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Two *CaMK* genes with different biochemical characteristics exist in *Magnaporthe oryzae*

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Abstract Two types of Ca^{2+}/calmodulin-dependent protein kinase (CaMK) were found in *Magnaporthe oryzae*. *MgCaMK1* and *MgCaMK2* were both cloned and sequenced. High similarities in amino acid sequence to other reported CaMKs in fungi suggested that CaMKs were relatively conserved. Both MgCaMK1 and MgCaMK2 have a serine/threonine protein kinase active site and a calmodulin (CaM)-binding domain. Southern blot analysis showed that *MgCaMK1* or *MgCaMK2* existed as a single copy in the *M. oryzae* genome. Subsequently, *MgCaMK1* or *MgCaMK2* was expressed in *Escherichia coli* BL21 via a pET-32a (+) plasmid. The purified proteins exhibited protein kinase activity. The autophosphorylation and substrate phosphorylation of MgCaMK1 exhibited a Ca^{2+}/calmodulin-dependent manner, and suggested that it belonged to the group of Ca^{2+}/calmodulin-dependent protein kinases. However, the autophosphorylation and substrate phosphorylation of MgCaMK2 exhibited a Ca^{2+}-dependent manner and suggested that it belonged to the group of Ca^{2+}-dependent protein kinases.

Keywords Autophosphorylation · Calmodulin (CaM) · Phosphorylation · Prokaryotic expression · Substrate

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Introduction

Blast fungus *Magnaporthe oryzae* causes serious disease in various types of the grass family, including rice, wheat, and barley, especially in cultivated rice, often cause worldwide devastating rice blast disease and threaten food security (Dean 1997; Talbot 1995). So *M. oryzae* has been used as a model fungus for investigating pathogen-host interactions (Dean et al. 2005; Ebbole 2007). To effectively control and reduce the damage of *M. oryzae*, it is important to understand the signal transduction pathways which regulate the development and pathogenicity of this fungus. Signal transduction mechanisms that regulate fungal proliferation and differentiation include highly conserved mitogen-activated protein (MAP) kinase, cyclic AMP (cAMP), and Ca^{2+} signalling cascades. Recent studies have provided evidence for the involvement of cAMP-dependent protein kinase, adenyl cyclase, and MAP kinase cascades in hyphal growth polarity, morphogenesis, conidiation, spore germination, and appressorium formation by *M. oryzae* (Kang et al. 1999; Xu and Hamer 1996; Xu, 2000; Lee and Dean 1993). With regard to Ca^{2+} signalling cascades, a large number of Ca^{2+}-signalling proteins have been identified and characterized in *Saccharomyces cerevisiae* and some filamentous fungus, but relatively few have been discovered in *M. oryzae*. Zelter et al. (2004) identified 42 Ca^{2+}-signalling related proteins in *M. oryzae* by comparative genomic analysis, most of which were previously unknown. Nguyen et al. (2008) found 37 calcium-signalling related genes involved in hyphal

growth, sporulation and pathogenicity in *M. oryzae*, using a high-throughput RNA-silencing system.

As an important member of the Ca^{2+} signalling cascades, Ca^{2+}/calmodulin-dependent protein kinase (CaMK) belongs to serine/threonine protein kinases. CaMK contains two major domains: an amino-terminal catalytic domain which is highly conserved, and a carboxy-terminal regulatory domain. The regulatory domain is consisted of overlapping auto-inhibitory and Ca^{2+}/CaM binding domains. The auto-inhibitory domain acts as a pseudosubstrate, mimicking substrate interaction with the catalytic domain and thus blocking substrate access to the catalytic site. The Ca^{2+}/CaM binding domain, consisted of approximately 20 amino acids, is located in the C-terminal portion of the enzyme. Once the Ca^{2+}/CaM binding domain combines Ca^{2+}/calmodulin, a conformational change in CaMK ensues the auto-inhibitory domain is removed from the catalytic domain, exposing the active site and enabling the binding of substrate and its subsequent phosphorylation.

Numerous reports also have been focused on *CaMK* genes in fungi. Two CaMKs, *cmk1 and cmk2*, have been discovered in *Saccharomyces cerevisiae*. The *cmk1* encodes an open reading frame that is homologous to the sequence of vertebrate typeII CaM kinases. The *cmk2* gene encodes another CaM-dependent protein kinase with high sequence similarity to CMKI (Pausch et al. 1991). In *Schizosaccharomyces pombe*, *cmk1* encodes a 335-amino acid protein with significant amino acid sequence homology to mammalian CaMK-I, including a conserved sequence for phosphorylation by CaM kinase kinase. Studies have shown that *cmk1* is a CaM-dependent protein kinase (Rasmussen 2000). In *Aspergillus nidulans*, the cDNA of CaMK (CMKa) contains an in-frame start codon, an open reading frame (ORF) of 1,242 bp and is polyadenylated. Southern blot analysis indicates that CaMK is encoded by a single-copy gene (Kornstein et al. 1992). Another two kinases, CMKB and CMKC possessing high sequence identity with mammalian CaM kinases (CaMKs) I/IV and CaMKKα/β respectively, have been identified (Joseph and Means 2000). In *Neurospora crassa*, amino acid alignment of CaMK (CaMK-I) shows that it shares 71% sequence identity to CaMKA of *A. nidulans* and 53% to CaMK-II of *S. cerevisiae* (Yang et al. 2001). The cDNA of *Colletotrichum gloeosporioides* (*CgCMK*) has been shown to contain the CaM binding domain and 11 conserved kinase domains (Kim et al. 1998). Tsai et al. (2002) described a CaMK gene (FCaMK) in *Arthrobotrys dactyloides*, a nematode-trapping fungus. The resulting 373-amino-acid protein had significant homology to mammalian CaMKs. FCaMK contained a serine/threonine kinase domain followed by a calmodulin-binding domain. Liz et al. (2007) cloned a CaMK gene (SSCMK1) from *Sporothrix schenckii*. Bioinformatic analyses of SSCMK1 showed that this protein had the distinctive features that characterized as a calcium/calmodulin protein kinase: a serine/threonine protein kinase domain and a calmodulin-binding domain.

With regards to *Magnaporthe oryzae*, Liu et al. (2009) reported that the disruption of a putative calcium/calmodulin-dependent kinase (MoCMK1) of *M. oryzae* could delay the conidial germination and appressorial formation, weaken the ability to infect the susceptible rice cultivar, and concluded that MoCMK1 played key roles in the pathogenicity of the rice blast fungus. Our research group (Ma et al. 2009) reported the cloning, prokaryotic expression, and bioactivity of the calmodulin gene of *Magnaporthe oryzae*, which was at upper reaches of MgCaMK in Ca^{2+} signalling cascade pathway. In this paper, we report two *CaMK* genes with different biochemical characteristics found in *Magnaporthe oryzae.*

Materials and methods

Mycelia preparation of *Magnaporthe oryzae*

M. oryzae strains PO-041 were cultured on plate with complete medium (CM) at 28°C for 5d (Talbot et al. 1993). The mycelia were then inoculated to flask with 150 mL soluble starch liquid medium (soluble starch 1%, yeast extract 0.5%, sugar 0.3%) and cultured with constant agitation (120 rpm) at 28°C for vegetative growth with 5 d. The mycelia were harvested by filtration through cotton gauze.

Gene cloning and sequence analysis

Total RNA of mycelia was extracted by using Trizol reagents (TaKaRa Biotechnology, China). Five hundred nanograms of total RNA was used as template in RT reaction mixture for cDNA synthesis. The RT reaction was prepared by oligo (dT) primers and M-MLV reverse transcriptase (Promega, USA) according

to the manufacturer's guidelines. Polymerase chain reaction (PCR) was subsequently performed through using the following primers with engineered *Eco*RI and *Bam*HI or *Hin*dIII sites (underlined):

MgCaMK1 RT-P1: 5′-GCGAATTCAGCCGGTACATTCGCAACAC-3′;
MgCaMK1 RT-P2: 5′-GCGGATCCCATGGCCGGAAAACAACAAA-3′.
MgCaMK2 RT-P1: 5′-GCGAATTCCCGTCCAACCCCATCGCTATC-3′;
MgCaMK2 RT-P2: 5′-GCAAGCTTCGGCCCTTCTCAGTATCCCAA-3′.

PCR condition was performed using the following thermal program: initial denaturation at 94°C for 5 min, 25 cycles of 94°C for 1 min, 60°C for 30 s, and 70°C for 1 min, with a final extension cycle at 72°C for 10 min. The amplification was purified and ligated into a pUCm-T vector (Sangon, Shanghai, China) and then transformed into *E. coli* DH5α. The positive clone (contain pUC-MgCaMK1/ pUC-MgCaMK2) was sequenced (Sangon, Shanghai).

The comparison of the amino acid sequence of the MgCaMK with other reported CaMKs was analyzed using DNAstar Software.

Identification of *MgCaMK* copy number in the genome

The copy number of *MgCaMK1* and *MgCaMK2* in the genome was determined by Southern blot analysis. Genomic DNA was prepared by CTAB methods. Forty micrograms of DNA was first digested by *Sal*I and *Eco*RI or *Bgl*II and *Eco*RI, then separated by agarose gel (0.8%) electrophoresis, and finally transferred onto nylon film (Hybond-N^+, Solarbio) by capillary methods. The following two pair of primers was designed to amplify the hybridization probe:

MgCaMK1 P-P1: 5′-GCCGTAGACTACCTACACAGCA-3′;
MgCaMK1 P-P2: 5′-TCCAGATGATCCTGCATTGC-3′.
MgCaMK2 P-P1: 5′-GGCATCAAAACATCCTTACG-3′;
MgCaMK2 P-P2: 5′-TCCGTATCCTGAGTCTTTGTG-3′.

The Southern Hybridization procedure was performed according to the protocol from Roche for the DIG High Prime DNA Labeling and Detection Starter Kit (Roche Ltd., Swiss)

Construction of *MgCaMK* prokaryotic expression plasmid

MgCaMK1/*MgCaMK2* was subcloned by using the pUC-MgCaMK plasmid as template. The subclone primers were as follows:

MgCaMK1 S-P1: 5′-GCGGATCCAGTGCCTTCAACATGAGC-3′;
MgCaMK1 S-P2: 5′-CCAAGCTTTGAGCCAGTGAAGCTCTTCC-3′.
MgCaMK2 S-P1: 5′-GCGGATCCACCGACATTTCCAGAGCCATG-3′;
MgCaMK2 S-P2: 5′-CCAAGCTTCCCCCGGCTTCCAACGCCG-3′.

*Bam*HI and *Hin*dIII sites (underlined) were added in the sense and antisense primers, respectively. PCR procedures were as described above. The purified *MgCaMK1* or *MgCaMK2* fragments after PCR were digested with *Bam*HI and *Hin*dIII, and then separately ligated into the *Bam*HI and *Hin*dIII-digested vectors pET32a (+) (Novagen) resulting in recombinant palsmids pET32a-*MgCaMK1* and pET32a-*MgCaMK2.*

Preparation of bacteria extract

The pET32a-*MgCaMK1* or pET32a-*MgCaMK2* recombinant plasmids were transformed into *E. coli* BL21 (DE3) after confirmation of the correct reading frame by sequence analysis. The fusion proteins Trx-MgCaMK1 and Trx-MgCaMK2 expressed by recombinant plasmids were performed as described previously (Ma et al. 2009). Trx-MgCaMK1 and Trx-MgCaMK2 were purified by HIS-Select™ Nickel Affinity Gel according to the protocol (Sigma, USA). The purified proteins were kept at −80°C for future experiments.

In-gel kinase activity assays

Autophosphorylation activity of the purified Trx-MgCaMK1 and Trx-MgCaMK2 was measured. Autophosphorylation was performed in 20 μl reaction mixture containing 25 mM Tris-HCl pH 7.5, 0.5 mM DTT, 10 mM $MgCl_2$, 5 μCi [γ-^{32}P] ATP, either 1 μg Trx-MgCaMK1 or Trx-MgCaMK2, respectively. The effects of activating factors on the enzymatic activty

were measured by adding either Ca^{2+} (2.5 mM $CaCl_2$) or CaM (1 μg), or both.

For substrate phosphorylation activity of the purified Trx-MgCaMK1 and Trx-MgCaMK2, 10 μg/μl histone III-s was used as the substrate. The reaction mixture was performed as described above. The effects of activating factors on the activty were measured by adding either Ca^{2+} (2.5 mM $CaCl_2$) or CaM (1 μg), or both. When CaM was used as the sole activating factor, 2.0 mM EGTA was included in the reaction mixture. After 30 min of incubation at 30°C, reactions were terminated by adding 4× sample buffer and boiling for 5 min. SDS-PAGE was performed then the gels were stained with 0.1% Coomassie brilliant blue R250. Autoradiography was analyzed by Typhoon 9210 Variable Mode Imager (Amersham Pharmacia Biotech).

Results

Analysis of *MgCaMK1* and *MgCaMK2*

Two CaMK genes, named *MgCaMK1* and *MgCaMK2*, were amplified using RT-PCR from *M. oryzae*. The entire open reading frame (ORF) of *MgCaMK1* contains 1,221 bp encoding 406 amino acid residues with predicted molecular weight of 45.6 KDa. The entire ORF of *MgCaMK2* is 1,260 bp encoding 419 amino acid residues with predicted molecular weight of 45.7 KDa.

The amino acid sequence alignment of MgCaMK1 with other CaMKs revealed that MgCaMK1 shared the highest similarity (76% identity) with NcCaMK (*Neurospora crassa*) and SsCaMK (*Sporothrix schenckii*), followed by AnCMKA (*Aspergillus nidulans*, 66% identity), ScCmk1 (*Saccharomyces cerevisiae*, 55% identity), ScCmk2 (*Saccharomyces cerevisiae*, 50% identity), AnCMKB (*Aspergillus nidulans*, 43% identity), RnCaMKI (*Rattus norvegicus*, 43% identity), HsCaMKI (*Homo sapiens*, 43% identity), CgCaMK (*Colletotrichum gloeosporioides*, 42% identity), and NcCaMKI (*Neurospora crassa*, 33% identity) (Fig. 1).

MgCaMK2 shared the highest similarity with AcCaMK (*Aspergillus clavatus*, 88% identity), followed by AdCaMK (*Arthrobotrys dactyloides*, 84% identity), CgCaMK (*Colletotrichum gloeosporioides*, 81% identity), NcCaMKI (*Neurospora crassa*, 78% identity), NcCaMK (*Neurospora crassa*, 44% identity), AnCMKB (*Aspergillus nidulans*, 66% identity), AnCMKA (*Aspergillus nidulans*, 32% identity), RnCaMKI (*Rattus norvegicus*, 45%identity), and HsCaMKI (*Homo sapiens*, 44% identity) (Fig. 2).

Using the ScanProsite motif search program, protein domain analysis of MgCaMK1 showed an ATP-binding region between amino acid L29 to K54 and a serine/threonine protein kinase active site between V138 and Y150. NCBI online analysis indicated that the catalytic loop of MgCaMK1 was between H140 and L149, while the activation loop was from D163 to S172 and from L177 to E190. Calmodulin Target Database analysis of MgCaMK1 indicated a putative CaM-binding domain from amino acid residues 295 to 313 (FMAKARLRR GIEMVKLANR). All these findings suggested that this novel gene encodes a Ca^{2+}/CaMK (Fig. 1).

In the case of MgCaMK2, the protein kinase ATP-binding region (L22 to K45) and the serine/threonine protein kinase active site (between I135 and F147) was also found. Conserved domain analysis of MgCaMK2 identified a catalytic loop between H137 and L146. The activation loop of MgCaMK2 was between D160 to E169 and L175 to E188. The putative CaM-binding domain of MgCaMK2 was located from amino acid residues 298 to 316 (ARRTLHAAIDTVRAINKLR) (Fig. 2). Twelve subdomains highly conserved in known protein kinases existed in the C-terminal catalytic domains of MgCaMK1 and MgCaMK2 (Figs. 1, 2).

A phylogenetic tree was constructed based on the full-length homologous sequence of several CaMKs or CDPKs from different species (Fig. 3). MgCaMK1 is considerably closer to that *of C. posadasii* than other CaMKs and CDPKs. MgCaMK2 is most closely related to that of *C. gloeosporioides*. These results also clearly indicated that MgCaMK1 and MgCaMK2 belonged to different members of the CaMK superfamily.

MgCaMK1 or *MgCaMK2* is a single copy gene in the *M. oryzae* genome

The Southern blot analysis on the copy number of *MgCaMK1* or *MgCaMK2* in the genome was shown in Fig. 4a, b. Hybridization of the *Eco*RI-digested genomic DNA with labeled probes revealed one specific band with a molecular weight of 10.6 kb. The *Sal*I digested genomic DNA presented two hybridized bands with molecular weights of 3.9 kb and 2.9 kb (Fig. 4a). Since there is no *Eco*RI recognition site and only one *Sal*I recognition site in

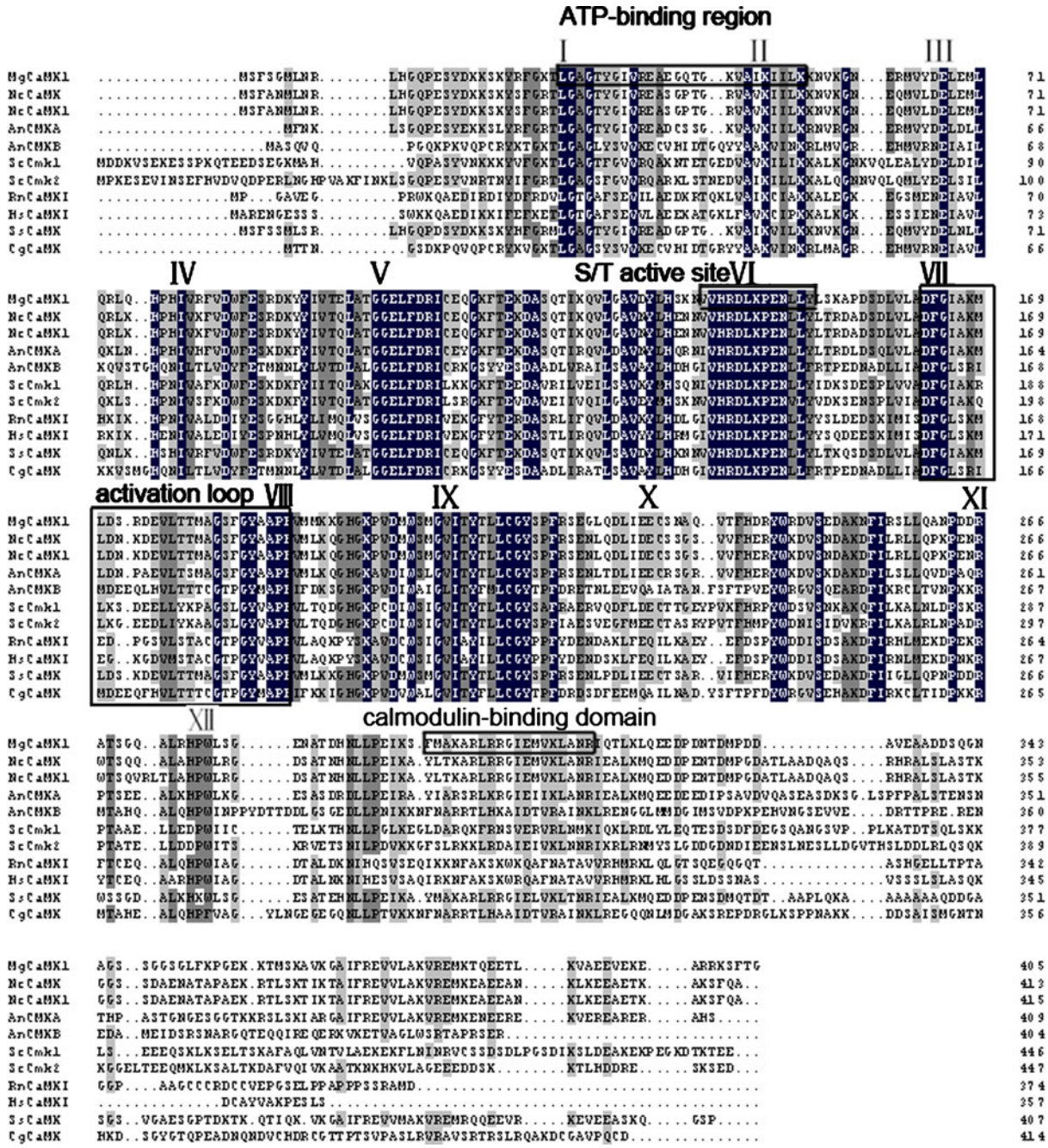

Fig. 1 Amino acid sequence alignment of MgCaMK1 with other CaMKs. The following abbreviations were used: NcCaMK: *Neurospora crassa* CaMK (XP_958895); NcCaMKI: *Neurospora crassa* CaMKI (XP_959927); AnCMKB: *Aspergillus nidulans* CMKB (AF156027); AnCMKA: *Aspergillus nidulans* CMKA (XP_660016); ScCmk1: *Saccharomyces cerevisiae* ScCmk1 (NP_116669); ScCmk2: *Saccharomyces cerevisiae* Cmk2 (NP_014626); RnCaMKI: *Rattus norvegicus* CaMKI (NP_604463); HsCaMKI: *Homo sapiens* CaMKI (NP_065130); SsCaMK: *Sporothrix schenckii* CaMK (AAV80434); CgCaMK: *Colletotrichum gloeosporioides* CaMK (AAC62515). *Black boxes* indicated identical residues, and *gray boxes* indicated conservative changes. The sequences in the box represented the protein kinase ATP-binding region (L29-K54), the serine/threonine protein kinase active site (V138-Y150), the activation loop (D163-E190) and the putative calmodulin-binding domain (F295-R313), respectively. Twelve distinct subdomains were indicated by *Roman numerals*

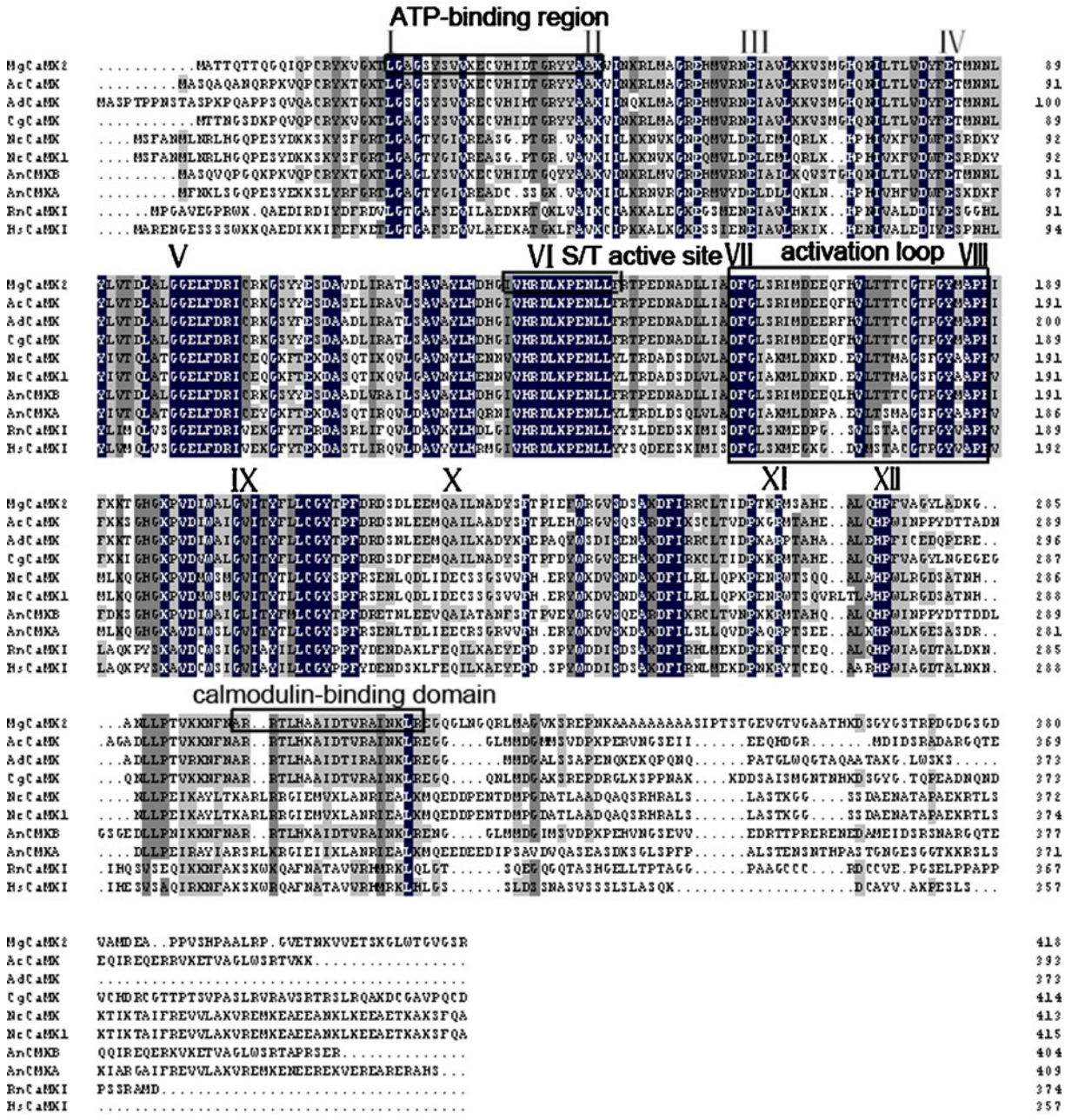

Fig. 2 Amino acid sequence alignment of MgCaMK2 with other CaMKs. The following abbreviations were used: AcCaMK: *Aspergillus clavatus* CaMK (XP_001270951); AdCaMK: *Arthrobotrys dactyloides* CaMK (AAG43970); CgCaMK: *Colletotrichum gloeosporioides* CaMK (AAC62515); NcCaMK: *Neurospora crassa* CaMK (XP_958895); NcCaMKI: *Neurospora crassa* CaMKI (XP_959927); AnCMKB: *Aspergillus nidulans* CMKB (AF156027); AnCMKA: *Aspergillus nidulans* CMKA (XP_660016); RnCaMKI: *Rattus norvegicus* CaMKI (NP_604463); HsCaMKI: *Homo sapiens* CaMKI (NP_065130). *Black boxes* indicated identical residues, and *gray boxes* indicated conservative changes. The sequences in the box represented the protein kinase ATP-binding region (L22-K45), the serine/threonine protein kinase active site (I135-F147), the activation loop (D160-E188) and the putative calmodulin-binding domain (A298-R316), respectively. Twelve distinct subdomains were indicated by *Roman numerals*

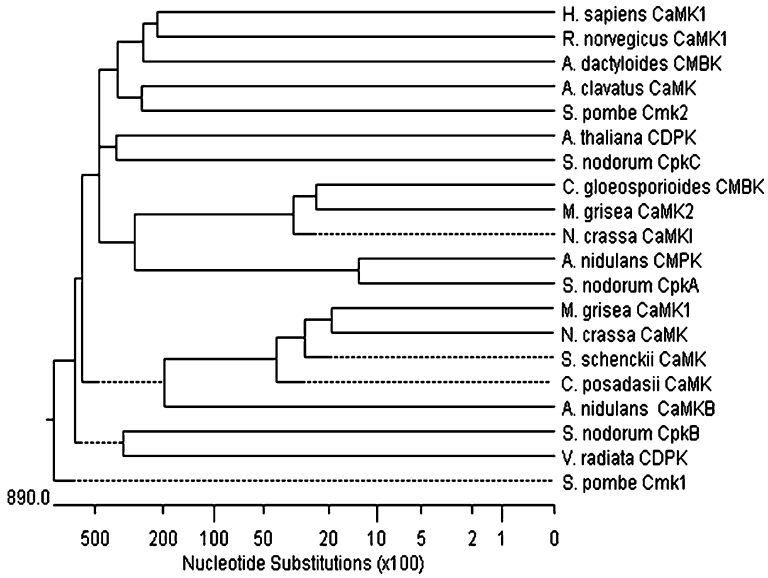

Fig. 3 Phylogenetic tree showing the relationship between MgCaMK1/MgCaMK2 with other CaMKs and CDPKs. Accession numbers: *A. clavatus* CaMK (XP_001270951); *A. dactyloides* CMBK (AAG43970); *A. nidulans* CaMKB (XP_660669); *A. nidulans* CMPK (XP_660016); *A. thaliana* CDPK (AAC69927); *C. gloeosporioides* CMBK (AAC62515); *C. posadasii* CaMK (EER29494); *H. sapiens* CaMK1 (NP_065130); *N. crassa* CaMK (XP_958895); *N. crassa* CaMKI (XP_959927); *R. norvegicus* CaMK1 (NP_604463); *S. pombe* Cmk1 (NP_593464); *S. pombe* Cmk2 (O42844); *S. schenckii* CaMK (AAV80434); *S. nodorum* CpkA (DQ397887); *S. nodorum* CpkB (SNU15778.1); *S. nodorum* CpkC (SNU09198.1); *V. radiata* CDPK (AAC49405)

the *MgCaMK1* sequence, we concluded that *MgCaMK*1 was a single copy gene in the *M. oryzae* genome.

In the case of *MgCaMK*2, hybridization of the *Eco*RI-digested genomic DNA with labelled probes showed one band with a molecular weight of 9.1 kb.

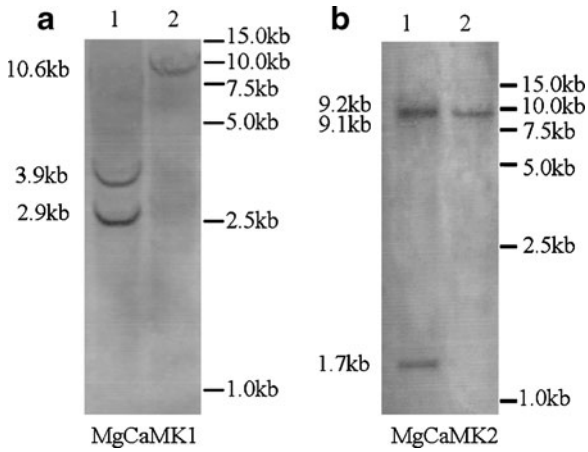

Fig. 4 Southern blot analysis of *MgCaMK1* and *MgCaMK2*. **a** Southern blot analysis of *MgCaMK1*. Lane 1, *M. oryzae* genomic DNA was digested by *Sal*I. Lane 2, Genomic DNA was digested by *Eco*RI. **b** Southern blot analysis of *MgCaMK2*. Lane 1, Genomic DNA was digested by *Bgl*II; Lane 2, Genomic DNA was digested by *Eco*RI

The *Bgl*II digested genomic DNA revealed two bands with molecular weights of 9.2 kb and 1.7 kb (Fig. 4b). As the same reasoning aforementioned, *MgCaMK2* also was a single copy gene in the genome.

In-gel kinase activity of Trx-MgCaMK1

The in-gel autophosphorylation and substrate phosphorylation activities of Trx-MgCaMK1 were analyzed. Several reaction conditions were tested, including the addition of Ca^{2+}, CaM and Ca^{2+}/CaM complex to the reaction buffer. When Ca^{2+}/CaM were added in the reaction mixture, Trx-MgCaMK1 displayed highest autophosphorylation kinase activity (Fig. 5a). The autophosphorylated activity of Trx-MgCaMK1 decreased to 45.8% when only Ca^{2+} was added to the reaction buffer. When EGTA and CaM were both added in the reaction mixture, the activity decreased to 18.4% (Fig. 5c). These results suggested that the autophosphorylation activity of MgCaMK1 depended on the presence of Ca^{2+} and CaM.

The substrate phosphorylation activity of Trx-MgCaMK1 was analyzed using histone III-s as its substrates. Several reaction conditions were tested, including the addition of Ca^{2+}, CaM and Ca^{2+}/CaM

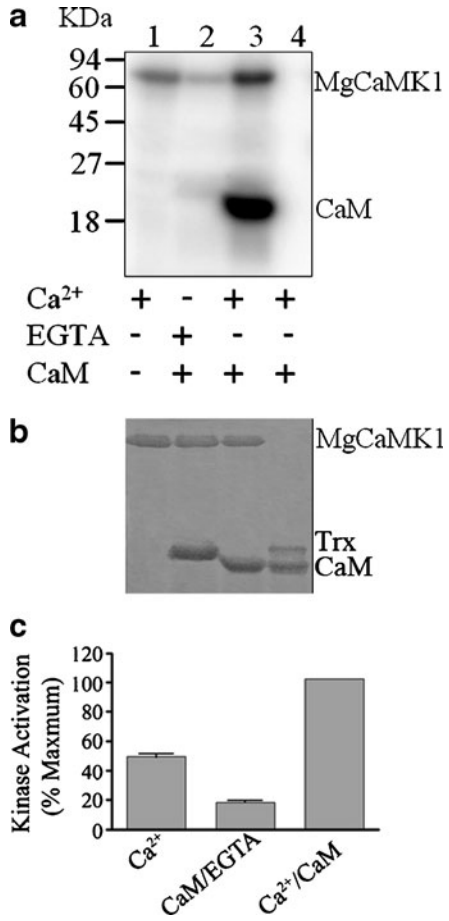

Fig. 5 Autophosphorylation activity analysis of the recombinant MgCaMK1. **a** 1-3, MgCaMK1 activity was determined in the presence of Ca^{2+}, EGTA/CaM, Ca^{2+}/CaM, respectively. 4, The activity of empty-vector control with Ca^{2+}/CaM. **b** The corresponding SDS-PAGE of MgCaMK1. **c** Densitometric analysis of autophosphorylation activity by Quantity One software

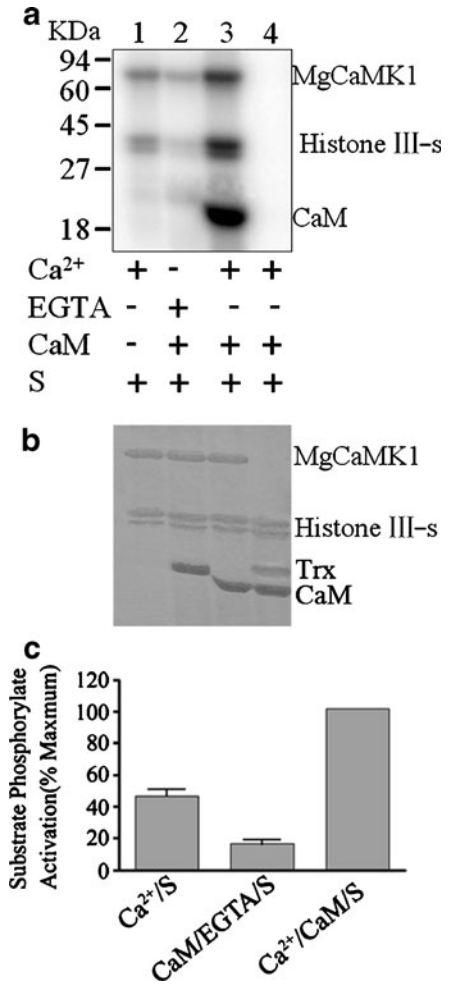

Fig. 6 Substrate phosphorylation activity analysis of the recombinant MgCaMK1. **a** 1-3, Histone III-s phosphorylated activity was determined in the presence of Ca^{2+}/S, EGTA/CaM/S, Ca^{2+}/CaM/S, respectively. Histone III-s was added as general substrate (S). 4, The activity of empty-vector control with Ca^{2+}/CaM/S. **b** The corresponding SDS-PAGE of MgCaMK1. **c** Densitometric analysis of histone III-s phosphorylated activity by Quantity One software

complex in the reaction buffer. When Ca^{2+}/CaM were added in the reaction mixture, the substrate phosphorylation activity of Trx-MgCaMK1 was at the highest level (Fig. 6a). The addition of Ca^{2+} alone reduced the phosphorylation activity to 46.7%. The addition of both EGTA and CaM decreased phosphorylation activity to 16.3% (Fig. 6c). These results suggested that the substrate phosphorylation activity of MgCaMK1 required both Ca^{2+} and CaM.

In-gel kinase activity of Trx-MgCaMK2

The in-gel kinase autophosphorylation activity of Trx-MgCaMK2 was analyzed. Several kinase reaction conditions were tested, including the addition of Ca^{2+}, CaM and Ca^{2+}/CaM complex in the reaction buffer. When Ca^{2+} (2.5 mM) was added alone, the kinase autophosphorylation activity of Trx-MgCaMK2 reached the highest level (Fig. 7a). The addition of EGTA and CaM decreased the enzyme activity to 40.5%. The addition of both Ca^{2+} and CaM reduced the autophosphorylation kinase activity to 53.1% (Fig. 7d). Considering CaM could competitively interact with Ca^{2+}, the concentration of Ca^{2+} was increased to 10 mM. However, the autophosphoryla-

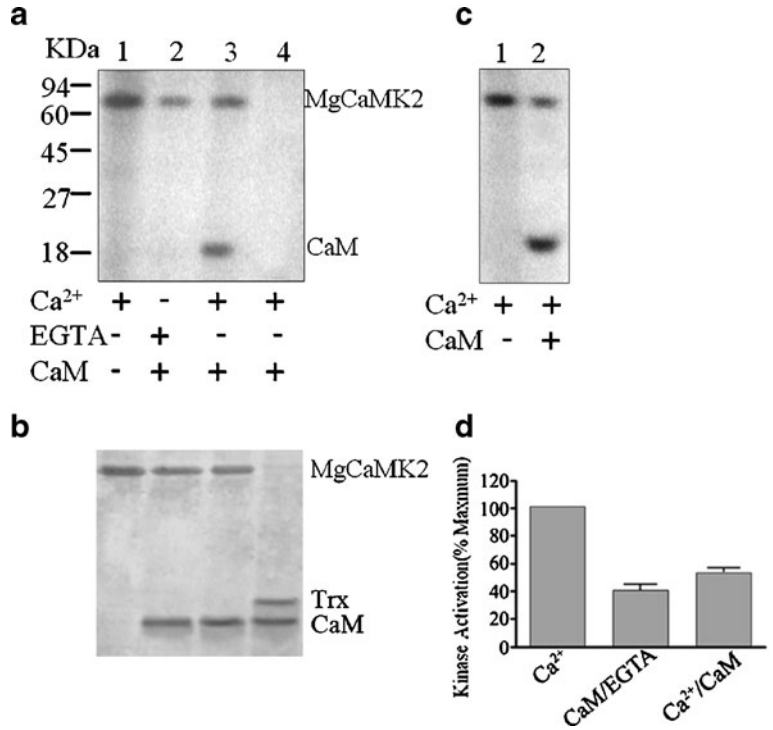

Fig. 7 Autophosphorylation activity analysis of the recombinant MgCaMK2. **a** 1-3, MgCaMK2 activity was determined in the presence of Ca^{2+} (2.5 mM), EGTA/CaM, Ca^{2+}/CaM, respectively. 4, The activity of empty-vector control with Ca^{2+}/CaM. **b** The corresponding SDS-PAGE of MgCaMK2. **c** MgCaMK2 activity was determined in the presence of Ca^{2+} (10 mM) and CaM. **d** Densitometric analysis of autophosphorylation activity by Quantity One software

tion activity of Trx-MgCaMK2 presented with Ca^{2+}/CaM still exhibited lower activity than that with Ca^{2+} alone (Fig. 7c). These results suggested that the autophosphorylation activity of MgCaMK2 mainly depended on the presence of Ca^{2+} and dropped significantly in the presence of CaM.

The substrate phosphorylayion activity of Trx-MgCaMK2 displayed similar trend as autophosphorylation activity. The addition of Ca^{2+} alone resulted highest substrate phosphorylation activity of Trx-MgCaMK2 (Fig. 8a). The addition of both EGTA and CaM decreased the enzyme activity to 52.5%. The addition of both Ca^{2+} and CaM reduced substrate phosphorylation activity to 64.1% (Fig. 8c). These results also suggested that the substrate phosphorylation activity of MgCaMK2 mainly depended on the presence of Ca^{2+} and was restrained by CaM. Consequently, MgCaMK2 could be a Ca^{2+}-dependent protein kinase (CDPK), but not a CaMK.

In our in-gel kinase assay, MgCaMK1 and MgCaMK2 not only phosphorylated common substrate histone III-s, but also phosphorylated CaM as their substrates (Figs. 5, 6, 7, 8). This resulted indicated that the phosphorylation of CaM and CaMKs themselves played an important role in the regulation of kinase activities and the signal translation between CaM and CaMK.

Discussion

In this study, *MgCaMK1* and *MgCaMK2* genes were cloned and identified from *M. oryzae* strain PO-041. Amino acid sequence alignment showed that MgCaMK1 and MgCaMK2 were homologous to the members of the fungus CaMK family. Both MgCaMK1 and MgCaMK2 had an ATP-binding region, a serine/threonine protein kinase active site and a CaM-binding domain.

The kinase activities are strictly regulated by phosphorylation and dephosphorylation. For CaMKs, not only the phosphorylation of Ca^{2+}/CaM but also CaMKs phosphorylated themselves play an important role in the regulation of kinase activities (Ishida et al. 2003). Our kinase activity assays revealed that the autophosphorylation and substrate phosphorylation

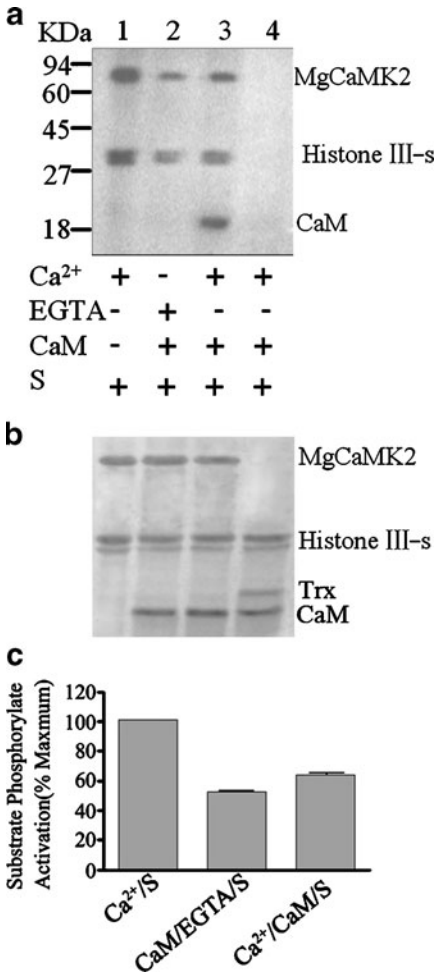

Fig. 8 Substrate phosphorylation activity analysis of the recombinant MgCaMK2. **a** 1-3, Histone III-s phosphorylated activity was determined in the presence of Ca^{2+}/S, EGTA/CaM/S, Ca^{2+}/CaM/S, respectively. Histone III-s was added as general substrate (S). 4, The activity of empty-vector control with Ca^{2+}/CaM/S. **b** The corresponding SDS-PAGE of MgCaMK2. **c** Densitometric analysis of histone III-s phosphorylated activity by Quantity One software

reactions of MgCaMK1 were dependent on both free Ca^{2+} and CaM under the presence of ATP. MgCaMK1 could phosphorylate histone III-s substrate as well as CaM of *M. oryzae*. MgCaMK2 could also phosphorylate histone III-s and CaM, but its phosphorylation activity was only dependent on Ca^{2+} but not CaM. Considering both CaM and CaMK could bind to Ca^{2+}, the concentration of Ca^{2+} was increased from 2.5 mM to 10 mM. Howerer, MgCaMK2 still exhibited Ca^{2+}-dependent property and its activity was restrained by CaM. This inhibitory effect of CaM on autophosphorylation and substrate phosphorylation activities suggested a potential regulatory pathway between MgCaMK2 and CaM. In addition, MgCaMK2 could also be a member of Ca^{2+}-dependent protein kinases (CDPKs). CDPKs have been identified in plants and some protozoans. However, CDPKs seem to be absent from the sequenced genomes of yeast, nematodes, fruit flies, and humans (Cheng et al. 2002). In plants, CDPKs contain Ca^{2+}-binding EF hands and directly bind to Ca^{2+}. This calcium-stimulated kinase activity does not dependent on CaM (Roberts and Harmon 1992). Harper et al. (1991) utilized phylogenetic analysis to show that the CDPK gene family arose through the fusion of a CaMK and a calmodulin. However, the analysis of protein domains and amino acid sequence alignment of MgCaMK2 indicated that it belonged to a member of CaMK family. The Ca^{2+}-dependent manner which the in-gel autophosphorylation and substrate phosphorylation activities had shown is unclear. It could be attributed to false folding of the protein or the inherent characteristic of CaMKs of *M. oryzae*. Further studies are needed to elucidate this issue.

A large number of multi-functional Ca^{2+}-dependent protein kinases have been previously identified and characterized in mammals. Studies showed that CaMKs play pivotal roles in Ca^{2+} signalling pathways, for instance, the regulation of the neuronal functions in learning, memory, and neuronal cell death (Ishida et al. 2003).

However, relatively fewer CaMKs have been discovered in fungi. In *S. cerevisiae*, a single deletion of CMK2, or both CMKI and CMK2 deletions were not lethal, although the loss of CMK2 caused a slower spore germination rate (Pausch et al. 1991). Deletion of the *CMK1* and *CMK2* genes decreases the LD_{50} of pheromone compared with that of a wild-type strain (Moser et al. 1996). In *S. pombe*, the mRNA for *cmk1* is expressed in a cell cycle-dependent manner, peaking at or near the G1/S boundary. Overexpression of *cmk1* in wild-type caused no apparent effects on growth and division (Rasmussen 2000). In *A. dactyloides*, Southern blot analysis detected a single copy of the *camk* gene, suggesting that FCaMK played an important role in Ca^{2+}calmodulin signalling in *A. dactyloides* (Tsai et al. 2002). In *C. gloeosporioides*, KN93, as a selective inhibitor of CaMK, inhibited 50% germination and appressorium formation, blocked melanization, and caused the formation of abnormal appressoria (Kim et al. 1998). In *N. crassa*, the purified CAMK-1 could specifically phosphory-

late FRQ, the circadian clock protein FREQUENCY. When the *camk-1* was disrupted by gene replacement, the *camk-1* null strains grew slowly, indicating that CAMK-1 plays an important role in growth and development of *N. crassa*. Analysis of the *camk-1* null strain revealed that the deletion of *camk-1* affected phase, period, and light-induced phase shifting of the circadian conidiation rhythm (Yang et al. 2001). In *S. schenckii*, CaMK inhibitors KN-62 and lavendustin C were found to inhibit budding by cells induced to re-enter the yeast cell cycle and to favor the yeast to mycelium transition (Liz et al. 2007). In *A. nidulans*, a conditional strain for the expression of CaMK was created. When grown under conditions that resulted in a 90% decrease in the enzyme, both nuclear division and growth were markedly slowed. The CaMK seems to be important for progression from G2 to mitosis (Dayton and Means 1996). The disruption of CMKa gene prevents entry of spores into the nuclear division cycle (Dayton et al. 1997). CMKB knockout is lethal to cells. Knockout of *cmk*C is not lethal, as spores lacking CMKC could germinate with delayed kinetics (Joseph and Means 2000). Three genes encoding different CaMKs were characterized in *Stagonospora nodorum.* Targeted gene disruption indicated that CpkA was not required for lesion development but was essential for sporulation at the completion of the infection cycle. CpkB and CpkC lacking strains appeared unaffected growth but delayed lesion development and sporulation during infection (Solomon et al. 2006). For *Magnaporthe oryzae,* Nguyen et al. (2008) analyzed six possible Ca^{2+}/CaM-dependent serine/threonine protein kinases (MGG00925.5, MGG09912.5, MGG06421.5, MGG08547.5, MGG01196.5 and MGG01596.5). The MGG09912.5 RNAi mutants showed strong growth defects and formed no conidia while the MGG_00925.5 mutants had mild growth defects and produced reduced conidia. A *MOCMK1* gene which had the same sequence with MGG_09912.5 was disrupted in *M. oryzae* and the phenotypes of mutants was different from MGG09912.5 RNAi mutants. The *MoCMK1* mutants had sparse aerial hypha, fewer conidia, delayed conidial germination and appressorial formation, weakened ability to infect the susceptible rice (Liu et al. 2009). These results indicated that Ca^{2+}/CaM-dependent protein kinases played important roles in the pathogenicity of the rice blast fungus.

In our study, two CaMK genes, *MgCaMK1* and *MgCaMK2* were isolated. Amino acid sequence analysis showed that MgCaMK1 had the same sequence with MGG_09912.5 and MoCMK1. MgCaMK2 had the same sequence with MGG_00925.5. This means that they are the same protein kinase respectively. Our kinase biochemical characterizatics assay supplemented the evidence of MgCaMK1 and MgCaMK2 were medium protein kinases in calcium/calmodulin-signal pathway. This signal pathway might participate in the pathogenicity of the rice blast fungus. Further, understanding genes and biochemistry property of the proteins in *M. oryzae* would facilitate searches of finding pathway targets to regulate fungi pathogenicity and exploiting new fungicides.

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References

Cheng, S. H., Matthew, R. W., Chen, H. C., & Jen, S. (2002). Calcium signaling through protein kinases. The arabidopsis calcium-dependent protein kinase gene family. *Plant Physiology, 129*, 469–485.

Dayton, J. S., & Means, A. R. (1996). Ca2+/Caimodulin-dependent kinase is essential for both growth and nuclear division in *Aspergillus nidulans*. *Molecular Biology of the Cell, 7*, 1511–1519.

Dayton, J. S., Sumi, M., & Nanthakumar, N. N. (1997). Expression of a constitutively active Ca2+/Calmodulin-dependent kinase in *Aspergillus nidulans* spores prevents germination and entry into the cell cycle. *The Journal of Biological Chemistry, 272*(6), 3223–3230.

Dean, R. A. (1997). Signal pathways and appressorium morphogenesis. *Annual Review of Phytopathology, 35*, 211–234.

Dean, R. A., Talbot, N. J., Ebbole, D. J., Farman, M. L., Mitchell, T. K., Orbach, M. J., et al. (2005). The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature, 434*, 980–986.

Ebbole, D. J. (2007). *Magnaporthe* as a model for understanding host-pathogen interactions. *Annual Review of Phytopathology, 45*, 437–456.

Harper, J. F., Sussman, M. R., Schaller, G. E., Putnam-Evans, C., Charbonneau, H., & Harmon, A. C. (1991). A calciumdependent protein kinase with a regulatory domain similar to calmodulin. *Science, 252*, 951–954.

Ishida, A., Shigeri, Y., Taniguchi, T., & Kameshita, I. (2003). Protein phosphatases that regulate multifunctional Ca2+/calmodulindependent protein kinases, from biochemistry to pharmacology. *Pharmacology & Therapeutics, 100*(3), 291–305.

Joseph, J. D., & Means, A. R. (2000). Identification and characterization of two Ca2+/CaM-dependent protein kinases required for normal nuclear division in *Aspergillus nidulans*. *The Journal of Biological Chemistry, 275*(49), 38230–38238.

Kang, S. H., Khang, C. H., & Lee, Y. H. (1999). Regulation of cAMP dependent protein kinase during appressorium formation in *Magnaporthe grisea*. *FEMS Microbiology Letters, 170*, 419–423.

Kim, Y. K., Li, D. X., & Kolattukudy, P. E. (1998). Induction of Ca2+-calmodulin signaling by hard-surface contact primes *Colletotrichum gloeosporioides* conidia to germinate and form appressoria. *Journal of Bacteriology, 180*, 5144–5150.

Kornstein, L. B., Gaiso, M. L., Hammell, R. L., & Bartelt, D. C. (1992). Cloning and sequence determination of a cDNA encoding *Aspergillus nidulans* calmodulin-dependent multifunctional protein kinase. *Gene, 113*(1), 75–82.

Lee, Y. H., & Dean, R. A. (1993). cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. *The Plant Cell, 5*, 693–700.

Liu, X. H., Lu, J. P., Dong, B., Gu, Y., & Lin, F. C. (2009) Disruptionof *MoCMK1*, encoding a putative calcium/calmodulin-dependentkinase, in *Magnaporthe oryzae*. *Microbiological Research*, 2006; doi:10.1016/j.micres.2009.08.007.

Liz, V. A., Shirley, V. B., Ricardo, R. G. M., & Nuri, R. V. (2007). Functional, genetic and bioinformatic characterization of a calcium/calmodulin kinase gene in *Sporothrix schenckii*. *BMC Microbiology, 7*(107), 1–12.

Ma, Z. B., Zhao, J. X., Wang, L. A., & Zheng, X. B. (2009). Cloning, prokaryotic expression, and bioactivityof the calmodulin gene of *Magnaporthe grisea*. *FEMS Microbiology Letters, 300*, 107–114.

Moser, M. J., Geiser, J. R., & Davis, T. N. (1996). Ca2+-calmodulin promotes survival of pheromone-induced growth arrest by activation of calcineurin and Ca2+-calmodulin-dependent protein kinase. *Molecular and Cellular Biology, 16*(9), 4824–4831.

Nguyen, Q. B., Kadotani, N., Kasahara, S., Tosa, Y., Mayama, S., & Nakayashiki, H. (2008). Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, *Magnaporthe oryzae*, using a high-throughput RNA-silencing system. *Molecular Microbiology, 68*(6), 1348–1365.

Pausch, M. H., Kaim, D., Kunisawa, R., Admon, A., & Thorner, J. (1991). Multiple Ca2+/calmodulin-dependent protein kinase genes in a unicellular eukaryote. *The EMBO Journal, 10*(6), 1511–1522.

Rasmussen, C. D. (2000). Cloning of a calmodulin kinase I homologue from *Schizosaccharomyces pombe*. *The Journal of Biological Chemistry, 275*(1), 685–690.

Roberts, D. M., & Harmon, A. C. (1992). Calcium-modulated proteins-targets of intracellular calcium signals in higher-plants. *Annual Review of Plant Physiology, 43*, 375–414.

Solomon, P. S., Rybak, K., Trengove, R. D., & Oliver, R. P. (2006). Investigating the role of calcium/calmodulin-dependent protein kinases in *Stagonospora nodorum*. *Molecular Microbiology, 62*(2), 367–381.

Talbot, N. J. (1995). Having a blast, exploring the pathogenicity of *Magnaporthe grisea*. *Trends in Microbiology, 3*, 9–16.

Talbot, N. J., Ebbole, D. J., & Hamer, J. E. (1993). Identification and characterization of MPG1, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *The Plant Cell, 5*, 1575–1590.

Tsai, P. J., Tu, J., & Chen, T. H. (2002). Cloning of a Ca(2+)/calmodulin-dependent protein kinase gene from the filamentous fungus *Arthrobotrys dactyloides*. *FEMS Microbiology Letters, 212*(1), 7–13.

Xu, J. R. (2000). Map kinases in fungal pathogens. *Fungal Genetics and Biology, 31*(3), 137–152.

Xu, J. R., & Hamer, J. E. (1996). MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes & Development, 10*(21), 2696–2706.

Yang, Y., Cheng, P., Zhi, G., & Liu, Y. (2001). Identification of a calcium/calmodulin-dependent protein kinase that phosphorylates the *Neurospora circadian* clock protein FREQUENCY. *The Journal of Biological Chemistry, 276*(44), 41064–41072.

Zelter, A., Bencina, M., Bowman, B. J., Yarden, O., & Read, N. D. (2004). A comparative genomic analysis of the calcium signaling machinery in *Neurospora crassa*, *Magnaporthe grisea*, and *Saccharomyces cerevisiae*. *Fungal Genetics and Biology, 41*(9), 827–841.