled and similar in length to those of 70-15. However, conidia produced by the mutant  $\Delta Mgrho3-22$  had reduced widths (Table 2) and appeared to be ellipsoid instead of pyriform (Fig. 3A). We also found that the Mgrho3 mutant appeared to have the hyperbranching phenotype in aerial hyphae and became curly and slightly swollen at the tip compared to those of 70-15 (Fig. 3B and C). Similar to 70-15, the Mgrho3 deletion mutant had one nucleus in each hyphal compartment (data not shown), suggesting that nuclear division and cytokinesis were normal in the



FIG. 2. The MgRho3 knockout construct and molecular confirmation. (A) Restriction map of the MgRho3 genomic region and knockout construct. Thick arrows indicate orientations of the MgRho3 and phosphotransferase (hph) genes. Restriction enzymes are abbreviated as follows: K, KpnI; S, SmaI; Sa, SalI; X, XhoI. The MgRho3 knockout construct pKOR3 was constructed by replacing the XhoI/SmaI 774-bp fragment of the MgRho3 ORF with the hph gene. (B) Total genomic DNA samples (5 µg per lane) isolated from M. grisea 70-15 (wild type [WT]), ΔMgrho3-22 (deletion mutant), MgRho3-Com (complemented transformant), and MgRho3-Ect (ectopic transformant) were digested with PstI and subjected to Southern blot analysis. The probe, a 599-bp PCR fragment amplified from 70-15 genomic DNA using primers S1 and S2, is part of the 0.7-kb MgRho3 fragment replaced by the 2.4-kb hph gene (top). The same blot (at the top of the panel) was stripped and rehybridized with a 421-bp probe amplified from 70-15 genomic DNA by primers S3/P1r (lower portion of the panels). This probe is located downstream of the SmaI gene deletion site (see Materials and Methods). (C) Total RNA samples (approximately 2 µg per reaction mixture) isolated from mycelia of M. grisea strains 70-15 (WT), ΔMgrho3-22 and MgRho3-Com were subjected to RT-PCR using gene-specific primers P6f/P6r. The RT-PCR product is 633 bp, as predicted.

Mgrho3 deletion mutant. Although MgRho3 is dispensable for septum formation in *M. grisea*, the average length of hyphal compartments (distance between two septa) was reduced in  $\Delta$ Mgrho3-22 (Fig. 3C). FM4-64 staining of hyphae in the wild type and the Mgrho3 deletion mutant showed that Spitzenkörper was still present at the hyphal tips, as shown in Fig. 4. This result matched our preliminary result (data not shown), that the total secreted protein was not affected in the mutant.

MgRho3 is essential for plant infection. To determine whether MgRho3 is involved in pathogenesis, we conducted

FIG. 1. Alignment and phylogenetic analysis of MgRho3 with other fungal Rho3 homologs. (A) An alignment of Rho3 homologs in fungi based on their amino acid sequences. Five GTP/GDP binding or hydrolysis domains are highlighted and labeled as G1 through G5. G1, GXXXXGKS/T; G2, core effector domain T; G3, DXXGQ/H/T; G4, T/NKXD; G5, C/SAK/L/T); CAAX box, a posttranslational prenylation motif. (B) Phylogenetic relationship of Rho3 homologs in fungi, calculated using the neighbor-joining method of parsimony distance in PHYLIP 3.65 (bootstrap values [percentages] are indicated at the nodes). The fungal source and accession number of each sequence are represented as follows: AfRho3, *Aspergillus fumigatus*, AAG12157; AnRho3, *Aspergillus nidulans*, XP\_660291; CaRho3, *Candida albicans*, EAK95917; EgRho3, *Eremothecium Gossypii*, AAG41252; EnRho3, *Emericella nidulans*, AAK55443; FgRho3, *Fusarium graminearum*, XP\_380346; HjRho3, *Hypocrea jecorina*, CAC20376; MgRho3, *Magnaporthe grisea*, MG07176.4); NcRho3, *Neurospora crassa*, CAE76595; ScRho1, *Saccharomyces cerevisiae*, BAA00897; SccRho3, *Schizophyllum commune*, Q9P8J9; SpRho1, *Schizosaccharomyces pombe*, O13928; UmRho3, *Ustilago maydis*, XP\_401685. The tree was rooted with AtROP1 (*Arabidopsis thaliana*, NP190698) as an outgroup member.

Strain	Saprophytic growth (mm/day) <sup>b</sup>	Conidiation $(10^5)^c$	Distance between two septa $(\mu m)^d$	Length/width ratio of conidia <sup>e</sup>	Lesions on 5-cm-long rice leaf tip <sup>f</sup>
70-15	$6.30\pm0.10$	$3.20\pm0.28$	$65.57 \pm 19.71$	$2.32\pm0.16$	$26.0 \pm 6.4$
$\Delta$ Mgrho3-22	$6.20 \pm 0.10$	$1.90 \pm 0.71$	$21.72 \pm 6.12$	$4.14 \pm 0.68$	$0.6 \pm 1.4$
MgRho3OE-16	$6.73 \pm 0.16$	$9.00 \pm 0.85$	$67.81 \pm 24.02$	$2.26 \pm 0.22$	$94.8 \pm 20.3$
MgRho3-Com	$6.40 \pm 1.73$	$3.30\pm0.71$	$56.74 \pm 12.45$	$2.41\pm0.23$	$21.0\pm3.6$

TABLE 2. Phenotypic analysis of MgRho3 mutants of M. grisea<sup>a</sup>

<sup>a</sup> Data provided in all columns are averages with standard deviations.

<sup>b</sup> Diameter of hyphal radii at day 8 after incubation on complete medium agar plates under room temperature conditions.

<sup>c</sup> Average numbers of conidia harvested from a 9-cm oatmeal agar plate at day 10 after incubation under room temperature conditions.

<sup>d</sup> Average distance between septa of mycelia in 50 measurements; stained by calcofluor.

<sup>e</sup> Conidial length/width ratio; average of 50 measurements.

<sup>f</sup> Lesion numbers determined 5 days after inoculation.

infection assays with 2-week-old rice seedlings. Rice leaves inoculated with the wild-type strain 70-15 developed typical blast lesions 7 days after inoculation (Fig. 5A). Under the same conditions, the Mgrho3 deletion mutant  $\Delta$ Mgrho3-22 showed dramatically reduced virulence, although a small proportion of inoculated leaves still produced a few lesions (Fig. 5A and Table 2). However, these brown lesions failed to produce any conidia when leaves were detached and incubated under highmoisture conditions for a prolonged period in a petri dish. So, these lesions are hypersensitive reaction-like lesions. The Mgrho3 deletion mutants were further confirmed to be nonpathogenic in wounding leaf (Fig. 5B) and root infection (Fig. 5C) assays. Under the same conditions, both the ectopic transformant (MgRho3-Ect) and the wild-type strain caused typical blast lesions on inoculated rice leaves (Fig. 5A). The other two Mgrho3 deletion mutants,  $\Delta$ Mgrho3-5 and  $\Delta$ Mgrho3-9, dis-



played the same defects in pathogenicity as  $\Delta Mgrho3-22$  (data not shown).

We reintroduced the wild-type Mg*Rho3* gene carried by plasmid pCOR3 into the mutant  $\Delta$ Mg*rho3-22* and obtained three Basta-resistant transformants carrying the transforming vector by PCR analysis. One of the resulting transformants, Mg*Rho3*-Com, contained a single copy of pCOR3 integrated into the genome (Fig. 2B) and was selected for further analysis. Similar to the wild-type strain, the transformant Mg*Rho3*-Com was able to penetrate and develop infectious hyphae in onion epidermal cells. This was further confirmed by its fully recovered pathogenicity in rice (Fig. 5; see also Fig. 7, below). These data indicated that deletion of Mg*Rho3* was directly responsible for the defects of the Mg*rho3* deletion mutants in plant infection.

MgRho3 is essential for the development of functional appressoria. To further determine the defects of the Mgrho3 deletion mutants, we assayed spore germination and appressorium development on the hydrophobic side of GelBond membranes. Almost all 70-15 conidia had germinated by 4 h, but only 66.5% of conidia of the mutant  $\Delta$ Mgrho3-22 produced germ tubes in the same time period under the same conditions, followed by 76.4% at 8 h and 100% at 24 h. At 8 h, all germ tubes had formed melanized appressoria in 70-15 and MgRho3-Com cultures, but only about 15.7% of the germ tubes of the Mgrho3 deletion mutant differentiated into appressoria. At 24 h, about 60% of the  $\Delta$ Mgrho3-22 germ tubes formed appressoria but were not fully melanized (Fig. 6A and



FIG. 3. Spore morphology, hyphal branching, and septation. (A) Conidia cultured on an oatmeal agar plate at day 10 after incubation were examined with differential interference contrast microscopy. (B) Branching patterns of mycelia of a complete medium culture at day 3 after incubation. Frequent branching happened at the terminal mycelia of  $\Delta$ Mgrho3-22. (C) Calcofluor staining of mycelia to show the distance between septa.

FIG. 4. Spitzenkörper localization of the Mg*rho3* deletion mutant. Young growing mycelia were stained with aqueous FM4-64 solution (7.5  $\mu$ M) and observed immediately under a confocal microscope. Arrows show the Spitzenkörper localization at the hyphal tips of both WT and Mg*rho3* deletion mutants.



FIG. 5. Infection assays of Mgrho3 deletion mutants. (A) Rice leaves of cultivar CO39 were sprayed with conidium suspensions from the wild-type strain (70-15),  $\Delta$ Mgrho3-22, MgRho3-Ect, and MgRho3-Com. Typical leaves were scanned 7 days after inoculation. (B) Wounded rice leaves of cultivar CO39 were inoculated with conidia ( $5 \times 10^4$  conidia/ml) from strains 70-15,  $\Delta$ Mgrho3-22, and MgRho3-Com. Typical leaves were photographed 5 days after inoculation. (C) A root infection assay was carried out as described elsewhere (9).

B). More appressoria developed upon prolonged incubation for up to 48 h; however, most of these germ tubes produced deformed and less-melanized appressoria. Most appressoria of the mutant  $\Delta Mgrho3-22$  were aberrant in morphology, more elongated, and less swollen than the wild type. Only about 9% of appressoria appeared to be normal (swollen and rounded) in morphology (Fig. 6A), but they were smaller in size (Fig. 6A). We further examined the turgor pressure of appressoria of  $\Delta$ Mgrho3-22 with the incipient cytorrhysis technique. Appressoria of  $\Delta Mgrho3-22$  were more osmotically sensitive and  $72.8\% \pm 10.6\%$  of appressoria collapsed when they were treated with 2 M glycerol for 20 min, but only  $12.7\% \pm 5.7\%$ appressoria of the wild-type 70-15 collapsed under the same conditions. The complemented strain was normal in germination and appressorium formation and turgor pressure establishment (Fig. 6A, B, and C). Furthermore, we measured the endogenous cAMP levels in Mgrho3 mutants. Interestingly, the intracellular cAMP level in the Mgrho3 deletion mutants was  $3,455.1 \pm 254.8$  fmol/mg of mycelia, which was 67% of that detected in the wild-type strain 70-15 (5,201.9  $\pm$  199.1 fmol/mg of mycelia).

In penetration assays with onion epidermal cells, we also observed that most appressoria formed on onion epidermis were aberrant in morphology and defective in plant penetration (Fig. 7). Only about 12% of the appressoria formed by the mutant  $\Delta Mgrho3-22$  were able to penetrate and form limited infectious hyphae in onion epidermal cells. Even after prolonged incubation for up to 72 h, penetration frequency and infectious hyphal growth were not increased in the Mgrho3 deletion mutant. Under the same conditions, over 85% of appressoria formed by the wild-type strain 70-15 penetrated into epidermal cells, and invasive hyphae had grown prosperously by 48 h (Fig. 7). The results indicate that the Mgrho3 deletion mutant is defective in developing functional appressoria and infectious hyphae. When 10 mM exogenous cAMP was supplied, defects of Mgrho3 deletion mutants for appressorium morphology, penetration, and plant infection could not



FIG. 6. Appressorium formation of the Mgrho3 deletion mutant on artificial hydrophobic surfaces. Conidia were incubated on the surface of hydrophobic GelBond films as described in Materials and Methods. Healthy appressoria from the wild-type strain (WT) and MgRho3-Com versus abnormal appressoria from the  $\Delta$ Mgrho3-22 strain were examined with differential interference contrast microscopy.



FIG. 7. Penetration assays of the Mgrho3 deletion mutant on onion epidermal cells. Conidial suspensions (around 1,000 conidia in 20  $\mu$ l) of wild-type strain 70-15 (WT),  $\Delta$ Mgrho3-22, MgRho3-Com, and MgRho3-Ect were inoculated on strips of onion epidermis as described by Xu et al. (44). Infectious hyphae were photographed 1 day after inoculation (top panel) and 2 days after inoculation (lower panel) by using differential interference contrast microscopy. A, appressorium; C, conidium; H, hypha; IF, infectious hypha.

be rescued (data not shown). It is likely that these defects are directly responsible for the loss of pathogenicity in the Mgrho3 deletion mutant.

Overexpression of MgRho3 enhances pathogenicity. To demonstrate that the expression level of MgRho3 affects its function, we used the RP27 promoter derived from the M. grisea ribosomal protein 27 gene constructed in pSM565 (4) to express MgRho3 in the wild-type strain 70-15 of M. grisea. Three MgRho3 overexpression mutants were identified by realtime PCR (Fig. 8A), showing a 6- to 10-fold increase in transcripts compared with the wild-type strain at vegetative hyphal stage and a 2-fold increase in infected rice leaves. All three overexpression transformants had similar phenotypes, but only data of transformant MgRho3OE-16 are presented here. In contrast to the MgRho3 deletion mutant  $\Delta$ Mgrho3-22, conidiation increased in transformant MgRho3OE-16, about three times more than that of the wild-type 70-15 strain (Table 2). Conidial morphology (Fig. 8C) and germination appeared to be normal in the transformant MgRho3OE-16. Interestingly, appressorium formation was faster in MgRho3OE-16 than in the wild-type strain. All the germ tubes formed appressoria after 4 h of incubation on the GelBond hydrophobic surface, which was significantly faster than that with the wild type. Infectious growth of MgRho3 overexpression mutants on onion epidermal cells was accelerated, and appressorium development and infectious growth were also advanced (Fig. 8D). On 2-week-old seedlings of rice cultivar CO39, MgRho3OE-16 caused more and larger lesions than the wild-type strain (Fig. 8B), with 94.8  $\pm$  20.3 lesions on a 5-cm-long rice leaf tip, while the wild type produced only  $26.04 \pm 6.4$  lesions (Table 2), suggesting that overexpression of MgRho3 increased the fungal virulence. The other two MgRho3 overexpression transformants had similar phenotypes and displayed enhanced virulence.

Expression of MgRho3-CA and -DN alleles results in distinct defects in appressorium development and pathogenicity. To further investigate the biological function of MgRho3, we constructed the MgRho3-CA and MgRho3-DN alleles and transformed them into the wild-type 70-15 strain. Conidia produced by transformants expressing MgRho3-CA had normal morphology but were defective in appressorium formation (Fig. 9A). Similar to the MgRho3 deletion mutant  $\Delta$ Mgrho3-22, transformants expressing the MgRho3-DN allele produced slender conidia (Fig. 9A) and deformed appressoria. Interestingly, both MgRho3-CA and MgRho3-DN transformants were defective in plant infection (Fig. 9). These results suggest that proper regulation of MgRho3 activity is critical for appressorium formation and plant infection.

# DISCUSSION

Rho3 is a member of the Ras superfamily of monomeric GTPases. It is known to be involved in the regulation of cell polarity, exocytosis, and vesicle secretion through its functional characterization in *S. cerevisiae* (2, 21, 23, 29). In *S. pombe*, Rho3 regulates cell division, cell separation, and polarized cell growth (38). In filamentous fungi, Rho3 homologs are not essential for establishing and maintaining polarized hyphal tip growth. Deletion of Rho3 reduces vegetative growth and protein secretion in *T. reesei* (37). In *M. grisea*, we showed that Mgrho3 deletion mutants have no obvious defects in vegetative growth. In contrast to irregular swelling observed in an Agrho3 mutant (19, 40), germ tubes and vegetative hyphae of the Mgrho3 mutants had basically normal morphology. However,



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FIG. 8. Morphology of MgRho3 overexpression mutants. (A) Total RNA samples (approximately 5  $\mu$ g per reaction mixture) isolated from mycelia of *M. grisea* strains 70-15 (wild type [WT]) and MgRho3 overexpression transformants (MgRho3OE-8, -12, and -16) were subjected to real-time PCR. (B) Rice leaves of cultivar CO39 were sprayed with conidial suspensions from the wild-type strain (70-15) and MgRho3OE-16. Typical leaves were scanned 7 days after inoculation. (C) Appressoria examined with differential interference contrast microscopy 8 h after conidia were incubated on the surface of hydrophobic GelBond films as described in Materials and Methods. (D) Conidial suspensions (around 1,000 condia in 20  $\mu$ l) of *M. grisea* strain 70-15 (WT) and MgRho3OE-16 were inoculated on strips of onion epidermis. Infectious hyphae were photographed 2 days after inoculation.

conidia produced by the Mg*rho3* mutants were narrower and appeared more slender than those of the wild-type strain (Fig. 3A). Conidium germination was delayed, but the germination pattern remained the same.

The Mgrho3 deletion mutant still formed appressoria, which

were morphologically abnormal and defective in plant penetration. In rice infection assays, the Mgrho3 deletion mutant is nonpathogenic, indicating that MgRho3 is a key regulator in appressorium penetration and infectious growth in *M. grisea*. To our knowledge, this is the first report that links the Rho3



FIG. 9. Morphology and infection assay of Mg*Rho3* dominant mutants. (A) Conidia of 70-15 (wild type [WT]), Mg*Rho3*-CA, and Mg*Rho3*-DN cultured on oatmeal agar plates for 10 days. (B) Conidial suspensions (around 1,000 conidia in 20 μl) of *M. grisea* strain 70-15 (WT) and Mg*Rho3* dominant mutants were inoculated on strips of onion epidermis. Infectious hyphae were photographed 2 days after inoculation. (C) Rice leaves of cultivar CO39 were sprayed with conidial suspensions from the wild-type strain (70-15), Mg*Rho3*-CA, and Mg*Rho3*-DN. A, appressorium; C, conidium; H, hypha; IF, infectious hypha. All were examined with differential interference contrast microscopy.

protein with fungal pathogenesis. The Rho3 homologs are well conserved in other plant pathogenic fungi (Fig. 1) and may be involved in various plant infection processes. Appressorium formation and invasive growth are two key steps in the infection cycle of many plant pathogenic fungi.

Two signaling pathways, the cAMP/protein kinase A (PKA) and PMK1 mitogen-activated protein kinase pathways, are known to regulate these processes in *M. grisea* (20, 42, 46). Unlike the cpkA and pmk1 mutants, the Mgrho3 mutants produce slender conidia (Fig. 3A) and abnormal appressoria (Fig. 6A). Our data showed that although the intracellular cAMP level in the Mgrho3 deletion mutant is lower than that of the wild-type strain, exogenous cAMP failed to rescue defects in appressorium development, penetration, and plant infection. These data suggest that the Mgrho3 mutant differs from the cpkA mutant (deleted of the catalytic subunit of PKA) in appressorial penetration and plant infection. About 12% of appressoria formed by the Mgrho3 mutants are able to penetrate and form infectious hyphae. Black specks were observed on rice leaves sprayed with the Mgrho3 mutants. The cpkA mutant, however, is blocked in penetration and causes rare blast lesions, possibly by penetrating through wounds (44). These data suggest that Rho3 may not directly activate the cAMP/ PKA pathway in regulation of appressorium development and normal turgor pressure establishment.

The cytorrhysis assay indicated that the Mgrho3 mutant had reduced appressorium turgor, which plays a key role in plant penetration in *M. grisea* (8). One possible explanation is that the slender conidia of the Mgrho3 mutant may have less carbon storage than normal pyriform conidia. In *M. grisea*, carbon storage, such as in glycogen and lipid bodies, plays an important role in appressorium turgor generation (12, 39). Therefore, it is likely that appressoria formed by the Mgrho3 mutants are defective in penetration because of the reduced turgor pressure.

However, it remains possible that failure in appressorial penetration in the Mgrho3 mutant is caused by defects in cytoskeleton reorganization associated with formation of the penetration peg. Small GTPases are well known to regulate actin cytoskeleton organization. In *M. grisea*, actin accumulates at the base of the appressorium and in the penetration peg (3), suggesting a reorganization of the actin cytoskeleton during such polarized growth. Mutants deleted of *MST12*, one of the putative downstream transcription factors of Pmk1, form melanized appressoria and have normal appressorium turgor pressure (26). Appressoria formed by the *mst12* mutant are defec-

tive in microtubule reorganization associated with penetration peg formation at late stages of appressorium formation. Clergeot et al. (6) speculated that PLS1 could be involved in a similar signaling pathway in fungi that controls actin cytoskeleton reorganization at the base of the appressorium before penetration peg emergence. MgRho3 may also play a role in regulation of the actin cytoskeleton, as in other organisms (36). It is well documented that Rho3 controls polarized cell growth in yeast through regulation of actin cytoskeleton and membrane trafficking, and a number of Rho3 effectors have been identified and are known to play an important role in these regulatory functions, including formin (For3), Myo2, Exo70, and Rgd1 (23, 28, 29, 30). In M. grisea, all these genes are well conserved, but none of them has been functionally characterized, so that results in S. cerevisiae will facilitate the study of MgRho3 effectors in M. grisea.

Finally, our results indicated that overexpression of wildtype MgRho3 increases the fungal virulence in rice. Interestingly, overexpression of wild-type Cdc42 has no morphological consequences in Penicillium marneffei (5) and no effects on pathogenicity in Claviceps purpurea (31). In contrast, overexpression of Cdc42 disturbs the normal pattern of budding site selection in S. cerevisiae (17) and induces growth tip enlargement in Wangiella dermatitidis (45). In M. grisea, MgRho3 is constitutively expressed in vegetative hyphae, conidia, germ tubes, and appressoria. Its expression peaks at the conidium germination stage (47). Our real-time PCR data showed that there was a twofold increase in infected rice leaves with the MgRho3 overexpression transformants, suggesting that the expression level of MgRho3 may be related to the fungal infectivity. Expression of either the dominant active or dominant negative MgRho3 allele caused dramatic defects in appressorium formation and plant penetration, and dominant negative MgRho3 also resulted in deformed conidia. In S. cerevisiae, transformants expressing a dominant active Rho3 displayed cold sensitivity and produced elongated, bent cells (2, 16). However, expression of a dominant negative Rho3 allele abolished its interaction with Exo70 and Myo2 and caused defects both in the actin cytoskeleton and exocytosis (29). In M. grisea, overexpression of wild-type MgRho3 only increases its expression level but still maintains the equilibrium between GTP- and GDP-bound forms. However, overexpression of a dominant active allele of MgRho3 may bypass the upstream activation signals and disturb the activation status.

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