Large-Scale Gene Disruption in *Magnaporthe oryzae* Identifies MC69, a Secreted Protein Required for Infection by Monocot and Dicot Fungal Pathogens

Hiromasa Saitoh1*, Shizuko Fujisawa1, Chikako Mitsuoka1, Akiko Ito1, Akiko Hirabuchi1, Kyoko Ikeda2, Hiroki Irieda2, Kae Yoshino2, Kentaro Yoshida1,3, Hideo Matsumura1*, Yukio Tosa4, Joe Win3, Sophien Kamoun3, Yoshitaka Takan4, Ryohei Terauchi1

1 Iwate Biotechnology Research Center, Kitakami, Iwate, Japan, 2 Laboratory of Plant Pathology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan, 3 The Sainsbury Laboratory, John Innes Centre, Norwich, United Kingdom, 4 Laboratory of Plant Pathology, Graduate School of Agricultural Sciences, Kobe University, Kobe, Japan

**Abstract**

To search for virulence effector genes of the rice blast fungus, *Magnaporthe oryzae*, we carried out a large-scale targeted disruption of genes for 78 putative secreted proteins that are expressed during the early stages of infection of *M. oryzae*. Disruption of the majority of genes did not affect growth, conidiation, or pathogenicity of *M. oryzae*. One exception was the gene *MC69*. The *mc69* mutant showed a severe reduction in blast symptoms on rice and barley, indicating the importance of *MC69* for pathogenicity of *M. oryzae*. The *mc69* mutant did not exhibit changes in saprophytic growth and conidiation. Microscopic analysis of infection behavior in the *mc69* mutant revealed that MC69 is dispensable for appressorium formation. However, *mc69* mutant failed to develop invasive hyphae after appressorium formation in rice leaf sheath, indicating a critical role of *MC69* in interaction with host plants. *MC69* encodes a hypothetical 54 amino acids protein with a signal peptide. Live-cell imaging suggested that fluorescently labeled MC69 was not translocated into rice cytoplasm. Site-directed mutagenesis of two conserved cysteine residues (Cys36 and Cys46) in the mature MC69 impaired function of MC69 without affecting its secretion, suggesting the importance of the disulfide bond in *MC69* pathogenicity function. Furthermore, deletion of the *MC69* orthologous gene reduced pathogenicity of the cucumber anthracnose fungus *Colletotrichum orbiculare* on both cucumber and *Nicotiana benthamiana* leaves. We conclude that *MC69* is a secreted pathogenicity protein commonly required for infection of two different plant pathogenic fungi, *M. oryzae* and *C. orbiculare* pathogenic on monocot and dicot plants, respectively.

**Introduction**

Rice blast, caused by an ascomycete fungus *Magnaporthe oryzae*, is the most severe fungal disease of rice throughout the world [1]. Genetic studies of this pathogen over the last two decades have made the *Magnaporthe*-rice pathosystem an excellent model for investigating fungus-plant interactions.

Plants are equipped to sense evolutionarily conserved microbial molecular signatures, collectively called Pathogen-Associated Molecular Patterns (PAMPs) or Microbe-Associated Molecular Patterns (MAMPs), and activate PAMP-Triggered Immunity (PTI) [2–4]. Pathogens are capable of inhibiting PTI on their host plants by delivering virulence effector proteins into host cells [5–9].

In *M. oryzae*, effector secretion machinery has recently been elucidated [10–12]. A Golgi-localized P-type ATPase-encoding gene, *MgAPT2* is required for exocytosis during plant infection. Further analysis suggested that *MgAPT2* is involved in secretion of a range of extracellular enzymes as well as an AVR effector for the rapid induction of host defense responses in an incompatible reaction in rice cultivar IR-68 [10]. Another study demonstrated that *M. oryzae* mutants with a defect in an ER chaperone-encoding gene, *LHS1*, have reduced activities of extracellular enzymes and secretion of AVR-Pita1 [13,14] blocking *Pi-ta* R-gene-mediated hypersensitive response. The contribution of *LHS1* to protein translocation and secretion of proteins, including effectors, revealed the importance of ER chaperones for successful disease development by rice blast fungus [12]. Live-cell imaging revealed development of the biotrophic interfacial complex (BIC), a structure that accumulates fluorescently labeled effectors secreted by invasive hyphae (IH). The examined BIC-localized secreted proteins were translocated into rice cytoplasm. By contrast, a
**Author Summary**

*Magnaporthe oryzae* causes the most devastating fungal disease in rice. *M. oryzae* secretes a plethora of effector proteins, including several avirulence proteins which are known to be recognized by host resistance proteins activating innate immunity. However, the effectors that are required for virulence activity have not been identified in *M. oryzae* to date except for an effector protein, Secreted LysM Protein 1 (Slp1) that was recently identified. We performed a large-scale disruption analysis of *M. oryzae* effector candidates and identified a small protein MC69, which is secreted by the fungus during infection. When MC69 is absent, pathogenicity is severely reduced after penetration into the host cells. Furthermore, deletion of the MC69 orthologous gene in *Colletotrichum orbiculare* reduced its pathogenicity in the host plants cucumber and *Nicotiana benthamiana*. Thus, MC69 is conserved in ascomycete fungi and is crucial for establishing compatibility. This is the first report of a single secreted protein that is indispensable for pathogenicity in both monocot and dicot pathogenic fungi. How MC69 contributes to pathogenicity or virulence is unknown but it could be required for the fungus to be a pathogen or might be a classical effector that acts on plant target molecules.

**Results**

Large-scale disruption analysis of *Magnaporthe oryzae* secreted protein genes

To search for effector protein genes of *Magnaporthe oryzae*, we carried out a large-scale targeted gene disruption analysis of the 78 putative secreted protein genes that are expressed during infection (Table 1). Initially we selected 1,306 putative secreted protein genes as described previously [20]. The 78 genes subjected to functional study were selected on the basis of their confirmed expression in the pathogen at the early stages of infection. We focused on secreted protein genes involved in the two stages of infection of *M. oryzae*. One is the appressorium formation stage and the other is the biotrophic invasion stage. Treatment with cyclic AMP (cAMP) induces appressorium formation on hydrophilic surface [24]. By SAGE (Serial Analysis of Gene Expression) of cAMP-treated conidia on hydrophilic membrane 6 h after the start of treatment [25], we identified several pathogenicity genes, e.g. *MPG1*, *MAS1* and *MAC1*, already characterized [26–28] and thought to be involved in pathogen-host interaction. Therefore, we assumed that a part of effector genes should be expressed during the appressorium formation. To achieve a high efficiency tag-to-gene annotation, we established SuperSAGE method that extracts a 26-bp tag from each cDNA [29]. SuperSAGE of the cAMP-treated *M. oryzae* strain 70-15 has been done in this study to search for novel effector candidates (Table S1). Furthermore, we also used the SuperSAGE data of invasive hyphae for searching new effectors [Supplemental Data Set 1 in [20]]. Indeed, this SuperSAGE analysis revealed that two *AVR* effector genes, *AVR-Poa* and *AVR-Pvi* were expressed at the stage of invasive hyphae (Supplemental Data Set 2 in [20]).

To investigate the function of the effector candidate genes, we generated disruption mutants for each of the selected 78 genes (Table 1) in *M. oryzae* by TAG-KO method [30,31]. To assess the virulence of each mutant, conidial suspension of each mutant was sprayed onto seedlings of a barley cultivar Nigrate, which is susceptible to the wild type *M. oryzae*. Blast phenotypes of barley infected by KO mutants of all the genes except for *MC69* gene were the same as that infected by wild-type strain Ina72. Similarly the 77 mutants did not show reduced virulence in a susceptible rice cultivar Shin No. 2. By contrast, we observed a dramatic reduction in disease symptoms on barley cotyledons and the susceptible rice cultivar, Shin No. 2, inoculated with all of the three independent *mc69* mutant lines (Figure 1A). Consequently, we identified the *MC69* gene (MGG_02848.6) as required for pathogenicity of *M. oryzae* after a large-scale targeted gene disruption analysis of the 78 putative secreted protein genes.

In summary, we found that targeted disruption of *MC69* affected pathogenicity of *M. oryzae* and disruption of the other 77 genes had no effect on its pathogenicity.

**MC69 is required for appressorial penetration and pathogenicity of *M. oryzae***

To investigate the physiological and molecular function of *MC69* in detail, we generated *MC69* disruptants in *M. oryzae* strain Ina72 by targeted gene disruption as described above. Colony growth, color and the production of conidia were the same as the wild-type strain (Figure S2A and B). We observed a remarkable reduction in disease symptoms on barley and rice inoculated with the *mc69* mutants compared to those inoculated with the wild type strain 4 and 7 days after inoculation suggesting an important role of *MC69* in fungal pathogenicity (Figure 1A). Subsequently, we performed a detailed phenotypic analysis of the *mc69* mutants. The *mc69* mutants exhibited a defect in appressorium-mediated

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biotrophy-associated secreted protein BAS4, which uniformly outlines the IH, was not translocated into the host cytoplasm [11]. These results suggest that BIC represents the site of effector translocation in rice blast disease [11].

Several effector protein genes have been cloned and characterized from *M. oryzae* but all of them were avirulence (AVR) effectors with no virulence function elucidated to date [13–20] except for a recently identified virulence effector protein, Slp1 [21]. Slp1 accumulates at the interface between the fungal cell wall and the rice plasma membrane, can bind to chitin, and is able to suppress chitin-induced plant immune responses, including generation of reactive oxygen species and plant defense gene expression [21]. Several effector candidates were identified by using interaction transcriptome in the biotrophic invasion of *M. oryzae* [22]. In the paper the authors have identified a known effector PWL2 as well as 58 candidate effectors showing >10-fold increase in the expression in the biotrophic invasive hyphae relative to control mycelia using *M. oryzae* oligoarrays. Four of these candidates were confirmed to be fungal biotrophy-associated secreted proteins [22]. However, virulence function of all the candidates has not been elucidated, and comprehensive gene disruption analyses of the candidates have not been carried out. Therefore, in this study we employed a large-scale disruption analysis of *M. oryzae* secreted protein genes to search for novel virulence effectors.

Whole-genome draft sequence of *M. oryzae* was published for the isolate 70-15, a laboratory strain [23]. The genome assembly consists of 37.8 Mb nucleotides encoding 11,109 predicted protein coding genes. We recently retrieved 1,306 putative secreted protein genes from the predicted proteome of 70-15 [20]. From these, a total of 78 genes expressed in the fungus were disrupted and analyzed. We found that disruptants of the 77 genes did not show change in pathogenicity as compared to the wild-type strains. Disruption of only one gene, *MC69*, showed a severe reduction in pathogenicity. Further analysis showed that MC69 protein is involved in the full pathogenicity of *M. oryzae* after the penetration stage of infection.
penetration in rice leaf sheath cells but neither in conidial germination nor appressorium formation (Figure 1B and C). We studied 200 appressoria of each mc69 mutant, 97% with failed penetration (no visible hyphae) and 1% with post-penetration blockage. Therefore, we conclude that MC69 is required for appressorial penetration and pathogenicity of M. oryzae. Although most of appressoria formed by the mc69 mutants could neither penetrate nor produce infectious hyphae in the inoculated rice leaf sheath cells, we further analyzed invasive growth at 50 appressorial penetration sites by rating the hyphal growth from the level 1 (low) to 4 (high; see Materials and Methods, Figure 1D). In Ina72 WT, 84% of penetration sites showed invasive growth levels 3 or 4, by contrast in the mc69 mutant infectious growth within the inner epidermal tissue was relatively limited (levels 2 and 3) 32 hours after inoculation, suggesting that the loss of MC69 also affects infectious growth to some extent at the post-penetration stage.

Table 1. Gene disruption analysis of 78 putative secreted protein genes.

| Mutant ID | Gene ID | cAMPa | IHb | Mutant ID | Gene ID | cAMPa | IHb |
|-----------|---------|-------|-----|-----------|---------|-------|-----|
| HS9       | MGG_04172.6 | +     | --  | MC28      | MGG_01609.6 | +     | +   |
| RT11      | MGG_08342.6 | +     | --  | MC33      | MGG_09378.6 | +     | --  |
| RT76      | MGG_05716.6 | +     | --  | MC45      | MGG_00380.6 | --    | +   |
| HM4       | MGG_04737.6 | +     | +   | MC47      | MGG_00269.6 | --    | +   |
| HM5       | MGG_09188.6 | +     | --  | MC48      | MGG_05989.6 | --    | +   |
| HM6       | MGG_05798.6 | --    | +   | MC52      | MGG_10171.6 | --    | +   |
| HM17      | MGG_09920.6 | +     | --  | MC55      | MGG_05366.6 | +     | --  |
| HM18      | MGG_05785.6 | +     | +   | MC56      | MGG_05608.6 | +     | --  |
| HM20      | MGG_03356.6 | --    | +   | MC57      | MGG_10877.5 | +     | --  |
| HM21      | MGG_00505.6 | +     | +   | MC58      | MGG_08275.6 | +     | +   |
| HM22      | MGG_07763.6 | --    | +   | MC59      | MGG_09246.6 | +     | --  |
| HM24      | MGG_02245.6 | --    | +   | MC61      | MGG_09716.6 | +     | --  |
| HM27      | MGG_10102.6 | --    | +   | MC62      | MGG_02296.6 | +     | --  |
| HM30      | MGG_05381.6 | +     | --  | MC63      | MGG_06069.6 | +     | +   |
| HM36      | MGG_09460.6 | +     | --  | MC65      | MGG_05912.6 | +     | --  |
| HM57      | MGG_00703.6 | +     | +   | MC69      | MGG_02848.6 | +     | --  |
| HM63      | MGG_00659.6 | +     | --  | MC70      | MGG_03347.6 | +     | --  |
| HM65      | MGG_03245.6 | +     | +   | MC71      | MGG_12906.6 | +     | --  |
| HM66      | MGG_02420.6 | --    | +   | MC72      | MGG_13009.6 | +     | --  |
| HM68      | MGG_01532.6 | +     | +   | MC73      | MGG_13275.6 | +     | --  |
| HM88      | MGG_06951.6 | +     | +   | MC79      | MGG_08041.6 | +     | +   |
| HM91      | MGG_00314.6 | +     | --  | MC81      | MGG_09875.6 | --    | +   |
| HM93      | MGG_01843.6 | +     | +   | MC82      | MGG_07560.6 | +     | +   |
| HM104     | MGG_02987.6 | +     | +   | MC83      | MGG_00052.6 | +     | +   |
| HM106     | MGG_03130.6 | +     | +   | MoCel12A  | MGG_00677.6 | +     | --  |
| HM108     | MGG_07312.6 | +     | --  | KY5       | MGG_10799.6 | +     | +   |
| Eco2      | MGG_00269.6 | --    | +   | KY8       | MGG_03844.6 | --    | +   |
| HMM14     | MGG_01872.6 | +     | +   | KY10      | MGG_03870.6 | --    | +   |
| HMM53     | MGG_06216.6 | +     | +   | KY22      | MGG_10291.6 | --    | +   |
| Taka1     | MGG_05232.6 | +     | --  | KY23      | MGG_10394.6 | --    | +   |
| Taka2     | MGG_00860.6 | +     | --  | KY45      | MGG_08298.6 | --    | +   |
| MC4       | MGG_06840.6 | --    | +   | KY51      | MGG_01387.6 | --    | +   |
| MC8       | MGG_07877.6 | +     | +   | KY55      | MGG_03338.6 | --    | +   |
| MC11      | MGG_03593.6 | +     | +   | AI9       | MGG_09742.6 | +     | +   |
| MC14      | MGG_09465.6 | +     | +   | AI41      | MGG_07704.6 | +     | +   |
| MC16      | MGG_00522.6 | +     | +   | AI43      | MGG_05092.6 | +     | +   |
| MC19      | MGG_05103.6 | +     | +   | AI44      | MGG_03316.6 | --    | +   |
| MC24      | MGG_04952.6 | --    | +   | AI58      | MGG_07645.6 | +     | --  |
| MC25      | MGG_03276.6 | --    | +   | AI59      | MGG_01064.6 | +     | +   |

*aExpressed gene in the cAMP-treated M. oryzae on dialysis membrane at 6 hpi in the Table S1.

bExpressed gene in the M. oryzae-infected rice leaf sheath at 40 hpi in the Supplemental data set 1 [20].

(+) or (-) indicate the expressed gene or not, respectively in the cAMP-treated or invasive hyphae of M. oryzae.

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Secreted Pathogenicity Protein of Fungal Pathogens

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To test whether the observed phenotypes of the mc69 mutants were solely caused by disruption of MC69, an intact copy of MC69 was introduced into the mc69 mutant mc69-87 (Figure S1B) for complementation. The MC69-reintegrated strain showed normal appressorial penetration rate and the strain developed blast disease symptoms on barley and rice leaves with a similar extent to the wild type (Figure 1E and F). These results demonstrate that disruption of MC69 gene caused defects in appressorial penetration and development of blast symptoms by M. oryzae.

MC69 is expressed in conidia and all stages of infection, secreted but not translocated into rice cells

MC69 was found in the SuperSAGE list of the cAMP-treated conidia (Table S1). MC69-EST was found in MGOS databases for mycelium, conidia, germinated conidia and appressoria [32]. RL-SAGE tags of MC69 were also found in the fungus grown on a minimum medium for three days [33]. These data suggest that MC69 is constitutively expressed in M. oryzae.

To investigate the expression pattern of MC69 in detail, we produced an M. oryzae strain Ina72 harboring a vector containing the MC69 promoter fused with a reporter protein gene mCherry (MC69p::mCherry; Figure 2A) generating WT+mCherry. The mCherry fluorescence was observed in all morphological stages with enhanced fluorescence in conidia before germination (0 h) and matured appressoria (12 h after incubation) on glass coverslips under confocal laser-scanning microscope (Figure 2B). To determine the mode of expression and spatial localization of the
Figure 2. Microscopic analysis suggests that MC69 promoter is constitutively active and MC69::mCherry fusion protein is secreted.

(A) and (D) Schematic diagrams of mCherry and MC69::mCherry fusion protein expression constructs. (B) and (E) Conidia from WT+mCherry and mc69+MC69::mCherry were harvested, and appressorium development was observed for 12 h on glass coverslips. Merged DIC and mCherry (red) images were taken. Scale bars = 10 μm. (C) and (F) Merged DIC and mCherry images of the rice leaf sheath cells infected with mCherry- and MC69::mCherry-expressing transformants 24 h and 48 h after inoculation. Scale bars = 20 μm. (G) Blast symptoms caused by Ina72 WT, mc69 and mc69+MC69::Cherry on rice (cv. Shin No. 2) 7 days after inoculation.

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MC69 protein, a construct \textit{MC69p::MC69::mCherry} was prepared (Figure 2D) and used for transformation of the \textit{mc69} mutant generating \textit{mc69+MC69::mCherry}, and the mCherry fluorescence was then observed on glass coverslips (Figure 2E). The transgenic \textit{M. oryzae} mutant \textit{mc69} expressing \textit{MC69::mCherry} restored pathogenicity (Figure 2G), showing that the fusion protein \textit{MC69::mCherry} is functional for infectivity. The mCherry fluorescence was detected in all the developmental stages like WT+\textit{mCherry}, but the intensity of fluorescence in the strain \textit{mc69+MC69::mCherry} was weaker than that of the strain WT+\textit{mCherry} presumably because of secretion and diffusion of \textit{MC69::mCherry} fusion protein (Figure 2B and E). To observe the fluorescence of WT+\textit{mCherry} and \textit{mc69+MC69::mCherry} in the infected tissues, we inoculated these conidial suspensions to rice leaf sheath. The mCherry fluorescence was detected in the invaded hyphae 24 and 48 hours after inoculation with WT+\textit{mCherry}, but not detected in that inoculation with \textit{mc69+MC69::mCherry} (Figure 2C and F). These results suggest that the \textit{MC69} gene is expressed throughout infection: conidia, infection-related morphogenesis and subsequent growth stage. The fluorescence from \textit{MC69::mCherry} fusion proteins was not detected in the invaded hyphae in planta presumably because they have been secreted.

To obtain direct evidence that the \textit{MC69} protein is actually produced in the invasive hyphae, we made \textit{mc69} mutant harboring a construct \textit{MC69p::MC69::HA (mc69+MC69::HA)} or a \textit{MC69p::MC69::3xFLAG (mc69+MC69::3xFLAG)} to perform immunodetection of \textit{MC69::HA} or \textit{MC69::3xFLAG} proteins in planta, respectively. Both of \textit{mc69+MC69::HA} and \textit{mc69+MC69::3xFLAG} restored appressorial penetration and invasive growth in rice leaf sheath (Figure S3A and C), showing that the fusion proteins \textit{MC69::HA} and \textit{MC69::3xFLAG} are functional for pathogenicity. To clarify whether the \textit{MC69::HA} and \textit{MC69::3xFLAG} are expressed or not, \textit{mc69+MC69::HA-} and \textit{mc69+MC69::3xFLAG-} infected rice leaf sheath extracts were analyzed by SDS-PAGE gel blot analysis. We extracted total protein at 24 and 48 hours after leaf sheath inoculation. Note that at 24 hours after inoculation, most conidia develop appressoria but hyphal invasion is still limited, whereas at 48 hours extensive hyphal growth develops. Both of \textit{HA-} and \textit{3xFLAG}-tagged \textit{MC69} were detected only faintly 24 hours after inoculation, but these proteins were abundant 48 hours after inoculation (Figure S3B and D) indicating that \textit{MC69} protein was indeed produced in invasive hyphae. Furthermore, we tried to express an \textit{AVR} effector gene, \textit{AVR-Pia [18,20]} from the \textit{MC69} promoter to see whether \textit{AVR-Pia} avirulence function is supported by \textit{MC69::mCherry} in rice cells, we added a modified small NLS from simian virus large T-antigen [35] at the C terminus of the \textit{MC69::mCherry} fusion downstream of the \textit{AVR-Pia} promoter (\textit{PW12p::MC69::mCherry::NLS}) and transformed \textit{M. oryzae} strain Sasa2 with the construct. A transformant \textit{M. oryzae} harboring \textit{PW12p::MC69::mCherry::NLS} was used as positive control. \textit{PW12::mCherry::NLS} exhibited significant fluorescence in BIC and in nuclei of invaded host cells at successful infection sites 24, 27 and 32 hours after inoculation (Figure 3A) whereas \textit{MC69::mCherry::NLS} did not show fluorescence in nuclei of the invaded rice cells, but showed weaker fluorescence in BIC than that of \textit{PW12::mCherry::NLS} (Figure 3B). To eliminate the possibility that the NLS influences BIC localization, a transformant strain harboring \textit{PW12p::MC69::mCherry} was inoculated to rice leaf sheath. The result showed that \textit{MC69::mCherry} was also detected in the BIC (Figure 3C). In addition, we observed \textit{mCherry} fluorescence with different pinhole settings to compare the signals in the BIC among the three strains 27 hours after inoculation. The result showed that BIC accumulation signals of \textit{MC69::mCherry::NLS} and \textit{MC69::mCherry} were significantly weaker than that of \textit{PW12::mCherry::NLS} (Figure S5). These results suggest that the \textit{MC69} does not translocate into the infected rice cells, but localizes in BIC, however the accumulation level of \textit{MC69} in BIC is significantly lower than that of \textit{PW12}.

**Two cysteine residues are essential for \textit{MC69} function**

\textit{MC69} homologs were found in other filamentous fungi \textit{Colletotrichum orbiculare} (AB669186), \textit{Glomerella graminicola} (EFQ29542), \textit{Verticillium albo-atrum} (EYE15898), \textit{V. dahlieae} (EY920493), \textit{Neurospora crassa} (NP_965292), \textit{N. tetrasperma} (EGO52621), \textit{Myceliotheca thermophila} (XP_006599994), \textit{Podospora anserina} (XP_00190740), \textit{Gossypium ciliare} (EFX05010), \textit{Fusarium oxysporum} (EGU75378), \textit{Gibberella zeae} (XP_3836669), \textit{Tischendorfia atrovirens} (EHK44387), \textit{T. virens} (EHK23962, Metarhizium acridum (EFY93067), \textit{M. anisopliae} (EFY97094) and \textit{Corpeps militaris} (EGX95034) (Figure S6 and S7). However, these amino acid sequences did not contain known domains/motifs that would allow the prediction of their function. Nevertheless, \textit{MC69} homologs contain two conserved cysteine residues in the mature protein region C-terminal to the signal peptide (Figure S6). A software DISULFIND [http://disulfind.dsi.unifi.it/; [36]] predicted that the two cysteine residues in mature \textit{MC69} can form a disulfide bond (Figure 4A). To test whether these cysteines are necessary for \textit{MC69} function, mutant alleles of \textit{MC69} were generated in which each or both of C36 and C46 were replaced with alanine (Figure 4B). Mutant alleles with one amino acid acid replacement (\textit{MC69(C36A); MC69(C46A)}) or two replacements (\textit{MC69(C36A,C46A)}) were expressed in the \textit{mc69} mutant (\textit{mc69+MC69(C36A); mc69+MC69(C46A) or mc69+MC69(C36A,C46A)}). In all cases, appressorial penetration rate and blast symptoms on barley and rice were slightly restored, but still significantly reduced as compared to the wild type (Figure 4C and D). In addition, we further analyzed invasive growth rating of the 50 appressorial penetration sites. Infectious growth of \textit{mc69+MC69(C36A); mc69+MC69(C46A)} and \textit{mc69+MC69(C36A,C46A)} within the inner epidermal tissue was slightly restored as compared to the \textit{mc69} mutant (Figure S8). These results indicate that C36 and C46,
presumably involved in disulfide bond, are necessary for MC69 to exert its pathogenicity in *M. oryzae*.

To see whether C36 and C46 are important for MC69 secretion/localization, spatial localization of the MC69(C36A) protein was tested by transforming *mc69* mutant with a construct MC69p::MC69(C36A)::mCherry, resulting in *mc69*+MC69(C36A)::mCherry (Figure 5A). We inoculated conidial suspension of the strain to rice leaf sheath to observe the mCherry fluorescence in the infected tissue. The mCherry fluorescence was detected in appressoria but not in the invaded hyphae 24 and 48 hours after inoculation (Figure 5B). The result suggests that the MC69(C36A)::mCherry protein was secreted into the plant and diffused below the detection limit like MC69::mCherry (Figure 2F and 5B). To clarify whether the MC69::mCherry and MC69(C36A)::mCherry are secreted or not, extracellular proteins secreted by *Magnaporthe* after liquid culture were analyzed by SDS-PAGE gel blot analysis. We used the wild-type strain expressing mCherry under MC69 promoter (WT+mCherry; Figure 2B and C) as negative control. Western blot analysis (Figure 5C) revealed the presence of mCherry-tagged MC69 and MC69(C36A)::mCherry were secreted or not, extracellular proteins secreted by *Magnaporthe* after liquid culture were analyzed by SDS-PAGE gel blot analysis. We used the wild-type strain expressing mCherry under MC69 promoter (WT+mCherry; Figure 2B and C) as negative control. Western blot analysis (Figure 5C) revealed the presence of mCherry-tagged MC69 and MC69(C36A) in the culture medium. Faint signals of cleaved mCherry were observed as well for the transformants mc69+MC69::mCherry and mc69+MC69(C36A)::mCherry. The molecular weight (MW) of the fusion proteins was around 30 kDa, in line with the predicted MW of mature MC69::mCherry. These data strongly suggest that both of MC69 and MC69(C36A) are secreted to the medium, and C36 is not important for MC69 secretion. It could be possible that mutation of the Cys residues may impact pathogenicity by reducing the stability of the protein after secretion in planta.

**MC69 is commonly required for pathogenicity in *M. oryzae***

To investigate whether pathogenicity function of MC69 is conserved in *M. oryzae*, we produced mc69 disruptants in other two Japanese field isolates of TH68-141 and Hoku1, in addition to the isolate Ina72. We used the MC69 knockout vector used for Ina72 to generate mc69 disruptants of both TH68-141 and Hoku1 isolates. Generated mc69 disruptants of the two isolates showed a reduced pathogenicity on barley leaves as compared to the wild type strains (Figure 6), indicating the importance of MC69 in virulence of TH68-141 and Hoku1.

The whole genome sequence of 70-15, a well-studied laboratory strain of *M. oryzae*, was published [23]. We found that 70-15 showed poor virulence as compared to the Japanese strains in the previous study. It caused intermediate responses in all of the 13 tested rice cultivars: infection caused reddish lesions of various sizes, but they did not further develop into typical susceptible brown spindle-shaped necrotic lesions [20]. To investigate whether MC69 is required for pathogenicity in 70-15, MC69 gene disruption analysis was performed in the 70-15 background (Figure S1). Two independent MC69-KO lines (mc69-119 and mc69-31) and wild-type 70-15 were sprayed onto barley cotyledons and rice leaves. The barley and rice infected by mc69 mutants showed much weaker symptoms as compared to the 70-15-infected plants (Figure 7A), indicating the importance of MC69 in virulence of 70-15. Appressorial penetration rates of the mutants in rice leaf sheath cells were significantly lower than that of 70-15 but the rates of germination and appressorium formation were same with the wild type (Figure 7B). In addition, we further analyzed invasive growth rating of 50 appressorial penetration

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**Figure 3. MC69::mCherry is not translocated into the rice cytoplasm.** Merged DIC and mCherry images of rice leaf sheath cells infected by *M. oryzae* Sasa2 strain harboring (A) PWL2::PWL2::mCherry::NLS, (B) PWL2::MC69::mCherry::NLS, and (C) PWL2::MC69::mCherry 24, 27 and 32 h after inoculation as observed by confocal laser scanning microscopy. Arrows indicate BICs and triangles indicate rice nuclei. Pinhole setting is 240 μm for all panels. Scale bar = 20 μm. doi:10.1371/journal.ppat.1002711.g003
Infectious growth of the mc69 mutants within the inner epidermal tissue was restricted as compared to the wild type (Figure S9). However, the colony growth and conidiation of the mutants on oatmeal agar media were similar to the wild type (Figure S2C and D). Thus, the mc69 mutants of 70-15 have a defect in appressorial penetration and development of blast symptoms, which is similar to the phenotype of the mc69 disruptants of Ina72. These results suggest that MC69 is commonly required for appressorial penetration and subsequent colonization in various M. oryzae strains.

An ortholog of MC69 is required for pathogenicity of Colletotrichum orbiculare

The importance of MC69 in M. oryzae raised a possibility that MC69 orthologs are also involved in pathogenicity of other fungal pathogens. To assess this point, we investigated whether MC69 ortholog is involved in pathogenicity of the cucumber anthracnose fungus C. orbiculare (Figure S6). A gene homologous to MC69 was isolated from C. orbiculare in this study. The isolated gene, designated CoMC69, comprises 220 bp interrupted by an intron and encodes a predicted protein of 54 amino acids (Figure 8A). Intron/exon organization in MC69 orthologs in filamentous fungi indicated that most of them have one intron only followed by an exon (140–156 bp) except for the genes in T. virens and Gibberella zeae (Figure S10). First exons in all genes encode a common region containing two conserved cysteine residues in the mature proteins (Figure S6).

To investigate whether CoMC69 is involved in the pathogenicity of C. orbiculare, we produced CoMC69 disruption mutants. The plasmid pCBGDMC69 was designed to replace the CoMC69 gene in the wild-type strain 104-T through double crossover homologous recombination (Figure S11A and B).

The colony morphology and conidiogenesis of Comc69 mutants grown on PDA medium were similar to that of 104-T (Figure S11C and data not shown). We next investigated their pathogenicity on host cucumber leaves. Conidial suspensions from the Comc69 mutants were spotted on detached cucumber leaves and incubated for 7 days. The Comc69 mutants exhibited clear reduction in lesion development in comparison with the wild-type strain 104-T (Figure 8B). C. orbiculare 104-T is able to infect Nicotiana benthamiana, which is not closely related to cucumber [37]. The Comc69 mutants also exhibited reduced pathogenicity on N. benthamiana (Figure 8C). These results indicate that CoMC69 is required for pathogenicity of C. orbiculare, suggesting conserved roles of the MC69 proteins in pathogenicity of both M. oryzae and C. orbiculare. To investigate the gene expression of CoMC69 in plant infection of C. orbiculare, we generated C. orbiculare strains carrying a reporter plasmid...
containing the 1.4 kb 5’ upstream region of CoMC69 fused with mCherry. As a result, we found the mCherry fluorescence in appressorium and primary intracellular hyphae of the transgenic C. orbiculare, indicating the expression of CoMC69 in the plant infection stage of C. orbiculare (Figure 8D).

Discussion

In this study, we show that MC69, a novel secreted protein of Magnaporthe oryzae, is essential for successful appressorium development and blast symptom development in rice and barley cultivars. The MC69 gene (MGG_02848.6) resides on chromosome VII of M. oryzae. The MC69 protein comprises 54 amino acids and is predicted to harbor a putative N-terminal secretion signal peptide (Figure 4B). MC69 seems to be a solitary gene without any paralogs in the genome. It lacks known sequence motifs associated with enzymatic function. Although MC69 homologs were found in other filamentous fungi [Figure S6], their functions are also not known. Expression of homologs were found in other filamentous fungi (Figure S6), their motifs associated with enzymatic function. Although without any paralogs in the genome. It lacks known sequence 

Figure 5. Secretion of MC69(C36A)::mCherry and MC69::mCherry fusion proteins. (A) Schematic diagram of MC69(36A)::mCherry fusion protein expression construct. (B) Merged DIC and mCherry images of rice leaf sheath cells infected by the MC69(C36A)::mCherry-expressing transformants 24 h and 48 h after inoculation. Scale bar = 20 μm. (C) Western blot probed with an anti-DsRed antibody. Samples were loaded as follows: lane 1, culture filtrate from mCherry-expressing strain; lane 2, culture filtrate from MC69::mCherry-expressing strain; lane 3, culture filtrate from MC69(C36A)::mCherry-expressing strain.

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Figure 6. MC69 is required for pathogenicity of other two different Japanese field isolates TH68-141 and Hoku1. Conidial suspension of wild-type strain TH68-141 (TH68-141 WT), the three independent mc69 mutants (mc69-49, mc69-55 and mc69-66), wild-type strain Hoku1 (Hoku1 WT) and the two independent mc69 mutants (mc69-75 and mc69-82) were inoculated on barley (cv. Nigrata) leaves and incubated for 5 days.

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Secreted Pathogenicity Protein of Fungal Pathogens

On the other hand, disruption of a total of 77 secreted protein genes in M. oryzae did not affect its pathogenicity within our experimental condition. Since there is no systematic bias in our selection of secreted protein genes for disruption, we extrapolate that 77/78 = 99% of secreted protein genes do not show clear reduction in pathogenicity even after knockout. Several secreted avirulence (AVR) effector genes have been isolated from M. oryzae, including PWL effectors [16,19], AVR-Pita [13,14], AVR1-CO39 [15], AVR-Pz-t [17], AVR-Pi, AVR-Pi and AVR P1/km/kp [18,20], but the virulence functions of the genes are still unknown. In fact, the AVR-Pita effector is dispensable for virulence on rice [14,39]. According to our results and available information on M. oryzae AVR effectors, we hypothesize two mutually non-exclusive
possibilities: (1) virulence contribution of most of effectors is too small to be detected by conventional assays; (2) effectors have redundant activities and more than one effector participate in the same virulence pathway.

A recent report of *M. oryzae* indicates that the fungus overcomes the first line of defense (PAMP-Triggered Immunity) by secreting an effector protein, Slp1 during invasion of new rice cells [21]. There are several reports for secreted effectors of other fungal pathogens. Pep1 of *U. maydis* was described above. Several hydrophobins or repellent genes that encode secreted proteins of *U. maydis* were examined for their roles in virulence. Single knock-outs of these genes did not affect virulence, but a double knockout of the repellent-encoding gene *Rsp1* and *Hum3* (a gene encoding a protein containing both, a hydrophobin domain and a repellent region) were arrested at an early stage of penetration. This indicates that Rsp1 and Hum3 are effectors with a partly redundant virulence function during the early stages of infection [40]. We speculate a similar situation may occur in *M. oryzae*. It would be a good way to focus on effector candidates exhibiting higher similarities and knockout or silence multiple genes simultaneously, to identify the multiple effectors that act redundantly.

We showed that *MC69* was required for pathogenicity of the additional three strains of *M. oryzae* in addition to the strain Ina72, indicating conserved roles of *MC69* in *M. oryzae*. To investigate whether the pathogenicity function of the *MC69* ortholog in the well-studied dicot fungal pathogen, we isolated an ortholog, *CaMC69* from the cucumber anthracnose fungus, *Colletotrichum orbiculare*. Notably, in *C. orbiculare* the deletion of *CaMC69* reduced pathogenicity on the hosts cucumber and *Nicotiana benthamiana* leaves (Figure 8).

Phylogenetic analyses were performed with *M. oryzae* *MC69* (*MoMC69*), with 16 homologs, from other phytopathogenic (*C. orbiculare*, *Gnomarella graminicola*, *Verticillium albo-atrum*, *V. dahiae*, *Grossmannia clavigera*, *Fusicoccum oxyacanthae* and *Gibberella zeae*), entomopathogenic (*Metarhizium acridum* and *M. anisopliae*), caterpillar killer (*Cordyceps militaris*), fungal parasite (*Trichoderma atroviride* and *T. virens*) or saprophytic (*Neurospora crassa*, *N. tetrasperma*, *Myceliophthora thermophila* and *Podospora anserina*) fungi (Figure S7). *MoMC69* and *CoMC69* are closely related to the *Verticillium* wilt pathogen *V. albo-atrum*, *V. dahiae* and the cereal plants anthracnose fungus *Gnomarella graminicola*. A conserved motif containing the two cysteine residues showed high homology among all *MC69* homologs (Figure S6). Thus, it will be interesting to determine whether genes orthologous to *Mcm69* also contribute to the pathogenicity of various plant, fungus, entomo or caterpillar pathogenic fungi. However, *MC69* orthology also occur in saprophytes suggesting that a possibility that the primary function of the protein is in relation to the structure or function of the fungus itself, and that the function must be intact for the fungus to succeed as a pathogen.

We generated transgenic rice overexpressing *MC69* to examine susceptibility to *M. oryzae* wild-type strain or the *mc69* mutant infection. However, overexpression of *MC69* in rice neither enhanced the pathogenicity of *M. oryzae* wild-type strain nor complemented the pathogenicity deficiency of the *mc69* mutant (data not shown). We hypothesize three possibilities why overexpression of *MC69* did not affect *M. oryzae* wild-type strain and the *mc69* mutant infection. One possibility is that the localization of *MC69* in the infection sites of *M. oryzae* in rice cells is important. We produced *M. oryzae* transformant harboring *PWL2::MC69:mCherry*. After inoculation of the strain to the rice leaf sheath, mCherry fluorescence was detected in biformic intercellular complex (BIC) (Figure 5C). However, *MC69:mCherry* fusion protein expressed by *MC69* promoter was not detected in BIC (Figure 2F). It might be because *MC69* promoter activity was weaker than *PWL2* promoter activity or *PWL2* promoter leads *MC69:mCherry* to BIC accumulation but *MC69* promoter did not. To test these possibilities, *mc69* mutant expressing *MC69*:EGFP fusion protein downstream of *MC69* promoter has been produced because EGFP fluorescence was relatively stronger than mCherry fluorescence. When the transformant was inoculated to the rice leaf sheath, *MC69*:EGFP was shown to be accumulated to the BIC (data not shown). These results indicate that BIC localization of *MC69* is important for virulence of *M. oryzae*. Therefore, ectopic overexpression of *MC69* in rice neither enhanced pathogenicity of wild-type strain nor complemented deficiency of *mc69* mutant of *M. oryzae* in trans because of the *MC69* protein would not be localized in BIC. The second possibility is that post translational modification of *MC69* protein in *M. oryzae* might be different from that in *plants* even though the secreted *MC69*:mCherry protein shows an expected molecular size (Figure 5C). The third possibility is that *MC69* affects the physiology of the fungus but does not directly affect the physiology of the plant so that expression of *MC69* in rice did not complement the defect in *mc69* mutant of *M. oryzae*. 

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**Figure 7.** *MC69* is necessary for appressorial penetration and pathogenicity of a laboratory strain 70-15. (A) *MC69* is required for pathogenicity of *M. oryzae* strain 70-15. Conidial suspension of the wild-type strain 70-15 (70-15 WT) and the *mc69* mutants (mc69-119, -31) were inoculated on barley (cv. Nigrate) and rice (cv. Shin No. 2) leaves, and incubated for 7 days. (B) Germination, appressorium formation and appressorial penetration of 70-15 WT and the *mc69* mutants. The ratio of germination was calculated as the mean percentage of conidia germinated after 32 h on rice (cv. Shin No. 2) leaf sheath cells. The mean percentage of appressorium formation on rice leaf sheath cells among the germinated conidia is presented. Three replicates of ~50 conidia were counted for each observation. The mean percentage of appressorial penetration by the *mc69* mutants is presented 32 h after inoculation. Standard errors are indicated by the vertical bars. doi:10.1371/journal.ppat.1002711.g007
Khang et al. (2010) demonstrated that BIC-localized secreted proteins PWL2 and BAS1 were translocated into the rice cytoplasm but a secreted protein BAS4, which uniformly outlines the invasive hyphae, was not [11]. Interestingly, when M. oryzae transformants secreted fluorescent MC69 fusion protein during epidermal cell invasion, the fluorescent protein was observed in BICs (Figure 3). To investigate whether this feature is specific to MC69 or not, we generated M. oryzae strains expressing fluorescence-labeled version of four putative secreted proteins (HMM14, MC55, GAS1 or GAS2) with NLS. HMM14 and MC55 are the secreted protein genes studied here (Table 1) and GAS1 and GAS2 are secreted protein genes involved in virulence of M. oryzae [28]. The generated strains were inoculated to rice leaf sheaths, and localization of each protein was investigated. The result showed that all four proteins were localized to the BICs, and both GAS1 and GAS2 were then translocated into the rice cytoplasm, which is similar to PWL2. By contrast, HMM14 and MC55 were not translocated into the rice cytoplasm like MC69 (data not shown). However, fluorescent signals of BIC accumulations of MC69::mCherry::NLS, MC69::mCherry, HMM14::mCherry::NLS and MC55::mCherry::NLS were significantly weaker than that of PWL2::mCherry::NLS, GAS1::mCherry::NLS and GAS2::mCherry::NLS (Figure 3, S5 and data not shown). These findings indicate that BIC accumulation level of secreted proteins might be important for translocation to the infected rice cells.

A virulence effector Slp1 sequesters chitin oligosaccharides to prevent PAMP-triggered immunity in rice, thereby facilitating rapid spread of the fungus within host tissue [21]. Slp1 contains two putative LysM domains, which have previously been shown to bind carbohydrates [41]. An effector known as Ecp6 that also contains LysM domains was identified from a fungal pathogen Cladosporium fulvum that causes leaf mold of tomato [42]. Another effector AVR4 of C. fulvum binds to chitin present in fungal cell walls and that, through this binding AVR4 can protect these cell walls against hydrolysis by plant chitinases [43]. Growth of the Δapp1 mutants of U. maydis are arrested during penetration of the epidermal cell and elicit a strong plant defense response such as formation of large papillae, induction of strong cell wall autofluorescence, H2O2 accumulation and defense related genes.

Figure 8. CoMC69 is involved in fungal pathogenicity of C. orbiculare. (A) Sequence alignment of MC69 between M. oryzae (Mo) and C. orbiculare (Co). Amino acid sequences were aligned using Clustal W program [52]. Identical amino acids are indicated as white letters on a black background. Similar residues are shown by gray background. Gaps introduced for alignment are indicated by hyphens. (B) Pathogenicity test of the Comc69 mutants on cucumber. Conidial suspensions were inoculated on detached cotyledons of cucumber (Cucumis sativa). On the left half of the cotyledons, the wild-type strain 104-T was inoculated as positive control. On the right half, the Comc69 strains (DMC1 and DMC2) were inoculated. Inoculated cotyledons were incubated for 7 days. (C) Pathogenicity test of the Comc69 mutants on N. benthamiana. On the left half of the detached leaves of N. benthamiana, the strain 104-T was inoculated as positive control. On the right half, the Comc69 strains (DMC1 and DMC2) were inoculated. Inoculated leaves were incubated for 7 days. (D) mCherry-based reporter assay for expression of the CoMC69 gene. Conidia from the C. orbiculare strain carrying the CoMC69 promoter-mCherry fusion gene (CoMC69p::mCherry) was inoculated onto the lower surfaces of cucumber cotyledons, and the inoculated plant was incubated for 4 days. a, appressorium; ih, intracellular hypha. Scale bars = 10 μm. doi:10.1371/journal.ppat.1002711.g008
expression [38]. We tried to elucidate the roles of MC69 by addressing differences in H$_2$O$_2$ accumulation and expression of defense related genes in m69 mutant and wild type M. oryzae. However, results showed no difference between m69 and wild type so that we have no evidence that MC69 suppresses plant defense responses at the moment (data not shown). Taken together, we demonstrated that MC69 has a pathogenicity function (required for the fungus to be a pathogen), but its function has yet to be elucidated.

Materials and Methods

Fungal strains, medium and transformation

All isolates of M. oryzae used in this study are stored at the Iwate Biotechnology Research Center. Fungal strains used were the wild-type strains 70-15, Ina72, TH68-141, Hokal1, Sasa2 and Ina86-137 [20]. To obtain protoplasts, hyphae of M. oryzae strains were incubated for 3 days in 200 mL of YM medium (0.5% yeast extract and 2% of glucose, w/v). Protoplast preparation and transformation were performed as described previously [44]. Hygromycin- or bialaphos-resistant transformants were selected on plates with 300 μg mL$^{-1}$ of hygromycin B (Wako Pure Chemicals, Osaka, Japan) or 250 μg mL$^{-1}$ of bialaphos (Wako Pure Chemicals). C. orbiculare (Berk. & Mont.) Arx (syn. C. lagenarium [Pass.] Ellis & Halst.) strain 104-T (MAFF240422) was used as the wild-type strain. All C. orbiculare strains were maintained on 5.9% (w/v) PDA (Difco Laboratories, Detroit, MI) at 24°C. Preparation of protoplasts and transformation of C. orbiculare were performed according to a method described previously [45].

SuperSAGE of cAMP-treated M. oryzae strain 70-15

Mycelia of M. oryzae were grown on oatmeal agar medium (30 g 1$^{-1}$ oatmeal, 5 g 1$^{-1}$ sucrose and 16 g 1$^{-1}$ agar). To enhance conidia formation, the fungus was first grown on oatmeal agar medium for 9 days at 25°C, and then exposed to Black Light Blue light (Toshiba FS20S/BL2 20W; Toshiba, Tokyo, Japan) for 4 days at 22°C, after aerial hyphae of the colonies had been washed away with sterilized distilled water. Conidia of M. oryzae were suspended in 50 mM cAMP to a final density of 1×10$^8$ conidia mL$^{-1}$. This suspension was then poured onto dialysis membranes (2.5 ml of suspension/25 cm$^2$ membrane surface; Spectra/Por, cutoff 1,000 Da; Spectrum Medical Industries, Terminal Annez, CA) and incubated at 25°C in dark [25]. Total RNA was extracted from germinating conidia incubated for 6 h on dialysis membranes, as described below. 32 sheets of membranes containing the germinating conidia were crushed and homogenized in liquid nitrogen with mortar and pestle. The homogenate was transferred to a centrifuge tube containing 40 ml of TRI Reagent (SIGMA-ALDRICH, St. Louis, MO), homogenized by vigorous shaking and incubated at room temperature for 5 min. Then 8 ml of chloroform was added, homogenized by vigorous shaking for 15 sec and incubated at room temperature for 3 min. After centrifugation at 1000 x g for 15 min at 4°C, the upper aqueous phase was transferred to a new centrifuge tube, and the total RNA was precipitated by the addition of 20 ml of isopropanol after incubation at room temperature for 10 min. The pellet was rinsed with 70% ethanol. SuperSAGE library was made from total RNA as described [46,47]. Di-tag fragments were sequenced by the 454 FLX sequencer (454 Life Sciences). Each 26-bp tag sequence was used for BLASTN search against M. oryzae 70-15 genome sequence. A total of 23,491 tags to comprising 26-bp sequence were recovered. Number of tags for each of putative secreted protein genes of M. oryzae is given in Table S1.

Plasmid construction

To construct the gene-disruption vector pGPSMC69-44, a 8.7-kb fragment containing the MC69 gene amplified with the primers MC69S1 (5’- ATATGACGGGAGACGCACTACAC-3’) and MC69AS1 (5’- CGTCACGGTCTTTTCTTTTTGTTGTCGGC-3’) was cloned into pCR-XL-TOPO to generate pXLMC69 using the TOPO XL, PCR Cloning Kit (Invitrogen, Carlsbad, CA). MC69 was mutated using an adaptation of the TAG-KO method using pGPS-HYG-CAM [30,31]. The pXLMC69 containing MC69 was used as the target. An insertion was formed within the coding region of MC69 (at 11 amino acids) in pXLMC69, which resulted in pGPSMC69-44 (Figure S1).

For complementation assay of an m69 mutant with MC69, a 5.7-kb fragment containing MC69 was amplified with the primers NMU1 (5’T-ATAAGAATCGGCGCCGTGATTCTCATGCTCC-3’) and XMG5L1 (5’T-GCCAGGCTTCTGGTGGG-3’). A 1.7-kb fragment containing a half 3’-terminal part of the MC69 ORF and terminator was amplified with the primers NMU3A1 and M13F (5’T-GCCAGGCTTCTGGTGGG-3’). The PCR product was digested with NotI and XbaI (exists in the middle of the PCR product after 1.1-kb far from the poly A signal recognition site of MC69) to generate 3.1-kb fragment containing MC69, and ligated to the same restriction sites of which carries the bialaphos-resistant (bar) gene [48], creating pCB1531-MC69.

To substitute a cysteine residue at 36 amino acids in MC69 by alanine, single point mutation was introduced in plasmid pCB1531-MC69 using a primer BMC36AUA4 (5’T-CAGGTCAAGAACGGCGGTCGTGAGC-3’) which was underlined in italics). A 1.7-kb fragment containing a half 3’-terminal part of the MC69 ORF and terminator was amplified with the primers PMC6AUA4 and M13F (5’T-GCCAGGCTTCTGGTGGG-3’). The PCR product was digested with NotI and XbaI, and exchanged to the BstEI/XbaI fragment of pCB1531-MC69, creating pCB1531-MC69 (C36A) (Figure 4B). To substitute a cysteine residue at 46 amino acids in MC69 by alanine, single point mutation was introduced in plasmid pCB1531-MC69 using a primer BMC46AUA5 (5’T-CAGGTCAAGAACGGCGGTCGTGAGC-3’) which was underlined in italics). A 1.7-kb fragment containing a half 3’-terminal part of the MC69 ORF and terminator was amplified with the primers PMC46AUA5 and M13F, and the PCR product was used as a template for another PCR with the primers BMCMC69 using a primer BMC36AUA4 (5’T-CAGGTCAAGAACGGCGGTCGTGAGC-3’) which was underlined in italics) and M13F (5’T-GCCAGGCTTCTGGTGGG-3’). The PCR product was digested with BstEI and XbaI, and exchanged to the BstEI/XbaI fragment of pCB1531-MC69, creating pCB1531-MC69 (C36A) (Figure 4B). To substitute a cysteine residue at 36 and 46 amino acids in MC69 by alanine, double point mutations were introduced in plasmid pCB1531-MC69 using a primer BMC36&46AUA4 (5’T-CAGGTCAAGAACGGCGGTCGTGAGC-3’) which was underlined in italics) and M13F. The PCR product was digested with BstEI and XbaI, and exchanged to the BstEI/XbaI fragment of pCB1531-MC69, creating pCB1531-MC69 (C36A,C46A) (Figure 4B). For construction of the MC69-EGFP gene fusion vector pCB1531-MC69-EGFP, a 1.7-kb fragment containing MC69 gene was amplified with the primers NMU1 and XMG5L1 (5’T-GCTCTAGACACACCGCACTACAC-3’) and XMG5L1 (5’T-GCCAGGCTTCTGGTGGG-3’). The PCR product encoding MC69-Gly$\_5$ was digested with NotI and XbaI, and exchanged to the
NotI/XbaI fragment of the Tef promoter in pBAGFP [49], generating pCB1531-MC69-EGFP. For construction of the MC69-m-Cherry gene fusion vector pCB1531-MC69-mCherry, a 0.7-kb mCherry cDNA fragment was amplified with the primers XmuU1 (5′-GGCTCTAGACATGGTACGCGAGGCCAGG-3′; XbaI site is underlined) and Bmn1L (5′-CGGATCCTCTAGGTACACGCTGCTC-3′; BamHI site is underlined) using pmCherry (Clontech, Mountain View, CA) as a template. The PCR product was digested with XbaI and BamHI, and exchanged to the XbaI/BamHI fragment of EGFP cDNA in pCB1531-MC69-EGFP, generating pCB1531-MC69-mCherry (Figure 2D), a 1.6-kb fragment of MC69 promoter (MC69p) and MC69-C364 ORF was amplified with the primers NMU1 and XM5G1L. The PCR product was digested with NotI and XbaI, and exchanged to the NotI/XbaI fragment of MC69p-MC69 in pCB1531-MC69-mCherry, generating pCB1531-MC69-mCherry (Figure 5A). A 1.4-kb fragment of MC69p was amplified with the primers NMU1 and XM5pL2 (5′-GGCTGACCTCTGCTAGGTACACGCTGCTC-3′; XbaI site is underlined). The PCR product was digested with NotI and XbaI, and exchanged to the NotI/XbaI fragment of MC69p-MC69 in pCB1531-MC69-mCherry, generating pCB1531-MC69-mCherry-stop (Figure 2A).

A 0.6-kb fragment of PWL2 promoter was amplified by the primers Pwp2L-2′ (5′-GAGGAGAAGGGCCCCTGTTACAAAGCGCGGTTGAAAGATTCCGTCG-3′; NotI site is underlined) and Pwp2L-2′ (5′-GAGGAGAAGGGCCCCTGTTACAAAGCGCGGTTGAAAGATTCCGTCG-3′; XbaI site is underlined). The PCR product was digested with NotI and BamHI, and exchanged to the NotI/BamHI fragment of Tefp-EGFP in pBAGFP [49], generating pCB-Ppwl2-mCherry. A modified SV40 NLS-epitope (5′-GAGAAAGGTAGATTATGGAGCTTAAG-3′) coding double stranded fragment [35] was produced annealing the oligos mSV40NLS (5′-GAGAAAGGTAGATTATGGAGCTTAAG-3′; BamHI site is underlined) and c-mSV40NLS (5′-GAGAAAGGTAGATTATGGAGCTTAAG-3′; XbaI site is underlined) as a spacer peptide between MC69 and HA tag. The PCR product was digested with XbaI and BamHI, and exchanged mCherry gene with the same sites of pCB1531-MC69-mCherry, generating pCB1531-MC69-HA (Figure S3).

To make the MC69-HA gene fusion construct pUC57-MC69-3xFLAG was custom-synthesized (GenScript, Piscataway, NJ). MC69-3xFLAG was amplified from pUC57-MC69-3xFLAG with the primers SMU2 (5′-GACTAGTTAAAAATGGAAGGCCGTCTTTCGTTCTCGCCG-3′; SpeI site is underlined) and BFL1 (5′-CGGATCCTCCAGCATGTCCTTTGAATTC-3′; BamHI site is underlined). The PCR product was digested with SpeI and BamHI, and exchanged mCherry gene at XbaI and BamHI sites of pCB1531-MC69-mCherry, generating pCB1531-MC69p-3xFLAG (Figure S3).

For construction of the MC69p::AVR-Pia expression vector pCB1531-MC69p-AVR-Pia, a 0.3-kb fragment containing AVR-Pia gene was amplified from pCB1004-pex22 [20] with the primers XP22U2 (5′-GCTCAGACCAATCGGTATAATTTTCGCAATTTTTC-3′; XbaI site is underlined) and BP22L2 (5′-CGGAGAAGGTAGATTATGGAGCTTAAG-3′; EcoRI site is underlined) as a template. The amplified fragment was digested with NotI and HindIII, and introduced into pCB1531-MC69p::AVR-Pia to produce pCB5MC69. The 3·kb fragment that contained the 5′-flanking region of CoMC69 was amplified with the PCR primers COMC5S (5′-ATAATCCCAGGCCTTCTCTCAGATGCAGGTGAGGGATGAGACCC-3′; BamHI site is underlined) and AP22L2 (5′-CGGAGAAGGTAGATTATGGAGCTTAAG-3′; EcoRI site is underlined) as a template. The amplified product was digested with XbaI and BamHI, and introduced into pCB-Pwp2l-mCherry-NLS, generating pCB-Pwp2l-PW2-mCherry-NLS (Figure 3A). A 0.2-kb MC69 cDNA fragment was amplified from the total cDNA with the primers XMU2 (5′-GCTCTAGAATTAAGAATGATGAGCAGGGCGCTC-3′; XbaI site is underlined) and XM1L (5′-CGGATCCTCTAGGTACACGCTGCTC-3′; BamHI site is underlined). The PCR product was digested with XbaI and Xhol, and introduced into pCB-Pwp2l-mCherry-NLS and pCB-Pwp2l-MC69-mCherry, respectively (Figure 2B and C).

To make the MC69-HA gene fusion vector pCB1531-MC69-HA, HA-tagged full cDNA of MC69 (MC69HA) was amplified from the total cDNA with the primers XM2U2 and BMHAL1 (5′-CGGATCCTCTAGGTACACGCTGCTC-3′; BamHI site is underlined) and HA tag sequence are indicated in lower cases and italics, respectively) which was designed with two glycine codons (underlined) as a spacer peptide between MC69 and HA tag. The PCR product was digested with XbaI and BamHI, and exchanged mCherry gene at the same sites of pCB1531-MC69-mCherry, generating pCB1531-MC69-HA-mCherry (Figure S3). MC69-3xFLAG gene fusion construct pUC57-MC69-3xFLAG was custom-synthesized (GenScript, Piscataway, NJ). MC69-3xFLAG was amplified from pUC57-MC69-3xFLAG with the primers SMU2 (5′-GACTAGTTAAAAATGGAAGGCCGTCTTTCGTTCTCGCCG-3′; SpeI site is underlined) and BFL1 (5′-CGGATCCTCCAGCATGTCCTTTGAATTC-3′; BamHI site is underlined). The PCR product was digested with SpeI and BamHI, and exchanged mCherry gene at XbaI and BamHI sites of pCB1531-MC69p-AVR-Pia, generating pCB1531-MC69p-3xFLAG (Figure S4).

CoMC69 was isolated from genome of C. orthobactica 104-T by PCR using degenerate primers designed in amino acid sequence of MC69 homologs in fungal pathogens including C. graminicola. To construct the gene replacement vector pGDCOMC69, the 3·kb fragment containing the 5′-flanking region of CoMC69 was amplified with the PCR primers COMC5S (5′-ATAATCCCAGGCCTTCTCTCAGATGCAGGTGAGGGATGAGACCC-3′; NotI site is underlined) and COMC3AS (5′-CCCAAGCTTTGCTTGGTGGAGAATGCG-3′; HindIII site is underlined) as a template. The amplified product was digested with NotI and HindIII, and introduced into pCB1636 [48], which contained the kph gene, to produce plasmid pCB5MC69. The 3·kb fragment that contained the 3′-flanking region of CoMC69 was amplified by PCR with the primers COMC3S (5′-GGGATCCTAGTCGAAGCTTCTTCTTTTGCGTTCTGAAAGG-3′; KpnI site is underlined). The amplified fragment was digested with ApaI and KpnI, and introduced into pCB5MC69 to produce pGDCOMC69 (Figure S11A). To generate the reporter construct pBATCoMC69pro-mCherry, the 1·kb 5′ upstream region of CoMC69 and mCherry were amplified using PCR with the two primer sets, (i) CoMC69pro-NotI-f (5′-ATAATAGTACGGCCGGTCTTCTTGTTCGTTCTC-3′; NotI site is underlined) and CoMC69pro-BamHI-r (5′-CGGATCCCTCTAGGTACACGCTGCTC-3′; BamHI site is underlined), and (ii) mCherry-BamHI-f (5′-CGGATCCCTCTAGGTACACGCTGCTC-3′; BamHI site is underlined) and mCherry-EcoRI-r (5′-CGGATCCCTCTAGGTACACGCTGCTC-3′; EcoRI site is underlined), respectively.
proteins were transferred on to Immobilon Transfer Membranes a 10–20% precast e-PAGEL (ATTO, Tokyo, Japan) and the scanning microscope (Olympus) with a Nikon 60 performed using an Olympus FluoView FV500 confocal laser-
cucumber cotyledons. Detection of mCherry fluorescence was the reporter strain were inoculated on the lower surfaces of 
C. orbiculare and/or with more than 2 branches within one cell; Level 4, branch; Level 2, invasive hyphae length is 10–20 branches; Level 3, invasive hyphae length is longer than 20 branches; Level 1, invasive hyphae are spread more than one cell (Figure 1D). To test fungal pathogenicity of C. orbiculare, conidial suspensions of tested C. orbiculare strains (approximately 5×10^5 conidia/ml) were spotted onto detached leaves of cucumber or N. benthamiana.

Confocal laser-scanning microscopy

Germinated conidia and appressoria were observed on glass coverslips, and invaded hyphae were observed in epidermal cells of rice leaf sheath. mCherry fluorescence was observed using an Olympus FV1000-D confocal laser-scanning microscope (Olympus, Tokyo, Japan) equipped with a Multi argon laser, an Olympus FluoView FV1000-D confocal laser-scanning microscope (Olympus, Tokyo, Japan) equipped with a Multi argon laser, and a Nikon 60× UPlanFLN (0.9 numerical aperture) oil-immersion objective (Nikon, Tokyo, Japan). Samples were mounted in water under cover slips and numerical aperture) oil-immersion objective (Nikon, Tokyo, Japan). Samples were mounted in water under cover slips and excited with the He/Ne laser. We used diachronic mirror DM440/543/633, SDM630 beam splitter, and emission filter BA560-600.

Preparation of M. oryzae-infected rice leaf sheath extract and Western blot analysis

Conidial suspension (1×10^6 conidia ml^-1) was injected into rice (cv. Shim No. 2) leaf sheath and placed in a dew chamber at 25°C for 32 h in the dark. The infected leaf sheaths were ground in liquid nitrogen, thawed in X ml of extraction buffer (250 mM Tris-HCl pH 7.5, 2.5 mM EDTA, 0.1% ascorbic acid (v/v), 1 mM PMSF, 0.01% PI cocktail (v/v) (SIGMA-ALDRICH), 0.1% Triton X-100 (v/v) for X mg sample, vortex for 10 min at 4°C, and centrifuged at 15,000 g for 20 min at 4°C in a micro-centrifuge. The crude extracts (15 μl per lane) were separated on a 10–20% precast e-PAGEL (ATTO, Tokyo, Japan) and the proteins were transferred to Immobilon Transfer Membranes (Millipore, Billerica, MA). The blots were blocked in 2% ECL Advance Blocking Agent (GE Healthcare, Buckinghamshire, UK) in TTBS (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20 (v/v)) for 1 h at room temperature with gentle agitation. For immunodetection, blots were probed with anti-HA (3F10)-HRP (Roche, Mannheim, Germany) or anti-FLAG M2-HRP (SIGMA-ALDRICH) in a 1:10,000 dilution in TTBS for 2 h. After washing the membrane for 10 min three times, the reactions were detected using an ECL Advance Western blotting detection reagents (GE Healthcare) and a Luminescent Image Analyzer LAS-4000 (Fujiﬁlm, Tokyo, Japan).
replicate experiments. Means are expressed as numbers of conidia ×10^3 of conidial suspension/cm² of culture.

(TIF)

**Figure S3** MC69 protein is produced in the invasive hyphae. (A,C) In planta growth of MC69::HA- and MC69::3xFLAG-expressing transformants (mc69+MC69::HA and mc69+MC69::3xFLAG) at the post-invasion stage. Invasive mycelia inside the rice (cv. Shin No. 2) leaf sheath cells were photographed 48 h after incubation. Scale bar = 20 μm. (B,D) Western blots probed with an anti-HA and an anti-FLAG antibodies. Protein extracts of rice leaf sheaths 24 h and 48 h after inoculation with Ina72 wild type (WT), mc69+MC69::HA and mc69+MC69::3xFLAG were loaded.

(TIF)

**Figure S4** AVR-Pia avirulence function is retained under the MC69 promoter. (A) The isolate Ina86-137 does not have AVR-Pia function and thus can cause disease on Sasanishiki harboring the R gene Pia. Ina86-137 strains transformed with AVR-Piap::AVR-Pia (+AVR-Piap::AVR-Pia) [20] or MC69::AVR-Pia (+MC69::AVR-Pia-1, -2) became incompatible with Sasanishiki. Both Ina86-137 wild type, Ina86-137 containing AVR-Piap::AVR-Pia, or MC69::AVR-Pia were able to cause disease on a rice cultivar Shin No. 2 lacking Pia, suggesting that the effect of transformation with AVR-Piap::AVR-Pia and MC69::AVR-Pia is Pia dependent. (B) Confirmation of active AVR-Pia transgene by RT-PCR in M. oryzae transformants during infection. RT-PCR analysis of Ina86-137 WT (lane 1), +AVR-Piap::AVR-Pia (lane 2), +MC69::AVR-Pia-1 and -2 (lane 3 and 4) with AVR-Pia- or Mg-Actin-specific primers [20].

(TIF)

**Figure S5** MC69::mCherry confers BIC localization with weaker fluorescence than that of PWL2::mCherry. Merged DIC and mCherry images of rice leaf sheath cells infected with M. oryzae Sasa2 strain harboring (A) PWL2::PWL2::mCherry::NLS (B) PWL2::PWL2::mCherry::NLS, and (C) PWL2::MC69::mCherry 27 h after inoculation as observed by confocal laser scanning microscopy. Arrows indicate BICs and triangles indicate rice nuclei. Pinhole settings are 80 μm for left panels and 240 μm for right panels. Scale bar = 20 μm.

(TIF)

**Figure S6** Predicted amino acid sequence alignment of MC69 with homologs from other filamentous fungi. Amino acid sequences of MC69 (Mo), MC69 homologs of Colletotrichum orbiculare (Co), Glomerella gaminicola (Gg), Verticillium albo-atrum (Va), V. dahliae (Vd), Neosporopsis crassa (Nc), N. tetrasperma (Nt), Myclobotrys thermophila (Ml), Podospora anserina (Pa), Grosmannia clavigera (Gc), Fusarium oxysporum (Fo), Gibberella zeae (Gz), Trichoderma atrovirens (Ta), T. virens (Tv), Metachizium acidum (Mac), M. anisopliae (Man), and Cordyceps militaris (Cm) were aligned using the Clustal W program [32]. Identical amino acids are indicated as white letters on a black background. Similar residues are shown on gray backgrounds. Gaps introduced for alignment are indicated by dashes. The predicted signal peptide and two conserved cysteine residues (C36 and C46) are indicated on top.

(TIF)

**Figure S7** Phylogenetic tree of M. oryzae MC69 protein sequence and 16 homologs from other fungi. Phylogenetic analyses were performed with M. oryzae MC69 (Mo), with 16 homologs are shown in Figure S6 legend.

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**Figure S8** Invasive growth rating of rice leaf sheath cells 32 h after inoculating with Ina72 WT, mc69, mc69+MC69, mc69+MC69(C36A), mc69+MC69(C46A), and mc69+MC69(C36A,C46A). For details of the invasive growth levels and rating see Materials and Methods.

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**Figure S9** Invasive growth rating of rice leaf sheath cells 32 h after inoculating with 70-15 WT and mc69-31. For details of the invasive growth levels and rating see Materials and Methods.

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**Figure S10** Intronic/exonic organization in M. oryzae MC69 gene and 16 orthologous genes from other fungi. Abbreviations of fungus names are shown in Figure S6 legend.

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**Figure S11** Gene disruption of CaMC69 in C. orbiculare. (A) CaMC69 locus and the gene disruption vector pGDCOMC69. By homologous recombination through double crossing over, the CaMC69 gene was replaced by a hygromycin resistance gene cassette (HYG). (B) Genomic PCR analysis of the comc69 mutants of C. orbiculare. Genomic DNAs were isolated from the wild-type strain 104-T and the comc69 strains (DMC1 and DMC2). The 0.3 kb product containing the entire CaMC69 gene was amplified from the genome DNA of 104-T with the two primers, indicated by arrows, COMC69F (5’-CGAAGAAGGACGCTATTTG-3’) and COMC69R (5’-CTCGAGGACTACAGCATG-3’). In contrast, the 1.6 kb product was amplified from the genome DNA of both comc69 strains, which is consistent with gene replacement shown in (A). Lane 1, λ Hind III marker; lane 2, 104-T; lane 3, DMC1; lane 4, DMC2. (C) Colony phenotype of the C. orbiculare mc69 mutants. The wild-type strain 104-T and comc69 mutants (DMC1 and DMC2) were grown on PDA for 12 days.

(TIF)

**Table S1** SuperSAGE result of cAMP-treated Magnaporthe oryzae strain 70-15.

(XLS)

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**Author Contributions**

Conceived and designed the experiments: H. Saitoh, Y. Takano, R. Terauchi. Performed the experiments: H. Saitoh, S. Fujisawa, C. Komakawa, C. Sayama, K. Yoshida, H. Matsumura, Y. Tosa, J. Win, S. Kamoun, R. Terauchi. Contributed reagents/materials/analysis tools: K. Yoshida, H. Matsumura, Y. Tosa, J. Win, S. Kamoun, R. Terauchi. Wrote the paper: H. Saitoh, Y. Takano, R. Terauchi.

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