Functional Analysis of *MoCMKK2* Gene in *Magnaporthe oryzae*

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Abstract Calicium is an important intracellular second messenger and the Ca^{2+}-signal transduction pathway is involved nearly every aspects of the cellular signal transduction network. In this study, the function of a calcium/calmodulin-dependent protein kinase kinase 2 (*MoCMKK2*) gene in the development of the rice blast fungus *Magnaporthe oryzae* was studied with a targeted gene disruption method. The sporulation of the null mutant was dramatically increased; however, the pathogenicity, mycelial growth rates, mycelial growth under starvation, conidial germination, appressorium formation and mating test of the *MoCMKK2* null mutant were comparable with those of the wild-type strain. These data suggest that the *MoCMKK2* gene is a negative factor to regulate sporulation. These findings will further our understanding of the disease infection cycle of *Magnaporthe oryzae*.

Keywords *Magnaporthe oryzae*; *MoCMKK2*; homologous combination knock-out strategy; sporulation

Magnaporthe oryzae (*M.oryzae*)(anamorph *Pyricularia grisea*), the casual agent of rice blast disease, is the most devastating phytopathogen of rice and causes severe loss of rice production all over the world. As a filamentous plant pathogen fungus, which has been fully sequenced, *M. oryzae* has become the model fungus for studying gene functions and pathogen-plant interactions [1]. *M. oryzae* undergoes a series processes to prepare for its invasion and forms a specialized infection structure called the appressorium, which is necessary for pathogenicity [2]. During the infection process, several signal pathways participate in external and internal signal transduction in response to physical and chemical stimulation.

One of the most important pathways is calcium-mediated signal transduction pathway. It regulates downstream reactions by modulating the cellular Ca^{2+} concentration ($[Ca^{2+}]_c$). Various physiological processes, such as differentiation, hormone signaling, cell cycle regulation, gene expression, apoptosis and stress responses are regulated by calcium-mediated signal transduction [3–6].

As for filamentous fungi, in which growth patterns and development are more complex, there is relative little information about the Ca^{2+}- signal mechanisms. In 2008, the research team of Nakayashiki studied a series of genes encoded Ca^{2+} signaling proteins by a new RNA-silencing method and the *MoCMKK2* gene (MGG_06421.6) which encoded the calcium/calmodulin-dependent protein kinase kinase 2 studied in this paper were also included [7]. The function of this gene was studied in this paper by targeted gene disruption method. Mutant analysis revealed that this gene was neither correlated with fungal development, stress nor pathogenicity. However, it does correlate with conidation, insofar as the mutation downregulated conidia yields which was remarkably different from the results of the research team of Nakayashiki. These results revealed that there were significant differences in the two kinds of gene function analysis methods.

1 Material and Methods

1.1 Fungal strains and culture condition

The wild-type strain Guy-11 of *M. oryzae* and

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mutant strains used in this study were cultured and maintained on complete medium (CM) [8]. For DNA isolation, the fungal strains were grown in 150 ml CM liquid medium at 28 ℃ for 4–5 days, with constant shaking (150 r/min). OCM (CM with 1 mol/L sucrose) was used for fungal transformation. The mating assay, which involved crossing with strain 2539, was performed on oat meal agar (OMA) medium (30 g oat in 1 L distilled water).

1.2 Construction of plasmids and transformation of the fungus

The 1.9 kb *MoCMKK2* gene (MGG_06421.6, http://www.broad.mit.edu/annotation/fungi/magnaporthe) was replaced with the 1.4 kb *HPH* gene to create the *Δmocmkk2* mutant. The knock-out vector for the *MoCMKK2* gene was constructed by inserting the up- and down- streams sequences of the *MoCMKK2* gene to the two sides of *HPH* gene in the pBS-HPH1 [9]. A 1.3 kb fragment of the upstream flanking sequence was amplified from genomic DNA of Guy-11 using the primers *MoCMKK2*upp1 (5'-CCctcgagTTGGGCGGTGATGAAGGATAAT-3') and *MoCMKK2*upp2 (5'-TTgtcgacGCCGGCGCACCAACAGAA-3'), and was cloned into the pBS-HPH1 *Xho*I and *Sal*I sites to generated pBS-HPH1- *MoCMKK2*up. The primer set *MoCMKK2*dnp1 (5'-GGgcggccgcAGCCACCGCATCAATCGTATCC-3') and *MoCMKK2*dnp2 (5'-TTcccgggGAACCCTCGCCAGCCTCAAT-3') was used to amplify the 1.3 kb downstream flanking sequence, which was inserted into the *Not*I and *Sac*I sites of pBS-HPH-*MoCMKK2*up to generate the gene deletion vector pBS-MoCMKK2. The resulting targeted knockout vector was linearized with *Xho*I, purified with a PCR product purification kit (Axgen, USA), and transformed into Guy-11 protoplasts by polyethylene-glycol-mediated fungal transformation [10].

The transformants were selected using OCM agar containing 200 μg/ml hygromycin B, and then screened with PCR using primers *MoCMKK2*yzp1 (5'-CGATCAATGCCCGGGTCTTCTA-3') and *MoCMKK2*yzp2 (5'-TCGGGCCTTTGAGTATGTTTGA-3'). The candidate mutants were verified by Southern blotting.

1.3 Nucleic acid manipulation and Southern blotting analysis

Cloning, restriction enzyme digestion, PCR, and fungal genomic DNA isolation were performed as described previously [11]. For the Southern blot analysis of the *Δmocmkk2* mutant, the genomic DNA was digested with *Hin*dIII, separated by electrophoresis on 0.7% agarose gel, transferred to a positively charged nylon membrane. The probe sequence was amplified from genomic DNA with *MoCMKK2*probep1 (5'-GGCGCCGCCATAGCCCCCATTC-3') and *MoCMKK2*probep2 (5'-TTACCGCCCAAGACCAAGCCAAGTTT-3') and labeled with digoxigenin (DIG). Hybridization was carried out with the DIG high prime DNA labeling and detection starter kit I (Roche Diagnostics, Germany), according to manufacturer's protocol.

1.4 Fungal development assays and infection assays

The myceilial growth rate was measured every 3 days for 12 days. For the conidiation assay, the conidia were collected and resuspended in 5 ml of sterile distilled water. The spore concentration was counted under a microscope with a hemocytometer. For the conidial germination and appressorium formation tests, the spore suspension was filtered through three layers of lens paper and diluted to 1×10^5 spore/ml. A 20 μl droplet was placed on a plastic coverlip with three replicates and incubated under moist conditions at 25 ℃. The conidial germination and appressorium formation rates were measured by the microscopic observation of more than 300 spores at 2, 4, 6 and 24 hpi (hours post inoculation), respectively. All the experiments described above were repeated at least three times.

For the explant infection assay, barley leaves (*Hordeum vulgare* cv. ZJ-8) were cut from 8-day-old barley seedlings, and a 20 μl droplet of spore suspension (1×10^4 spore/ml) was placed on each leaf. The leaves were put on 4% (*W/V*) water agar plates and incubated at 25 ℃ for 4 days to observe the disease symptoms. For the rice infection assay, the spore suspension (1×10^4 spore/ml) containing 0.2% (*W/V*) gelatin was sprayed on 14-day-old rice seedlings (*Oryza sativa* cv. CO-39)

with an artist's airbrush. The plant were incubated in a dew chamber at 25 ℃ for 48 h under constant dark conditions and then moved to a growth chamber under a 12 h/12 h light/dark cycle. The disease symptoms were monitored for 7 days after inoculation and disease severity was recorded as described by Bonman *et al.* [12].

2 Results

2.1 Gene disruption of *MoCMKK2*

To investigate the function of *MoCMKK2*, we generated the null mutants by targeted gene replacement. The protoplasts of the wild-type strain Guy-11 were transformed with pBS-*MoCMKK2*, which has been linearized by *Xho*I. About 200 transformants were obtained and screened by PCR. Two independent transformants *Δmocmkk2*-26 and *Δmocmkk2*-48 were confirmed by Southern hybridization, and one ectopic transformant, which was designated as ectopic-2, was chosen as the ectopic control. The two mutants and the ectopic transformant were purified by single spore isolation. As expected, a 6.2 kb fragment was detected in the wild-type strain while a 3.5 kb fragment was detected in the two transformants, *Δmocmkk2*-26 and *Δmocmkk2*-48 (Fig.1). The ectopic transformant produced two bands, indicating the *hph* cassette was inserted randomly into the wild-type Guy-11. Bands shift from 6.2 kb to 3.5 kb in the wild type and the two transformants indicated the single insertion of the *hph* cassette in the target region.

2.2 Vegetative growth and developmental assays of *Δmocmkk2* mutant

The colony characteristics of the *Δmocmkk2* mutant were not significantly different from those of the wild-type strain on PDA medium. On CM, colonies of *Δmocmkk2* mutants appeared darker than those of the wild type Guy-11 and the ectopic strain. The aerial hypha of *Δmocmkk2* mutants were compacted on CM, in contrary to the wild type Guy-11 and the ectopic strain (Fig.2).The growth rates of the *Δmocmkk2* mutant on CM, CM-N (CM without nitrogen source), CM-C (CM without carbon source) and CM-hypertonic (CM with 1 mol/L sorbitol) were not remarkably different from those of the wild-type strain ($P \leqslant 0.05$). In the mating test against strain 2539, the *Δmocmkk2* mutant produced normal perithecia as did the wild-type strain (Fig.3). The conidial germination rates and the appressorium formation rates of the *Δmocmkk2* mutant were nearly the same with those of the wild-type strain (Table 1) after 2, 4, 6, and 24 h, respectively ($P \leqslant 0.05$).

In the sporulation assays, the conidia were collected from 11-day-old CM plates and resuspended in 5 ml of sterile distilled water. The sporulation yields of the *Δmocmkk2* mutants were five times than that of the

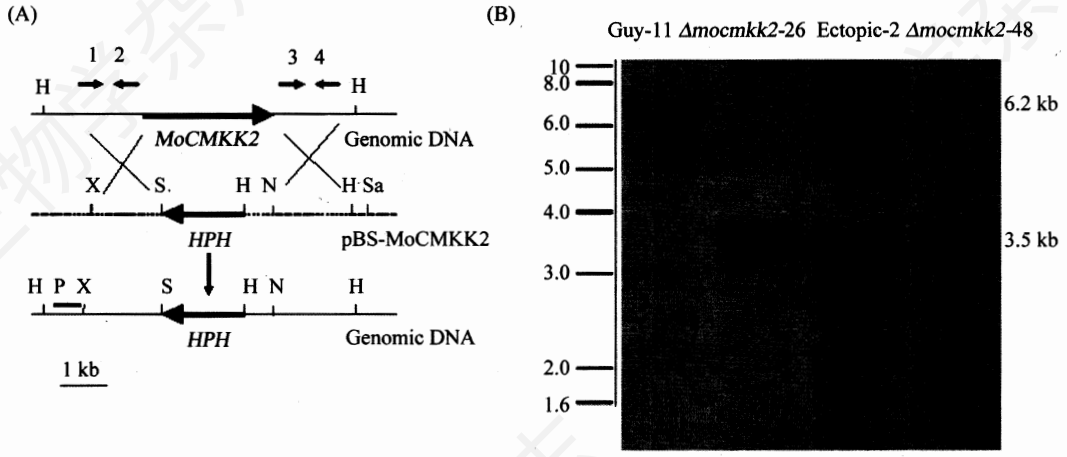

Fig.1 Targeted gene deletion of *MoCMKK2* in *M. oryzae*

A: *MoCMKK2* locus and gene deletion vector (pBS-MoCMKK2). Large arrows indicate orientations of the *MoCMKK2* and *HPH* genes. H: *Hin*dIII; N: *Not*I; S: *Sal*I; Sa: *Sac*I; X, *Xho*I; P: DNA Southern blot probe. 1, 2: *MoCMKK2*upp1 and *MoCMKK2*upp2; 3, 4: *MoCMKK2*dnp1 and *MoCMKK2*dnp2; B: Southern blot analysis of *Δmocmkk2* mutant and its rescued transformant. All genomic DNA samples were digested with *Hin*dIII, fractionated, and probed with a 700 bp fragment of the probe sequence amplified form Guy-11 genomic DNA shown in (A). The DNA size markers are the 1 kb Plus DNA Ladder (Invitrogen), and are listed in kilobases (kb) to the left of (B).

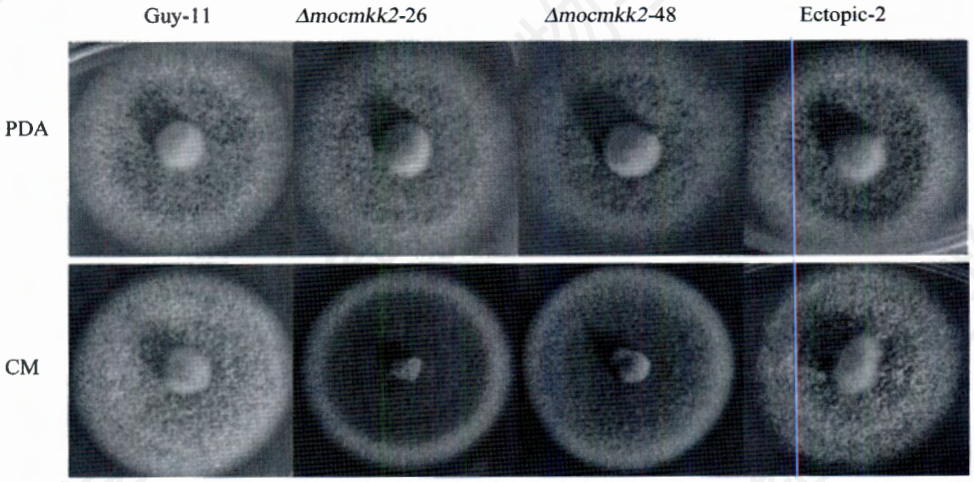

Fig.2 Colony characteristics of the *Δmocmkk2* mutants on PDA and CM

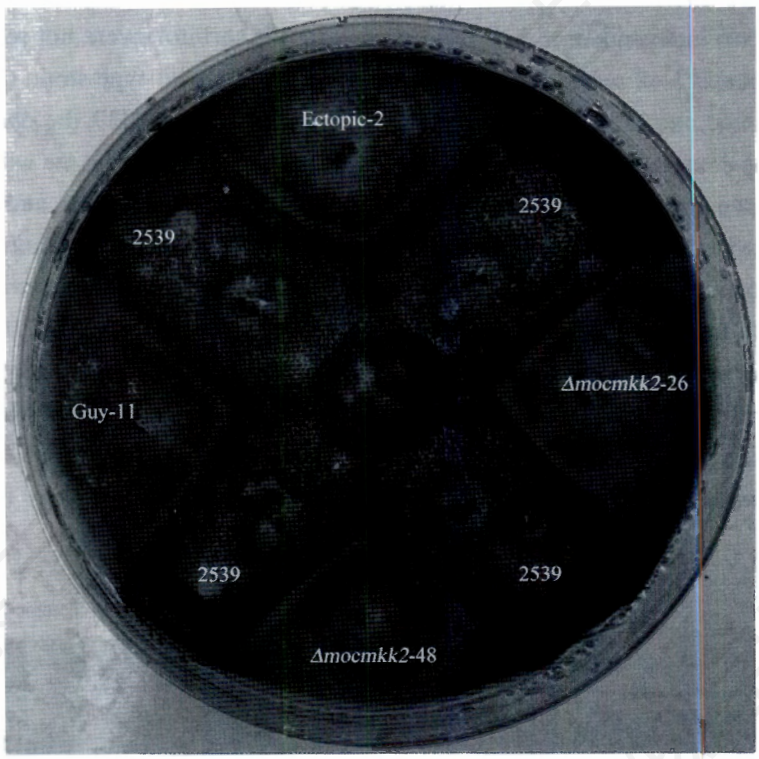

Fig.3 Mating test of the *Δmocmkk2* mutants

Table 1 Developmental characteristics of *Δmocmkk2* mutant

Strain	Sporulation (1×10^5/cm^2)	Conidial germination (%)		Appressorial formation (%)	
		2 hpi	4 hpi	6 hpi	24 hpi
Guy-11	2.29±0.22[a]	92.58±0.14[a]	98.18±2.94[a]	85.37±1.51[a]	98.93±0.25[a]
Δmocmkk2-26	11.92±0.24[b]	92.78±0.62[a]	97.20±1.01[a]	83.36±2.73[a]	97.36±0.77[a]
Δmocmkk2-48	10.28±0.47[b]	93.15±0.88[a]	97.24±0.46[a]	83.42±0.36[a]	97.21±0.81[a]
Ectopic-2	2.41±0.30[a]	93.00±1.41[a]	97.88±1.13[a]	84.32±1.73[a]	99.12±0.46[a]

a, b in the column denoted that the results estimated by Duncan's test ($P \leqslant 0.05$).

wild type (Table 1), whereas there was no significant difference between those of the wild type and the ectopic mutant. These results imply that the *MOCMKK2* gene has a key role in the sporulation of *M. oryzae*.

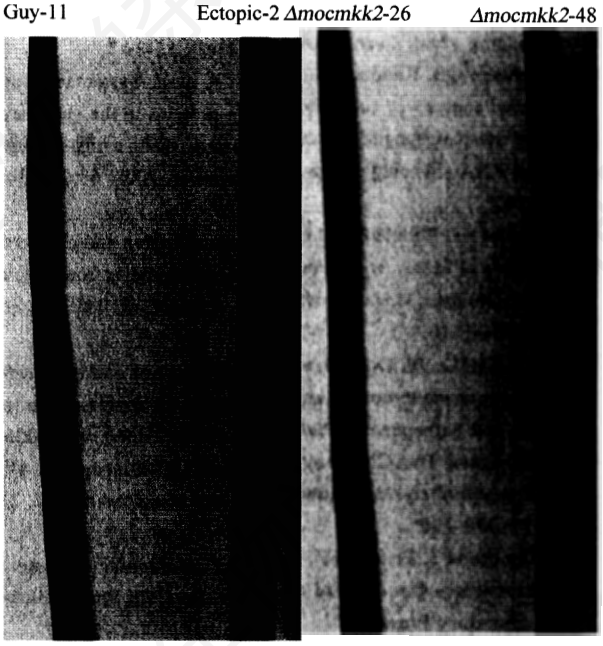

Fig.4 Pathogenicity assays of *Δmocmkk2* mutants on rice

2.3 Pathogenicity assays

The role of *MoCMKK2* gene in pathogenesis was investigated with infection assays on barley and the susceptible rice. To clearly distinguish the pathogenicity difference between the wild-type strain and the mutants, we reduced the standard inoculation concentration from 1×10^5 spore/ml to 1×10^4 spore/ml. However, in both of the droplet inoculation of barley and the spray inoculation of rice, no detectable differences were observed among the wild-type strain, the *Δmocmkk2* mutants and the ectopic mutant (Fig.4).

3 Discussion

In the present study, we analyzed the function of *MoCMKK2* gene by disruption of this gene, which encodes the calcium/calmodulin-dependent protein kinase kinase 2 protein. The *Δmocmkk2* mutants did not correlated with growth rate, conidial germination, appressorial formation, or pathogenicity. The *Δmocmkk2* mutants also did not lose the capacity to mate with strain 2539, which is the opposite mating type to the wild-type strain Guy-11. The mycelial growth on CM of *Δmocmkk2* mutants and the wild type were comparable. By a thorough analysis of the sporulation, we found that this gene was correlated with the conidial production in *M. oryzae*. The *Δmocmkk2* mutants produced a large number of conidia, five times more than that were produced by the wild-type strain. These results demonstrate that this gene correlates with the downregulation of sporulation, which has also been reported in the previous studies of the Ca^{2+} signaling pathway that regulates sporulation [13].

In *M. oryzae*, limited information has been available about the role of the Ca^{2+} signaling pathway or any specific gene related to Ca^{2+}-signaling transduction in its whole development. Previous studies of *M. oryzae* have only indicated that a calcium/calmodulin-dependent signaling system is involved in appressorium formation and conidial germination [14–16]. Using a high-throughput RNA-silencing system, the research team of Nakayashiki performed a genome-wide functional analysis of Ca^{2+} signaling proteins that had been identified with a comparative genomics approach [7,17]. The *MoCMKK2* gene (MGG_06421.6) studied in this paper was also included in their research. In their studies, mutation of this gene caused defects in its growth rate and sporulation, which were about 4/5 and 1/5 those of the parent strain, respectively. However, we obtained the different results, in which the *Δmocmkk2* mutants displayed the same growth rate as the wild-type stain, but the numbers of conidia of the *Δmocmkk2* mutants were five times higher than those of the wild-type strain. This significant difference in two mutations of the same gene is attributed to the different knockout strategies. Using RNA-silencing approach, the suppression of the expression of a specific gene can offer a clue to the function of the gene. However, it is sometimes difficult to interpret the results because the mutation is incomplete or reversible mutation. Furthermore, genes in a conserved gene family might be knocked down totally, which will also make the results difficult to interpret [18]. Unlike RNA-silencing, the homologous recombination knockout strategy disrupts the targeted gene completely and facilitates the analysis of the function of the gene without other interference. Targeted gene disruption by homologous recombination has become much easier than previously, especially for the fungi whose genome sequences were available. In contrast, the RNA-silencing method has some advantages over the conventional gene

knockout strategy [19]. For example, RNA silencing can be used to investigate the lethal gene that cannot be knocked out completely, because it only reduces the expression level of the gene, which will not lead to death.

Sporulation is significant to phytopathology researchers since spores are one of the most important dissemination ways of fungal diseases. Moreover, it is a key step for fungi to complete the life cycles. Generally, sporulation occurs under conditions where nutrients are limited. But fungi do not produce spores infinitely under poor nutrients conditions, because overproduction of spores will metabolize too much nutrients and the spores do not have enough energy to release and survive. Therefore, there must be some genes like *MoCMKK2* gene which negatively regulate the conidiogenesis process.

Sporulation is quite a complex and underestimated developmental process, which is regulated by various factors. However, the actual role of *MoCMKK2* in sporulation is still unclear. Future studies will focus on the interactions of *MoCMKK2* and other CaM protein kinase genes, which should facilitate us to further the understanding of the conidiogenesis process of *M.oryzae*.

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稻瘟菌 *MoCMKK2* 基因的功能分析

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摘要 钙离子是非常重要的第二信使，钙离子信号途径几乎参与到细胞生长发育过程中的各个过程。本研究中主要通过同源置换的基因敲除方法对稻瘟病菌中依赖钙离子/钙调蛋白的蛋白激酶激酶2 (*MoCMKK2*)基因的功能进行了分析。结果发现，*MoCMKK2* 基因缺失突变体的产孢量显著上升；而在致病性、菌丝生长速率、饥饿条件下的生长、分生孢子萌发率和附着孢形成率等方面跟野生型没有差别。上述结果显示，该基因是一个影响产孢量的负调控基因。这一发现为后续稻瘟菌钙离子信号途径相关基因的研究打下了基础。

关键词 稻瘟病菌; *MoCMKK2*; 同源重组基因敲除策略; 产孢

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