Bypassing Both Surface Attachment and Surface Recognition Requirements for Appressorium Formation by Overactive Ras Signaling in *Magnaporthe oryzae*

Xiaoying Zhou,¹ Xinhua Zhao,¹ Chaoyang Xue,¹ Yafeng Dai,² and Jin-Rong Xu¹

¹Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, U.S.A.; ²State Key Laboratory of Crop Stress Biology for Arid Areas, Northwestern A&F University, Yangling, Shaanxi 712100, China

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Magnaporthe oryzae forms a highly specialized infection structure called an appressorium for plant penetration. In *M. oryzae* and many other plant-pathogenic fungi, surface attachment and surface recognition are two essential requirements for appressorium formation. Development of appressoria in the air has not been reported. In this study, we found that expression of a dominant active *MoRAS2*^{G18V} allele in *M. oryzae* resulted in the formation of morphologically abnormal appressoria on nonconductive surfaces, in liquid suspensions, and on aerial hyphae without attachment to hard surfaces. Both the Pmk1 mitogen-activated protein kinase cascade and cAMP signaling pathways that regulate surface recognition and appressorium morphogenesis in *M. oryzae* were overactivated in the *MoRAS2*^{G18V} transformant. In mutants deleted of *PMK1* or *CPKA*, expression of *MoRAS2*^{G18V} had no significant effects on appressorium morphogenesis. Furthermore, expression of dominant *MoRAS2* in *Colletotrichum graminicola* and *C. gloeosporioides* also caused the formation of appressorium-like structures in aerial hyphae. Overall, our data indicate that *MoRAS2* functions upstream from both the cAMP-PKA and Pmk1 pathways and overactive Ras signaling leads to improper activation of these two pathways and appressorium formation without surface attachment in appressorium-forming pathogens.

In many plant-pathogenic fungi that form a highly specialized infection structure called an appressorium for plant penetration, surface attachment and surface recognition are two essential requirements for appressorium development. In the rice blast fungus *Magnaporthe oryzae*, a model for studying fungus–plant interactions, several signal transduction pathways have been shown to be involved in the regulation of

appressorium formation and penetration (Ebbole 2007; Wilson and Talbot 2009). Whereas cAMP signaling is important for initial surface recognition and appressorium turgor generation, the *PMK1* mitogen-activated protein kinase (MAPK) regulates late stages of appressorium development, penetration, and invasive growth. The cAMP-PKA pathway also is important for the generation of appressorium turgor pressure that is important for plant penetration (Li et al. 2012; McDonough and Rodriguez 2012; Wilson and Talbot 2009).

Although components of the *MST11-MST7-PMK1* MAPK cascade is well conserved between *M. oryzae* and the budding yeast *Saccharomyces cerevisiae* (Li et al. 2012), they regulate different processes in these two fungi and differ in upstream regulators and downstream targets. Unlike the yeast pheromone response pathway, two PAK kinases and MgCdc42 are not involved in the activation of the Mst11-Mst7-Pmk1 cascade in *M. oryzae* (Li et al. 2004; Park et al. 2006). Therefore, the activation of *MST11* mitogen-activated protein/extracellular signal-regulated kinase remains unclear. However, it has been reported that Mst50 directly interacted with both Mst11 and Mst7, and therefore functions as an adaptor protein for the Pmk1 cascade (Park et al. 2006). Similar to the *pmk1* deletion mutant, the *mst50* mutant was defective in appressorium formation and plant infection. Interestingly, both Mst50 and Mst11 interact with *MoRas1* and *MoRas2*, two Ras proteins in *M. oryzae*, by yeast two-hybrid assays (Park et al. 2006), suggesting a role of Ras signaling in the activation of the *PMK1* pathway.

Ras proteins are low molecular weight GTP-binding proteins that switch between the active GTP and inactive GDP-bound statuses. They regulate cellular responses to external stimuli by activating various downstream signaling pathways. In *S. cerevisiae*, the two Ras proteins Ras1 and Ras2 both control the activities of adenylate cyclase (Tamanai 2011). Intracellular acidification or exogenous glucose increases the Ras-GTP level which, in turn, activates the downstream cAMP-PKA pathway. In addition, Ras2 functions upstream from both cAMP signaling and Kss1 MAPK pathways to regulate pseudohyphal growth (Tamanai 2011). Whereas the Ras2Val19 strain carrying the G19V mutation was stimulated in the Cdc42-Kss1-dependent filamentous growth pathway (Mosch et al. 1996), expression of the *RAS2*Val19 allele in the *gpa2* deletion mutant suppressed its defects in pseudohyphal growth (Lorenz and Heitman 1997). In the fission yeast *Schizosaccharomyces pombe*, a single Ras gene regulates the downstream Byr2 MAPK pathway for mating responses and the Scd1 morphogenetic pathway (Onken et al. 2006). Ras proteins also have been

Corresponding author: J.-R. Xu; Telephone: +1.765.496.6918; Fax: +1.765.496.6918; E-mail: jinrong@purdue.edu

X. Zhou and X. Zhao contributed equally to this work.

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shown to play various roles in fungal development and pathogenesis processes in different fungi. In all the fungal pathogens that have been studied, Ras signaling is either essential or important for virulence (Bluhm et al. 2007; Fortwendel et al. 2004; Muller et al. 2003; Waugh et al. 2002).

In this study, we functionally characterized the two *Ras* genes that interacted with both Mst50 and Mst11 (Park et al. 2006). Whereas the *Moras1* deletion mutant had no obvious defects other than minor reduction in conidiation, *MoRAS2* appeared to be an essential gene. In transformants expressing the dominant active *MoRAS2*^{G18V} allele, conidiation was significantly reduced. The *MoRAS2*^{G18V} transformant had increased Pmk1 phosphorylation and an elevated intracellular cAMP level in aerial hyphae. It formed morphologically abnormal appressoria on both hydrophilic and hydrophobic surfaces and appressorium-like structures in conidium suspensions and on aerial hyphae. Interestingly, expression of *MoRAS2*^{G18V} in *Colletotrichum graminicola* and *C. gloeosporioides* also resulted in the formation of appressorium-like structures in aerial hyphae. These observations indicate that expression of dominant active *MoRAS2* may overstimulate both the cAMP signaling and Pmk1 MAPK pathways and bypass the requirements for both surface attachment and surface recognition for appressorium morphogenesis in *M. oryzae* and other appressorium-forming fungi.

RESULTS

RAS2 is an essential gene in *M. oryzae*.

Similar to their orthologs in other fungi, *MoRAS1* (MGG_09499.6) and *MoRAS2* (MGG_06154.6) have five fingerprint sequence motifs (G1 to G5) for GTP/GDP-binding and a C-terminal CAAX motif. To determine their functions in *M. oryzae*, we attempted to generate the *Moras1* and *Moras2* deletion mutant by gene replacement (Supplementary Fig. S1). After screening over 400 transformants from independent transformations, we failed to identify *Moras2* deletion mutants. On the other hand, two *Moras1* mutants generated in this study were only slightly reduced in conidiation but normal in growth, appressorium formation, and plant infection. Therefore, *MoRAS2* appears to be an essential gene whereas *MoRAS1* may play only a minor role in conidiation. Studies in several filamentous fungi have shown that, although they are highly similar to each other, the two Ras paralogs have distinct functions and one often plays a more critical role than the

other (Fernandes et al. 2008; Fillinger et al. 2002; Fortwendel et al. 2004; Nichols et al. 2009). Deletion of the major *Ras* gene but not the minor one is lethal in *Aspergillus nidulans*, *Penicillium marneffeii*, and *C. trifolii* (Boyce et al. 2005; Chen et al. 2006; Fillinger et al. 2002).

Abnormal appressoria formed by the *MoRAS2*^{G18V} transformants.

The dominant active allele of *MoRAS2* was constructed by introducing the G18V mutation, which is equivalent to the G19V mutation in yeast (Hlavata et al. 2003). When the *M. oryzae* genome was first released, the start codon of this gene was not annotated properly and its open reading frame (ORF) had five extra amino acid residues at the N terminus. Strain DA66 was one of the transformants of the wild-type strain 70-15 expressing the *MoRAS2*^{G18V} allele (Table 1). The expression level of *MoRAS2* in aerial hyphae increased approximately twofold in DA66 in comparison with 70-15 (Supplementary Fig. S2). On oatmeal agar (OTA) plates, DA66 had no obvious defects in growth rate but was reduced in the production of aerial hyphae. Colonies of DA66 were heavily melanized in the center (Fig. 1A) and significantly reduced in conidiation (Table 2; Fig. 1A).

On hydrophobic surfaces, the *MoRAS2*^{G18V} transformant DA66 was normal in conidium germination and efficiently formed melanized appressoria (Table 2). However, appressoria formed by DA66 were morphologically abnormal. Compared with normal dome-shaped appressoria, the majority of appressoria formed by DA66 had irregular shapes (Fig. 1B). Some of them appeared to be formed subapically or in chains. These observations indicated that expression of *MoRAS2*^{G18V} may result in improper regulation of appressorium morphogenesis.

Transformant DA66 formed appressoria on hydrophilic surfaces.

Recognition of hydrophobic surfaces is an important signal for appressorium formation in *M. oryzae*. On the hydrophilic surface of GelBond membranes, germ tubes of 70-15 rarely formed appressoria after incubation for 24 h. Under the same conditions, over 90% of the DA66 germ tubes formed appressoria (Table 2; Fig. 1C). Because DA66 formed appressoria efficiently on both hydrophobic and hydrophilic surfaces, expression of the *MoRAS2*^{G18V} allele must be sufficient to bypass the requirement for attachment to hydrophobic surfaces for appressorium formation.

Table 1. Wild-type and mutant strains of *Magnaporthe oryzae* used in this study

Strain	Genotype description	Reference
70-15	Wild-type, <i>MAT1-1</i>	Chao and Ellingboe 1991
Guy11	Wild-type, <i>MAT1-2</i>	Leung et al. 1988
Ku80	Δ <i>Mgku80</i> mutant of Guy11	Villalba et al. 2008
nn78	Δ <i>pmk1</i> mutant of Guy11	Xu and Hamer 1996
I-27	Δ <i>cpkA</i> mutant of 70-15	Mitchell and Dean 1995
DA5	<i>MST7</i> ^{S212D T216E} transformant of 70-15	Zhao et al. 2005
DA66	<i>MoRAS2</i> ^{G18V} transformant of 70-15	This study
HZ90	<i>MoRAS2</i> ^{G18V} transformant of 70-15	This study
HZ91	<i>MoRAS2</i> ^{G18V} transformant of 70-15	This study
HZ92	<i>MoRAS2</i> ^{G18V} transformant of 70-15	This study
CX77	Δ <i>ras1</i> mutant of 70-15	This study
DAG-1	Transformant of DA66 expressing <i>GAS1</i> -GFP	This study
DAG-6	Transformant of DA66 expressing <i>GAS1</i> -GFP	This study
DA1-3	<i>MoRAS2</i> ^{G18V} transformant of <i>Colletotrichum graminicola</i>	This study
DA2-2	<i>MoRAS2</i> ^{G18V} transformant of <i>C. gloeosporioides</i>	This study
DA4-1	<i>MoRAS2</i> ^{G18V} transformant of <i>Fusarium graminearum</i>	This study
DA-P1	<i>MoRAS2</i> ^{G18V} transformant of the <i>pmk1</i> mutant nn78	This study
DA-P2	<i>MoRAS2</i> ^{G18V} transformant of the <i>pmk1</i> mutant nn78	This study
DA-C1	<i>MoRAS2</i> ^{G18V} transformant of the <i>cpkA</i> mutant I-27	This study
DA-C2	<i>MoRAS2</i> ^{G18V} transformant of the <i>cpkA</i> mutant I-27	This study

Expression of *MoRAS2*^{G18V} bypasses surface attachment for appressorium formation.

In *M. oryzae*, it has been reported that attachment to hard surfaces in the initial 2 h is required to form appressoria (Liu et al. 2007). To test whether transformant DA66 could form appressoria without surface attachment, conidia of both DA66 and 70-15 were incubated in hanging drops to avoid direct contact with hard surfaces (Fig. 2A). After incubation for 24 h,

Fig. 1. Abnormal colony and appressorium morphology of the *MoRAS2*^{G18V} transformant DA66. **A**, One-week-old oatmeal agar cultures of the wild-type strain 70-15 and DA66. Enhanced melanization was observed in the center of DA66 colonies (arrow). **B**, In comparison with 70-15, DA66 formed morphologically abnormal appressoria on hydrophobic surfaces. **C**, On the hydrophilic surface of GelBond membranes, 70-15 failed to form appressoria. Bar = 10 μ m.

conidia of 70-15 aggregated at the bottom of conidium drops and produced long germ tubes without tip differentiation (Fig. 2B). Under the same conditions, DA66 conidia germinated and formed morphologically abnormal appressoria (Fig. 2B). These results indicated that dominant active *MoRAS2* bypassed the surface attachment requirement for appressorium formation.

The same hanging drop method was used to assay for appressorium formation with conidia of transformant DA5 that expresses the dominant active *MST7*^{S212D T216E} allele (Zhao et al. 2005). Although transformant DA5 formed appressoria on both hydrophobic and hydrophilic surfaces (Zhao et al. 2005), no appressoria were observed in the conidium hang-

Fig. 2. Appressorium formation assays with hanging drops of conidium suspensions. **A**, Drops (20 μ l) of conidium suspensions (2×10^4 spores/ml) were placed over concave slide glasses and incubated upside down in a moisture chamber. **B**, After incubation for 36 h, conidium suspensions were examined under subjective lens of different magnifications. Conidia of 70-15 produced long germ tubes. Germ tubes of DA66 tended to be short and often formed melanized appressoria of abnormal morphology at the tip. Bar = 10 μ m.

Table 2. Growth rate, conidiation, and appressorium formation of *Magnaporthe oryzae* strains used in this study^x

Strain	Growth rate (mm/day) ^z	Conidiation ($\times 10^4$ conidia/plate)	Appressorium formation (%) ^y	
			Hydrophobic	Hydrophilic
70-15	3.1 $\pm$ 0.2 A	250.2 $\pm$ 66.2 B	93.6 $\pm$ 6.0 A	1.2 $\pm$ 1.0
DA66	2.9 $\pm$ 0.3 A	5.6 $\pm$ 3.6 E	92.9 $\pm$ 5.5 A	90.9 $\pm$ 7.2
DA-P1	2.1 $\pm$ 0.2 B	240.7 $\pm$ 93.8 B	0	0
DA-C1	3.2 $\pm$ 0.2 A	390.3 $\pm$ 57.8 A	42.1 $\pm$ 1.9 C	1.8 $\pm$ 0.3
Guy11	3.1 $\pm$ 0.2 A	480.4 $\pm$ 37.8 A	96.9 $\pm$ 2.5 A	1.0 $\pm$ 0.9

^x Numbers followed by the same letter indicate that there was no significant difference. Different letters were used to mark statistically significant differences ($P = 0.05$).

^y Percentages of germ tubes formed appressoria on the hydrophobic and hydrophilic surfaces of GelBond membranes.

^z Average growth rate and (mean $\pm$ standard deviation) were calculated from three independent measurements.

ing-drop assays (Supplementary Fig. S3). These observations indicated that constitutive activation of the Pmk1 MAPK by *MST7^{S212D T216E}* is not sufficient to bypass the surface attachment requirement for appressorium formation. Therefore, expression of the dominant active *MoRAS2* allele must have additional effects other than overstimulation of the Pmk1 pathway in *M. oryzae*.

Appressorium-like structures formed by the *MoRAS2^{G18V}* transformant in the air.

Because of enhanced melanization, we examined aerial hyphae and conidiophores harvested from the center of 2-week-old OTA cultures of strain DA66. Interestingly, the formation of melanized, appressorium-like structures were observed in aerial hyphae (Fig. 3A). Similar to appressoria formed by DA66 in hanging drop assays, these melanized structures developed in the air had irregular morphology. Most of them were formed at the tip or subapical regions of aerial hyphae or conidiophores (Fig. 3A). A septum was formed to delimit these irregular melanized structures, which is similar to appressorium differentiation by germ tubes (Saunders et al. 2010). Cryo-scanning electron microscopy (SEM) examination confirmed the formation of appressorium-like structures by transformant DA66 in the air (Fig. 3B).

GAS1 is expressed in appressorium-like structures formed in the air.

To further confirm that melanized hyphal tips were appressorium-like structures, a *GAS1*-green fluorescent protein (GFP) reporter construct generated in a previous study (Xue et al. 2002) was transformed into strain DA66. Although *GAS1* transcripts were present in other stages (Soanes et al. 2012), GFP signals were increased and became visible in the cytoplasm in late stages of appressoria in the *GAS1*-GFP trans-

formants (Xue et al. 2002). Two zeocin-resistant transformants of DA66 carrying a single copy of the *GAS1*-GFP construct (DAG-1 and DAG-6) were identified. Approximately $31.2 \pm 5.6\%$ of appressorium-like structures formed in the air by these two transformants had GFP signals in the cytoplasm (Fig. 4A), which is similar to the expression of *GAS1*-GFP in appressoria formed by the wild type (Xue et al. 2002). When aerial hyphae were fixed and examined by transmission electron microscopy (TEM), appressorium-like structures formed by DA66, similar to appressoria formed by germ tubes on hydrophobic surfaces, had the melanin layer (Fig. 4B). These data further indicate that these appressorium-like structures formed in the air have basic characteristics of appressoria.

MoRas2 functions upstream from the Pmk1 MAPK pathway.

Among the three *M. oryzae* genes (*MST50*, *MST11*, and *MAC1*) with the Ras association (RA) domain, two are compo-

Fig. 4. Expression of *GAS1*-green fluorescent protein (GFP) and formation of a melanin layer in melanized hyphal compartments. **A**, Appressorium-like structures formed in aerial hyphae of DAG-1, a transformant of DA66 expressing the *GAS1*-GFP construct, were examined by differential interference contrast and epifluorescence microscopy. **B**, Aerial hyphae of DA66 were examined with a dissecting microscope and by transmission electron microscopy. Arrows pointed to melanized appressorium-like structures and the melanin layer. Bar = 10 μ m.

Fig. 3. Aerial hyphae and conidiophores were harvested from the center of 1-week-old oatmeal agar cultures and examined by **A**, differential interference contrast microscopy and **B**, cryo-scanning electron microscopy. Bar = 10 μ m. Whereas abundant conidia were formed by 70-15, transformant DA66 rarely produced conidia but developed appressorium-like structures (arrows) at the tip of aerial hyphae and conidiophores.

Fig. 5. Appressorium formation assays with transformants of the $\Delta pmk1$ and $\Delta cpkA$ mutants expressing the *MoRAS2^{G18V}* allele. **A**, The $\Delta pmk1$ /*MoRAS2^{G18V}* transformant failed to form appressoria on hydrophobic or hydrophilic surfaces. **B**, The $\Delta cpkA$ /*MoRAS2^{G18V}* transformant formed appressoria on hydrophobic but not hydrophilic surfaces.

nents of the Pmk1 MAPK pathway (Park et al. 2006; Zhao et al. 2005). To determine the functional relationship between MoRas2 and Pmk1, we transformed the *MoRAS2*^{G18V} allele into the *pmk1* mutant nn78 (Xu and Hamer 1996). The resulting *pmk1/MoRAS2*^{G18V} transformants DA-P1 and DA-P2 (Table 1), like the *pmk1* mutant, failed to form appressoria on hydrophobic or hydrophilic surfaces (Fig. 5A; Table 2), indicating that Pmk1 is required for improper appressorium morphogenesis in the *MoRAS2*^{G18V} transformant.

To further investigate the role of MoRas2 in appressorium development, we assayed the activation of Pmk1 with an anti-TpEY antibody. In proteins isolated from vegetative hyphae harvested from 2-day-old liquid complete medium (CM) cultures, no significant difference in the phosphorylation of Pmk1 was observed between the wild type strain 70-15 and transformant DA66 (Fig. 6A). However, in proteins isolated from aerial hyphae harvested from 2-week-old OTA cultures, the phosphorylation level of Pmk1 was significantly higher in DA66 than in 70-15 (Fig. 6A), which is consistent with the observa-

tion that DA66 but not 70-15 formed appressorium-like structures in the air. In the same protein samples, the phosphorylation level of the Mps1 MAPK was similar between 70-15 and DA66, indicating that *MoRAS2*^{G18V} specifically overstimulated the Pmk1 but not Mps1 MAPK pathway.

MoRas2 is also involved in cAMP signaling.

In *M. oryzae*, the Mac1 adenylate cyclase (Choi and Dean 1997) is one of the three proteins that have the RA domain. To determine the functional relationship between MoRas2 and cAMP signaling, we transformed the *MoRAS2*^{G18V} allele into the *cpkA* mutant I-27 (Mitchell and Dean 1995). The resulting transformant, DA-C1, formed appressoria on hydrophobic but not on hydrophilic surfaces (Fig. 5B; Table 2). In addition, transformant DA-C1, similar to the *cpkA* mutant, tended to produce smaller appressoria. Therefore, expressing the dominant active *MoRAS2*^{G18V} allele failed to suppress the defects of the *cpkA* mutant in appressorium formation, indicating that *MoRAS2* also functions upstream from the cAMP signaling pathway.

To confirm this observation, we measured the intracellular cAMP level and PKA activities in the *MoRAS2*^{G18V} transformant. In proteins isolated from vegetative hyphae, the intracellular cAMP level was similar between transformant DA66 and 70-15 (Fig. 6B). However, in comparison with the wild type, transformant DA66 had an elevated intracellular cAMP level in aerial hyphae harvested from 2-week-old OTA cultures. In addition, PKA activities were significantly higher in transformant DA66 than in 70-15 (Fig. 6C). These results confirmed that expression of the *MoRAS2*^{G18V} allele stimulated the cAMP-PKA pathway in *M. oryzae*.

Transformants expressing the *MgRAS2*^{G18V} allele are nonpathogenic.

Because the *MgRAS2*^{G18V} transformant rarely produced conidia, we conducted infection assays with culture blocks. On seedlings inoculated with culture blocks of transformant DA66, no lesions were formed on the intact rice leaves (Fig. 7A). On wounded leaves, DA66 also failed to cause blast lesions outside the wounding sites (Fig. 7B). Under the same conditions, 70-15 caused lesions on both intact and wounded leaves (Fig. 7). Therefore, although it was overstimulated in appressorium formation, the *MgRAS2*^{G18V} transformant was defective in plant infection.

Expression of *MoRAS2*^{G18V} also stimulates the formation of aerial appressoria in other appressorium-forming fungi.

MoRAS2 orthologs are well conserved in filamentous ascomycetes. To determine whether the regulatory function of *MoRAS2* in appressorium formation is conserved in other plant-pathogenic fungi, we transformed the *MoRAS2*^{G18V} allele into the wild-type strains of *C. graminicola* and *C. gloeosporioides*. The resulting transformants of *C. graminicola* and *C. gloeosporioides*, DA1-3 and DA2-2, respectively (Table 1), contained a single copy of *MoRAS2*^{G18V}. They had no obvious changes in growth rate or colony morphology. Similar to DA66 in *M. oryzae*, melanized appressorium-like structures were observed on the tip of aerial hyphae in the *MoRAS2*^{G18V} transformants DA1-3 and DA2-2 (Fig. 8).

We also transformed the *MoRAS2*^{G18V} construct into a non-appressorium-forming fungus, *Fusarium graminearum*. The resulting transformant DA4-2 (Table 1) was confirmed by polymerase chain reaction (PCR) to contain the *MoRAS2*^{G18V} construct. It grew slightly more slowly than the wild-type strain on V8 agar plates but failed to produce melanized appressoria in aerial hyphae, which is not surprising because *F. graminearum* doesn't form appressoria. Therefore, expression of

Fig. 6. Assays for Pmk1 activation and intracellular cAMP. **A**, Western blot analysis for the expression and activation of Pmk1. A similar band was detected by an anti-Pmk1 antibody in proteins isolated from aerial hyphae and liquid CM cultures of 70-15 and DA66. When detected with an anti-TpEY antibody, Pmk1 but not Mps1 phosphorylation was increased in proteins isolated from aerial hyphae of DA66. **B**, An elevated intracellular cAMP level was detected in aerial hyphae but not liquid CM cultures of DA66. **C**, Assays for PKA activities in proteins isolated from aerial hyphae with the PepTag A1 PKA substrate peptide. On a 0.8% agarose gel, phosphorylated peptides in DA66 samples migrated toward the anode (+). In 70-15 samples, unphosphorylated peptides migrated toward the cathode (-). N = nonphosphorylated sample control and P = phosphorylated sample control.

MoRAS2^{G18V} only resulted in appressorium formation on aerial hyphae in appressorium-forming fungi.

DISCUSSION

Like many other fungi, the *M. oryzae* genome contains two *Ras* genes. Whereas deletion of *MoRAS1* had no obvious phenotypes, *MoRAS2* appeared to be an essential gene and expression of a dominant active allele of *MoRAS2* resulted in pleiotropic defects. Therefore, *MoRAS2* must play a more critical role than *MoRAS1* in growth and development in *M. oryzae*, which is similar to what has been reported in other filamentous fungi, including *A. nidulans*, *Cryptococcus neoformans*, and *Schizophyllum commune* (Fillinger et al. 2002; Schubert et al. 2006; Waugh et al. 2002). In all these fungi, one of the two paralogous *Ras* gene is more important or has a higher expression level than the other. In contrast, both *Ras1* and *Ras2* function to activate adenylate cyclase in *Saccharomyces cerevisiae*. Although none of them is essential, the *ras1 ras2* double mutant is not viable (Tamanai 2011). In *Ustilago maydis*, two *Ras* proteins have distinct functions. Whereas *Ras2* controls filamentous growth, *Ras1* is involved in pheromone responses (Lee and Kronstad 2002; Muller et al. 2003).

The cAMP signaling and Pmk1 MAPK pathways are known to be involved in surface recognition and appressorium morphogenesis in *M. oryzae* (Ebbole 2007; Li et al. 2012). In aerial hyphae of the *MoRAS2*^{G18V} transformant, Pmk1 phosphorylation and intracellular cAMP levels were increased, indicating

that expression of *MoRAS2*^{G18V} overstimulated both cAMP signaling and Pmk1 MAPK pathways, which is consistent with the formation of appressoria on hydrophilic surfaces and in the air. Therefore, *MoRAS2* is likely involved in both Pmk1 and cAMP signaling pathways in *M. oryzae*. In *S. cerevisiae*, expression of *Ras2*^{V19} increased the intracellular cAMP level and stimulated filamentous growth but had no effect on the mating pheromone pathway (Mosch et al. 1999; Tamanai 2011). In *Candida albicans*, *Ras1* is known to regulate both cAMP and MAPK pathways as well (Zhu et al. 2009). In *Cryptococcus neoformans*, *Ras1* likely mediates cross-talk between the Cek1 MAPK and cAMP signaling pathways. The *ras1* deletion mutant was defective in mating, agar adherence, and virulence. Its defect in mating was fully suppressed by overexpression of *GPB1* that regulates the Cpk1 MAPK pathway but only partially rescued by exogenous cAMP (Shapiro et al. 2011).

Because the phosphorylation level of Mps1 was not affected in the *MoRAS2*^{G18V} transformant, *MoRAS2* likely plays a specific role in the activation of the Pmk1 pathway. Interestingly, Pmk1 activation and the intracellular cAMP level were not affected in vegetative hyphae harvested from liquid CM cultures of the *MoRAS2*^{G18V} transformant. Consistent with that, we noticed that melanized appressorium-like structures were not formed at the tip of vegetative hyphae in liquid cultures. It is likely that melanized hyphal tips were related to aerial hyphal growth or conidium development, which does not occur in liquid cultures. Expression of *MoRAS2*^{G18V} resulted in the formation of appressorium-like structures instead of conidia, which was consistent with the fact that the *MoRAS2*^{G18V} transformant rarely produced conidia (Fig. 3; Table 2). In *Neurospora crassa*, the *band* mutation that resulted in dominant active *Ras1*^{bd} also affected cell differentiation and asexual development (Belden et al. 2007). In *A. fumigatus*, development of conidiophore and sterigmata was affected in the transformant expressing a dominant active *RasA* (Fortwendel et al. 2004).

Although appressorium formation was enhanced, the *MoRAS2*^{G18V} transformant was nonpathogenic in infection assays with intact and wounded leaves (Fig. 7). Similar phenomenon was observed in the transformant expressing the

Fig. 7. Plant infection assays with the *MoRAS2*^{G18V} transformant. **A**, Intact and **B**, wounded rice leaves were inoculated with water agar (control) or culture blocks of the wild type (70-15) and *MoRAS2*^{G18V} transformant (DA66) strains. Symptoms were examined 6 days postinoculation.

Fig. 8. Expression of the *MgRAS2*^{G18V} allele in *Colletotrichum graminicola* and *C. gloeosporioides*. Aerial hyphae from 2-week-old cultures of the *MgRAS2*^{G18V} transformants of **A**, *C. graminicola* and **B**, *C. gloeosporioides* were examined by dissecting (left) and differential interference contrast microscopy. Arrows point to melanized appressoria. Bar = 30 μ m.

MST7^{DA} allele that functions upstream from the Pmk1 MAPK (Zhao and Xu 2007). Because constitutive activation of the Pmk1 MAPK cascade resulted in loss of pathogenicity, it is likely that Pmk1 MAPK needs to be properly regulated during different infection stages. Appressorial penetration, development of invasive hyphae, and invasive growth may require the coordinated regulation between cAMP signaling and Pmk1 MAPK pathways in *M. oryzae*. Dominant active Ras mutations also have been shown to affect virulence in other fungal pathogens, including *C. neoformans* and *U. maydis* (Lee and Kronstad 2002; Lengeler et al. 2000; Xie et al. 2013).

One phenomenon we observed in the *MoRAS2^{G18V}* transformant is that it was relatively unstable during vegetative growth. The efficiency of appressorium formation on hydrophilic surfaces and the formation of melanized hyphal tips were often reduced in subcultures. In *M. oryzae*, the mutants blocked in the Pmk1 MAPK cascade, such as the *pmk1*, *mst7*, and *mst11* mutants, are stable. However, mutants defective in cAMP signaling, such as the *mgb1*, *mac1*, and *cap1* mutants, are known to produce spontaneous suppressors (Fang and Dean 2000; Nishimura et al. 2003; Zhou et al. 2012). Spontaneous mutations that occurred in the cAMP-PKA pathway or other downstream effectors of MoRas2 may partially suppress the phenotypes of the *MoRAS2^{G18V}* transformant. However, post-translational modifications such as phosphorylation also are known to affect Ras activities (Tamanoi 2011), and the A59T mutation in the dominant active H-Ras attenuates the efficiency of fibroblast transformation as a suppressor (Zisoulis and Kansas 2004). Therefore, suppressors of dominant active MoRas2 in *M. oryzae* may result from postmodifications or point mutations of *MoRAS2^{G18V}*.

Due to the observation of the instability of the *MoRAS2^{G18V}* transformants, all the data presented in this publication were generated with subcultures from the original transformants. Although Table 1 listed only four of them, we isolated over 10 *MoRAS2^{G18V}* transformants from different transformants. All of them had similar phenotypes, suggesting that it is unlikely for the observed phenotypes to be caused by positional effects of the *MoRAS2^{G18V}* integration sites. Nevertheless, quantitative reverse-transcriptase PCR assays showed that the expression level of *MoRAS2* in aerial hyphae increased approximately two-fold in the *MoRAS2^{G18V}* transformant. Although the transforming *MoRAS2^{G18V}* construct was under the control of its native promoter, it is possible that some of the phenotypes observed in these transformants are related to the expression of *MoRAS2*.

Surface attachment and recognition are critical for appressorium development in *M. oryzae* and other fungal pathogens (Lee et al. 2003; Wilson and Talbot 2009). In this study, we found that the *MoRAS2^{G18V}* transformant efficiently formed appressoria on hydrophilic surfaces and in hanging conidium drop assays. Expression of *MoRAS2^{G18V}* also resulted in the formation of appressorium-like structures on aerial hyphae (Fig. 4). These results indicated that the requirements for both surface attachment and surface recognition for appressorium formation were bypassed by dominant active MoRas2. Expression of the *MoRAS2^{G18V}* allele in *C. graminicola* and *C. gloeosporioides* also resulted in the formation of melanized, appressorium-like structures at the tip of aerial hyphae. However, appressorium-like structures were not observed on aerial hyphae of *F. graminearum* transformants expressing *MoRAS2^{G18V}*. Although the formation of infection hyphae or infection cushions on plant surfaces have been reported in *F. graminearum* (Boenisch and Schafer 2011; Wanjiu et al. 2002), we failed to observe any changes in hyphal tip growth or morphology in aerial hyphae of the *MoRAS2^{G18V}* transformants of the wild type strain PH-1. These results indicated that Ras proteins in appressorium-forming fungi may have a conserved role in both

surface attachment and surface recognition for regulating appressorium formation by coordinated regulation of cAMP signaling and Pmk1 MAPK pathways. Therefore, it will be important to further characterize Ras signaling in *M. oryzae* and the relationship between Ras proteins and the RA domain-containing proteins Mac1, Mst50, and Mst11, which are the key components of these two pathways.

MATERIALS AND METHODS

Strains and culture conditions.

The wild-type and mutant strains of *M. oryzae* (Table 1) were cultured on OTA plates at 25°C under fluorescent light, as described (Xu and Hamer 1996). For fungal transformation, hygromycin (Calbiochem, La Jolla, CA, U.S.A.) and zeocin (Invitrogen, Carlsbad CA, U.S.A.) were added to the final concentrations of 250 and 300 µg/ml, respectively. Monoconidial culture isolation, measurement of growth rate, and conidiation were performed as previously described (Li et al. 2004; Park et al. 2006). Vegetative hyphae from 3-day-old 5× YEG (0.5% yeast, 1% glucose) cultures were used for DNA and RNA isolation. For SEM examination, blocks of OTA cultures were frozen with liquid nitrogen and examined at −140°C in a JEOL JSM-840 scanning electron microscope (Kong et al. 2012). For TEM examination, samples were treated and examined with a Philips/FEI CM-100 transmission electron microscope (FEI Company, Hillsboro, OR, U.S.A.), as described (Mares et al. 2006).

Targeted deletion of the *Ras* genes in *M. oryzae*.

To delete the *MoRAS1* gene, the upstream and downstream flanking sequences were amplified with primer pairs RAS1-1F/RAS1-2R and RAS1-3F/RAS1-4R, respectively (Supplementary Table S1). The resulting PCR products were digested and ligated with the hygromycin resistance cassette (*hph*) released from pCX63m as described (Zhao et al. 2004). The gene-replacement construct was amplified with primers RAS1-1F and RAS1-4R and directly transformed into protoplasts of 70-15. Hygromycin-resistant transformants were screened by PCR and confirmed by Southern blot analysis. A similar strategy was used to generate gene-replacement constructs for the *MoRAS2* gene.

Generation of the *MoRAS2^{G18V}* allele and transformants.

The yeast gap repair method (Bruno et al. 2004) was used to generate the dominant active allele of *MoRAS2*. PCR products amplified with primer pairs RDAF1/RDAR1 and RDAF2/RDAR2 were co-transformed into the yeast strain XK1-25 with *Xho*I-digested plasmid pYP1 (Zhou et al. 2011). The underlined nucleotides in the sequences of primers RDAR1 and RDAF2 were the mutation sites. The *MoRAS2^{G18V}* construct named pHZ90 was isolated from the resulting Trp+ transformant and confirmed by sequencing analysis. After transformation of 70-15 with pHZ90, bleomycin-resistant transformants, including strains DA66, HZ90, HZ91, and HZ92 (Table 1), were confirmed by PCR to contain the *MoRAS2^{G18V}* construct.

Plasmid pHZ90 also was transformed into the *pmk1* mutant nn78 (Xu and Hamer 1996) and the *cpkA* mutant I27 (Mitchell and Dean 1995) to generate transformants DA-P1 and DA-C1, respectively. Transformation of *C. graminicola*, *C. gloeosporioides*, and *F. graminearum* with pHZ90 was performed as described (Armesto et al. 2012; Thon et al. 2000; Zhou et al. 2010). The resulting bleomycin-resistant transformants were confirmed by PCR to contain the *MoRAS2^{G18V}* construct.

Generation of GFP fusion transformants,

The *GAS1*-GFP fusion construct pHZ104 (Xue et al. 2002) also was transformed into protoplasts of DA66. The resulting

zeocin-resistant transformants were assayed by PCR and GFP signals for the presence of the transforming *GAS1*-GFP fusion construct.

Appressorium formation, penetration, and plant infection assays.

Conidia were harvested from 10-day-old OTA cultures and resuspended to 5×10^4 conidia/ml. For appressorium formation assays, 50- μ l droplets of conidium suspensions were placed on glass cover slips (Fisher Scientific, St. Louis) or GelBond membranes (Cambrex, East Rutherford, NJ, U.S.A.) and incubated at 25°C (Liu et al. 2007). Rice seedlings (2 weeks old) of 'CO-39' or 'Nipponbare' were used for spray infection assays. For infection assays with culture blocks, rice seedlings were inoculated with 1- to 2-mm² blocks from 10-day-old OTA cultures. Lesion formation was examined 5 days postinoculation.

Assays for the TEY (Thr-Glu-Tyr) phosphorylation, intracellular cAMP, and PKA activities.

Fungal biomasses were harvested from 2-week-old OTA plates or 2-day-old liquid CM cultures and used for protein isolation or cAMP extraction (Nishimura et al. 2003; Zhao et al. 2005). Total proteins were separated on a 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred to nitrocellulose membranes for Western blot analysis. TEY phosphorylation was assayed with the Phospho-Plus p44/42 MAPK antibody kit following the manufacturer's protocol (Cell Signaling Technology, Danvers, MA, U.S.A.). Intracellular cAMP was assayed as described (Nishimura et al. 2003; Zhao et al. 2005) with the cAMP enzyme immunoassay system (GE Healthcare Biosciences, Pittsburg, PA, U.S.A.). PKA activities were assayed with the PepTag nonradioactive PKA assay kit (Promega Corp., Madison, WI, U.S.A.) as described (Adachi and Hamer 1998).

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