

## Functional Analysis of *MCNA*, a Gene Encoding a Catalytic Subunit of Calcineurin, in the Rice Blast Fungus *Magnaporthe oryzae*

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*Magnaporthe oryzae*, the causal agent of rice blast, forms a specialized infection structure, called an appressorium, which is crucial for penetration and infection of the host plant. Pharmacological data suggest that calcium/calmodulin-dependent signaling is involved in appressorium formation in this fungus. To understand the role of the calcium/calmodulin-activated protein phosphatase on appressorium formation at the molecular level, *MCNA*, a gene encoding the catalytic subunit of calcineurin, was functionally characterized in *M. oryzae*. Transformants expressing sense/antisense RNA of *MCNA* exhibited significant reductions in mycelial growth, conidiation, appressorium formation, and pathogenicity. cDNA of *MCNA* functionally complemented a calcineurin disruptant strain (*cmp1::LEU2 cmp2::HIS3*) of *Saccharomyces cerevisiae*. These data suggest that calcineurin A plays important roles in signal transduction pathways involved in the infection-related morphogenesis and pathogenicity of *M. oryzae*.

**Keywords:** Calcineurin, *Magnaporthe oryzae*, calmodulin, protein phosphatase, sense/antisense, appressorium

*Magnaporthe oryzae* (Hebert) Barr (anamorph *Pyricularia oryzae* Sacc.) is a filamentous heterothallic ascomycete fungus that is best known as the causal agent of rice blast, the most destructive disease of cultivated rice throughout the world [29, 41]. The infection process of *M. oryzae* begins with adhesion of conidia through spore tip mucilage released from the periplasmic space at the apex of the conidia [11, 17]. Conidia then germinate, and the tip of the germ tube develops a highly melanized and dome-shaped infection structure, the appressorium. A penetration peg from the appressorium invades the plant cuticle, and infectious hyphae develop in host epidermal cells. The environmental cues triggering appressorium formation of *M. oryzae* are

known to be surface hydrophobicity [19, 20] and hardness of the contact surface, as well as cutin monomers from the plant surface [3, 10].

Over the past decade, evidence has accumulated that cAMP-dependent and mitogen-activated protein kinase (MAPK) signaling pathways are involved in regulating appressorium formation of this fungus. A series of elegant experiments have identified and functionally characterized the genes involved in these signaling pathways for appressorium formation [1, 18, 40]. Pharmacological data also suggest that the calcium/calmodulin-dependent signaling pathway is involved in appressorium formation of this fungus [18]. Differential expression of the calmodulin gene, *cam*, in *M. oryzae* was implicated in appressorium formation as well as conidium attachment on plant surfaces [23]. *CYP1*, a gene encoding cyclophilin, a cellular target of cyclosporin A (CsA), was reported as a virulence factor regulating appressorial turgor generation [38], but little is known about calcium/calmodulin signaling in infection-related morphogenesis in this fungus.

Calcineurin is the only calcium/calmodulin-activated protein phosphatase and is composed of a heterodimer of two subunits: an  $\alpha$ -catalytic subunit A (calcineurin A, CNA: 57–71 kDa) and a  $\beta$ -regulatory subunit B (calcineurin B, CNB: 19 kDa). The ubiquitous presence of this protein phosphatase throughout the animal and plant kingdoms has prompted investigations of its structure and function [8, 33]. Calcineurin A is required for environmental stress adaptation, such as to ions and salt in the budding yeast *Saccharomyces cerevisiae* [24, 25, 27], and the regulation of alkaline pH-mediated growth and virulence in the human pathogens *Candida albicans* and *Cryptococcus neoformans* [2, 28]. Calcineurin A also plays an important role in controlling the pathogenicity of *Aspergillus fumigatus* [35] and is essential for cell cycle progression in *Aspergillus nidulans* [32]. More recently, the roles of calcineurin A were characterized by inducible antisense RNA expression strategies in *Sclerotinia sclerotiorum* and *Neurospora crassa* [5, 12, 31]. Calcineurin A regulates the

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hyphal branching pattern in *N. crassa* [5, 31], and sclerotium development was significantly affected in transformants expressing antisense RNA of *CNA* in *S. sclerotiorum* [12].

To investigate the role of calcineurin A on infection-related morphogenesis and pathogenicity in *M. oryzae* at the molecular level, a gene (*MCNA*) encoding a calcineurin catalytic subunit was functionally characterized by sense/antisense RNA expression. The data suggest that calcineurin A plays significant roles in mycelial growth, conidiation, appressorium formation, and pathogenicity in this plant pathogenic fungus.

## MATERIALS AND METHODS

### Fungal Strain and Cultural Conditions

*M. oryzae* isolate 70–15 was obtained from Dr. A. H. Ellingboe (University of Wisconsin, Madison, WI, U.S.A.) and used throughout this study. This fungus was grown on oatmeal agar media (OMA) under continuous fluorescent light at 22°C for 10 days to induce conidiation. Protocols for protoplast formation and transformation were used from established procedures [21].

### Vector Construction

Molecular techniques were performed according to standard methods [34]. For the yeast complementation assay, cDNA of *MCNA* was amplified by PCR (CNAFF: 5'-ATGGAAGATGGCACCCAGG-3'; CNAFR: 5'-TCACGTGCTCAACCTGCG-3') and inserted into the EcoRI site of the yeast expression vector pYES2 (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) under the control of the *GAL1* promoter. This construct, named pYMCNA, was transformed into the calcineurin disruptant strain of *S. cerevisiae* DHT22-1a (*cmp1::LEU2 cmp2::HIS3*) [13]. For *MCNA* sense/antisense vector construction, *MCNA* (MG07456.5) was isolated by PCR amplification with the CALIF (5'-GAAYAAGCAGTTCTTCTGYA-3') and CALIR (5'-TGGCKTKCTTAATWCCYTCS-3') primer pair and cloned into pGEM-T (Promega, WI, U.S.A.), and the construct was named pJC05. A 1.2-kb BamHI fragment of pJC05 was inserted into pAN7-1 containing the hygromycin B resistance gene, and the resulting construct was named pJC08. A 2.4-kb BglII and a BamHI fragment of pAN7-1, a *gpd* promoter, were inserted into the BamHI site of pJC13. The orientation of the BamHI fragment was determined by restriction enzyme analysis and sequencing. Antisense and sense *MCNA* vectors, pJH20 and pJH22, respectively, were transformed into 70–15 protoplasts. Transformants were confirmed by Southern blotting analysis.

### Expression Assay

Total RNA was extracted from axenically grown fungal mycelia, as described previously [7]. A total of  $1 \times 10^7$  conidia were inoculated into the complete medium [36], and grown for 4 days at 160 rpm at 25°C. Mycelia were obtained through filtration, washed three times with sterile distilled water, and transferred to either CM-C (complete medium) (CM-glucose, yeast extract, peptone, and casamino acids) or CM-N (CM-sodium nitrate, yeast extract, and casamino acids) [36].

Mycelia were incubated in these media for 16 h at 160 rpm at 25°C and were used for total RNA extraction. RT-PCR experiments were carried out using the Superscript First-Strand Synthesis System

in accordance with the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.). RT-PCR was performed with 10 pmol of each primer for 20 or 25 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. CNARTPF (5'-AGCCACGAGGAGGTGTC-3')/CNARTPR (5'-TTTCTTGCGTGCTACTGCTA-3') and CNAFF/CNAFR PCR primer pairs were used for real-time PCR and RT-PCR. The  $\beta$ -tubulin gene was included as a control.

### Calcineurin Activity Assay

The wild-type strain and transformants of *M. oryzae* were grown for 3 days in complete media (CM) (25°C, pH 6.5). Cytoplasmic extracts were prepared by homogenizing the mycelia using liquid nitrogen in buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10  $\mu$ g/ml each of leupeptin and pepstatin. Calcineurin activity was measured according to the method of Pallen and Wang [30]. Phosphatase activity was assayed in 1 ml of a reaction mixture containing 25 mM Tris (pH 7.2), 25 mM MES (pH 7.0), 2.4 M *p*-nitrophenyl phosphate, and 1 mM  $MnCl_2$ . The reaction was initiated by addition of *p*-nitrophenyl phosphate followed by incubation at 30°C for 10 min, and terminated by the addition of 10  $\mu$ l of 13% (w/v)  $KH_2PO_4$ . The absorbance of the samples was measured at a wavelength of 405 nm. One unit of enzyme activity was defined as *p*-nitrophenyl phosphate per min per mg protein [14].

### Mycelial Growth, Conidiation, Appressorium Formation, and Pathogenicity Assays

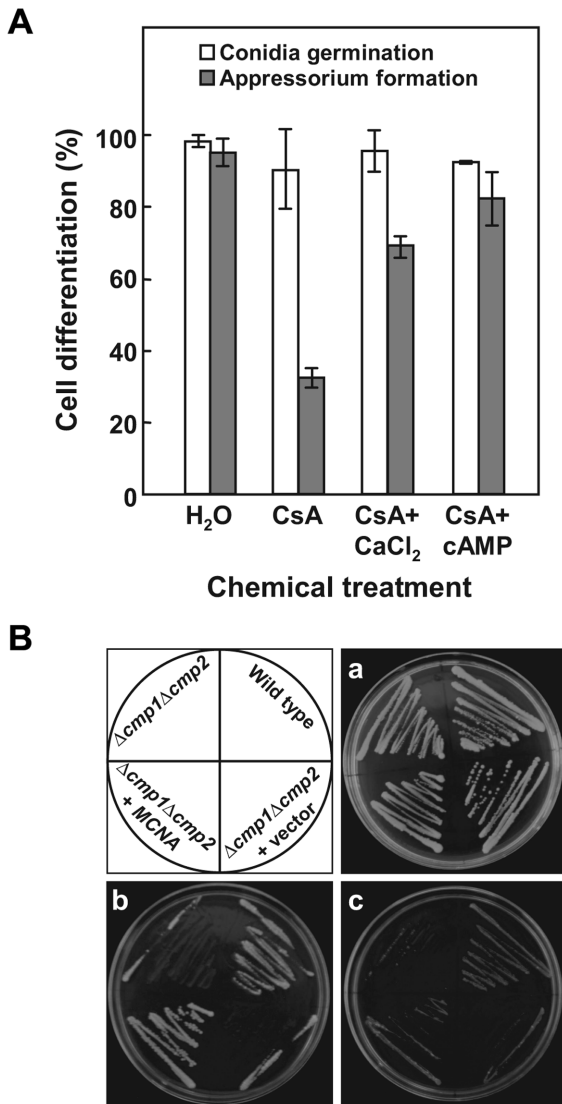
Mycelial growth was measured on CM under different pH conditions (pH 4.5, pH 6.5, pH 8.5, and pH 10.5), 4, 6, and 10 days after inoculation. Conidiation was measured in 10-day-old cultures on OMA. Experiments were repeated three times with three replicates.

Conidia harvested from 8-day-old cultures on OMA plates were used for appressorium formation on the hydrophobic side of GelBond films or plastic microscope coverslips as previously described [17]. A conidial suspension ( $5 \times 10^5$  conidia/ml) with 250 ppm Tween-20 was sprayed onto susceptible rice seedlings (cv. Nackdongbyeo). Disease severity was measured 7 days after inoculation and calculated using the modified equation of Ordóñez [17].

## RESULTS AND DISCUSSION

### Effects of Cyclosporin A on Appressorium Formation in *M. oryzae*

To investigate whether calcineurin A is involved in infection-related morphogenesis in *M. oryzae*, we examined the effects of cyclosporin A (CsA), a calcineurin A immunosuppressant, on conidial germination and appressorium formation [37]. Conidia germination was unaffected by CsA even at 20  $\mu$ M, but appressorium formation was significantly reduced by CsA in a dose-dependent manner. A reduction of about 60% in appressorium formation was observed in the presence of 10  $\mu$ M CsA (Fig. 1A). Addition of exogenous  $CaCl_2$  and cAMP restored appressorium formation that had been inhibited by CsA (Fig. 1A). These data strongly suggest that calcineurin-mediated calcium signaling is involved in appressorium formation and possibly in cross-talk between



**Fig. 1.** Effects of cyclosporin A and yeast complementation. **A.** Effects of cyclosporin A on conidial germination and appressorium formation in *M. oryzae*. Final concentrations of cyclosporin A (CsA), CaCl<sub>2</sub>, and adenosine 3':5'-cyclic monophosphate (cAMP) were 10  $\mu$ M, 10 mM, and 100  $\mu$ M, respectively. Error bar means standard deviation of three replicates. **B.** Complementation of *S. cerevisiae* calcineurin A disruptant strain DHT22-1a ( $\Delta cmp1\Delta cmp2$ ) by the *MCNA* gene. a, YPGal; b, YPGal+NaCl (0.8 M); c, YPGal + LiCl (50 mM).

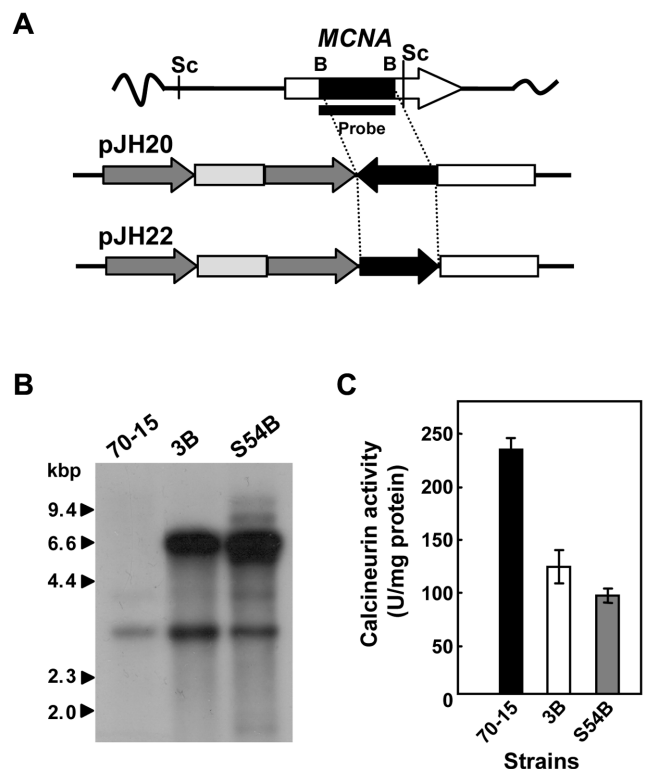
calcium- and cAMP-dependent signaling in this fungus. Cross-talks between cAMP (PKA) and calcium-dependent signaling pathways have been reported in several biological systems including animal cells, yeast, and fungi [4, 16, 26].

#### Characterization of the *MCNA* Gene

The *MCNA* (MGG07456.5) gene of *M. oryzae* wild-type strain 70-15 was isolated by using PCR amplification with degenerate primers CAL1F/CAL1R. The *MCNA* of *M. oryzae* contains a putative open reading frame (ORF) of 1608 bp encoding a product of 546 amino acids with two

introns. *MCNA* is highly homologous to calcineurin A in other filamentous fungi, but less to those in yeasts; *N. crassa* (88%), *A. nidulans* (80%), *S. sclerotiorum* (75%), *C. neoformans* (61%), *C. albicans* (54%), and *S. cerevisiae* (51%). Comparative analysis of three conserved domain regions, including calmodulin B binding, calmodulin binding, and autoinhibitory domains, clearly showed that *MCNA* belongs to the fungal clade (data not shown). Increased transcription of *MCNA* was detected under conditions of nitrogen and carbon starvation but not in complete medium (data not shown).

To investigate the functional homology between *MCNA* and calcineurin genes in other organisms, a yeast expression vector, *pYMCNA*, containing *MCNA* was transformed into *S. cerevisiae* calcineurin A disruptant strain DHT 22-1a (*cmp1::LEU2 cmp2::HIS3*). All strains including the control transformant grew well on YPGal medium, but only the transformant containing *MCNA* could grow on media in



**Fig. 2.** Construction and characterization of transformants expressing the antisense/sense *MCNA* gene.

**A.** Construction of antisense/sense expression vectors of *MCNA*, pJH20 and pJH22. The *gpd* promoter, hygromycin resistance gene (*HPH*), and *trpC* terminator are indicated by gray, light gray, and white, respectively. The direction of the arrow indicates antisense or sense expression. **B.** Southern blotting analysis of transformants containing pJH20 and pJH22. Genomic DNA was digested with *SacI* and hybridized with part of the ORF as a probe. **C.** Calcineurin activities of antisense/sense transformants, 3B and S54B. One unit of acid phosphatase activity was defined as 1 nmol of *p*-nitrophenol released from *p*-nitrophenyl phosphate (PNPP) per minute per mg protein. Error bar means standard deviation of three replicates.

the presence of 0.8 M NaCl and 50 mM LiCl (Fig. 1B). These observations suggest that *MCNA* of *M. oryzae* is the functional homolog of calcineurin A of *S. cerevisiae*. Similar results regarding salt stress tolerance have been reported in *S. cerevisiae* with *cnaA* of *A. oryzae* [13].

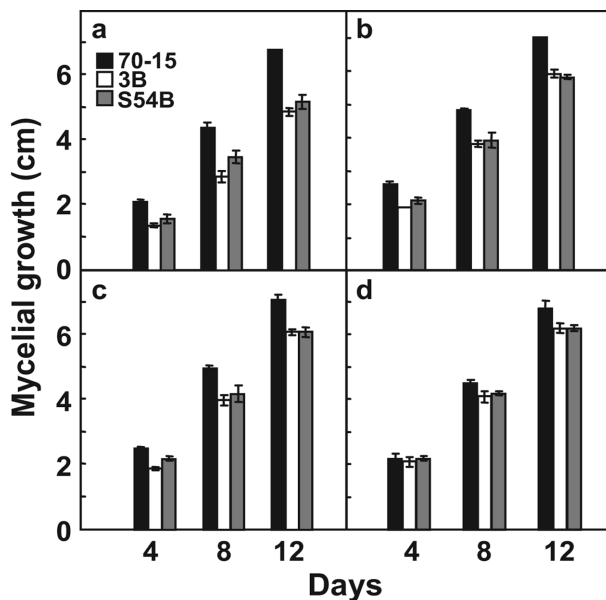
#### Antisense/Sense Expression of the *MCNA* Gene

To better understand the function of *MCNA* in more detail, a cosuppression strategy was applied [6, 15]. Antisense and sense expression vectors of *MCNA*, pJH20 and pJH22, respectively, were constructed (Fig. 2A) and transformed into wild-type 70–15 protoplasts. The transformant 3B contained the antisense construct of *MCNA* (pJH20) and S54B possessed the sense construct (pJH22) (Fig. 2B). To verify the effects of antisense/sense expression of *MCNA*, we examined the calcineurin activities in these transformants (Fig. 2C). Calcineurin activities in both transformants were decreased significantly to 50% as compared with the wild type (Fig. 2C). This clearly indicated that RNA silencing mutants attenuated the expression of *MCNA* in *M. oryzae*. S54B, the sense *MCNA* transformant, showed a greater reduction in calcineurin activity than the antisense *MCNA* transformant, 3B (Fig. 2C). A similar silencing phenomenon was previously reported in *M. grisea* [15]. In *S. sclerotiorum* and *N. crassa*, however, gene silencing of calcineurin A homologs, *CNA1* and *CNA-1*, was effective when an antisense expression strategy was used [12, 31]. The hairpin RNA structure is known to be the most efficient approach for gene silencing in higher organisms as well as in fungi

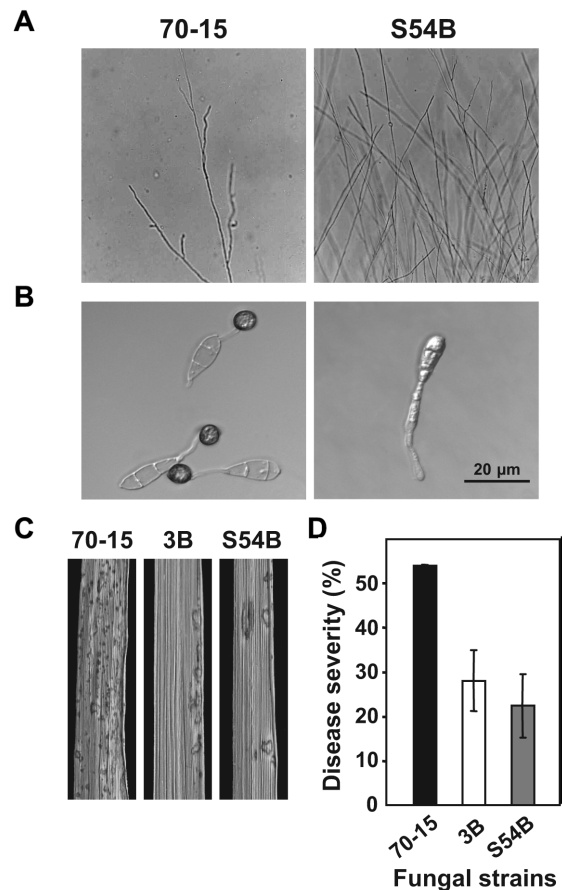
[9, 15, 39]. In *Cryphonectria parasitica*, a sense transformant of *cpg1*, a G-protein subunit, also altered colony morphology and reduced *CPG1* accumulation, whereas no such differences were observed between an antisense *cpg1* transformant and non-transformed wild-type strain [6]. We have shown that a single antisense/sense RNA expression, using part of a gene, is also a useful strategy for homology-dependent transcriptional gene silencing in fungi.

#### Effects of Antisense/Sense *MCNA* Expression on Mycelial Growth Rate and Appressorium Formation

As calcineurin is required for pH-mediated regulation in *C. albicans* and *C. neoformans* [2, 28], we examined the effects of pH on mycelial growth of transformants. The mycelial growth rates of transformants 3B and S54B were reduced compared with that of the wild-type on CM at 25°C, but was not significantly affected by pH (Fig. 3). These observations indicate that *MCNA* is not involved in pH-mediated regulation



**Fig. 3.** Effects of pH on mycelial growth of transformants. Fungal strains including 70–15, 3B, and S54B were grown on complete media under various pH conditions. Colony diameter was measured after incubation for 4, 8, and 12 days. Error bar means standard deviation of three replicates. a, pH 4.5; b, pH 6.5; c, pH 8.5; d, pH 10.5.



**Fig. 4.** Phenotypes of transformants expressing antisense/sense *MCNA*, 3B and S54B.

**A.** Hyphal branching pattern. Wild-type (70–15) and transformants were grown in water agar media at 25°C for 48 h. **B.** Appressorium formation. Conidial suspensions were incubated on the hydrophobic surfaces of coverslips for 16 h. **C and D.** Pathogenicity test. Rice seedlings were spray-inoculated with  $5 \times 10^5$  conidia/ml and disease severity was measured 7 days after inoculation.

of mycelial growth rate in *M. oryzae*. However, extensive hyphal branching and loss of apparent dominance of the main hyphae were observed in antisense/sense transformants, as also observed in the *cna-1* antisense transformant of *N. crassa* [31] (Fig. 4A). It seems that extensive hyphal tip branching led to arrest of hyphal elongation, resulting in a reduction in the mycelial growth rate of the transformants.

The average conidia production showed more than 70% reduction in transformants 3B ( $343,000 \pm 25,000$  conidia/ml) and S54B ( $620,000 \pm 66,000$  conidia/ml) compared with that of the wild type ( $1,697,000 \pm 172,000$  conidia/ml) on OMA at 25°C. The effects of antisense/sense *MCNA* expression on conidial germination and appressorium formation were examined. Conidial germination was unaffected, but the frequency of appressorium formation was significantly decreased in 3B ( $56.3 \pm 6.3\%$ ) and S54B ( $49.1 \pm 5.2\%$ ) compared with that of wild-type 70–15 ( $95.2 \pm 3.7\%$ ) on hydrophobic surfaces. However, swollen germ tube tips were observed in these transformants on inductive hydrophobic, but not on hydrophilic surfaces (Fig. 4B). These observations indicate that *MCNA* is required for appressorium formation but not for surface recognition. Addition of exogenous  $\text{CaCl}_2$  restored appressorium formation in the 3B and S54B transformants ( $98.4 \pm 1.1\%$  and  $91.1 \pm 8.2\%$ , respectively). These results, together with pharmacological data, clearly indicate that calcineurin A-mediated calcium signaling plays an important role in appressorium development in *M. oryzae*. Interestingly, addition of cAMP also restored defects in appressorium formation in 3B and S54B by  $95.0 \pm 2.8\%$  and  $94.1 \pm 1.3\%$ , respectively. These observations strongly suggest the existence of cross-talk between calcium- and cAMP-dependent signaling for appressorium formation in this fungus. Increased expression of *MCNA* in the *magB* mutant further supports this hypothesis. *magB* encoding the G-protein  $\alpha$ -subunit has been reported to regulate the cAMP/PKA signaling pathways for appressorium formation in *M. oryzae* [22].

### Effects of Antisense/Sense *MCNA* Expression on Pathogenicity

Conidial suspensions of antisense/sense transformants and wild-type strains of *M. oryzae* were sprayed onto rice leaves. Typical diamond-shaped and gray-centered lesions developed on rice leaves when inoculated with the wild type, but much fewer and smaller lesions were observed when inoculated with 3B and S54B (Fig. 4C). The disease severities on rice leaves inoculated with 3B and S54B were less than 30% compared to wild-type 70–15 (Fig. 4D). The reduced pathogenicity of antisense/sense *MCNA* transformants seems to be due, at least in part, to the reduced frequency of appressorium formation and invasive growth into the rice plant. Although the detailed mechanisms remain to be elucidated, calcineurin A genes have been reported as virulence factors in *C. albicans*, *C. neoformans*, and *S. sclerotiorum* [2, 12, 28].

In summary, calcineurin A-mediated signaling plays significant roles in cell differentiation in *M. oryzae*, including hyphal growth/branching, appressorium formation, and pathogenicity. Deciphering the cross-talk between calcium- and cAMP-dependent signaling for infection-related morphogenesis will provide new insights into disease control strategies.

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### REFERENCES

- Adachi, K. and J. E. Hamer. 1998. Divergent cAMP signaling pathways regulate growth and pathogenesis in the rice blast fungus *Magnaporthe grisea*. *Plant Cell* **10**: 1361–1374.
- Bader, T., B. Bodendorfer, K. Schroppel, and J. Morschhauser. 2003. Calcineurin is essential for virulence in *Candida albicans*. *Infect. Immun.* **71**: 5344–5354.
- Bae, C. Y., S. Kim, W. B. Choi, and Y. H. Lee. 2007. Involvement of extracellular matrix and integrin-like proteins on conidial adhesion and appressorium differentiation in *Magnaporthe oryzae*. *J. Microbiol. Biotechnol.* **17**: 1198–1203.
- Bencina, M., M. Legisa, and N. D. Read. 2005. Cross-talk between cAMP and calcium signalling in *Aspergillus niger*. *Mol. Microbiol.* **56**: 268–281.
- Bok, J. W., T. Sone, L. B. Silverman-Gavrila, R. R. Lew, F. J. Bowring, D. E. Catcheside, and A. J. Griffiths. 2001. Structure and function analysis of the calcium-related gene spray in *Neurospora crassa*. *Fungal Genet. Biol.* **32**: 145–158.
- Choi, G. H., B. Chen, and D. L. Nuss. 1995. Virus-mediated or transgenic suppression of a G-protein alpha subunit and attenuation of fungal virulence. *Proc. Natl. Acad. Sci. USA* **92**: 305–309.
- Davis, K. R. and F. M. Ausubel. 1989. Characterization of elicitor-induced defense responses in suspension-cultured cells of *Arabidopsis*. *Mol. Plant Microbe Interact.* **2**: 363–368.
- Dickman, M. B. and O. Yarden. 1999. Serine/threonine protein kinases and phosphatases in filamentous fungi. *Fungal Genet. Biol.* **26**: 99–117.
- Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**: 806–811.
- Gilbert, R. D., A. M. Johnson, and R. A. Dean. 1996. Chemical signals responsible for appressorium formation in the rice blast fungus *Magnaporthe grisea*. *Physiol. Molec. Plant Pathol.* **48**: 335–346.

11. Hamer, J. E. and N. J. Talbot. 1998. Infection-related development in the rice blast fungus *Magnaporthe grisea*. *Curr. Opin. Microbiol.* **1**: 693–697.
12. Harel, A., S. Bercovich, and O. Yarden. 2006. Calcineurin is required for sclerotial development and pathogenicity of *Sclerotinia sclerotiorum* in an oxalic acid-independent manner. *Mol. Plant Microbe Interact.* **19**: 682–693.
13. Juvvadi, P. R., M. Arioka, H. Nakajima, and K. Kitamoto. 2001. Cloning and sequence analysis of *cnaA* gene encoding the catalytic subunit of calcineurin from *Aspergillus oryzae*. *FEMS Microbiol. Lett.* **204**: 169–174.
14. Juvvadi, P. R., Y. Kuroki, M. Arioka, H. Nakajima, and K. Kitamoto. 2003. Functional analysis of the calcineurin-encoding gene *cnaA* from *Aspergillus oryzae*: Evidence for its putative role in stress adaptation. *Arch. Microbiol.* **179**: 416–422.
15. Kadotani, N., H. Nakayashiki, Y. Tosa, and S. Mayama. 2003. RNA silencing in the phytopathogenic fungus *Magnaporthe oryzae*. *Mol. Plant Microbe Interact.* **16**: 769–776.
16. Kafadar, K. A. and M. S. Cyert. 2004. Integration of stress responses: Modulation of calcineurin signaling in *Saccharomyces cerevisiae* by protein kinase A. *Eukaryot. Cell* **3**: 1147–1153.
17. Kim, S., I. P. Ahn, H. S. Rho, and Y. H. Lee. 2005. *MHP1*, a *Magnaporthe grisea* hydrophobin gene, is required for fungal development and plant colonization. *Mol. Microbiol.* **57**: 1224–1237.
18. Lee, S. C. and Y. H. Lee. 1998. Calcium/calmodulin-dependent signaling for appressorium formation in the plant pathogenic fungus *Magnaporthe grisea*. *Mol. Cells* **8**: 698–704.
19. Lee, Y.-H. and R. A. Dean. 1993. cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. *Plant Cell* **5**: 693–700.
20. Lee, Y.-H. and R. A. Dean. 1994. Hydrophobicity of contact surface induces appressorium formation in *Magnaporthe grisea*. *FEMS Microbiol. Lett.* **115**: 71–75.
21. Leung, H., U. Lehtinen, R. Karjalainen, D. Skinner, P. Tooley, S. Leong, and A. Ellingboe. 1990. Transformation of the rice blast fungus *Magnaporthe grisea* to hygromycin B resistance. *Curr. Genet.* **17**: 409–411.
22. Liu, S. and R. A. Dean. 1997. G protein alpha subunit genes control growth, development, and pathogenicity of *Magnaporthe grisea*. *Mol. Plant Microbe Interact.* **10**: 1075–1086.
23. Liu, Z. M. and P. E. Kolattukudy. 1999. Early expression of the calmodulin gene, which precedes appressorium formation in *Magnaporthe grisea*, is inhibited by self-inhibitors and requires surface attachment. *J. Bacteriol.* **181**: 3571–3577.
24. Mendoza, I., F. J. Quintero, R. A. Bressan, P. M. Hasegawa, and J. M. Pardo. 1996. Activated calcineurin confers high tolerance to ion stress and alters the budding pattern and cell morphology of yeast cells. *J. Biol. Chem.* **271**: 23061–23067.
25. Mendoza, I., F. Rubio, A. Rodriguez-Navarro, and J. M. Pardo. 1994. The protein phosphatase calcineurin is essential for NaCl tolerance of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **269**: 8792–8796.
26. Moore, T. M., P. M. Chetham, J. J. Kelly, and T. Stevens. 1998. Signal transduction and regulation of lung endothelial cell permeability. Interaction between calcium and cAMP. *Am. J. Physiol.* **275**: L203–L222.
27. Nakamura, T., Y. Liu, D. Hirata, H. Namba, S. Harada, T. Hirokawa, and T. Miyakawa. 1993. Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. *EMBO J.* **12**: 4063–4071.
28. Odom, A., S. Muir, E. Lim, D. L. Toffaletti, J. Perfect, and J. Heitman. 1997. Calcineurin is required for virulence of *Cryptococcus neoformans*. *EMBO J.* **16**: 2576–2589.
29. Ou, S. H. 1985. *Rice Diseases*. Commonwealth Mycological Institute, Kew, U.K.
30. Pallen, C. J. and J. H. Wang. 1983. Calmodulin-stimulated dephosphorylation of para-nitrophenyl phosphate and free phosphotyrosine by calcineurin. *J. Biol. Chem.* **258**: 8550–8553.
31. Prokisch, H., O. Yarden, M. Dieminger, M. Tropschug, and I. B. Barthelmess. 1997. Impairment of calcineurin function in *Neurospora crassa* reveals its essential role in hyphal growth, morphology and maintenance of the apical  $Ca^{2+}$  gradient. *Mol. Gen. Genet.* **256**: 104–114.
32. Rasmussen, C., C. Garen, S. Brining, R. L. Kincaid, R. L. Means, and A. R. Means. 1994. The calmodulin-dependent protein phosphatase catalytic subunit (calcineurin A) is an essential gene in *Aspergillus nidulans*. *EMBO J.* **13**: 3917–3924.
33. Rusnak, F. and P. Mertz. 2000. Calcineurin: Form and function. *Physiol. Rev.* **80**: 1483–1521.
34. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY, Cold Spring Harbor, NY.
35. Steinbach, W. J., R. A. Cramer, Jr., B. Z. Perfect, Y. G. Asfaw, T. C. Sauer, L. K. Najvar, *et al.* 2006. Calcineurin controls growth, morphology, and pathogenicity in *Aspergillus fumigatus*. *Eukaryot. Cell* **5**: 1091–1103.
36. Talbot, N. J., D. J. Ebbole, and J. E. Hamer. 1993. Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *Plant Cell* **5**: 1575–1590.
37. Viaud, M., A. Brunet-Simon, Y. Brygoo, J. M. Pradier, and C. Levis. 2003. Cyclophilin A and calcineurin functions investigated by gene inactivation, cyclosporin A inhibition and cDNA arrays approaches in the phytopathogenic fungus *Botrytis cinerea*. *Mol. Microbiol.* **50**: 1451–1465.
38. Viaud, M. C., P. V. Balhadere, and N. J. Talbot. 2002. A *Magnaporthe grisea* cyclophilin acts as a virulence determinant during plant infection. *Plant Cell* **14**: 917–930.
39. Wesley, S. V., C. A. Helliwell, N. A. Smith, M. B. Wang, D. T. Rouse, Q. Liu, *et al.* 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J.* **27**: 581–590.
40. Xu, J. R. and J. E. Hamer. 1996. MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes Dev.* **10**: 2696–2706.
41. Yi, M. and Y. H. Lee. 2008. Identification of genes encoding heat shock protein 40 family and the functional characterization of two Hsp40s, MHF16, and MHF21, in *Magnaporthe oryzae*. *Plant Pathol. J.* **24**: 131–142.