Development of a novel RNA silencing system for functional genomics in the rice blast fungus, Magnaporthe oryzae

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Development of a novel RNA silencing system for functional genomics in the rice blast fungus, *Magnaporthe oryzae*

イネ科植物いもち病菌における RNA サイレンシングを用いた機能ゲノム学システムの構築

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Contents

List of symbols

RdRp: ENA dependent RNA polymerase

siRNA: small interfering RNA

RISC: RNA induced silencing complex

dsRNA: double-stranded RNA

HDGS: homology dependent gene silencing

TGS: transcriptional gene silencing

PTGS: post transcriptional gene silencing

MIP: methylation induced premeiotically

MSUD: meiotic silencing by unpaired DNA

RNAi: RNA interference

hp dsRNA: hairpin double-stranded RNA

ihp dsRNA: intron hairpin double-stranded RNA

eGFP: enhance green fluorescence protein

PKS : polyketide synthase

MPG1: manose-1-phosphate guanyltransferase

MAC1 : Magnaporthe adenyl cyclase

CPKA : cAMP dependent protein kinase A

MAPK : Mitogen activated protein kinase

REMI : The refraction microtremor

PEG : polyethylene glycol

PKC: protein kinase C

CAM: calmodulin

ER: endoplasmic reticulum

PLC: phospholipase C

CNA: calcineurin A (catalytic subunit)

CNB: calcineurin B (regulatory subunit)

HACS: high-affinity uptake calcium

LACS: low-affinity uptake calcium

DAG: diacylglycerol

$InsP_3R$: inositol-(1,4,5)-triphosphate receptor

CsA: cyclophilin A

MEN: mitotic exit network

HOG: high osmolarity glycerol

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February 2008, Kobe, Japan

Nguyen Bao Quoc

CHAPTER I

General Introduction

1. An overview of RNA silencing

The stories of RNA silencing began from a study of target mRNA inhibition induced by a construct expressing complementary RNA strand of β-galactosidase mRNA under the control of promoter P_L in cell-free extracts. When antisense mRNA of β-galactosidase occurred, the synthesis of β-galactosidase protein was mostly inhibited (98%) (Pestka *et al.* 1984). This was called "antisense technology" that was also discovered in plants when the napoline gene in tobacco cells was suppressed (Rosthstein *et al*., 1987). However, scientists at this time could not imagine what mechanisms suppressed the expression of the target genes. In the beginning of 1990s, the first reports of gene silencing on pigmentation of petunia flower were published in international journals (Napoli *et al.*, 1990; Van der Krol *et al.*, 1990). Scientists tried to deepen the purple color of pentunia petals by overexpressing the *Chalcone synthase* transgene (CHS) involving in the anthocyanin pathway under the control of the 35S promoter. However, instead of expected deep purple color, appearance of variegated or even white color of petunia flowers indicated that the CHS gene was suppressed. This surprising observation was named "cosuppression" due to the inhibition of both the transgene and homologous endogenous gene. The other coincidence has also been observed in *Neurospora crassa* when Romano and Machino carried out a study to develop orange color of *N.crassa* by an introduced gene involved in a carotenoid synthesis pathway (1992). Most of *N.crassa* transformants showed "null" phenotype instead of expected deeper orange. "Quelling" was named for this phenomenon that can be observed at some next generations and then was back to the normal coloration. By 1995, Guo and Kemphues provided the first evidence that sense RNA can trigger gene silencing that is similar with silencing efficiency caused by antisense RNA (asRNA) in *Caenorhabditis elegans*. In this experiment, asRNA or sense RNA (sRNA) was injected in *C.elegans* to inhibit the *par-1* gene for identification of its function. The products of the *par-1* gene disappeared by both of sense and antisense RNA. These results cause the confusion with previous observations because scientists expected that asRNA can combine with sense RNA of the *par-1* gene to inhibit its transcription. Three years later, Fire and his colleagues explained this phenomenon by studies on *C.elegans* in 1998. The purpose of those is to check mutually interaction between sense and antisense RNAs for inhibiting gene expression. They recognized that double-stranded RNA (dsRNA) inhibited gene silencing 10 folds in comparison with either of sense or antisense RNA alone. Moreover, when dsRNA molecule was eliminated, silencing phenomenon gradually lost even using sense or antisense RNAs. Therefore, those results indicated that the key factor causing gene silencing is dsRNA

and the scientific term" RNAi" was named for this phenomenon. By this approach, Richard Cathew and his co-workers suppressed about 20 genes in *Drosophila* including *frizzled 2* and *wingless* involved in wing development. They has also proved that the injection of dsRNA into embryos of fruit fly induces more efficient silencing than feeding of yeast containing dsRNA. At the same time, Hannon's group at Cold Spring Habor Laboratory, New York has succeed in inhibiting the expression of cycling E and mycE involving in a developmental pathway and cell division of *Drosophila* by RNAi. Therefore, although some precise functions remain unclear, molecular mechanisms of RNA silencing is divided into two phases based on genetic studies in *C.elegans* and plants, and on biochemical studies in *Drosophila* (Bass, 2000). In the first phase, dsRNA "trigger" is cleaved by a member of the RNase-III family of nuclease called DICER (Bernstein *et al.*, 2001; Hammond *et al.*, 2001; Ketting, R.F. *et al.*, 2000) into 21-25 nucleotides shorter fragments called small interfering RNAs (siRNAs) (Elbashir *et al*., 2001; Zamore *et al.*, 2000). In the later phase, siRNAs are then incorporated into a multi-protein complex, known as the RNA-induced silencing complex (RISC) (Hanmond *et al.*, 2000). Activated RISC uses a single-stranded siRNA as a guide to target cognate mRNA for degradation by Slicer endoribonuclease (Elbashir *et al*, 2001). The degradation occurs near the middle of the region bound by the siRNA strand (Fig. 1).

Since the discovery of "cosuppression" in petunia, homology-dependent gene silencing (HDGS) has become more popular in plants. There are two concepts of HDGS including transcriptional gene silencing (TGS), by which the transcription of transgenes is suppressed because the promoters are inhibited by DNA methylation, and post transcriptional gene silencing (PTGS), by which the transcripts of transgenes are degraded by RNA silencing pathway. Through the discovery of involvement of dsRNA in the degradation or destruction of target mRNA, it is believed that molecular mechanisms underlying 'quelling', 'cosuppression', 'RNAi' or 'PTGS' are quite similar. As a generic term to describe all phenomena, 'RNA silencing' is proposed.

2. RNA silencing in plant pathogenic fungi

Plant parasitic fungi have been considered as the first pathogens in the history of science causing serious damages to plant crops. Until now, there are over 100,000 estimated fungal species that are the principal pathogenic agents causing diseases in plants. Each of pathogenic fungi can attack one to many different kinds of plants and vice versa. They attack living plants through various specialized ways to take nutrients. For example, by using infection structures such as plant penetrating hypha for the first

step of penetration or fungal molecules like melanin, glycerol, hydrophobin, enzymes and etc. for cell wall degradation and destruction of host plants.

There are many approaches to control plant fungal diseases such as plant selection, soil health, sanitation, crop rotation, watering, fungicide or pesticides in traditionally. However, improvement of plant resistance to fungal pathogens has remained a major challenge to ensure food supply for an increasing population in the world. For this purpose, the complexity of plant-fungus interactions and pathogenicity should be understood and elucidated, especially in the post genomic era when the genome sequences of plant important fungi are going to be known. To do this, molecular strategies such as R gene mediated resistance with "gene-for-gene" theory (Flor, 1971), the use of R gene pyramiding in a cultivar, transgenic technology, studies on broad-spectrum resistance pathways like the induced systemic resistance (ISR) pathway, the insect-responsive pathway involving jasmonic acid (JA), and the systemic acquired resistance (SAR) pathway that describes enhanced resistance ability of plants against pathogens. Functional identification of pathogenicity genes in a genomics world of fungal pathogen is also important to contribute the understanding plant-fungal interactions. In the past, forward genetic (conventional genetic) like gene replacement, insertional mutagenesis, nucleotide-based approaches, microarray analysis, serial analysis of gene expression, two-hybrid system, protein arrays, expression analysis (reviewed in Lorenz, 2002; Sweigard and Ebbole, 2001) were used for high-throughput analysis of gene function in filamentous fungi. However, recent genome sequencing efforts have been gradually finished that open a new trend of reverse genetics used to identify gene functions in the genomes of plant pathogenic fungi. Discovery of the first gene silencing phenomenon in *Neurospora crassa* (Romano and Macino, 1992) has contributed basic backgrounds for research and applications in plant pathogenic fungi so far. Demonstrations of gene silencing phenomena in phytopathogenic fungi such as C*ladosporium fulvum* (Hamada and Spanu, 1998); *Fusarium oxysporum* f-sp *lycopersici* (Ito *et al.*, 2002); *Magnaporthe oryzae* (Kadotani *et al.*, 2003, Nakayashiki *et al.*, 2005); *Venturia inaequalis* (Fitzgerald *et al*., 2004); *Phytopthora infestans* (Whisson *et al.* 2005); *Ophiostoma floccosum, O. piceae* (Tanguay *et al.*, 2006); and *Bipolaris oryzae* (Moriwaki *et al.*, 2007) have opened new opportunities not only for basic research but also its applications to reduce crop losses in cultivation.

3. Mechanisms of RNA mediated gene silencing in filamentous fungi

Since the discovery of quelling phenomenon in *Neurospora crassa,* it has been considered as an excellent model to study mechanisms of RNA mediated gene

silencing. The stories of RNA silencing mechanisms in fungi have been gradually elucidated due to the production of dsRNA causing the degradation of endogenous mRNA was explained by Fire and Mello in 1998. The identification of siRNA (small interfering RNAs) processed by dsRNA in mRNA destruction (Zamore *et al.*, 2000) has led to many efforts in elucidation of enzymes that is responsible for this function. One of recently identified enzymes is RNAse III family member, named "Dicer", functioning in producing short RNA duplexes of 21-23 nucleotides which target the selective destruction of homologous RNAs. In fungi, Dicer-like proteins have been recently identified in *Neurospora crassa* with *DCL1*, *DCL2* and *Magnaporthe oryzae* with *MDL1*, *MDL2* in which *DCL2* and *MDL2* were considered as major proteins in the dsRNA processing into siRNAs (Catalanotto *et al.,* 2004; Kadotani *et al.,* 2004, Murata *et al*., 2007). Following the identification of Dicer like proteins in fungi, other important enzymes such as Argonaute proteins containing two conserved domains, namely the PAZ and Piwi domains that are responsible for binding siRNA and cleaving the target mRNA by RNase H endoribonucleases respectively (Song *et al.,* 2004), and RNA-dependent RNA polumerase (RdRp) that functions in converting "aberrant" RNA into dsRNA, have been elucidated in many organisms. In *Neurospora crassa*, two Argonaute proteins including *QDE-2* that required for quelling (Fagards *et al.,* 2000) and *QDE-1* that is responsible for RdRp (Cogoni and Macino, 1999) were identified in the post-transcriptional gene silencing mechanism. In *Schizosaccharomyces pombe*, a single Dicer, RdRp and Argonaute protein (*Dcr1, Rdp1*, and *Ago1*) was demonstrated in both transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (Sigova *et al.,* 2004). Recently, together with the completion of genomic sequencing projects of fungi, homologues of components involving in RNA silencing pathway have been found with identified numbers of Dicer-like proteins, Argonaute, and RdRp proteins in a wide range of fungi in which RNAi activity has been reported by using a comparative genomics approach and phylogenetic studies (Nakayashiki *et al.,* 2006). Until now, RNA silencing mechanism in filamentous fungi can understand as a homology dependent gene silencing (HDGS) that was indicated that gene inactivation can be gained through some strategies of transcriptional gene silencing such as repeat-induced point mutation, MIP (methylation induced premeiotically or transnuclear transcriptional gene silencing) and post-transcriptional gene silencing such as quelling or meiotic silencing by unpaired DNA (MSUD) (reviewed in Carlo Cogoni, 2001; Hitoshi Nakayashiki, 2005). Moreover, RNA silencing phenomena have been predicted in many fungal species including *Ascomycota, Basidiomycota,* and *Zygomycota* based on molecular evolution and diversification of RNA silencing

proteins in fungi (Nakayashiki *et al.,* 2006).

4. Applicable trends of RNA silencing in plant pathogenic filamentous fungi

Recently, functional genomics research has been in progress in filamentous fungi by using a variety of functional genomics tools to understand molecular mechanisms of fungal pathogenesis. In post genomic era, when the number of sequenced fungal genomes such as *Neurospora crassa* (Galagan *et al.,* 2003)*, Magnaporthe oryzae* (Dean *et al.,* 2005)*, Aspergillus oryzae* (Machida *et al.,* 2005), *Phanerochaete chrysosporium* (Martinez *et al.,* 2004), *Aspergillus fumigatus* (Nierman *et al.,* 2005)*, Phaeosphaeria nodorum, Sclerotinia sclerotium, Botrytis cinerea, Fusarium graminearum,* and *Nectria haematococca* has been increased remarkably due to recent advanced sequencing technologies, RNA silencing has been widely used for identify gene functions in the genomes of filamentous fungi. However, most of them have just demonstrated in initial assessment of RNA silencing by using hairpin or intron hairpin dsRNA constructs that showed highly efficient in plant filamentous fungi by using eGFP gene as a model gene as well as other endogenous genes (table 1). However since the hairpin vectors require two steps of orientated cloning, they are not feasible on a global scale. Other strategy in RNA interference approach for this applicable trend is to use dual promoter system expressing dsRNA under the control of two opposing promoters. In *Magnaporthe oryzae,* although silencing efficiency of dual promoter system is not higher than that of single promoter system, it seems to be high enough to explore gene functions in fungal genomes. Moreover, dual promoter system showed its applicability in functional genome-wide screens by using co-silencing method. Herein, eGFP gene was used as an indicator gene and GFP silenced transformants were chosen to analyze phenotypic changes. Functional identification of 37 calcium signaling related genes in the rice blast fungus by using this strategy indicated that this system is very useful for gene functions in the genome at the large scale because it saved laborious time and easily inserts target genes or cDNA libraries (Quoc *et al.*, 2008)

One of potential applications of RNA silencing approach in phytopathogenic fungi is to elucidate molecular mechanisms involving fungal infection on the host plants. The infection process of fungi is composed of several key factors such as conidiation, conidia dispersal, conidia attachement to the host surface, appressorium formation, penetration and invasive growth. Of these factors for the infection process of plant pathogenic fungi, the cyclic AMP pathway (cAMP) was known to be important for host surface recognization in the first step of fungal infection and the

deletion of several important genes for example a hydrophobin (*MPG1)*, a membrane binding protein *PTH11,* the adenyl cyclase (*MAC1)*, the catalytic subunit of protein kinase A (*CPKA)* and the heterotrimeric GTP-binding proteins-α-subunit (*MAGB*) in *M.oryzae* involving this pathway will lead to reduced disease symptom. Other transduction pathway, MAPK signaling also plays an important role in fungi and the deletion of its genes is well characterized in surface recognition, appressorium formation, invasive growth and pathogenicity. Previously, "Forward Genetics" approaches such as REMI, tranposon tagging, T-DNA and etc. were used to demonstrate gene functions involving in above signaling transduction pathways. However, when genome sequences of several fungi have been identified, "Reverse Genetics" approaches should be better for functional genome-wide screens. By comparative analysis of gene homologous through genomic sequence data of several fungi, it has become convenient for the scientists to identify the sequence of target genes on each organism. Then they will be suppressed quickly at the large scale by using RNA silencing method. For example, the knock down of *MPG1* involving in *Magnaporthe oryzae* hydrophobin caused the decrease of pathogenicity in rice by using silencing construct pSilent-1 (Nakayashiki *et al.*, 2005) or the suppression of alpha-tomatine involving in cell wall degraded enzyme by RNA silencing can lead to the limitation of infection on tomato (Ito *et al.*, 2002). Moreover, RNA silencing has been applied for elucidating preinfection structures formation in *Phytopthora infestans* causing late blight on potato. For this purpose, the function of *P.infestan Cdc14* proteins involving in mitosis and the cell cycle was demonstrated in sporulation but not hyphal growth by using homology-dependent gene silencing (Ah Fong *et al.,* 2003). RNA silencing was also used for functional identification of *P.infestans* G proteins including α-subunit, *Pigpa1,* and β-subunit, *Pigpb1*. The zoospores produced by the *Pigpa1*-silenced mutants decreased about 20-45% in comparison with the wildtype strain resulting in the reduction of pathogenicity on leaves ranging from 3% to 14% compared with 77% in the wildtype strain (Latijinhouwers *et al.,* 2004). Meanwhile, the *Pigpb1* silenced mutants produced many abnormal zoospores leading to the influence of fungal development and sporangium formation (Latijinhouwers *et al.,* 2003).

Recently the functions of genes involving Ca^{2+} signaling machinery in the rice blast fungus, *Magnaporthe oryzae,* were also demonstrated by RNA silencing tool through co-silencing strategy (Quoc *et al*., 2008). In spite of the appearance of single gene silencing and co-suppression due to sequence similarity, the results in this study indicates that the applicability of RNA silencing on functional genome-wide screens is

very convenient for the scientists to understand the interaction between the host and phytopathogenic fungi.

Table 1. RNA silencing mediated hairpin or intron hairpin dsRNA constructs have been used in various phytopathogenic fungi.

No.	Phytopathogenic fungi	Transformation method	Name of silencing constructs	dsRNA system	Gene models	References
1	*Magnaporthe oryzae*	PEG	pEGFP-SA pSilent-1	hp dsRNA ihp dsRNA	eGFP, PKS MPG1	Kadotani *et al*., 2003 Nakayashiki *et a*l., 2005
2	*Venturia inaequalis*	PEG	JK1-GFP JK4-THN JK1-THN-GFP	hp dsRNA	GFP THN	Fitzgerald *et al*., 2004
3	*Cladosporium fulvum*		pHCF-1	Anti-sense	HCf-1	Hamada and Spanu, 1998
4	*Phytophthora infestan*	*in vitro*		Synthetic dsRNAs	GFP, *inf1* *cdc14*	Whisson *et al*., 2005
5	*Aspergillus flavus* *Aspergillus parasiticus* *Fusarium graminearum*	PEG	pTMH44.2 pTMH52.1 pTRM-*tri6*-*Hyg*	hp dsRNA	*A.f.aflR* *F.g.tri6*	McDonald *et al*., 2005
6	*Ophiostoma floccosum* *Ophistoma piceae*	*Agrobacterium*	Of-PKS1-1 Op-PKS1-1	hp dsRNA	PKS1	Tanguay *et al*., 2006
7	*Bipolaris oryzae*	REMI	pSilentPKS1-2	ihp dsRNA	PKS1	Moriwaki *et al*., 2007

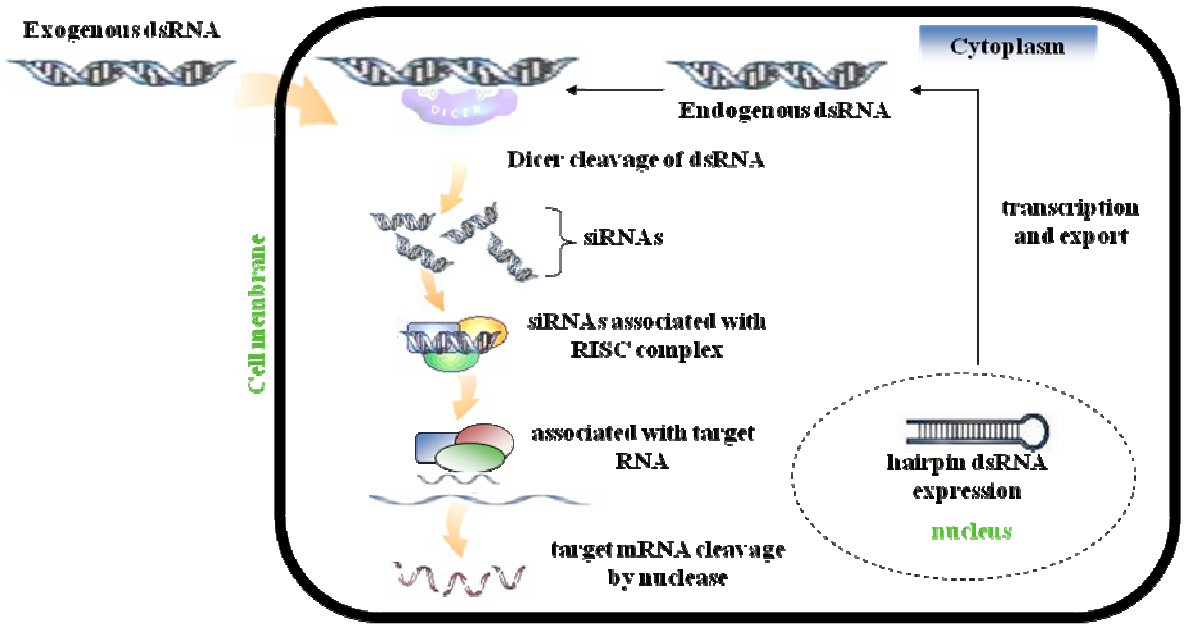


Figure 1: Molecular mechanism of RNA silencing

CHAPTER II

A dual promoter system for genome-wide gene function analysis by an RNA silencing approach in the rice blast fungus, *Magnaporthe oryzae*

I. Introduction

RNA silencing nowadays is one of hottest new research fields in biology that has coalesced from independent studies on various organisms during the last decades. RNA silencing offers efficient tools that knock-down or suppress the expression of genes through the delivery of 21-23 nucleotides short interfering RNA (siRNA) into a living cell. Recently, RNA silencing has been demonstrated in fungi such as *Neurospora crassa,* C*ladosporium fulvum, Cryptococcus neoformans, Mucor circinelloides, Magnaporthe oryzae, Venturia inaequalis, Histoplasma capsulatum, Aspergillus fumigatus, Aspergillus flavus, Aspergillus parasitius, Aspergillus graminearum, Phytopthora infestans, Ophiostoma floccosum, O. piceae, Bipolaris oryzae* (Romano and Macino, 1992; Hamada and Spanu, 1998; Liu *et al.*, 2001; Ito *et al.*, 2002; Nicolaùs *et al.*, 2003; Kadotani *et al.*, 2003, Nakayashiki *et al.*, 2005; Fitzgerald *et al*., 2004; Rappleye *et al*.2004; Mouyna *et al.*, 2004; McDonald *et al.*, 2005; Whisson *et al.* 2005; Tanguay *et al.*, 2006, and Moriwaki *et al.*, 2007). Most of those studies were carried out using the silencing constructs that produce hpRNA or ihpRNA. Hairpin or intron hairpin RNA expressing constructs, however, have some drawbacks such as two cloning steps with inverted repeats, difficulty in sequencing, time-consuming, and costly usage of the oligos for generation.

In previous study, we already established the silencing vector, pSilent-Dual1 (pSD1) that carries two convergent opposing RNA polymerase II promoters, *Aspergillus nidulans TrpC* (PtrpC) and *gpd* (P*gpd)*, can induce RNA silencing pathway in the rice blast fungus, *Magnaporthe oryzae* and can be a feasible alternative to existing systems for exploring gene function in filamentous fungi (Quoc *et al*., 2008). Although silencing efficiency of pSD1 is lower than hpRNA-or ihpRNA RNA silencing vectors, this silencing vector was shown to work efficiently on a large scale of endogenous or exogenous genes that is generally limited in hairpin or intron hairpin silencing contructs. Furthermore, high-throughput application of pSD1 for exploring gene function in ascomycete fungi was also described in systematic functional analysis of calcium signaling proteins in the gemone of rice blast fungus, *Magnaporthe oryzae.* (Quoc *et al.*, 2008).

In this work, we show the correlation of not only between the length of inserts and silencing efficiency induced by two dual promoter silencing vectors, pSD1 and pSD2 (inverted PtrpC promoter plasmid), but also co-silencing between the indicator gene, GFP and the target. This assessment will indicate whether silencing vectors can be applied for further genome-wide RNAi screening in the rice blast fungus, *Magnaporthe oryzae* particularly and generally in other filamentous fungi.

II. Materials and Methods

2.1. Fungal strains, culture condition and transformation.

A *Magnaporthe oryzae* strain used in this study was the wheat (*Triticum aestivum* (L.)) infecting isolate of *M.oryzae,* Br48 (Urashima *et al.*, 1999). It was maintained on potato dextrose agar (PDA) for several months. For long-term storage, *M.oryzae* was cultured on sterile barley seeds soaked with sucrose and then dried in a plastic box with silica gel and kept in 4^0C as previously described (Nakayashiki *et al.*, 1999). For DNA or RNA extraction and protoplast preparation, *M.oryzae* cultures were grown in 100 ml of CM liquid broth (0.3% casamino acids, 0.3% yeast extract, 0.5% scucrose) at 26^0C.

Fungal protoplasts were prepared by digesting fungal cell wals with lysing enzymes (Sigma) and fungal transformation was performed via polyethylene glycol (PEG)-mediated method as previously described (Nakayashiki *et al.,* 1999). For secondary screening, antibiotic resistant fungal colonies were transferred to 96-wells micro-plates containing CM or PDA agar media at concentrations of 400 μg/ml hygromycin B. After 3 days culture, GFP fluorescence of transformants was measured by Multilabel Counter, Arvo-SX (PerkinElmer, USA) with 485 nm excitation and 535 nm emission wavelengths.

2.2. Construction of silencing vectors

To generate two dual promoter vectors, pSilent-Dual1 (pSD1) constructed with the two RNA polymerase II promoters, the *Aspergilus nidulans trpC* promoter (PtrpC) and *Aspergilus nidulans gpd* promoter (Pgpd) and pSilent-Dual2 (pSD2) constructed with the two *Aspergilus nidulans trpC* promoters (PtrpC) in the inverted repeat orientation, three PCR-amplified fragments of above promoters and a neomycin-resistant gene cassette, were subsequently cloned into pBluescript SK+II (pBS) (Statagene La Jolla, CA, USA). Those fragment were amplified with the high fidelity DNA polymerase, KOD Plus (Toyobo, Japan) and sets of primers listed in the table 1 using pSH75, pNOM102, pII99 as templates, and inserted into the SalI/ClaI, SacI/NotI for pSD1, SalI/ClaI, NotI/BamHI for pSD2, and XhoI site in pBS, respectively (Fig.1)

Various dual promoter system based silencing vectors expressing duplicated GFP-RNA in different sizes and orientations and duplicated PKS-RNA were also obtained by inserting green fluorescent protein (eGFP) fragments (0.72 kb, 0.41 kb and 0.26 kb) and a 0.55 kb fragment of the polyketide synthase like gene (PKS) amplified

from pEGFP75 and a template of *Magnaporthe oryzae* genomic DNA. Then, they were inserted into the *Sma*I site of dual promoter vectors. For a co-silencing approach, a 0.41 kb fragment of the eGFP gene was first inserted at the SmaI site in both of silencing vectors, and a fragment of endogenous gene, cutinase xylanase (XYL) and Ca^{2+} permeable channel protein (MGG05643.5) are subsequently inserted at the EcoRV site in the resulting plasmid. The primer sequences of all genes used in study were reported in table 1.

2.3. Imaging and measurements of GFP fluorescence

After culturing on 96 wells micro-plates of PDA media containing appropriate antibiotics for 3 days the GFP fluorescence of transformants was measured by Wallac 1420 Multilabel Counter, Avro-SX (PerkinElmer, USA) and imaged by the ProXPRESS Proteomic Imaging System and ProFinder software (PerkinElmer) with 485 nm excitation and 535 nm emission wavelengths. The fluorescent images were also recorded by the PENTAMAX camera and analyzed with METAMORPH software (Nippon Roper, Chiba, Japan).

2.4. Phenotypic and enzymatic assays of the PKS and cutinase xylanase mutants

The *PKS* (melanin polyketide synthase) gene was chosen to determine whether the dual promoter system could be used to control the expression of endogenous genes in the rice blast fungus. Transformation was obtained via PEG method as previously described (Nakayashiki *et al.,* 1999). The transformants were picked up randomly and grown on the PDA media containing 400 μg/ml hygromycin B. After culturing for 10 days, silencing efficiency of the *PKS* gene was evaluated by classifying melanin colors of fungal colonies into 5 categories (Fig. 3)

To evaluate the activity of endogenous enzyme cutinase xylanase in the rice blast fungus, the cutinase xylanase gene (*XYL*) was chosen and cloned into the silencing construct, pSD1-$GFP_{0.4}$. Tranformation was carried out using PEG method as previously described (Nakayashiki *et al.,* 1999). The transformants were picked up randomly and grown in 96 wells plate containing PDA media with 400 μg/ml hygromycin B. After culturing for 4-5 days, 5 mM of 4-methylumbelliferyl-beta-D-xylopyranoside was mixed with umbelliferone buffer (50 mM Na_2PO_4+NaH_2PO_4, 1 mM beta-ME, 5 mM EDTA) and added into the each well of 96-wells plates. Enzymatic activity was measured by Wallac 1420 Multilabel Counter, Avro-SX (PerkinElmer, USA) with 365 nm excitation and 460 nm emission

wavelengths after 2 hr of incubation at 30^0C.

2.5. Southern hybridization

Fungal genomic DNA was extracted from transformants by a phenol extraction method as described previously (Nakayashiki *et al*., 1999) or by Plant Genomic DNA Extraction Miniprep System (Viogene, USA) according to the manufacturer's instruction. Southern blot analysis was performed using the dioxetane chemiluminescence system Gene Image™ (Amersham Biosciences, Piscataway, NJ, USA). Genomic DNA was digested with one or more appropriate restriction enzymes and then fractionated genomic DNAs were run on a 0.7% Tris-acetate EDTA agarose gel and transferred to Hypond N^+ (Amersham Bioscience). Membranes were pre-hybridized for 2 hrs at 68^0C and then the labeled probe made from Dig-High Prime (Roche, Cat.No. 1 585 606) was added into prehybridization solution for an additional 16 hrs of hybridization. After hybridization, they were washed in 2xSSC, 0.1% SDS at room temperature for 5 min, twice in 2x SSC, 0.1% SDS at 68^0C for 20 min and then twice for 20 min in 0.1xSSC containing 0.1% SDS at 68^0C. The hybridization signals were detected using Anti-Digoxigenin–AP, Fab fragments (Roche, Cat.No. 1 093 274) and *CDP-Star Ready to use* (Roche Molecular Biochemicals) according to the manufacturer's guides.

2.6. Northern hybridization

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNAs (16 µg/lane) were separated by electrophoresis on a 1.2% formadehyde agarose gel and transferred to Hypond N^+ membrane (Amersham Bioscience). Pre-hybridization and hybridization was performed in ULTRAhyb™ (Ambion) at 68^0C. RNA probes were prepared with Dig RNA Labeling Mix, (Roche, Cat.No. 1 277 073), using the T3 and T7 transcriptional system (Roche, Mannheim, Germany). Blots were washed twice in 2x SSC containing 0.1% SDS for 20 minutes and then twice in 0.1% SSC containing 0.1% SDS at 68^0C. Hybridization signals were detected using Anti-Digoxigenin –AP, Fab fragments (Roche) and CDP-Star Ready to use (Roche) according to the manufacturer's guides.

2.7. Quantitative RT-PCR (qRT-PCR)

Total RNAs (1 µg) were reverse-transcribed into first-strand cDNA by PrimeScript RT reagent Kit (Takara Shuzo, Japan) and amplified by qPCR Mastermix for SYBR Green I (Nippongene, Japan) using specific primer sets of each target gene

and two house-keeping genes (actin and EF1). Fluorescence from DNA-SYBER Green complex was monitored by ABI PRISM 7000 Sequence Detection System (Applied Biosystems) throughout the PCR reaction. The level of target mRNA, relative to the mean of both reference housekeeping genes, was calculated by the comparative Ct method described by the manufacturer (Applied Biosystems).

III. Results

3.1. Silencing efficiency of dual promoter vectors, pSD1 and pSD2 correlates with the length of inserts

To evaluate the efficiency of gene silencing by using dual promoter system in the rice blast fungus, *Magnaporthe oryzae,* different sizes (0.7 kb, 0.4 kb and 0.2 kb) of the enhanced green fluorescence protein (eGFP) selected as a model gene, were inserted at *SmaI* site in multi cloning site (MCS) of RNAi mediated dual promoter vectors, pSD1 and pSD2 respectively, resulting a library of pSD1-GFP and pSD2-GFP (Fig. 2). Those following plasmids and pSD1, pSD2 as negative controls, respectively, were introduced into the eGFP expressing *M..oryzae* strain. Transformants, produced by PEG mediated method, were randomly collected and transferred on PDA media containing hygromycin B in 96 well plates. After 3-4 days culturing, GFP fluorescence of the transformants was measured using Wallac 1420 Multilabel Counter, Avro SX (PerkinElmer, USA). Silencing efficiency was evaluated by the classification of transformants into 5 categories based on the intensity of their GFP fluorescence relative to the original strain. As shown in the figure 2, dual promoter vectors can efficiently suppress GFP fluorescence expression with various degrees in comparison with the empty vector as a control. In transformants introduced by pSD1 based silencing vectors, 39% reduction of eGFP expression was observed at strong silencing levels (0-20%) when using a 412 bp eGFFP gene in sense orientation. This was compared with 16% and 28% reduction of eGFP expression at the same degree when dsRNAs corresponding to 720 bp and 264 bp eGFP genes in sense orientation were respectively delivered. In contrast, high and moderate silencing levels of GFP fluorescence (0-20% and 21-40%) was also observed with 27% of total transformants introduced by pSD2 vectors when the full-length eGFP sequence (720 bp) was used. The proportion of transformants showing at the same degrees of silencing decreased with 17% and 13% of total transformants when using 412 bp and 264 bp eGFP genes respectively.

Although the GFP fluorescence silencing ability of pSD1 based silencing

constructs with the sense orientation of *eGFP* gene under the control of *Pgpd* seems to be slightly higher than that of them with the antisense orientation of *eGFP* gene under the control of *PtrpC*, the proportion of transformants showed that various silencing degrees induced by dual promoter system, did not differ much between sense and antisense orientations of 720- and 264-bp eGFP genes in pSD1 particularly as well as between dual promoter silencing constructs, pSD1 and pSD2 (Fig. 2). This could be concluded that oriented insertion of target genes is not required in dual promoter silencing vectors because dsRNA of eGFP gene created from a pool of sense and antisense strands of RNA driven from two opposing promoters can cause a reduction in various degrees of target genes in the rice blast fungus. Furthermore, the results described in these experiments indicated that effective size of target genes should be limited at approximately 500 bp or smaller to gain a better silencing efficiency when using dual promoter system as the literature suggestion.

3.2. Silencing of M.oryzae endogenous gene by dual promoter system

To further examine the potency of convergent promoter vector, pSD1, for controlling the expression of endogenous genes in *M.oryzae*, a polyketide synthase (PKS)-like gene (MG072194) was chosen as a target for silencing. The PKS gene is involved in melanin production which is known to be an essential factor for pathogenicity, i.e. appressorial function, in the phytopathogenic fungus *M.oryzae* (Howard *et al*., 1996; Kubo *et al.*, 1991; Langfelder *et al.,* 1998). An approximately 0.55 kb fragment of the PKS gene was amplified by KOD-Plus and cloned into the vector, pSD1, at the *SmaI* site. The resulting plasmid was introduced to *M.oryzae* strain by a polyethylene glycol mediated transformation as previously described.

To evaluate the effectiveness of pSD1-PKS for controlling expression of the PKS gene, the wildtype strain and Dicer mutant were also used as shown in the experiment of the eGFP gene (Quoc *et al.,* 2008). Based on the melanin color of transformants observed on PDA media, they were categorized into five classes from white colonies (completely silenced phenotype) to dark colonies (non-silenced phenotype). The results depicted that RNA silencing of the endogenous gene, PKS, in *M.oryzae* was also successfully induced by the pSD1 based construct. Since wildtype transformants showed various levels of melanin colors but almost none of transformants of the Dicer mutant showed decreased melanin colors. Herein, of the 70 wildtype transformants produced, 5.7% showed complete white phenotype (level 5), and 24% and 28% fell into level 4 and level 3 silencing, respectively. Eighty four percent of colonies transformed by the empty vector, pSD1, showed no silencing in

melanin color. Similar to silencing with the eGFP gene (Quoc *et al*., 2008) the silencing of PKS was not observed in the Dicer mutant strain. The results indicated that over 90% of transformants with pSD1-PKS showed melanin colors corresponding to level 1 (72%) and level 2 (21%). This rate was compared to 90% of the control colonies transformed by empty vector, demonstrated similar to one observed in transformants with the empty vector (90% fell into level 1) (Fig. 3).

3.3. Molecular analysis of silenced and non-silenced *M.oryzae* transformants

Genomic Southern analysis was carried out to examine the status of integrated vectors such as pSD1-GFP and pEGFP75 in transformants with silenced and non-silenced phenotype. Intact pSD1 in the genomic DNA is essential for driving sense and antisense RNA to form long dsRNA processed to the effector siRNAs. In addition, detection of intact pEGFP75 in the transformant's genome is also important since genetic changes in the pEGFP75 loci should affect GFP fluorescence in the transformants. To address this, Southern blot analyses were performed with the eGFP and PKS genes. In the case of the eGFP gene, genomic DNA of six transformants each from those with strong and weak GFP fluorescence was extracted and digested with *SacI* and *KpnI*, then followed by blotting and probing with the gpd promoter (Pgpd). A hybridizing band of approximately 1.7 kb indicating the full-length of pSilent-Dual-GFP expression cassette was detected in all the silenced transformants used but not in non-silenced transformants (Fig. 4A). This was probably due to the integration of the plasmid with interruption of the transcriptional unit of the eGFP gene or some rearrangement that might have occurred after the integration event (Kadotani *et al*., 2003). The pEGFP75 was also detected at 2.8 kb in all the transformants examined. The 2.8 kb fragment corresponds to the entire transcriptional unit of the eGFP gene in pEGFP75 when digested with *EcoRI and XbaI,* therefore indicating that all of the transformants retain the intact eGFP gene (data not shown). We next examine the expression of the eGFP gene in both of silenced and non-silenced transformants to determine if the loss of GFP fluorescence observed on silenced transformants has a correlation with a lower level of eGFP mRNA accumulation. Total RNA of the parent transformant and transformants with or without the intact silencing vector pSD1-GFP was isolated using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Northern blot analysis revealed that reduced levels of eGFP mRNA accumulation were detected in the silenced transformants, and were in parallel with the presence of pSD1-GFP in the genome. Different levels of eGFP mRNA accumulation observed in the silenced transformants may be the cause of various degrees of

silencing. In contrast, high levels of eGFP mRNA were produced in the control sample (eGFP expressing *M.oryzae* strain) and the non-silenced transformants without intact pSD1-GFP (Fig. 4A).

Southern and Northern analyses were also used to examine transformants with pSD1-PKS. Genomic southern blots with *SacI* and *KpnI* digestion and a Pgpd probe indicated that a hybridizing 1.0 kb band, corresponding to one of two parts of full-length of pSD1-PKS, were detected in the genome of silenced transformants (Fig. 4B). In contrast, pSD1-PKS was not detected in the genomic DNA of non-silenced transformants. PKS mRNA of both silenced and non-silenced transformants was examined by northern analyses. The results indicated that PKS mRNA accumulation was significantly reduced in the six silenced transformants compared to the six non-silenced transformants (Fig. 4B).

3.4. Co-silencing as an effective mean of multi-genes silencing induced by dual promoter system

As described above, dual promoter system can be used to inhibit the expression of target genes via the RNA silencing pathway. To examine the applicability of this system for further functional genome-wide screening in *M.oryzae*, a co-silencing method was attempted with the eGFP and cutinase xylanase (XYL) genes. For this purpose, silencing construct, pSD1-$GFP_{0.41}$ with sense orientation was chosen and a 0.7 kb XYL gene encoding xylanase XYL2 (Wu *et al.*, 1995) was cloned into above silencing constructs at the *EcoRV* site and then introduced into the eGFP--expressing *M.oryzae* strain through polyethylene glycol mediated transformation. Evaluation of GFP fluorescence of the resulting transformants was performed as described previously. Xylanase activity was also investigated at the same 96-wells-plate which was used for GFP fluorescence evaluation through various degrees of Umbelliferyl fluorescence. Herein, the substrate 4-Methylumbellyferyl-β-D-xyloyranoside (MX) was used because xylanase enzyme can cut the hydrogen (H) bond at the position of β-1,4 to release 4-methylumbelliferyl measured by Wallac 1420 Multilabel Counter, Avro-SX (PerkinElmer, USA) with 365 nm excitation and 460 nm emission wavelengths after 2 hr of incubation at 30^0C. Silencing efficiency of xylanase enzyme was evaluated by the classification of transformants into 5 categories based on the intensity of their Umb. fluorescence relative to the original strain. The correlation between eGFP and XYL genes was analyzed by Sigma Plot Statistic Program with linear regression method. The result showed that R value of pSD1-$GFP_{0.41}$/$XYL_{0.7}$ is nearer to 1 (R=0.67) (Fig. 5A). This

value was also confirmed more at molecular level through the performance of quantitative RT-PCR (qRT-PCR) that clearly affirmed effective correlation of mRNA concentration between GFP and the target MGG05643.5 described by R=0.71 (Fig. 5B). Those equations of both experiments indicated a good description between eGFP gene (the independent variable) and target genes (the dependent variable) that can be used at the global scale for functional genome-wide screening in the rice blast fugus, *M.oryzae.*

IV. Discussion

RNA silencing technique showed in this study has been developed as an alternative method to overcome the drawbacks of single promoter system for assessing gene functions in various species of fungi. Here, we demonstrated the relationship among silencing efficiency and the length, orientations of inserts in dual promoter system. In contrast to the single promoter system, silencing capability of both dual promoter vectors, pSD1 and pSD2, can be achieved mainly at moderate silencing levels more than at strong silencing level due to remarkable contribution of GFP and PKS genes silenced transformants at 21-60% and level 3, 4 respectively compared to wildtype phenotypes. Furthermore, the number of GFP fluorescence silenced transformants was higher for dual promoter silencing constructs with approximately 500 bp GFP fragments that was consistent with the results for not only silencing of endogenous gene, polyketide synthase (PKS) in this study but also higher activity of Dicer enzyme on 500 bp dsRNAs in humans and 670 bp PKS1 dsRNA in phytopathogenic fungus, *Ophiostoma piceae* (Bernstein *et al.*, 2001; Tanguay *et al.*, 2006). Previously, silencing efficiency of pSD1 was showed to be a little lower than that of hpRNA- or ihpRNA- expressing RNA silencing vector (Quoc *et al.*, 2008). This was probably due to the difference in dsRNA formation that requires physical annealing of two difference RNA molecules in the target cells in the pSD1 system while that in the hpRNA systems is achieved by self-folding of inverted repeats within an RNA molecule (Dubief *et al*., 2003). It is also possible that this drawback is caused by the domination of one promoter, leading to the production of only sense or antisense RNAs (Tran *et al.*, 2003). However, this is unlikely to be the case because silencing efficiency was not remarkably increased with pSD2, which carries two opposing TrpC promoters. Although silencing efficiency was involved in the orientation of inserts that was observed clearly at approximately 500 bp in length when induced by pSD1 based silencing constructs, it seem be not clear about the influence of inserts' orientation to silencing efficiency in the case of 720 and 264 bp GFP fragments in this study.

One strategy can be used to overcome the major drawback of dual promoter system involving in silencing efficiency is co-silencing method. Although simultaneous inhibition was applied for some organisms such as mammalian cell, pathogenic fungi, etc. using hairpin vectors (Yu *et al.*, 2003; Liu *et al.,* 2001; Mounya *et al.,* 2004), there's no evaluation about the correlation levels amongst them in details. In this study, transcription fusion of the target genes such as cutinase xylanase (XYL) and MGG05643.5 to an eGFP gene fragment that functioned as an indicator of the silencing level in the resulting transformants resulted in simultaneous silencing of both genes effectively even though single gene silencing sometimes occurs at a fixed rate. Therefore, this strategy can reduce laborious work, time and the number of selectable markers significantly during functional screening in silencing experiments at the large scale that was demonstrated in systemic functional analysis of Ca^{2+} signaling proteins in the genome of the rice blast fungus, *Magnaporthe oryzae* (Quoc *et al.*, 2008).

In short, the scope described in this study introduced a novel method that promises a feasible alternative to other system for systemic approaches in functional genomics. This system can be applied easily and quickly for generating cDNA silencing libraries that maybe useful to study the function of a gene family as a group due to simultaneously silencing of all family members, essential genes that cannot be knocked out due to lethality or sequence restrictions and some genes that have no obvious phenotype *in vitro*. Therefore, the association of this approach with other currently employed methods such as gene disruption techniques or sequence-specific mutagenesis will provide deepen insights of gene functions in the genome of not only rice blast fungus, *Magnaporthe oryzae* but also other filamentous fungi.

Table 01: Sets of gene-specific primers used in this study

Primer designation	DNA-Sequence	Target
PtrpC-ClaI-R	5'-TCAT'CGATGCTTGGGTAGAATAGGTAAG-3'	*trpC* promoter
PtrpC-SalI-F	5'-GGCCG'TCGACGACGTTAACTGATATTGAAG-3'	
Pgpd-SacI-F	5'-TTAGAGCTCGCCCCGAAGTGGAAAGGCT-3'	*gpd* promoter
Pgpd-NotI-R	5'-TAGCGGCCGCAAATAAAGGTTCTTGGATGG-3'	
GFP_{720}-HindIII-F	5'-CCCAAGCTTATGGTGAGCAAGGGCGAGGAG- 3'	GFP gene
GFP_{720}-HindIII-R	5'-CCCAAGCTTCCCTTGTACAGCTCGTCCAT -3'	
GFP_{412} -HindIII-F	5'-TGAAGCTTCCACCTACGGCAAGCTGACCC -3'	
GFP_{412} -ClaI-R	5'-CTGATCGATCCGTCCTCGATGTTGTGGCGG-3'	
GFP_{264} -SpeI-F	5'-CCCACTAGTCGATGCCACCTACGGCAAGCT-3'	
GFP_{264} -XbaI-R	5'-CCCTCTAGAATGCTGTTCACCAGGGTGTCG-3'	
GFP_{80} - F	5' -CTACCCCGACCACATGAAGCAGCACGACT-3'	
GFP_{80} - R	5'-AGAAGATGGTGCGCTCCTGGACGTAGCCTT-3'	
MGPKS-ApaI-F	5'-GCGGGGCCCCAAGCTGGCCATTGTCGGTAT-3'	PKS gene
MGPKS-HdIII-R	5'-CGCAAGCTTGCCCACAGCGAAGAGCAGGCA-3'	
MGXYL-Bm-F	5'-ATCGGATCCTCAGCTGTAAGGACGCGTCCC-3'	Cutinase
MGXYL-Bm-R	5'-ATCGGATCCTCCAACACAGGCAGGAACGGG-3'	xylanase gene
$MGG05643.5_{100}$ -F	5'-ATGTCTCAGCGTGTCGTCAACATCAGTGGT-3'	Ca^{2+} permeable
$MGG05643.5_{100}$ -R	5'-CAAAATGCCGCCTTTCGGCGCTAGGT-3'	channel
Mg-Actin -F	5'-GCGGTTACACCTTCTCTACCAC-3'	Actin
Mg-Actin -R	5'-AGTCTGATCTCCTGCTCAAAG-3'	
Mg-EF1 - F	5'-GTGACCACATGATCTCGGAGT-3'	EF1
Mg-EF1 - R	5'-CCTTCTTACCGTCCTTGTTGAC-3'	

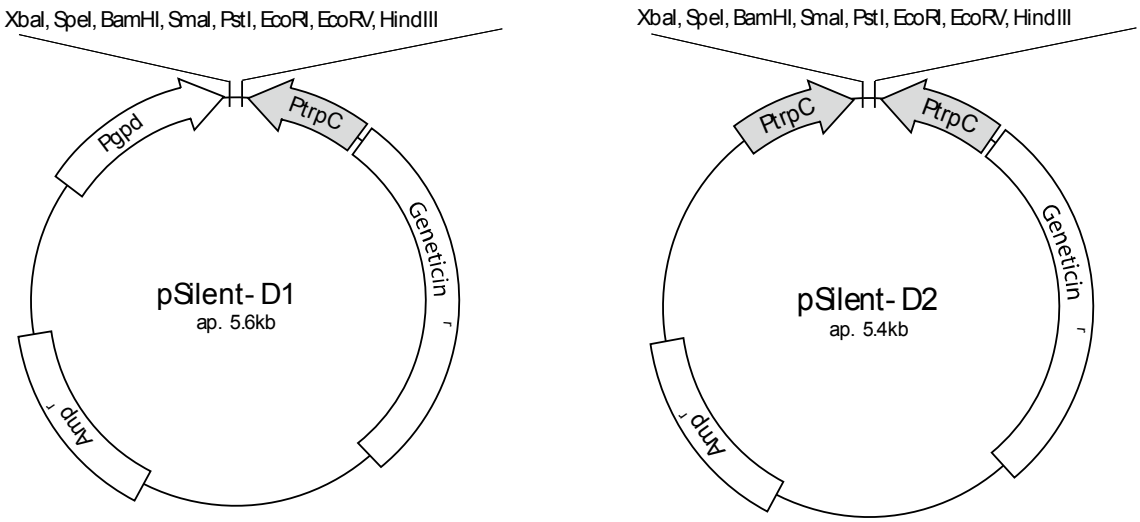


Figure 1. Schematic diagram of dual promoter silencing vectors, pSD1 and pSD2. PtrpC, *Aspergillus nidulans* trpC promoter; Pgpd, *A. nidulans* gpd promoter; Amp^r, ampicillin-resistant gene.

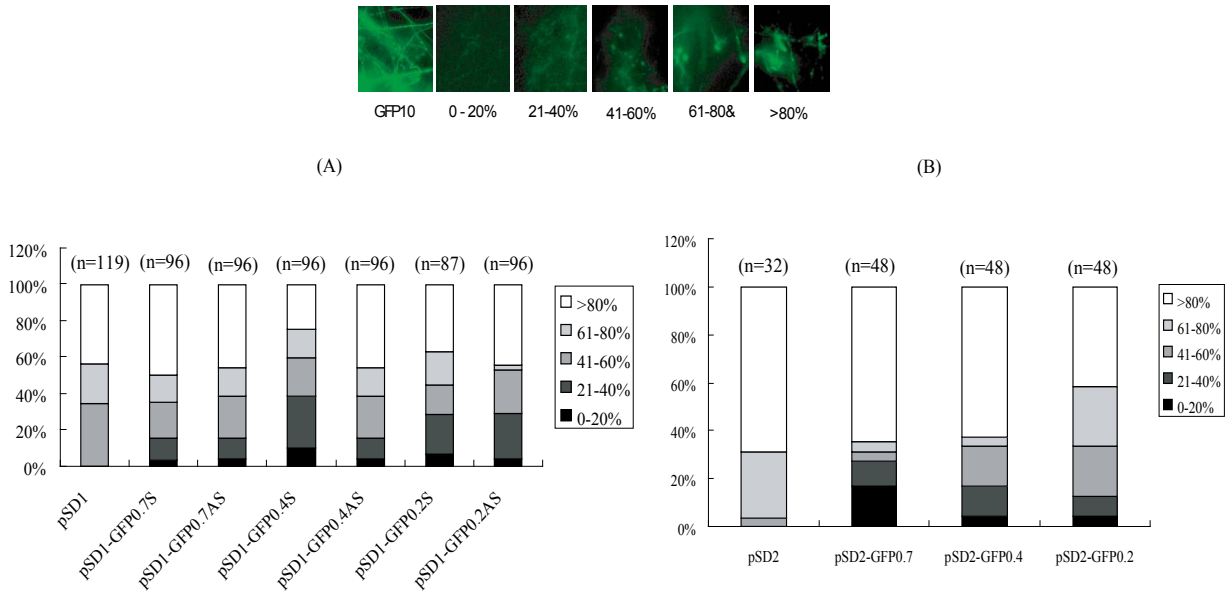


Figure 2. Relationship among silencing efficiency with the length of inserts and their orientations. Six pSD1 based silencing constructs that contain 0.72, 0.41 and 0.26 kb fragments of the eGFP gene in sense and antisense orientation respectively, three pSD2 based silencing vectors with similar above different sizes of eGFP gene and pSD1, pSD2 as a negative control, were introduced into eGFP-expressing *M.oryzae* strain with the wildtype background. At least seventy transformants each were picked up and classified into 5 categories (0-20%; 21-40%, 41-60%, 61-80% and >80%) based on relative fluorescence to the parent strain.

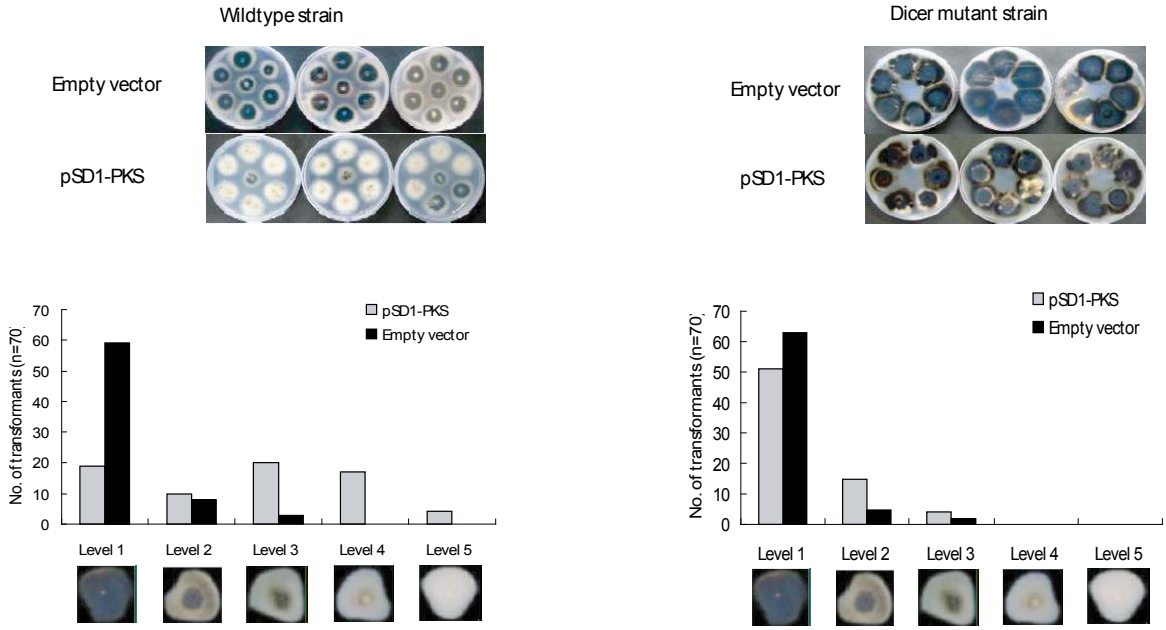


Figure 3. The silencing vector, pSD1-PKS, can knock-down the phenotype of the *PKS* gene in the wildtype strain of *M.oryzae* at various melanin levels but not in Dicer mutant strain. Transformants were picked up and grown in PDA media containing 400 µg/ml hygromycin B within 10 days. Based on the melanin color of transformants observed on PDA media, they were categorized into five levels from dark colonies (non-silenced phenotype) to white colonies (completely silenced phenotype). Efficiency of *PKS* gene's suppression was showed in the graph.

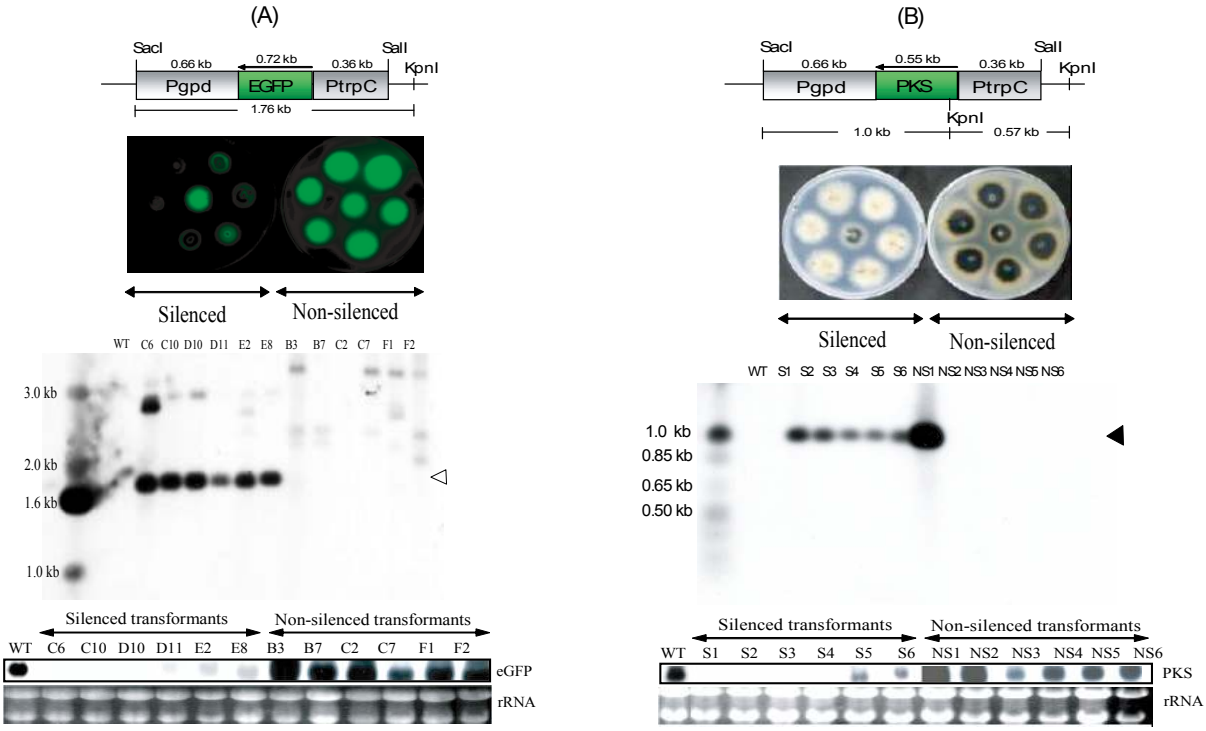


Figure 4. Southern and Northern analyses of the *M.oryzae* transformants with silenced and non-silenced phenotypes of *EGFP* and *PKS* genes. For Southern analysis, Genomic DNA was digested with SacI and KpnI and probed with a gpd promoter (Pgpd). (A) An open triangle indicates the 1.76 kb fragment corresponding to the full-length of pSilent-Dual-GFP expression cassette. (B) A closed triangle indicates the 1.0 kb band representing a part of full-length of the *PKS* silencing construct, pSilent-Dual-PKS. Northern blot analyses of silenced and non-silenced transformants containing *EGFP* and *PKS* silencing constructs. Total RNA was extracted and loaded in each lane of 1.2% formaldehyde agarose gel. Equivalent loading of total RNA in all lanes was confirmed by ethidium bromide-stained rRNA bands (bottom panel). (A) *EGFP* probe was used to detect the accumulation of *EGFP* mRNA in all transformants. The resulting RNA blot showed major reduction of *EGFP* mRNA in most of silenced transformants, but not in non-silenced transformants. (B) Identical RNA blot was hybridized with *PKS* probe. mRNA reduction of endogenous gene, *PKS*, was also detected in all silenced transformants, but not in non-silenced ones.

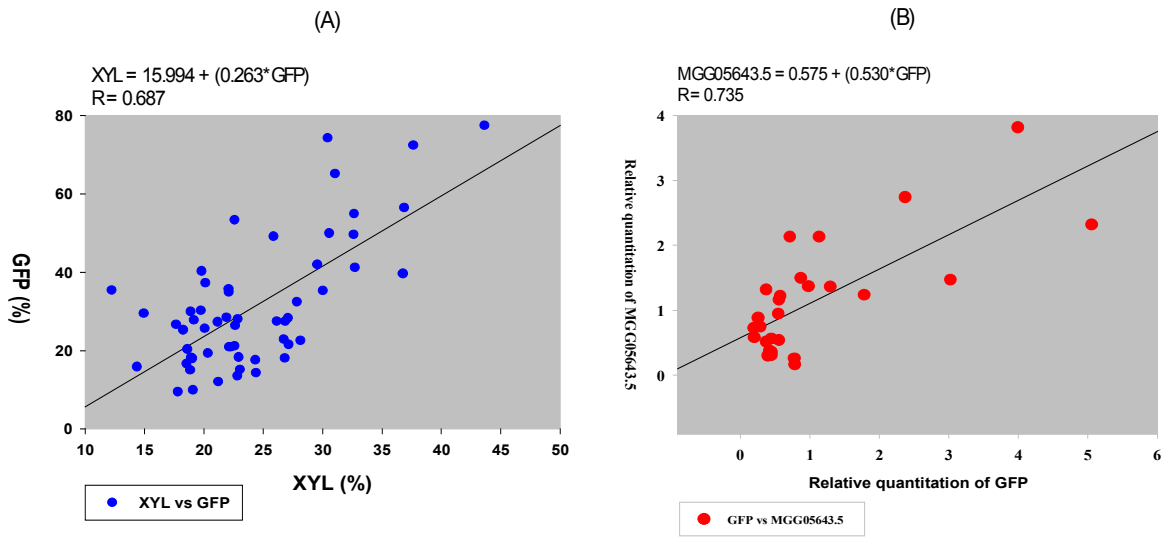


Figure 5. Correlative analysis of co-silencing method by using phenotypic assay with *eGFP* and cutinase xylanse (*XYL*) genes and quantitative RT-PCR (qRT-PCR) with eGFP and Ca^{2+} permeable channel (MGG05643.5). Linear regression method was used by Sigma Plot Statistic Program for analyzing the correlation between *eGFP* and above targets whose their R value is 0.68 (A) and 0.73 (B) respectively. Specific primers of actin and EF1-α genes were used as a control.

CHAPTER III

Systematic functional analysis of calcium signaling proteins in the genome of the rice blast fungus, *Magnaporthe oryzae,* using a high-throughput RNA silencing system

I. Introduction

The filamentous fungus, *Magnaporthe oryzae* is one of the most devastating threats to rice production worldwide. Because of its genetic tractability and availability of genome sequences for both rice and the fungus, the rice-blast system has been considered as a leading model to study plant–fungal pathogen interactions (Dean *et al.,* 2005). The infection process of *M. oryzae* involves a set of developmental events: production of asexual spores, conidia; germination of conidia; formation of a specialized infection structure called an appressorium; generation of great turgor pressure within the appressorium; formation of the infection peg to penetrate the host cell wall, and subsequent growth of invasive hyphae in the host cells (Howard and Valent, 1996). The morphological and physiological transitions during the process are stimulated through perception of environmental signals and are mediated by a variety of intracellular signal transduction pathways.

One such pathway is calcium-mediated signal transduction. In eukaryotic cells, the resting cytosolic free Ca^{2+} concentration $[Ca^{2+}]_c$ is maintained at an extremely low level, primarily by the activity of Ca^{2+}-pumps and -transporters. External signals such as hormones and environmental factors cause transient increases in $[Ca^{2+}]_c$ which serve as a second messenger to activate a number of diverse downstream signaling molecules exemplified by protein kinase C (PKC) and Ca^{2+}/calmodulin (CAM)-binding kinases (Bush, 1993; Gehring *et al.,* 1990). A variety of fundamental physiological processes such as differentiation, circadian rhythm, cell cycle, and stress responses have been shown to be regulated by Ca^{2+}-mediated signal transduction in many eukaryotic organisms (Sanders *et al.,* 2002; Berridge *et al.,* 2003; Kraus and Heitman, 2003; Hepler, 2005).

In filamentous fungi, relatively little information is available on the molecular mechanisms of Ca^{2+}-mediated signal transduction. Nevertheless, molecular genetic and pharmacological studies in filamentous fungi have provided evidence for the involvement of Ca^{2+} in the fundamental physiological processes as well as in fungal specific development such as sporulation, spore germination, hyphal orientation, hyphal branching and appressorium formation (Greene *et al.*, 2002; Joseph and Means, 2002; Adamikova *et al.*, 2004; Nelson *et al.*, 2004; Bencina *et al.*, 2005; Steinbach *et al.*, 2006; Brand *et al.*, 2007). By using a comparative genomics approach, Zelter *et al.*, 2004 have identified Ca^{2+} signaling proteins on a genome-wide scale in *Neurospora crassa*, *Magnaporthe oryzae*, and the budding yeast *Saccharomyces cerevisiae*. The identified proteins include most but not all of the components of Ca^{2+}-mediated signal transduction known in higher eukaryotes. Of interest, larger numbers of Ca^{2+} signaling

proteins in *N. crassa* and *M. oryzae* than in *S. cerevisiae* implied a greater complexity of the Ca^{2+} signaling machinery in filamentous fungi than yeasts.

In this study we performed genome-wide functional analysis of Ca^{2+} signaling proteins in *M. oryzae* using an RNA silencing approach. The target includes 37 genes encoding fundamental Ca^{2+} transport proteins (for example. Ca^{2+}-permeable channels, -pumps and -transporters), Ca^{2+} signal mediator proteins (phospholipase C, CAM, calcineurin, calnexin) as well as other Ca^{2+} binding proteins. The gene set includes more than twenty genes whose orthologs have never been genetically examined in filamentous fungi. RNA silencing or RNA interference (RNAi) offers efficient tools for knocking-down the expression of a gene of interest in a living cell. An advantage of the RNA silencing approach is its possible applicability to essential genes, which cannot be knocked-out. Some fundamental Ca^{2+} signaling proteins such as phospholipase C, CAM, calcineurin, and the Ca^{2+}/CAM-dependent kinase (Rad53) are known to be essential genes in *S. cerevisiae* or other fungi (Allen *et al.*, 1994; Chung *et al.*, 2006; Rasmussen *et al.*, 1990, 1994). Therefore, RNA silencing may offer a uniform platform for a systematic study of Ca^{2+} signaling proteins. To date, gene knock-down experiments by RNA silencing were mostly carried out using silencing vectors producing hairpin or intron-containing hairpin RNA (hpRNA or ihpRNA) in filamentous fungi (Liu *et al.*, 2001; Kadotani *et al.*, 2003, Fitzgerald *et al.*, 2004; Rappleye *et al*., 2004; Mouyna *et al.*, 2004; McDonald *et al.*, 2005; Tanguay *et al.*, 2006). Since construction of a hpRNA- or ihpRNA-expressing vector requires two steps of oriented cloning, its applicability is generally limited to a small or moderate scale. To overcome the limitations of hpRNA-expression vectors, we have established a dual promoter system of RNA silencing in filamentous fungi, allowing construction of silencing vectors in a high-throughput manner. The silencing vector, pSilent-Dual1 (pSD1) carries two convergent opposing RNA polymerase II promoters, *Aspergillus nidulans TrpC* (P*trpC*) and *gpd (*P*gpd*), and multi-cloning sites between the promoters. Independent transcription of a target gene from each promoter produces a pool of sense and anti-sense RNAs in the cell, which would combine together into long dsRNAs to be processed into siRNAs by Dicer. This system allowed us to identify novel Ca^{2+} signaling-related genes involved in various biological aspects of *M. oryzae* such as growth, sporulation, appressorium formation, and pathogenicity.

II. Material and Methods

2.1. Fungal strains, culture condition and transformation

The *M. oryzae* strain used in this study was Br48 (wheat infecting isolate) (Urashima *et al.*, 1999). Br48-GFP is a transformant of Br48 with the eGFP gene. Br48-GFP had no detectable phenotype with regard to growth rate, pigmentation, sporulation, germination, appressorium formation, conidial morphology and pathogenicity. They were maintained on potato dextrose agar (PDA) for several months. For long-term storage, *M. oryzae* was cultured on sterile barley seeds soaked with sucrose and then dried in a plastic box with silica gel and kept in 4^0C as previously described (Nakayashiki *et al*., 1999). For DNA or RNA extraction and protoplast preparation, *M. oryzae* cultures were grown in 100 ml of CM liquid broth (0.3% casamino acids, 0.3% yeast extract, 0.5% scucrose) at 26^0C.

Fungal spheroplasts were prepared by digesting fungal cell walls with lysing enzymes (Sigma) and fungal transformation was performed via a polyethylene glycol (PEG)-mediated method as previously described (Nakayashiki *et al.,* 1999). For secondary screening for silenced transformants, antibiotic resistant fungal colonies were transferred to 96-wells micro-plates containing CM or PDA agar media at concentrations of 400 μg/ml hygromycin B. After 3 days culture, GFP fluorescence of the transformants was measured by the multilabel counter, Arvo-SX (PerkinElmer, USA) with 485 nm excitation and 535 nm emission wavelengths.

2.2. Growth rate, Sporulation, conidial germination and appressorium formation

The growth rate of *M. oryzae* transformants was estimated from an average colony size (diameter) of three replicates at 3, 6 and 9 days after inoculation on PDA plate media. Colony diameter was serially measured on a diameter line drawn on the underside of the culture plates. For sporulation assay, *M. oryzae* transformants were grown on oat agar media at 25^0C for 6 days. To promote sporulation, aerial hyphae were washed off by rubbing mycelial surfaces with cotton balls and the cultures were kept at 25^0C for 2 days under near-ultraviolet radiation provided by fluorescent lamps (FL20s BLB; National, Osaka, Japan). Ten millilitters of distilled water was added per plate, and mycelial surface was rubbed by cotton balls to collect spores. The spore concentration was estimated by counting spores in the suspension under a microscope with a hemocytometer. For conidial germination and appressorium formation assays, a drop of spore suspension (10^5 spore/ml) was pipetted onto a glass surface, and then placed in a humid plastic box at room temperature. After 12 hours incubation, conidial germination and appressorium formation were checked by microscopic observation of at least 100 spores with three replicates. The experiments were repeated at least three

times independently.

2.3. Pathogenicity tests

Three test plants, barley cv. Nakaizumi and two cultivars of common wheat, Norin 4 and Chinese Spring were used in a seedling inoculation test. Wheat and barley seedlings were grown in plastic pots filled with a mixture of Sakata Supermix soil (Sakata Co.,Yokohama, Japan) and peat moss (3:1) in a greenhouse (25 to 30^0C) for 3 weeks. Preparation of conidial suspension and inoculation were carried out as previously described (Murakami *et al.*, 2000). Inoculated seedlings were incubated in a dark moistened box for 24 hours at 29^0C and then returned to a greenhouse. At 5 to 7 days after inoculation, symptoms were evaluated and rated by 6 progressive grades from 0 to 5 as previously described (Murakami *et al.*, 2000). Mutants showing symptoms rated less than grade 3 were regarded to be significantly reduced in pathogenicity.

2.4. Construction of silencing vectors

To construct the dual promoter vector, pSD1, three PCR-amplified DNA fragments, the *Aspergilus nidulans trpC* promoter (*PtrpC*), the *Aspergilus nidulans gpd* promoter *(Pgpd*) and a neomycin-resistant gene cassette, were subsequently cloned into pBluescript SK+II (pBS) (Statagene, La Jolla, CA, USA). Those fragments were amplified with the high fidelity DNA polymerase, KOD Plus (Toyobo, Japan) and sets of primers listed in supplementary table 1 using pSH75, pNOM102, pII99 as templates (Gritz and David, 1983; Roberts *et al.,* 1989; Beck *et al.,* 1982), and inserted into the SalI/ClaI, SacI/NotI and *Xho*I sites in pBS, respectively (Fig. 1).

Typically, pSD1-based silencing vectors were constructed by an insertion of a PCR-amplified fragment at the *Eco*RV site in the multi-cloning site of pSD1. A coding fragment (mostly 0.5 to 0.6 kb in length) in a target gene was amplified with a set of gene specific primers listed in Supplementary Table 2 and a blunt-end producing DNA polymerase such as KOD plus (Toyobo, Japan) using an ordinary three steps PCR program with an appropriate annealing temperature for the primers. For a co-silencing approach with the eGFP gene, a 0.41 kb eGFP fragment was first inserted at the *Sma*I site in pSD1, resulting in pSD1G. A fragment of a target gene is subsequently inserted at the *Eco*RV site in pSD1G.

2.5. Northern hybridization

Total RNA was isolated using the RNeasy Plant Mini kit (Qiagen) according

to the manufacturer's instructions. Sixteen micrograms of total RNA were separated by electrophoresis on a 1.2% formaldehyde denatured agarose gel and transferred to Hypond N^+ membrane (Amersham Bioscience). Pre-hybridization and hybridization were performed in ULTRAhyb^{TM} (Ambion) at 68^0C. RNA probes were prepared with Dig RNA Labeling Mix (Roche) using the T3 and T7 transcriptional systems. The blots were washed twice in 2x SSC containing 0.1% SDS for 20 minutes and then twice in 0.1% SSC containing 0.1% SDS at 68^0C. Hybridization signals were detected using Anti-Digoxigenin–AP, Fab fragments (Roche) and CDP-Star Ready to use (Roche) according to the manufacturer's guides.

For isolation and detection of siRNAs, frozen mycelia (200 mg) were ground to a fine powder with a mortar and pestle in liquid nitrogen, and 0.2 ml of Tri-Reagent (Sigma) were added. The homogenates were mixed with 0.2 ml choloroform-isoamylalchol (24:1). The mycelial debris was, then, pelleted by centrifugation at 10,000 X g at 4 C for 15 min. The supernatant was carefully removed and equal volume of isopropanol was added. Total RNA was recovered by centrifugation at 10,000 X g for 15 min at 4 C. The pellet was washed with 75% ethanol and resuspended in DEPC-treated water. High molecular weight RNAs were removed by selective precipitation in the presence of 5% polyethylene glycol 8000 and 0.5M NaCl, and small RNAs were then precipitated from the supernatants by adding 3 volume of absolute ethanol and 0.1 volume of 3 M sodium acetate (pH 5.0). Small RNAs was re-suspended in DEPC water and separated on a 12.5% acrylamide/8 M urea denaturing gel after quantified by spectrophotometric analysis. The detection of siRNAs was carried out mainly as previous described by Dalmay *et al.*, 2000. A DNA oligo (25-mer) was used as a molecular weight marker. Equal loading of small RNAs were estimated by ethidium bromide staining of predominant RNAs in the fraction.

2.6. Quantitative RT-PCR (qRT-PCR)

Total RNAs (1 μg) were reverse-transcribed into first-strand cDNA by PrimeScript RT reagent Kit (Takara Shuzo, Japan) and amplified by qPCR Mastermix for SYBR Green I (Nippongene, Japan) using specific primer sets of each target gene and two house-keeping genes (actin and EF1). Fluorescence from DNA-SYBER Green complex was monitored by ABI PRISM 7000 Sequence Detection System (Applied Biosystems) throughout the PCR reaction. The level of target mRNA, relative to the mean of both reference housekeeping genes, was calculated by the comparative Ct method described by the manufacturer (Applied Biosystems).

III. Results

3.1. pSilent-Dual1 (pSD1), an RNA silencing vector with a convergent opposing promoters system for filamentous fungi

To circumvent the limitations of hpRNA- or ihpRNA-expressing RNA silencing vectors, we have developed the RNA silencing vector pSD1 with two convergent opposing RNA polymerase II promoters, P*trp*C and P*gpd,* for filamentous fungi (Fig. 1). To assess the applicability of pSD1 for inducing RNA silencing, we first used the enhanced green fluorescence protein (eGFP) gene as a model. A 0.72 kb fragment of the eGFP gene was inserted at the *Sma*I site of pSD1, resulting in pSD1-GFP. Three plasmids, pSD1-GFP, pST-CUT, which is a pSilent-1 derivative expressing hairpin eGFP RNA (Nakayashiki et al. 2005), and pSD1 as a negative control, were introduced into an eGFP-expressing *M. oryzae* strain. An eGFP-expressing *modcl2* (formerly *mdl2*) knockout mutant was also used as a recipient strain for transformation with pSD1-GFP and pSD1. *Modcl2* is a Dicer-like protein responsible for the so far known siRNA biogenesis in *M. oryzae* (Kadotani *et al.,* 2004; Murata *et al.*, 2007). At least 70 transformants each were detected in every transformation, and subsequently classified into five classes (0–20%, 21–40%, 41–60%, 61–80%, > 81%) based on the intensity of their GFP fluorescence relative to the parent strain. Figure 2 shows the percentage of the transformants belonging to each class of the total transformants. Among pSD1-GFP transformants with the wild-type background, approximately 8% displayed strong silencing of GFP fluorescence (0–20%) and 32% showed significantly decreased levels of GFP fluorescence (21–40%) whereas the vast majority of transformants with the empty vector, pSD1 exhibited GFP fluorescence comparable (> 60%) to the parent strain (Fig. 2). With the Dicer mutant background, almost all transformants either with pSD1-GFP or pSD1 showed strong GFP fluorescence, suggesting that GFP silencing induced by pSD1-GFP was Dicer-dependent. To support this, the accumulation of GFP siRNAs was detected in pSD1-GFP- transformants but not in wild-type strain (Fig. 3A). We also examined the expression level of eGFP mRNA in silenced and non-silenced pSD1-GFP transformants. As expected, reduced eGFP mRNA accumulation was observed at varying levels among the silenced transformants while no reduction in eGFP mRNA accumulation was detected in the non-silenced transformants (Fig. 3B). These results clearly indicated that pSD1-GFP induced silencing of the eGFP gene through the RNA silencing machinery.

Previously, we reported that more than 30% of transformants with pST-CUT

showed strong silencing (0–20% GFP fluorescence relative to the parent strain) (Nakayashiki *et al.,* 2005). In this experiment, a comparable level of GFP silencing was observed in pST-CUT transformants. The efficiency of GFP silencing induced by pST-CUT seemed to be significantly higher than that by pSD1-GFP. This is consistent with a much higher level of siRNA accumulation in pST-CUT transformants than that in a pSD1-GFP transformant (Fig. 3A). Therefore, a pSD1-based vector induced RNA silencing at a practical level but with a little lower efficiency than did a vector derived from pSilent-1.

3.2. Functional analysis of calcium-signaling proteins in *M. oryzae* using the RNA silencing vector pSD1

Zelter *et al.*, 2004 have identified 48, 42, and 40 Ca^{2+} signaling proteins in the genomes of *N. crassa*, *M. oryzae*, and *S. cerevisiae,* respectively. In our study, 37 out of the 42 *M. oryzae* Ca^{2+} signaling proteins were analyzed. These included all the genes listed in their paper but did not include some downstream Ca^{2+}/CAM binding proteins that appear only in their web database. The 37 genes include ones encoding phospholipase C, calreticulin/calnexin, calpactin I heavy chain, calmodulin, calcineurin (regulatory, catalytic), Ca^{2+} exchangers, Ca^{2+} permeable channels, Ca^{2+} pumps, and Ca^{2+}/CAM binding proteins.

To facilitate screening of silenced transformants, we adopted a co-silencing strategy with the eGFP gene as an indicator of silencing in the pSD1 system (Fig. 4). Thereby, a target gene is transcriptionally fused to an eGFP fragment in pSD1, and then introduced into Br48-GFP, which is a transformant of the Br48 strain with the eGFP gene. Silencing of the GFP and target genes was expected to occur simultaneously since their RNAs were transcribed as a single fusion RNA. For this purpose, four transformants with reduced levels of GFP fluorescence were picked up for each target gene, and subjected to further screening for silenced transformants by northern blot analysis. Only transformants that showed reduced levels of target gene mRNA expression in northern analysis were subjected to phenotypic assays of growth rate, pigmentation, sporulation, germination, appressorium formation, conidial morphology and pathogenicity against three different host plants. To save space, selective data of the phenotypic analyses of a representative transformant for each gene are given in Table 1. All the results of the phenotypic analyses and northern blots are provided as Supplementary Table 2 and Supplementary Figure 1, respectively. Figure 5 shows examples of northern blots and some phenotypic analyses of knock-down mutants of calcium pump (MGG04066.5), calmodulin-like (MGG06884.5) and

calpactin heavy chain-like (MGG06847.5) genes. As often reported with knock-down mutants by RNA silencing, in every case, different levels of gene silencing were observed among transformants (Fig. 5). Major phenotypic changes observed in the silenced transformants are described below with respect to the functional category of the proteins.

Ca^{2+}-permeable channels: Ca^{2+}-permeable channels catalyze passive influx of Ca^{2+} across cell membranes into the cytosol from extracellular and intracellular compartments. Three different groups of Ca^{2+}-permeable channels corresponding to the *S. cerevisiae* genes, *Cch1*, *Mid1*, and *Yvc1* (*Trpy1*) were identified as MGG05643.5, MGG04001.5, and MGG09828.5*,* respectively, in *M. oryzae* (Zelter *et al.,* 2004). *Cch1* encodes a plasma membrane-located protein showing sequence similarity to the □1 catalytic subunit of voltage-gated Ca^{2+}-permeable channels. The most striking structural characteristics of this group of proteins are four hydrophobic repeat units, each consisting of six transmembrane domains, that probably assemble into tetramers to form the core of the Ca^{2+} channel. MGG05643.5 is the *Cch1* ortholog in *M. oryzae*. The *mgg05643.5*-knockdown mutants showed a drastic reduction in the rates of sporulation and appressorium formation, and produced malformed conidia (Table 1, see Supplemental Table 1). The rates of growth and germination were also slightly lower in the mutants than in wild-type. Unexpectedly, the *mgg05643.5* knock-down mutants were still infectious to the host plants though at a lower level, indicating that the appressoria formed were functional despite the low formation rate.

Mid1 encodes a stretch-activated permeable channel, which opens in response to mechanical deformation of the membrane. In budding yeast cells, Mid1p was reported to be located in the plasma membrane and the endoplasmic reticulum (ER) membrane (Yoshimura *et al.*, 2004). Knock-down of MGG04001.5, the *Mid1* ortholog in *M. oryzae*, resulted in only a relatively slight change in the phenotypes examined. The rates of sporulation, melanization and appressorium formation were moderately decreased in the mutants but they were almost fully pathogenic against the host plants (Table 1, see Supplemental Table 1).

Yvc1 or *Trpy1* encodes a transient receptor potential-like Ca^{2+}-permeable channel that mediates Ca^{2+} release from the vacuole into the yeast cytoplasm. Recently, Yvc1p has been shown to be a vacuolar mechanosensitive channel responding to an osmotic upshock (Zhou *et al.*, 2003). The knock-down mutant of MGG09828.5 showed severe growth defects on PDA; it produced significantly fewer conidia, some of which were malformed, and also produced significantly fewer appressoria (Table 1).

The mutant retained reduced pathogenicity on barely but appeared to be non-pathogenic on the wheat cultivars. Overall, the Ca^{2+}-permeable channels seemed to be more involved in fungal development (sporulation and/or appressoria formation) than in fungal pathogenicity against the host.

Ca^{2+} pumps: Ca^{2+} pumps belong to the superfamily of P-type ATPases that hydrolyze ATP to drive the active transport of Ca^{2+} from the cytoplasm either out of the cell or into internal stores such as the ER or Golgi. Relatively large numbers of P-type ATPases (12 proteins) were found in the *M. oryzae* genome compared to *S. cerevisiae* (5 proteins) and *N. crassa* (9 proteins) genomes (Zelter *et al.,* 2004). In some cases, a high degree of nucleic acid sequence similarity was detected between overall sequences of the *M. oryzae* ATPases. Due to the sequence-specific nature of RNA silencing, we observed that some groups of closely-related genes were silenced simultaneously at varying degrees (Table 2). Therefore, it is to be noted that the phenotypes described below with the knock-down mutants of those genes resulted from silencing of multiple genes as shown in Table 2.

Pmc1 encodes a vacuolar membrane-located Ca^{2+} pump that works in concert with the vacuolar membrane Ca^{2+}/H^{+} antiporter, Vcx1p, in *S. cerevisiae* cells (Denis and Cyert, 2002). *M. oryzae* has three apparent *Pmc1* homologs, MGG02487.5, MGG04890.5 and MGG07971.5. MGG07971.5 showed further genetic distance from the others in the phylogenetic analysis by Zelter *et al.*, 2004. The knock-down mutants of MGG02487.5, MGG04890.5 and MGG07971.5 all showed a significantly lower growth rate on PDA and a reduction in mycelial melanization (Table 1). None of the mutants formed conidia, except one of the two *mgg07971.5* mutants (see Supplemental Table 1). The *mgg07971.5* mutant produced a few malformed conidia that germinated at a significantly lower rate. However, no appressoria were formed by the mutant on a glass surface. Accordingly, the *mgg07971.5* mutant almost completely lost its pathogenicity against the host plants.

The yeast *Pmr1* gene encodes a Golgi membrane ion pump responsible for transporting Ca^{2+} and Mn^{2+} into the Golgi apparatus, and appears to be the major player for Ca^{2+} homeostasis in budding yeast (Mandal *et al.*, 2003). MGG11727.5 is an apparent *Pmr1* ortholog in *M. oryzae*. Another *M. oryzae* Ca^{2+} pump, MGG04550.5, also belongs to the Type P_{2A} ATPase family as does *Pmr1*. Knock-down of those *M. oryzae Pmr1* homologs resulted in no conidiation and significant slow-growth phenotypes as observed with the knock-down mutants of *M. oryzae Pmc1* homologs. Defects during infection processes were not examined due to there being no conidia

formed by the mutants. Those results indicated that Ca^{2+} pumps played a crucial role in sporulation as well as in normal cell growth in *M. oryzae*. Consistent with the results obtained in *M. oryzae*, growth defects on rich media were also observed in knockout mutants of *Pmr1*-like genes in other fungi. These include *AFPMR1* in *Aspergillus fumigatus* (Soriani *et al.*, 2005), *KlPMR1* in *Kluyveromyces lactis* (Uccelletti *et al.*, 1999), *PMRA* in *A. niger* (Yang *et al.*, 2001), *YlPMR1* in *Yarrowia lipolytica* (Park *et al.*, 1998), and *PpPMR1* in *Pichia pastoris* (Dux and Inan, 2006).

The other *M. oryzae* P-type ATPases were homologous to yeast *Spf1*, *Neo1*, *YOR291W,* and the *Ena1-Ena5* gene family. Even though those proteins had high sequence similarity with Ca^{2+}–ATPases, some of them would transport cations other than Ca^{2+}. *Spf1* protein is mainly localized to the ER and is likely to be involved in the maintenance of ion homeostasis related to the secretory pathway (Cronin *et al.*, 2002). The knock-down mutant of MGG12000.5, the possible *M. oryzae* ortholog of *Spf1*, had defects in growth, sporulation, germination, appressorium formation and pathogenicity (Table 1). In particular, the germination rate of the mutant was lowest among those tested in this study (see Supplemental Table 1). *Neo1* is an essential gene in *S. cerevisiae.* The *Neo1* protein has been shown to localize to the ER and Golgi complex and to be required for a retrograde transport pathway between these organelles in *S. cerevisiae* (Hua and Graham, 2003). MGG04066.5 is the closest homolog of *Neo1* in the *M. oryzae* genome. The *mgg04066.5* knock-down mutants exhibited severe growth defects and barely formed conidia (Table 1). The conidia germinated almost normally but most of them failed to form appressoria. Consequently, the mutants lost pathogenicity against the host plants. MGG06925.5 is the possible ortholog of yeast *YOR291W*, which encodes a putative cation translocating ATPase in *S. cerevisiae* that belongs to the P_5 ATPase family. Similar to the knock-down mutants of the *Pmc1*- and *Pmr1*-like genes, the *mgg06925.5* knock-down mutants exhibited no conidiation and slow-growth phenotypes (Table 1). The amino acid sequences of MGG02074.5, MGG05078.5, MGG10730.5 and MGG13279.5 fell into the *Ena1-Ena5* gene family that consists of Na^{+}-ATPases. Therefore, those genes may not be involved in Ca^{2+} signaling pathways in *M. oryzae*. The knock-down mutants of MGG02074.5, MGG05078.5, and MGG10730.5 showed similar phenotypes, severe growth defects, low sporulation and appressorium formation, except that only the *mgg10730.5* mutants retained pathogenicity against the host even though the degree of virulence of the mutants was reduced (Table 1, see Supplemental Table 1). The *mgg13279.5* knock-down mutant also exhibited severe growth defects but the pathogenicity of the mutant was not examined because there were no conidia formed.

Ca^{2+} exchangers: Ca^{2+} exchangers are electrogenic ion-exchange membrane proteins that maintain a steady level of calcium by removing Ca^{2+} from the cytoplasm to either the outside of the cells or into internal organelles. By molecular phylogenetic and domain analyses, two classes of six possible Ca^{2+} exchangers, five Ca^{2+}/H^{+} antiporters and one Ca^{2+}/Na^{+} antiporter, were identified in the *M. oryzae* genome (Zelter *et al.,* 2004). The *S. cerevisiae Vcx1* and *N. crassa CAX* genes, both of which encode a Ca^{2+}/H^{+} antiporter, were reported to be localized in the vacuolar membrane. However, intracellular localization of the *M. oryzae* Ca^{2+} exchangers is so far unknown. The knock-down mutants of all the *M. oryzae* Ca^{2+} exchangers except MGG01638.5 exhibited very similar phenotypes such as severe defects in appressorium formation, moderate reduction in sporulation and mycelial pigmentation, a slightly slower growth on PDA and almost normal pathogenicity against the host plants (Table 1, see Supplemental Table 1). Relatively higher appressorium formation was observed with the mgg04159-knock-down mutant. The *mgg01638.5* knock-down mutant showed severe growth defects and no conidia formation. Due to high sequence similarity between MGG08710.5 and MGG04159.5, the genes were silenced simultaneously (Table 2). Therefore, the phenotypes of *mgg08710.5* and *mgg04159.5* knock-down mutants resulted from silencing of both, but not one, of the genes.

Phospholipase C (PLC): A phospholipase is an enzyme that converts phospholipids into fatty acids and other lipophilic substances. By cleaving phosphatidyl inositol 4, 5-bisphosphate ($PIP_{2)}$, PLC releases two important second messengers in the calcium signaling pathway; inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). In animal and plant systems, IP_3 induces Ca^{2+} release from intracellular store sites such as ER to the cytoplasm through its binding to a specific receptor (IP3R) on the Ca^{2+} store sites. DAG, on the other hand, functions as an activator of protein kinase C (PKC), which mediates further signaling pathways by phosphorylation of a broad range of target proteins. In filamentous fungi such as *N. crassa* and *M. oryzae*, however, genes encoding IP3R proteins were not found in their genomes (Zelter *et al.*, 2004). Therefore, it is unclear, so far, how Ca^{2+} is released from the intracellular Ca^{2+} stores and if IP_3 is involved in the process in fungi.

Zelter *et al*., 2004 identified 4 homologs (MGG02444.5, MGG05332.5, MGG05905.5, MGG02682.5) of PLC-δ one of the three phosphatidyl inositol-specific PLC isoforms, in the *M. oryzae* genome. The knock-down mutants of MGG02444.5 and MGG02682.5 formed no conidia (Table 1), even though their mRNA expression levels were only partially decreased (see Supplemental Figure 1), suggesting a crucial

role of those genes in the sporulation pathway of the fungus. The growth rate and colony pigmentation were also reduced in the *mgg02444.5* and *mgg02682.5* knock-down mutants. The knock-down mutants of the other two PLC-like genes, MGG05332.5 and MG05905.5, showed relatively milder phenotypic changes. The knock-down mutants showed a slight growth delay at 3 days incubation but not in later stages, and retained, albeit reduced, spore formation ability and pathogenicity against the host plants (see Supplemental Table 1). Their spores germinated normally but malformed spores were often observed. Appressorium formation was significantly suppressed in those two mutants. Knock-out mutants of *S. cerevisiae PLC1*, *N. crassa NcPLC1*, and *Cryphonectria parasitica CPLC1* commonly showed growth defects (Gavric *et al.*, 2007; Chung *et al.*, 2006) as did most of the PLC knock-down mutants of *M. oryzae*. *CaPLC1*, one of the three PLC-like genes in *Candida albicans* was shown to be an essential gene, and its conditional mutant also exhibited growth defects (Kunze *et al.*, 2005). Interestingly, the *C. parasitica CPLC1* mutant expressed reduced appressorium formation and virulence as shown with the *mgg05332.5* and *mgg05905.5* mutants.

Calcium signal transducers (calmodulin, phosphatase, kinase): Calmodulin (CAM) is the primary transducer of cytosolic Ca^{2+} changes in eukaryotes. CAM undergoes a conformational change upon binding to Ca^{2+}, and regulates a multitude of different CAM-binding proteins that are mostly unable to bind calcium themselves. CAM is an essential gene in *S. cerevisiae* and *A. nidulans* (Rasmussen *et al.*, 1990). Two knockdown mutants of *M. oryzae* CAM (MGG06884.5) were obtained. The level of gene silencing was strong in one mutant (S3) but very weak in the other (S2) (see Supplemental Figure 1). The strongly silenced mutant showed severe growth defects, low conidium production, low appressorium formation, and almost no pathogenicity against the host plants (Table 1). In contrast, the weakly silenced mutant grew almost normally in rich media and was pathogenic against the host plants with lower virulence (see Supplemental Table 1). Interestingly, sporulation and appressorium formation were severely affected in the latter mutant despite the low level of silencing, indicating that CAM plays a crucial role in those biological processes. By pharmacological approaches using CAM antagonists such as W7, W5, R24571, TFP, chlorpromazine, 48/80, and calmidazolium, involvement of CAM in growth and/or infection structure developments were also shown previously in various fungi such as *Colletotrichum gloeosporioides, C. coccodes, C. dematium, C. trifolii, Cochliobolus miyabeanus, Erysiphe pisi, N. crassa, C. albicans, Blastocladiella emersonii, Zoophthora radicans,*

Paracoccidioides brasiliensis, M. oryzae, Cercospora nicotiana, Phytophthora infestans, and *Fusarium graminearum* (Uhm *et al.*, 2002; Ahn *et al.*, 2003, 2007; Singh *et al.,* 2001; Warwar *et al.*, 1995; Rao *et al.*, 1997; Roy and Datta, 1987; Simao and Gomes, 2001; Sato *et al.*, 2004; Magalhaes *et al.*, 1991; Carvalho *et al.*, 2003; Lee and Lee, 1998: Liu *et al.*, 1999; Chung, 2003; Judelson *et al.*, 2002, Robson *et al.*, 1991). Our results provided genetic evidence that supports the findings of previous pharmacological studies.

Calcineurin is a conserved Ca^{2+}/CAM-dependent serine/threonine protein phosphatase in eukaryotes that regulates a variety of physiological processes such as cell cycle progression, polarized growth, and adaptation to stresses. Calcineurin consists of two subunits, catalytic subunit A (calcineurin A, CNA) and regulatory subunit B (calcineurin B, CNB). Calcineurin A is an essential gene in *A. nidulans* (Rasmussen *et al.*, 1994). In the *M. oryzae* genome*, S. cerevisiae CNA*- and *CNB*-like genes, MGG074563.5 and MGG06933.5, respectively, were identified (Zelter *et al.*, 2004). Knock-down of the *CNA*-like gene (MGG074563.5) caused only mild growth retardation and moderately reduced sporulation and appressorium formation while knock-down of the *CNB*-like gene (MGG06933.5) resulted in severe defects in vegetative growth, colony pigmentation, sporulation and appressorium formation (Table 1). In addition, the knock-down mutant of the *CNB*-like gene completely lost pathogenicity against the host plant, indicating that the regulatory subunit B plays a more crucial role in the pathogenicity of the fungus than does the catalytic subunit A. Reduced virulence and/or growth were also observed in knock-out mutants of a *CNB*-like gene in *N. crassa* (Koth and Free, 1998) and *C. neoformans* (Fox e*t al.*, 2001). Our results were consistent with previous inhibitor experiments suggesting the involvement of calcineurin in fungal infection-related developments and virulence in *M. oryzae* and other phytopathogenic fungi (Ahn *et al.*, 2007; Lee and Lee, 1998; Viaud *et al.*, 2002, 2003).

We analyzed six possible Ca^{2+}/CAM-dependent serine/threonine protein kinases; MGG00925.5, MGG09912.5, MGG06421.5, MGG08547.5, MGG01196.5 and MGG01596.5. MGG00925.5 and MGG09912.5 were possible orthologs of *S. cerevisiae Cmk1* and *Cmk2,* which are CAM-dependent protein kinases likely playing a role in the stress response. Knock-down of MGG09912.5 more severely affected the phenotypes examined than did that of MGG00925.5 (Table 1, see Supplemental Table 1). The *mgg09912.5* mutants showed strong growth defects and formed no conidia while the *mgg00925.5* mutants had relatively mild growth defects and produced conidia at reduced levels. The *mgg00925.5* mutants caused a few visible symptoms on

the host plants. However, the lesions were mostly not progressive, indicating that the mutants had only reduced virulence in the host cell. Growth defects by knock-out of *Cmk*-like genes were also previously reported in *A. nidulans* (Kornstein *et al.*, 1992; Dayton and Means, 1996; Dayton *et al.*, 1997), *N. crassa* (Yang *et al.*, 2001), and *Stagonospora nodorum* (Solomon e*t al.*, 2006).

The closest *S. cerevisiae* homologs of the other four *M. oryzae* kinases, MGG06421.5, MGG08547.5, MGG01196.5 and MGG01596.5 are *Tos3*, *Rck2*, *Kin4*, and *Dun1*, respectively. The *Tos3* kinase phosphorylates and activates the *Snf1p* kinase, which is an AMP-activated serine/threonine protein kinase playing a key role in the cellular response to nutrient stress. *Rck2* is a mitogen-activated protein kinase-activated protein kinase, and is required for reprogramming of ribosomes during oxidative stress. The *Rck2* kinase was originally identified as a suppressor of cell cycle checkpoint mutations in fission yeast, and later was shown to be involved in the high osmolarity glycerol (HOG) pathway in *S. cerevisiae* (Bilsland *et al.*, 2000, 2004). *Kin4* inhibits the mitotic exit network (MEN) in response to spindle alignment defects (Pereira *et al.*, 2005; D'Aquino *et al.*, 2005). *Dun1* and *Rad53* are related kinases involved in the *Mec1/Rad53/Dun1* pathway that plays a central role in the cell cycle checkpoint response. The pathway governs the transcriptional induction of DNA repair and ribonucleotide reductase genes upon DNA damage (Zhou *et al.*, 1993; Chen *et al.*, 2007). The knockdown mutants of the *M. oryzae Rck2*- and *Dun1*-like genes (MGG08547.5 and MGG01596.5) showed similar phenotypes; growth defects in rich media and no conidium formation (Table 1). Knockdown of the *M. oryzae Kin4*-like gene, MGG01196.5, caused defects in vegetative growth and appressorium formation but, interestingly, not in sporulation (Table 1). The *mgg01196.5* mutant retained pathogenicity against barley with a reduced virulence, but appeared to be no more pathogenic against the wheat cultivars even though some lesions were formed on the cultivars. The knockdown mutants of the *Tos3*-like gene, MGG06421.5, were fully pathogenic against the host plants despite slower growth on rich media and moderately reduced sporulation.

Other Ca^{2+}/CAM binding proteins: Calreticulin and calnexin are related proteins located in the endoplasmic reticulum in higher eukaryotes. Calreticulin is soluble protein in the ER lumen while calnexin is an ER membrane-integrated protein. Both proteins have a Ca^{2+} binding domain and function as a molecular chaperone and as a component of the ER quality control machinery. In the *M. oryzae* genome, only one calreticulin/calnexin-like gene, MGG01607.5 was identified (Zelter *et al.,* 2004).

One of the two knock-down mutants of MGG01607.5 that showed stronger silencing of the gene had severe growth defects in rich media and formed no conidia (see Supplemental Table 1 and Supplemental Figure 1). The other mutant with moderate silencing also had milder growth defects and formed a few conidia that germinated at an approximately 50% lower rate than those of the wild-type, but failed to form appressoria. Calpactin-I is a tetramer consisting of two heavy (36 kD) and two light (11 kD) chains, and is a calcium-dependent membrane- and cytoskeletal-binding protein. Calpactin I is thought to promote membrane interactions during exocytosis as it can aggregate secretory chromaffin granules. Only one homolog of calpactin heavy chain (MGG06847.5) was identified in the *M. oryzae* genome. Knock-down of MGG06847.5 mostly resulted in severe growth defects, no mycelial pigmentation and no conidium formation (Table 1). One *mgg06847.5* mutant with a moderate silencing level produced conidia at a low level but none of them formed appressoria, and therefore, had no pathogenicity against the host plants (see Supplemental Table 1 and Supplemental Figure 1). MGG06180.5 is a possible ortholog of the *S. cerevisiae End3* gene that is an EH domain-containing protein involved in endocytosis, actin cytoskeletal organization and cell wall morphogenesis. In *A. nidulans*, a possible ortholog of *End3* is the *SagA* gene that was shown to affect sensitivity to DNA-damaging agents. The knock-down mutant of MGG06180.5 exhibited a reduced fitness in the rich media and formed no conidia (Table 1).

3.3. Simultaneous silencing of homologous genes in the genome

Since RNA silencing serves as a sequence-dependent gene inactivation system, it is known that transcripts homologous to the target are often suppressed simultaneously. To examine the level and extent of simultaneous silencing of homologous genes in the knock-down mutants used in this study, Quantitative RT-PCR (qRT-PCR) and northern analyses were performed. Homologous genes to each target gene were screened by BLAST search against the *Magnaprothe* database with a cutoff E-value of 1e-3. Since it has been reported that silencing target is sometimes extended from the trigger to its flanking sequences, we used entire coding sequences as query for the BLAST searches. Nine Ca^{2+} pumps, two Ca^{2+} exchangers and one Ca^{2+}/CAM kinase had homologous gene(s) in the genome (Table 2). Most of the homologous genes were other members in the same gene family with the target but some of them seemed to be more distantly related, and were even not included in the *Magnaporthe* Ca^{2+} signaling genes identified by Zelter *et al.*, 2004.

Approximately three-fourths (22/30) of the homologous genes examined were

significantly silenced together with the target gene (Table 2). The other one-fourth homologous genes that were not silenced showed relatively low sequence similarity with the corresponding target gene. In addition, the highly homologous genes (E-value < 1e-9) were strongly silenced at comparable levels to the 'true' target without exception. Therefore, it seemed that the level of simultaneous silencing correlated, to some extent, with the degree of sequence similarity to the target gene. The correlation, however, did not seem to simply depend on the sequence similarity since various levels of gene expression were observed among homologous genes with similar levels of sequence similarity especially when the similarity is relatively low (E-value > 1e-7). It is noteworthy that in some cases the mRNA expression of a non-silenced homologous gene was highly up-regulated in the knock-down mutants compared to in wild-type. This may due to possible functional compensation against the reduced gene expression of the target and its homologous gene(s), which is often reported with a functionally-related homologue with the target gene in a knock-out mutant.

IV. Discussion

4.1. Calcium signaling machinery for infection-related development and pathogenicity in *M. oryzae*

In this study, we have analyzed 37 Ca^{2+} signaling proteins in *M. oryzae* using the RNA silencing vector, pSD1 with a convergent pol II promoter system. This is the first systematic functional analysis of Ca^{2+} signaling proteins in filamentous fungi. The genes examined included almost all fundamental elements involved in upstream Ca^{2+} signaling and its movement across the plasma membrane and between intracellular compartments. Based on the statistical significance of the results, we concluded that at least 26, 35, 22 and 15 of the 37 genes were involved in cell growth, sporulation, appressorium formation and pathogenicity, respectively, in *M. oryzae* (Table 1, Fig. 6). There were two degrees of involvement: (1) as a single gene (indicated by a black superscript notation in Fig. 6), and (2) as a gene family (indicated by a red superscript notation in Fig. 6). We also suggest that more six genes (MGG05643.5, MGG11454.5, MGG01193.5, MGG02682.5, MGG06421.5, MGG01607.5) were possibly involved in cell growth since their knock-down mutants showed significant slow growth at 3 or 6 days after culture (Supplemental Table 1). It should be noted that the pathogenicity of 14 of the 35 sporulation mutants was not examined due to complete lack of conidia (Table 1). In a broader sense, the 14 genes are also involved in the pathogenicity of the fungus since those sporulation mutants would not be pathogenic under natural conditions. In Figure 6, as a summary, we present an overview of the Ca^{2+} signaling machinery in *M. oryzae* by applying the information from studies in yeasts and mammalian cells.

Despite the previous pharmacological and genetic studies on Ca^{2+} signaling in fungal development and pathogenicity, only limited information was available about the role of a specific Ca^{2+} signaling gene in those processes. Our results include a number of novel findings on genetic aspects of Ca^{2+} signaling in filamentous fungi. For example, this study provides first evidence that *Spf1*- and *Neo1*-like Ca^{2+} pumps, calreticulin, and calpactin heavy chain are essential for fungal pathogenicity. *Pmc1*-like Ca^{2+} pumps are also shown to be crucial for fungal pathogenicity as a group in this study. Surprisingly, in addition, 35 of the 37 genes examined were more or less involved in sporulation, and 22 of the 23 genes were associated with appressorium formation. It would be more informative to note that the genes not involved in sporulation and appressorium formation were MGG01381.5 (Ca^{2+} transporter) and MGG01196.5 (*Kin4*-like Ca^{2+}/CAM-dependent protein kinase), and MGG06421.5

(*Tos3*-like Ca^{2+}/CAM-dependent protein kinase), respectively. Particularly, knockdown of *Pmc1*- *Pmr1*- *Spf1*- and *Ena*-like Ca^{2+} pumps (MGG04550.5, MGG04890.5, MGG11727.5, MGG02487.5, MGG06925.5, MGG12005.5, MGG013279.5), phospholipase C (MGG02444.5, MGG02682.5), Ca^{2+} transporter (MGG01638.5), and Ca^{2+}/CAM-dependent protein kinases (MGG00925.5, MGG08547.5, MGG01596.5, MGG06180.5) resulted in a complete loss of sporulation. Therefore, infection-related development seemed to be extremely sensitive to disturbance in calcium homeostasis. Since most of the sporulation and appressorium mutants also showed growth defects, the phenotypic defects in the infection-related development might, at least partly, arise from a disorder of the fungus's basic metabolism. However, growth defects did not always correlate with sporulation defects (for example, MGG01196.5) and vice-versa, sporulation defects did not always accompany growth defects (for example, MGG05332.5). Therefore, some specific Ca^{2+} signaling also appeared to be required for sporulation or appressorium formation, respectively. In support of this, sporulation and appressorium formation were likely to be partly mediated by discrete Ca^{2+} signaling pathways in addition to a common pathway, since the knockdown mutants of MGG01381.5, MGG01196.5, and MGG06421.5 mentioned above showed defects in only one of the two infection-related developments.

An interesting finding in this study was that defects in the infection-related developments did not necessarily result in loss of pathogenicity of *M. oryzae.* This was of particular note with the mutants of Ca^{2+} transporters and permeable channels. Most of those mutants exhibited a considerable decrease in appressorium formation and sporulation rates without a severe reduction in pathogenicity against the host plants. This was also the case with some other mutants of phospholipase C (MGG05332.5, MGG05905.5), calcineurin catalytic subunit (MGG07456.5), *Tos3*-like kinase (MGG06421.5), *Kin4*-like kinase (MGG01196.5), and *Ena1*-like P-type ATPase (MGG010730.5). The results suggested that those genes were transiently required for the infection-related developments but not for fungal virulence after the invasion into the host cell. In our experiments, the rate of appressorium formation was examined on a slide glass and recorded after 12 h incubation. In some cases, the rate of appressorium formation increased when the incubation time was prolonged. Therefore, some of the mutations might cause only a delay in appressorium formation. It was also possible that some chemical or physical signals from the host leaves might compensate for the signaling defects caused by the mutations upon inoculation tests. In addition, it is noteworthy that some of the mutants (for example, MGG01638.5, MGG09828.5 and MGG01196.5 and MGG010730.5) retained almost full pathogenicity despite quite

severe growth defects on rich media, indicating that fungal growth on rich media did not always mimic that in the host cell.

Overall, our results showed that most of the Ca^{2+} signaling proteins were likely to be involved in multiple signaling pathways rather than a specific pathway in *M. oryzae,* since most of the knock-down mutants exhibited multiple phenotypic defects. This is consistent with the widely accepted model that Ca^{2+} signaling mediates a variety of fundamental physiological processes in eukaryotic organisms. Nevertheless, some proteins mentioned above appeared to rather specifically function in a given or limited pathway(s). To elucidate a more precise function of each Ca^{2+} signaling protein, the intracellular location of each protein should be determined and measurements of intracellular free Ca^{2+} in each mutant should be analyzed in future studies.

4.2. pSD1 as a feasible alternative to existing systems for exploring gene function in filamentous fungi

Here, we have demonstrated that the RNA silencing vector pSD1 can be a powerful tool for loss of function analysis in *M. oryzae.* Since the promoters used in pSD1 are known to be effective in a wide range of ascomycete fungi, the pSD1 system could also be applicable for that class of fungi. The greatest merit of the pSD1 system is that it allows single step non-oriented cloning for vector construction. This advantage includes not only fewer cloning steps but also construction of multiple silencing vectors in a single transformation experiment. We often use mixed PCR fragments of several different genes for ligation to obtain multiple pSD1-based silencing vectors at a time. Potentially, when cDNA inserts were made from a normalized mRNA pool, a silencing library consisting of thousands of genes might be constructed in a single transformation. Since the length of inserts in pSD1 greatly affected the efficiency of silencing induction by the constructs (Quoc et al. unpublished data), one of the problems in such work would be how to adjust the length of cDNAs. Nevertheless, the ease of handling of pSD1 will provide new high-throughput applications for exploring gene function in ascomycete fungi.

A major drawback of pSD1 is its lower silencing efficiency compared to hpRNA- or ihpRNA-expressing RNA silencing vectors. Formation of dsRNA in the pSD1 system requires physical annealing of two different RNA molecules in the target cells while that in the hpRNA systems is achieved by self-folding of inverted repeats within an RNA molecule. The difference in dsRNA formation between the systems can be a major cause of the different silencing efficiencies. Since different types of

promoters were used in pSD1, it also might be possible that the domination of one promoter caused uneven transcription of sense and antisense RNAs, resulting in lower production of dsRNA (Tran *et al.*, 2003). However, this is unlikely to be the case because silencing efficiency was not remarkably increased with pSilent-Dual2 (pSD2), which carries two opposing TrpC promoters (Quoc *et al.*, unpublished data).

In this study, however, this drawback of pSD1 was overcome by transcriptional fusion of the target gene to an eGFP gene fragment that functioned as an indicator of the silencing level in the resulting transformants. The simple and convenient screening for strongly silenced transformants made up for the relatively low frequency of occurrence of strongly silenced transformants by pSD1-based vectors. It should be noted, however, that the silencing level of a target gene did not always correlate with that of the eGFP gene. In some cases, we observed silencing of only one of the two genes. Another advantage of simultaneous silencing of transcriptionally fused genes is its potential use for multiple gene targets. Using hpRNA-expressing vectors, simultaneous silencing of multiple endogenous genes has been shown in some fungi (Liu *et al.,* 2001; Mounya *et al.,* 2004). This strategy will be effective when a redundantly functioning gene family is subjected to functional analysis.

One of the advantages of exploring gene function by RNA silencing is its applicability to essential genes. For example, the function of essential genes in fungal pathogenicity remains largely unknown since conventional approaches such as gene disruption or mutant screening are not available because of lethality. In this study, for instance, imperfect silencing with reduced levels of gene expression by pSD1-based vectors enabled us to show that calmodulin and calcineurin (catalytic subunit), which are known to be essential genes in *S. cerevisiae* or *A. nidulans*, were involved in infection-related developments (sporulation and appressorium formation) in addition to cell growth on rich media. Therefore, pSD1 would provide a powerful tool to analyze essential genes in ascomycete fungi as an alternative to conventional approaches to such genes (i.e. using temperature-sensitive mutants).

The major disadvantages of RNA silencing include the difficulty of data interpretation due to incomplete silencing and/or off-target effects, which describe unexpected changes in the expression pattern of genes that have partial sequence similarity to the target. As described above, the results obtained with *M. oryzae* knock-down mutants in this study were mostly consistent with those previously observed in knock-out mutants of the corresponding gene and/or pharmacological studies in yeast or other fungi. In addition, even though the levels of silencing sometimes differed significantly among knock-down mutants of a certain gene, similar

phenotypes, albeit at varying degrees, were usually observed among them. Therefore, the biological processes in which the gene is involved could be clearly determined.

In this study, however, we were not able to exclude off-target effects especially within a gene family (Table 2). We detected simultaneous silencing in at least four groups of genes, the *Ena1-Ena5* ATPases (MGG07971.5, MGG02487.5, MGG04890.5, MGG04550.5, MGG10027.5, and MGG00529.5), *Pmc1*-like Ca^{2+}-ATPases (MGG13279.5, MGG05078.5, MGG010730.5, and MGG02074.5), *Neo1*-like Ca^{2+}-ATPases (MGG04066.5 and MGG02767.5) and Ca^{2+} exchangers (MGG08710.5 and MGG04159.5). The pSD1 vector requires an insert of at least 300-500 bp long for efficient silencing induction (Quoc *et al.*, unpublished data). Therefore, in some cases, it was not possible to choose a portion of a gene to be inserted in pSD1 that exhibits no significant sequence similarity to other genes. In addition, our results suggested that silencing target might be extended from the trigger to its flanking coding sequences in *M. oryzae*. Therefore, RNA silencing by the pSD1 system would not be suitable to precisely analyze the function of an individual family member. However, it would be useful to analyze the function of a gene family as a group since it can silence all family members simultaneously.

Table 1. Phenotypic characterization of RNAi mutants of calcium signaling proteins in *Magnaporthe oryzae*

Gene	Mutant name	RNAi effects					
		Growth rate	Sporulation	Appressoria	Path[1].		
					B[2]	N[3]	C[4]
Br48-GFP (parent strain)		51.05 ± 4.33	162.33 ± 12.50	86.00 ± 5.66	5	5	5
Ca^{2+} permeable channel							
MGG05643.5	S2	41.44 ± 5.48	22.00 ± 14.17**	5.5 ± 3.54**	4	3	2
MGG12128.5	S1	41.56 ± 4.83	61.67 ± 10.97**	31.50 ± 7.78*	5	4	4
MGG09828.5	S2	25.00 ± 6.39**	32.00 ± 9.17**	31.00 ± 11.31*	3	2	1
Ca^{2+} pump							
MGG04550.5	S1	39.72 ± 2.26*	0.00 ± 0.00**	ND		ND[5]	
MGG04890.5	S2	40.83 ± 1.92*	0.00 ± 0.00**	ND		ND	
MGG11727.5	S4	33.44 ± 3.21**	0.00 ± 0.00**	ND		ND	
MGG10730.5	S3	34.56 ± 6.42*	37.00 ± 1.00**	20.00 ± 9.89*	4	3	2
MGG02074.5	S4	33.94 ± 0.48**	32.00 ± 6.25**	8.00 ± 1.41**	0	0	0
MGG04066.5	S1	30.78 ± 2.26**	8.00 ± 2.65**	4.00 ± 2.83**	0	0	0
MGG05078.5	S2	32.78 ± 1.92**	21.33 ± 8.51**	5.5 ± 4.95**	0	0	0
MGG06925.5	S4	40.55 ± 4.6*	0.00 ± 0.00**	ND		ND	
MGG07971.5	S2	38.06 ± 5.18*	41.33 ± 15.01**	0.00 ± 0.00**	1	0	0
MGG02487.5	S3	41.72 ± 3.01*	0.00 ± 0.00**	ND		ND	
MGG13279.5	S4	29.11 ± 5.03**	0.00 ± 0.00**	ND		ND	
MGG12005.5	S4	33.88 ± 2.84**	27.33 ± 8.51**	0.00 ± 0.00**	2	0	0
Ca^{2+} exchanger							
MGG11454.5	S2	48.33 ± 0.58	44.66 ± 11.15**	10.00 ± 1.41**	5	5	5
MGG01193.5	S3	39.34 ± 7.42	36.00 ± 20.30**	12.5 ± 4.95**	5	4	4
MGG08710.5	S4	48.72 ± 2.79	76.00 ± 19.00**	5.5 ± 4.95**	5	5	5
MGG04159.5	S3	42.06 ± 2.8*	37.33 ± 31.64**	24.00 ± 5.66**	5	5	5
MGG01381.5	S2	46.05 ± 8.94	158.67 ± 16.29	3.5 ± 2.12**	5	5	4
MGG01638.5	S3	30.55 ± 1.34**	0.00 ± 0.00**	ND		ND	
Phospholipase C-□							
MGG02444.5	S3	39.50 ± 4.16*	0.00 ± 0.00**	ND		ND	
MGG05332.5	S2	46.89 ± 3.15	15.66 ± 3.22**	7.00 ± 2.83**	4	2	1
MGG05905.5	S2	47.55 ± 3.7	53.66 ± 17.21**	11.5 ± 2.12**	5	3	2
MGG02682.5	S1	43.55 ± 4.38	0.00 ± 0.00**	ND		ND	
Calmodulin							
MGG06884.5	S1	32.33 ± 2.08**	24.00 ± 7.00**	9.00 ± 4.24**	2	0	0
Calcineurin B (regulatory)							
MGG06933.5	S3	34.55 ± 3.41**	7.00 ± 3.00**	7.00 ± 1.41**	1	0	0
Calcineur A (catalytic)							
MGG07456.5	S4	40.56 ± 2.16*	39.00 ± 10.44**	47.00 ± 4.24*	5	5	5

To be continued

Ca^{2+} and CAM binding							
MGG00925.5	S2	40.83 ± 0.28**	9.33 ± 5.03**	8.5 ± 0.7**	3	2	2
MGG09912.5	S4	20.00 ± 3.04**	0.00 ± 0.00**	ND		ND	
MGG06421.5	S2	40.72 ± 5.11	37.67 ± 5.86**	89.00 ± 7. 07	5	5	5
MGG08547.5	S4	40.22 ± 2.50*	0.00 ± 0.00**	ND		ND	
MGG01196.5	S4	39.83 ± 4.48*	211 ± 19.69	11.5 ± 0.71**	4	4	4
MGG01596.5	S3	34.48 ± 2.75**	0.00 ± 0.00**	ND		ND	
MGG06180.5	S1	39.00 ± 4.92*	0.00 ± 0.00**	ND		ND	
Calreticulin/Calnexin							
MGG01607.5	S2	42.33 ± 3.32	11.66 ± 1.52**	00.00 ± 0.00	0	0	0
Calpactin heavy chain							
MGG06847.5	S2	36.38 ± 3.69*	31.00 ± 10.14**	0.00 ± 0.00**	0	0	0

Path[1], pathogenicity; B[2], Barely cv. Nakaizumi; N[3], Wheat cv. Norin 4; C[4], Wheat cv. Chinese Spring; ND[5], not determined; *, $p < 0.05$ from parent strain (t test); **, $p < 0.01$ from parent strain (t test)

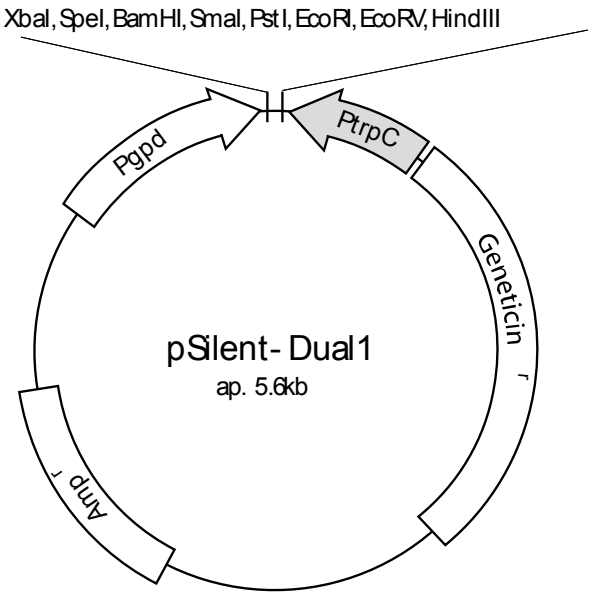
Table 2. Simultaneous silencing of homologous genes in the knock-down mutants

knock-down mutant	gene	Similarity (E-value)	mRNA expression[1] (fold)
Ca^{2+} pumps			
mgg04550.5	MGG04550.5	-	0.56 ± 0.06*
	MGG04994.5	1.60E-07	0.36 ± 0.01*
	MGG07971.5	2.72E-06	1.09 ± 0.18
	MGG07200.5	9.88E-06	0.31 ± 0.02*
	MGG13279.5	6.10E-04	0.57 ± 0.01*
mgg04890.5	MGG04890.5	-	N.D.[2]
	MGG02487.5	9.16E-10	0.14 ±1.83e-3*
mgg10730.5	MGG10730.5	-	0.45 ± 0.04*
	MGG02074.5	3.75E-24	0.38 ± 0.26*
	MGG13279.5	7.86E-10	0.41 ± 0.21*
	MGG05078.5	7.30E-04	3.98 ± 0.93
mgg02074.5	MGG02074.5	-	0.05 ±7.37e-3*
	MGG10730.5	3.75E-24	0.10 ± 0.01*
	MGG13279.5	1.92E-07	0.02 ± 0.01*
	MGG05078.5	1.18E-05	0.19 ± 0.02*
	MGG07200.5	7.31E-04	0.48 ± 0.09*
mgg04066.5	MGG04066.5	-	0.46 ± 0.18*
	MGG02767.5	2.32E-04	0.43 ± 0.17*
mgg05078.5	MGG05078.5	-	0.30 ± 0.13*
	MGG02074.5	1.87E-04	0.91 ± 0.43
	MGG13279.5	7.40E-04	0.49 ± 0.31*
mgg07971.5	MGG10730.5	7.40E-04	0.48 ± 0.12*
	MGG07971.5	-	0.59 ± 0.15*
	MGG02487.5	3.75E-06	0.51 ± 0.01*
	MGG04550.5	3.75E-06	1.12 ± 0.07
	MGG10027.5	9.15E-04	0.47 ± 0.05*

	MGG00529.5	9.15E-04	2.73 ± 0.59
	MGG02487.5	-	0.20 ± 0.1*
mgg02487.5	MGG04890.5	1.04E-09	N.D.[2]
	MGG07971.5	3.96E-06	0.11 ± 0.05*
	MGG10027.5	1.57E-05	1.22 ± 0.3
	MGG13279.5	-	0.39 ± 0.09*
	MGG10730.5	7.83E-10	0.45 ± 0.04*
mgg13279.5	MGG02074.5	1.91E-07	5.07 ± 2.21
	MGG05078.5	7.27E-04	0.70 ± 0.02*
	MGG04550.5	7.27E-04	0.77 ± 0.20
Ca^{2+} transporters			
mgg08710.5	MGG08710.5	-	0.43 ± 0.02*
	MGG04159.5	5.70E-10	0.63 ± 0.24*
mgg04159.5	MGG04159.5	-	0.30 ± 0.21*
	MGG08710.5	4.38E-10	0.27 ± 0.03*
$Ca^{2+}$and CAM binding protein			
mgg08547.5	MGG08547.5	-	0.53 ± 0.11*
	MGG00183.5	3.85E-04	1.43 ± 0.47

*, significantly reduced ($p < 0.05$ from wild type); 1, relative fold to wild type; 2, not reproducibly detected

(A)



(B) Multiple cloning site region of pSD1

	XbaI	SpeI	BamHI	SmaI*	PstI	EcoRI	EcoRV*	HindII
(gpd Promoter) ----	*GCTCTAGA*	*ACTAGT*	*GGATCC*	*CCCGGG*	*CTGCAG*	*GAATTC*	*GATATC*	*AAG*

CTTAT ---- (PtrpC Promoter)

Figure 1. Schematic diagram of the RNA silencing vector, pSilent-Dual1 (A) and the nucleic acids sequence in the multiple cloning sites (B). PtrpC, *Aspergillus nidulans* trpC promoter; Pgpd, *A. nidulans* gpd promoter; Amp^r, ampicillin-resistant gene.

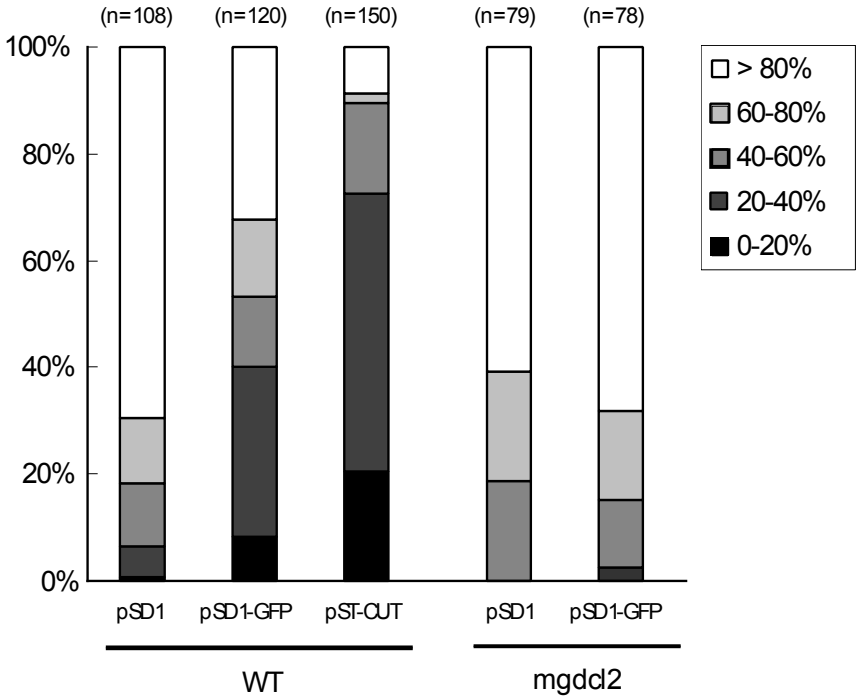


Figure 2. Comparison of silencing efficiency between single and dual promoter silencing systems in *M.oryzae*. Three plasmids, pSD1-GFP, which is pSD1 containing a 0.72 kb fragment of the eGFP gene as an insert, pST-CUT, which is a pSilent-1 derivative expressing hairpin eGFP RNA (Nakayashiki *et al.,* 2005), and pSD1 as a negative control, were introduced into two eGFP-expressing *M. oryzae* strains with different genetic backgrounds (wild-type [WT] and modcl2 mutant). At least seventy transformants each were picked up and classified into 5 categories (0-20%; 21-40%, 41-60%, 61-80% and >80%) based on relative fluorescence to the parent strain.

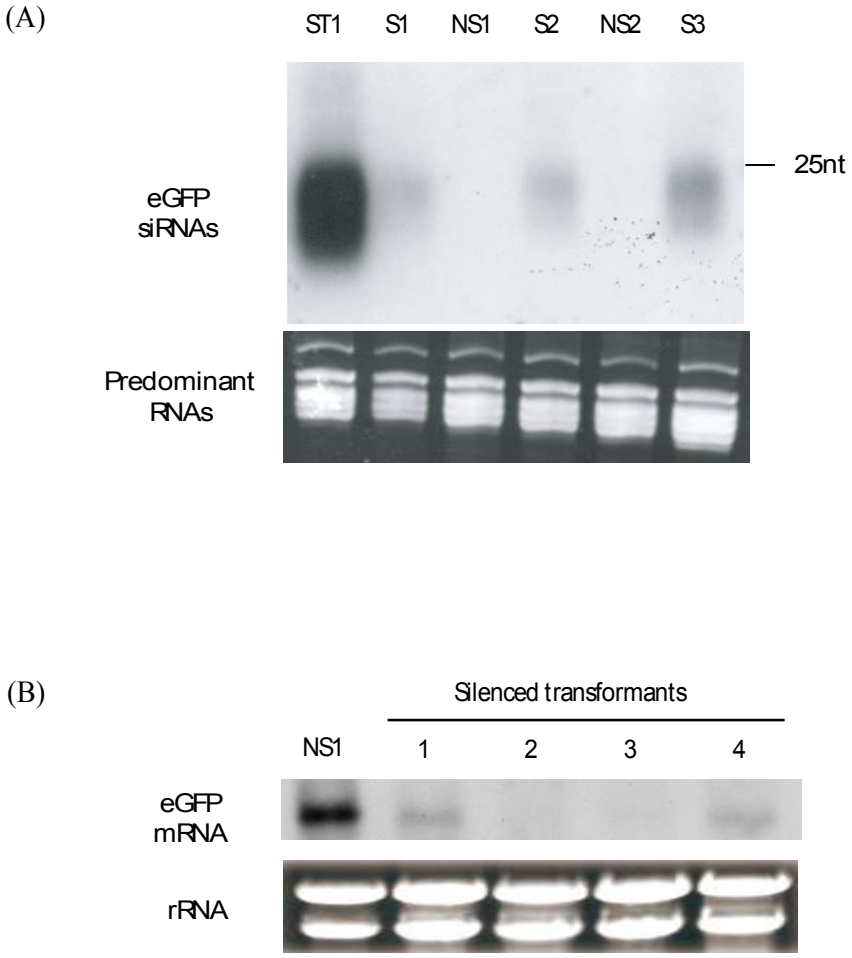


Figure 3. Northern blot analysis of siRNAs (A) and mRNA (B) of the eGFP gene in *M. oryzae* transformants with pSD1-GFP. A,Transformants with pSD1-GFP showing silenced phenotype (S1-S3) and non-silenced phenotype (NS1-NS2) were subjected to the analysis. To compare the levels of siRNA accumulation, one transformants with pST-CUT (ST1) was also employed. DNA oligonucleotides (25 mer) were used as molecular size makers for siRNA analysis. B, Four silenced transformants (1-4) and one non-silenced transformant (NS1) were subjected to the analysis. Equal loading of total RNA was estimated by ethidium bromide staining of predominant RNAs (A) or rRNAs (B).

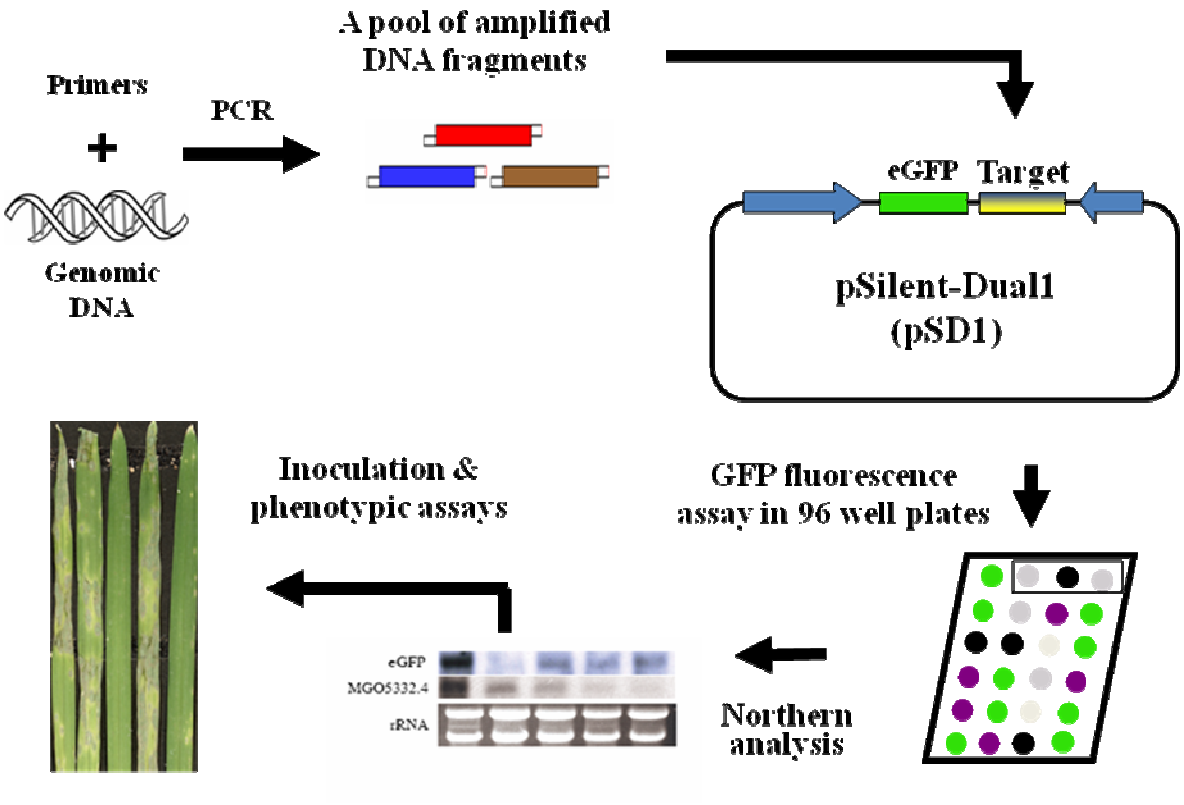


Figure 4. Schematic diagram of the high-throughput RNA silencing system by pSilent-Dual1 (pSD1). A pool of DNA fragments were amplified by PCR using specific sets of primers and genomic DNA, and inserted into pSD1G (pSD1 with a fragment of the eGFP gene). Resulting recombinant plasmids were screened by colony PCR, and each of them was introduced into a GFP-expressing *Magnaporthe oryzae* strain. The *M. oryzae* transformants were grown in a 96-well plate, and GFP fluorescence of the colonies was measured using a fluorescent plate reader. Northern blot or qPCR analysis was performed with *M. oryzae* transformants showing low or no GFP fluorescence, and ones with a reduced mRNA level were further subjected to phenotypic analyses.

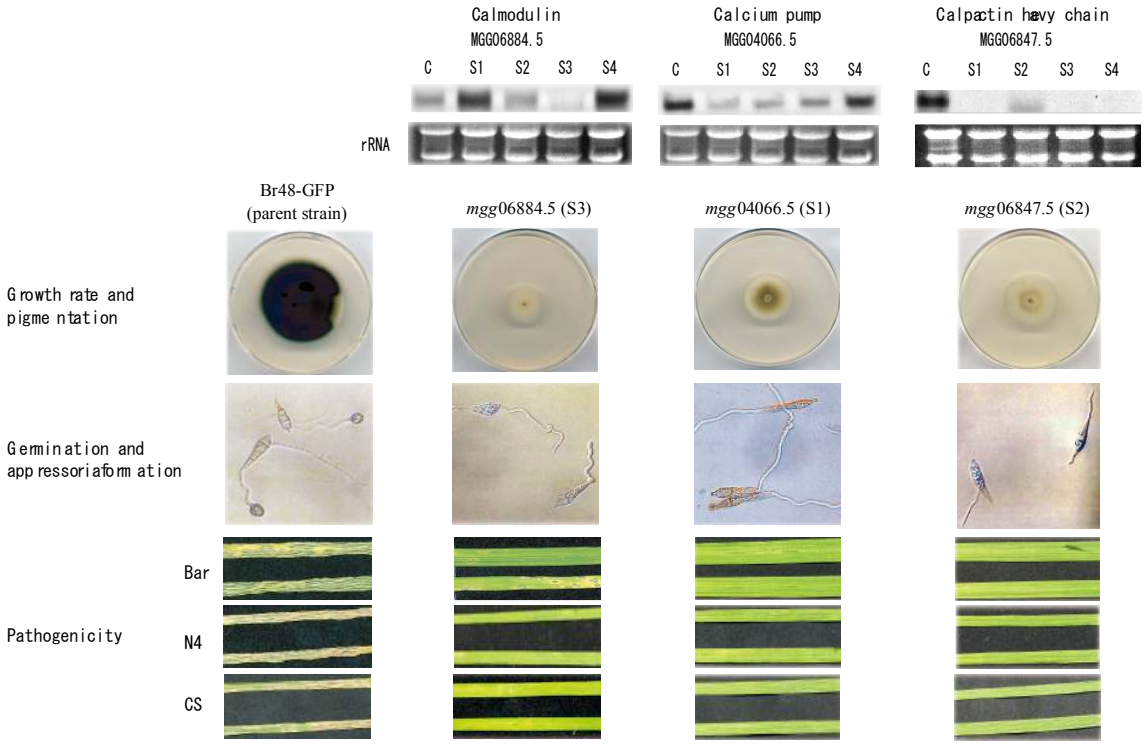


Figure 5. Phenotypic assays of RNAi mutants of three calcium signaling protiens, MGG06884.5 (calmodulin), MGG04066.5 (calcium pump) and MGG06847.5 (calpactin heavy chain). C, Br48-GFP (parent strain); S1-S4, transformants with a pSD1-based silencing construct; Bar, Barely cv. Nakaizumi; N4, Wheat cv. Norin 4; CS, Wheat cv. Chinese Spring.

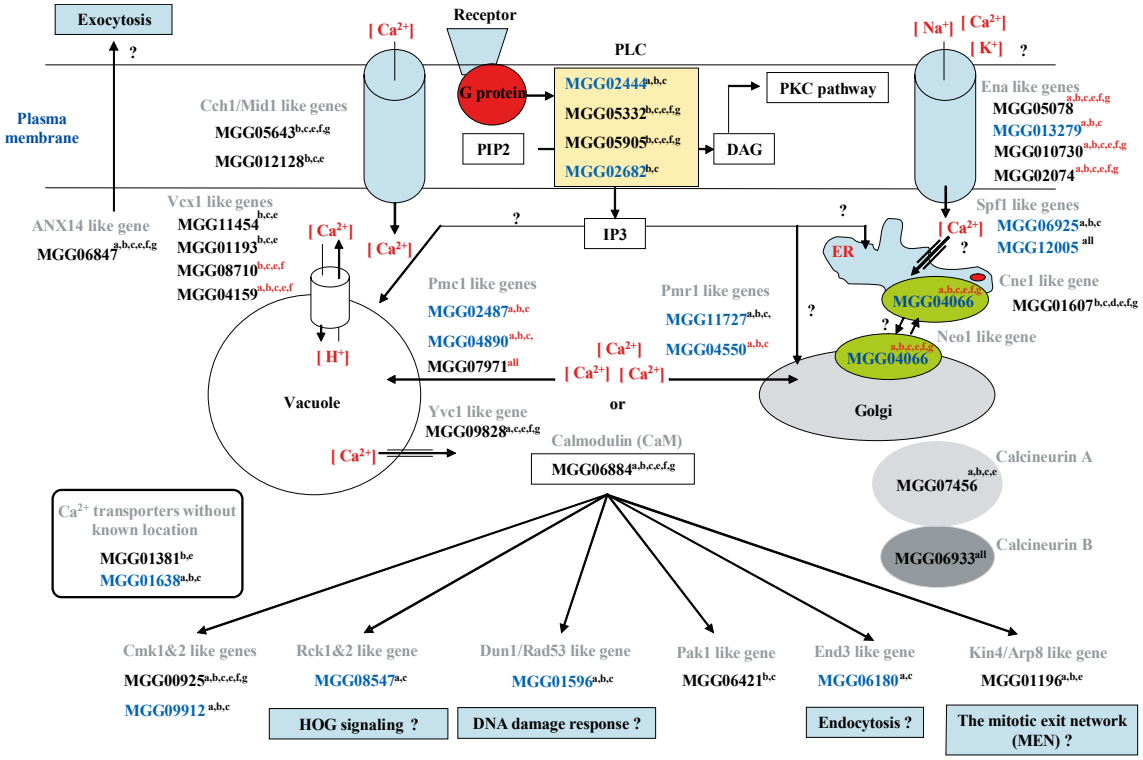


Figure 6. A comprehensive model of calcium signaling and transport pathways in *M. oryzae*. The model was drawn by applying the information from studies in yeasts and mammalian cells. Genes given in blue have no data on conidial germination, appresorium formation, conidial morphology; and pathogenicity due to a complete loss of sporulation when knocked-down. Phenotypic defects observed in knock-down mutants were indicated by superscript notation on the MG numbers. Red superscript notations indicated that the phenotypes resulted from silencing of multiple genes listed in Table 2. a, growth rate; b, pigmentation; c, sporulation; d, conidial germination; e, appresorium formation; f, conidial morphology; g, pathogenicity

CHAPTER IV

Ca^{2+} signaling system from yeast to fungi

I. Introduction

Since the discovery of Ca^{2+} involvement in heart muscle activity over 100 years ago (Ringer, 1883), Ca^{2+} has been known as second messengers in numerous signal transduction pathways as well as its relationship to physiological disorders in all organisms. In all cell systems, Ca^{2+} plays a crucial role not only in fundamental physiological responses but also in specific development. In plants, many Ca^{2+}-related disorders such as blossom-end-rot (BER) in tomatoes, peppers and melons, tip-burn in lettuce, bitter pit in apples, internal browning (IB) in pineapples, twins in pineapples, internal brown fleck in potatoes were observed because of their roles in growth, development and productivity of plants involving in growth of cells and tissues where it can involve in photomorphogenesis, embryogenesis, the self-compatibility response in pollen-pistel interaction, perception of symbiotic signals, hypersensitive responses induced by pathogen and elicitors, gravitropism and phototropism, assembling and disassembling of cytoskeleton elements, perception of red and blue light, cyclosis and movement of stomatal cells (reviewed in Medvedev, 2005). In human beings, calcium homeostasis has been considered as one of important factor critically involving in several important human calcium dependent diseases such as Alzhemer (Bernstein *et al.*, 2005), diabetes, hypertension, heart and bone diseases. In fungi, the involvement of Ca^{2+} in the fundamental physiological processes as well as in fungal specific development such as sporulation, spore germination, hyphal orientation, hyphal branching and appressorium formation (Cruz *et al*., 2002; Brand *et al*., 2007; Solomon *et al.*, 2006; Warwar and Dickman, 1996).

There are many key components in the system of calcium signaling from yeast to plant and animal cells such as phosphilipase C, calcium permeable channel, calcium pump, calpactin heavy chain (annexin), careticulin/calnexin, calmodulin, calcineurin A (catalytic), calcineurin B (regulatory), calcium transporter, calcium and CAM binding proteins, and mitochondrial Ca^{2+} homeostasis. Not look like in plant cells, a relatively little information of Ca^{2+} signaling until now has been demonstrated in fungal cell. Therefore, this review summarizes an overview of Ca^{2+} homeostasis considering in both physiological processes and pathogenicity consequences linked to Ca^{2+} signaling disturbance particularly in the model yeasts and phytopathogenic fungus, *Magnaporthe oryzae* by using forward and reverse genetic approaches and suggests possible pathways for controlling fungal growth and virulence by manipulating the interactions between Ca^{2+} signaling proteins and other intracellular signals.

II. Calcium measurement in fungi

The maintainence of steady level of the intracellular free Ca^{2+}concentration plays an important role for normal development of organisms. The understanding of cellular and subcellular Ca^{2+} activity indicates the involvement of Ca^{2+} in different intracellular signaling responses. For this purpose, calcium quantification is carried out in many studies to highlight molecular mechanism involved in different process. Until now, many attempts to measure [Ca^{2+}] at cellular and subcellular level have been established by using chemical fluorescent and bioluminescent calcium indicators due to their Ca^{2+} binding affinity (reviewed in Takahashi *et al*., 1999). The development of various calcium probes can be classified as fluorescent dyes (Indo-1, Fura-2, fura-4, calcium green, etc.) that are able to chelate Ca^{2+} causing a change in their fluorescence spectra and as florescent proteins (aequorin, yellow camaleons, pericams, etc.) that can be easily targeted to different intracellular organelles. Both kinds of probe have been applied mainly for Ca^{2+} measurement from yeast, fungi, plant and animal cells (Bush and Jones, 1987). In filamentous fungi, two methods including Ca^{2+} selective microelectrodes and fluorescent dyes have been previously reported in the mycelial fungus *Neurospora crassa* and *Trychophyton rubrum* (Miller *et al*., 1990; Levina *et al.*, 1995; Silverman-Gavrilla and Lew, 2000, 2001, 2002; Inoue *et al.*, 1996, 1998*)*. However, those methods can not be selectively targeted to specific cellular compartment even though they are cheap, easy to use and calibrate. Therefore, another method using calcium sensitive photoproteins that exhibited successfully functional expression in endoplasma reticulum (ER) (Miyawaki *et al.*, 1997; Foyouzi-Youssefi *et al.*, 2000), the nucleus (Miyawaki *et al.*, 1997), the golgi (Griesbeck *et al.*, 2001), mitochondria (Nagai *et al.*, 2001; Arnaudeau *et al.*, 2001), and the plasma membrane (Isshiki et al., 2004), has recently demonstrated in *Phyllosticta ampelicidia* and *Aspergillus nidulans* (Shaw *et al.*, 2001; Greene *et al.*, 2002). One of fluorescent proteins is aequorin from a jellyfish, *Aequorea Victoria*, to investigate bioluminescence due to the presence of its calcium sensitivity and green fluorescent protein (GFP). This protein contains three EF-hand Ca^{2+} binding sites where occupied by Ca^{2+} converting into apoaequorin-coelenteramide complex emitting blue light in vitro (λ_{max}= 469nm). In *S.cerevisae*, apoaequorin cDNA expression system is used in monitoring $[Ca^{2+}]_i$ changes in reponse to extracellular stimuli such as mating pheromone, α-factor, and glucose with *in vivo* and *in vitro* measuring methods (Nakajima Shimada *et al.*, 1991). To date, aequorin based Ca^{2+} measurement protocols have been demonstrated in some filamentous fungi such as *N.crassa, A.niger, A.awamori* and *P.ampelicida*by by using codon optimization of the aequorin gene for measuring an increasing $[Ca^{2+}]_c$ concentration caused by three external stimuli as mechanical perturbation,

hypoosmotic shock and high external calcium (Nelson *et al.*, 2004; Shaw *et al*., 2001). Therfore, this protein is so useful in fungal Ca^{2+} signaling studies by using stable transformation techniques because the transgene aequorin can be stably expressed in the cytoplasm, easily targeted to different intracellular organelles by appropriate targeting signal sequences or fusion constructs as demonstrated in plant cell (Mithofer and Mazars, 2002; Plieth, 2001). Recently, Ca^{2+} signaling proteins have been identified in some filamentous fungi such as *Neurospora crassa, Magnaporthe oryzae*, *Aspergillus nidulans* by using a comparative analysis of their genomes with the genome of *S.cerevisae* (Zelter *et al.*, 2004). Systemic functional analysis of calcium signaling proteins in the genome of the rice blast fungus, *Magnaporthe oryzae* revealed that most of *M.oryzae* Ca^{2+} signaling was likely to be involved in multiple signaling pathways rather than a specific pathway in yeast (Quoc *et al*., 2008). Due to the advantages of aequorin based Ca^{2+} measurement system, the applications of this aproach for $[Ca^{2+}]_c$ measurement in those fungi will elucidate more understanding on the functions of calcium signaling proteins as well as Ca^{2+} levels in cell compartments involving in fungal development.

III. Ca^{2+} transport pathways in fungi

1. Ca^{2+} transport across the plasma membrane

Many acitivities of Ca^{2+} transport at the plasma membrane for controlling the changes of Ca^{2+} concentrations between the extracellular space and the cytoplasm involving in the operation of many types of Ca^{2+} channels encoded by many Ca^{2+} signaling proteins such as Ca^{2+} permeable channels, Ca^{2+}-ATPase and calpactin heavy chain (annexin). In yeast, Ca^{2+} permeable channels including two plasma membrane located proteins, CCH1 (a voltage-gated channel) and MID1 (stretch-activated channel), and one vacuole located protein, YVC1 (vacuolar mechanosensitive channel), have been known as groups of Ca^{2+} signaling proteins involving in Ca^{2+} influx across cell membranes into the cytosol from extracellular and intracellular compartments. Morover, Cch1p-Mid1p Ca^{2+} permeable channels have been described as high-affinity calcium uptake system (HACS) and other plasma membrane located protein, Fig1p, is required for low-affinity calcium influx system (LACS) in *S.cerevisae* (Locke *et al.*, 2000; Muller *et al.*, 2001; 2003). Cch1p-Mid1p proteins control adequate levels of Ca^{2+} in the cytoplasm and their activity of HACS was stimulated in *pmr1* mutants and in wildtype strains overexpressing either Pmc1p or Vcx1p due to the reduction of $[Ca^{2+}]_c$. However, when intracellular Ca^{2+} stores become empty how they release the

signal to activate the activity of Ca^{2+} permeable channel at the plasma membrane still maintain a question for further elucidating their mechanism. In fungi, although genes encoding those systems have been demonstrated in *N.crassa, M.oryzae* (Zelter *et al*., 2004) and *C.albicans* (Brand *et al.*, 2007), the functions of Cch1p-Mid1p have just been described in *M.oryzae* and *C.albicans* (Quoc *et al.*, 2008; Brand *et al.*, 2007) indicating their involvement in fungal development. However, further studies should be carried out to determine their location and concentration of intracellular free Ca^{2+} in each mutant that resulting in the influence of fungal development.

Another pathway that could be involved in Ca^{2+} transport at plasma membrane is plasma membrane Ca^{2+}-ATPase (PMCA). In yeast, PMCA is encoded by a family of four genes (PMCA1-PMCA4) like in human. They are responsible for the high affinity expulsion of Ca^{2+} from the cytosol and are stimulated by interaction with calmodulin (Carafoli, 1992). However, PMCA related genes in yeast contain high sequence similarity with Pmc1-, Pmr1-, Ena1-5-like genes. In filamentous fungi, many PMCA like genes have been identified in *N.crassa, M.oryzae* by using genomic comparative analyis (Zelter *et al.*, 2004). One type of fungal PMCA related to the cellular plasma membrane located Ena1-5 of *S.cerevisae* which has been described in yeast (Haro *et al.*, 1991; Garciadeblas *et al.*, 1993), *Hortae werneckii* (Gorjan and Plemenitas, 2006), *Schwanniomyces occidentalis* (Banuelos & Rodriguez-Navarro, 1998), halotolerant *Debaryomices hansenii* (Almagro *et al*., 2001), *Schizosaccharomyces pombe* (Benito *et al.*, 2002), salt tolerant *Zygosaccharomyces rouxi* (Watanabe *et al*., 1999), a third NcENA ATPase was identified in salt tolerant *Neurospora crassa* (Benito *et al.*, 2000) and phytopathogenic fungus, *Fusarium oxysporum* var. *lini* (Cabello-Hurtado, 2000). In *Magnaporthe oryzae,* at least four homologs to ENA1 and ENA5 of *S.cerevisae*, of which two CTA3 like genes could be K^+ATPase, appear to encode Na^+ pumps (Rodriguez-Navarro *et al*., 1994; Benito *et al.*, 2000; Benito *et al.*, 2002; Zelter *et al.*, 2004). Therefore, it will be important to determine of these proteins have a role in K^+ or Na^+ transport at the plasma membrane of *M.oryzae* particularly and filamentous fungi generally.

2. Ca^{2+} transport in intracellular organelles

At the plasma membrane, GDP dissociates from G protein complex when receiving many extracellular signals. Then the Gα subunit and Gβ/ɤ complex dissociate from the receptor, Gα will bind with GTP. The G-alpha and GTP complex will bind to phospholipase C (PLC) and induces a conformational change that activates the enzyme. A phospholipase is an enzyme that converts phospholipids into fatty acids and other

lipophilic substances. By cleaving phosphatidyl inositol 4,5-bisphosphate (PIP_2), PLC releases two important second messengers in the calcium signaling pathway, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Berridge and Irvine, 1989). In plant and animal cells, an increase of Ca^{2+} concentration in the cytoplasm is due to the release Ca^{2+} from intracellular Ca^{2+} stores such as endoplasmic reticulum (ER), plant hypocotyl microsomes, vacuoles and tonoplast vesicles requiring the occurrence of inositol-(1,4,5)-triphosphate receptors ($InsP_3R$) on the Ca^{2+} store sites or Ca^{2+} influxes from the plasma membrane (Berridge and Ivrine, 1989; Drobak and Ferguson, 1985; Alexandre *et al.*, 1990; Allen *et al.*, 1995). In fungi, IP_3 activates Ca^{2+} release from vacuole of *Candida albicans, S.cerevisae* and *N.crassa* (Calvert and Sanders, 1995; Belde *et al.*, 1993; Cornelius *et al.*, 1989). In filamentous fungi such as *N. crassa* and *M. oryzae*, however, genes encoding IP3R proteins were not found in their genomes (Zelter *et al*., 2004). Although IP_3 activated Ca^{2+} channel in *N.crassa* membranes was identified (Silverman Gavrila and Lew, 2002), it is unclear, so far, how Ca^{2+} is released from the intracellular Ca^{2+} stores and if IP_3 is involved in the process in fungi. Since the discovery of PLC in the slime mold, *Dictyostelium discoideum* (Drayer and Van Haastert, 1992) and yeast, *S.cerevisae* (Fankhauser *et al*., 1995; Yokoo *et al*., 1993), the identification of PLC encoding genes has been demonstrated in diverse eukaryotic organisms other than mammals. In filamentous fungi, the first evidence on the occurrence of PLC encoding genes that contain the two conserved regions, X and Y domains, was reported in three species including *Botryotinia fuckeliana*, *Aspergillus nidulans* and *Neurospora crassa* (Jung *et al.*, 1997). Together with the achievements of genome sequence in various fungi such as *N.crassa, M.oryzae, A.fumigatus* and etc., genomic comparative analyis of the calcium signaling machinery indicated the occurrence of multiple copies of PLC genes in several species of filamentous fungi more than in yeast (Zelter *et al*., 2004). For examples, three genes in *N.crassa* with NCPLC1, NCPLC2, NCPLC3; three genes in *Candida albicans* with CaPLC1, CaPLC2, CaPLC3; four genes in *M.oryzae* with MGG02444.5, MGG05332.5, MGG05905.5, MGG02682.5. Although functional characterization of PLC genes have been described in *C.albicans, C.parasitica, N.crassa, M.oryzae , C. gloeosporioides, C. miyabeanus, C. coccodes* and *C.dematium* (Kunze *et al.*, 2005; Gavric *et al.*, 2007; Chung *et al.*, 2006; Kim *et al.*, 1998; Uhm *et al.*, 2002; Ahn *et al.*, 2003, 2007, Quoc *et al.*, 2008), it is important to identify which gene actually involves in PLC pathway at the plasma membrane as well as the measurement of $[Ca^{2+}]_c$ in filamentous fungi.

Vacuole, golgi apparatus, endoplasmic reticulum (ER) and mitochondria has been considered as major sites of Ca^{2+} intracellular storages that maintain cytosolic

Ca^{2+} levels for the development of organisms. The regulation of Ca^{2+} homeostasis was well-defined in many Ca^{2+} stores of yeast that has been considered as a good model where to study the roles of many Ca^{2+} -pumps, - transporters, -permeable channel and other Ca^{2+} signaling proteins localizing at the plasma membrane. Like the mammalian endoplasmic and sarcoplasmic reticulum for Ca^{2+} storage, vacuolar Ca^{2+}, the major intracellular Ca^{2+} store in yeast, plays a key role insequestering Ca^{2+} into the vacuole by two transporters such as Vcx1p, a low-affinity Ca^{2+}/H^{+} exchanger that rapidly sequesters Ca^{2+} into the vacuole and Pmc1p, a high-affinity Ca^{2+}-ATPase required for maintaining low $[Ca^{2+}]_{cyt}$ (Cunningham and Fink, 1994, 1996; Pozos *et al.*, 1996; Miseta *et al.*, 1999). Moreover, the activity of those proteins was also regulated by calcineurin (CaN) that in the activated form requires Ca^{2+} and calmodulin. Then it causes increased transcription of the vacuolar Ca^{2+}-ATPase PMC1 and represses the biochemical activity of the H^{+}/Ca^{2+} exchanger VCX1 (Cunningham and Fink, 1996). The vacuolar Ca^{2+} in yeast was also released by Yvc1p, a vacuolar membrane protein with homology to transient receptor potential (TRP) channel under a hypertonic shock. Low cytosolic Ca^{2+} is restored and vacuolar Ca^{2+} is replenished through the activity of Vcx1p, a Ca^{2+}/H^{+} exchanger (Denis and Cyert, 2002). In fungi, the routes for vacuolar Ca^{2+} release are not well characterized. However, the homologs of vacuolar membrane-located Ca^{2+} pump (PMC1), a H^{+}/Ca^{2+} antiporter (VCX1) and transient receptor potential channel (YVC1) have been identified in some filamentous fungi such as *M.oryzae, N.crassa. A.fumigatus* (Zelter *et al.*, 2004). The knock-down mutants of three PMC1 like genes (MGG02487.5, MGG04890.5, and MGG07971.5) showed many phenotypic changes more significantly than the knock-down mutants of four VCX1 like genes (MGG01193.5, MGG08710.5, MGG04159.5 and MGG2.175) in the rice blast fungus. Therefore, *M.oryzae* PMC1 like mutants triggered by activated calcineurin, could not replenish Ca^{2+} not only in vacuoles but also in golgi apparatus due to sequence homology between PMC1 and PMR1 like genes (Quoc *et al.*, 2008). Other Ca^{2+} pumps in the superfamily of P-Type Ca^{2+} ATPase encoded a Golgi membrane Ca^{2+} and Mn^{2+} ion pump, the PMR1 gene of the yeast *Saccharomyces cerevisae,* is required for normal secretory pathway that was named secretory pathway Ca^{2+} ATPase (SPCA) as well as for preventing Mn^{2+} intoxication (Antebi and Fink, 1992; Wuytack *et al.*, 2003). The characterization of gene homologous to the *S.cerevisae PMR1* has recently been reported for the phenotypic changes of their mutants such as growth retardation, cell shape, glycosylation and virulence in a number of other fungi such as *Aspergillus fumigatus* (Soriani *et al*. 2005), *Kluyveromyces lactis* (Uccelletti *et al*., 1999), *A. niger* (Yang *et al.*, 2001), *Yarrowia*

lipolytica (Park *et al.*, 1998), *Pichia pastoris* (Dux and Inan, 2006), *Candida albicans* (Bates *et al.*, 2005). However, more PMR1 like genes identified in *M.oryzae, N.crassa, A.fumigatus* indicated a greater complexity of golgi membrane Ca^{2+} pump in filamentous fungi than in yeast. Therefore, further studies will elucidate the involvement of each PMR1 like genes or this gene family in Golgi function and intracellular Ca^{2+} transport pathways of those fungi. In yeast, Neo1p has been known as one of five genes encoding P-type ATPases that are potential aminophospholipid tranlocases (APTs) or flipases (Zachowski *et al.*, 1989; Auland *et al.*, 1994; Tang *et al*., 1996; Gomes *et al*, 2000). This protein has been shown to localize to the ER and Golgi complex and to be required for retrograde transport pathway between these organelles in *S.cerevisae* (Hua and Graham, 2003). In fungi, the existence of a family of APTs required for rice blast disease and induction of host resistance, has been reported in *M.oryzae* (Gilbert *et al.*, 2006). Although *M.oryzae* Neo1 like gene (MGG04066.5) required for cell growth, pigmentation, sporulation, appressorium formation, conidia morphology and virulence (Quoc *et al.,* 2008), this mutant phenotypes were resulted from simultaneous silencing with the Drs2-related P-type ATPases called MgAPT2 (MGG02767.5) with a role in exocytosis required for plant tissue colonization due to the sequence similarity. Therefore, the characterization of individual gene (MGG04066.5) by using other strategies such as gene disruption will contribute in the elucidation of the secretory pathway caused by the family of APTs (DNF1, DNF2, DNF3, DRS2 and NEO1 like genes) in *M.oryzae.*

The endoplasmic reticulum (ER) was also considered as a source of releasable Ca^{2+} into the cytosol in response to various cellular demands. Cellular Ca^{2+} homeostasis at ER is maintained through the action of sarcoplasmic/endoplasmic Ca^{2+} ATPases (SEARCAs) that are highly conserved in eukaryotic organisms. Although SEARCA protein does not exist in the genome of *S.cerevisae*, P-type ATPase (Cod1/Spf1), mainly localized to the ER is likely to be involved in the secretory pathway (Cronin *et al*., 2002). Spf1 like genes in both families of basidiomycete and ascomycete plant pathogen, *Ustilago maydis* and *M.oryzae* required for growth at high temperature, hypha development, sporulation, melanization, appressorium formation, conidial morphology and virulence (Adamikova *et al*., 2004; Quoc *et al.*, 2008). The role of SEARCA was also confirmed the involvement of Spf1 like genes in stress tolerance and virulence in *Cryptococcus noformans* (Fan *et al.*, 2007). In yeast, mutants lacking Cch1p, Mid1p, calmodulin or calcineurin, died rapidly in response to ER stress agents (Bonilla *et al.*, 2002). This phenomenon can be observed in the basidiomycetous fungal pathogen, *Cryptococcus neoformans* that endoplasmic

reticulum Ca^{2+}-ATPase, Eca1, is likely involved in maintaining ER function, stress tolerance and virulence acting in parallel with Ca^{2+} calcineurin signaling (Fan *et al*., 2007). However, a little evidence reveals the roles for Ca^{2+} in fungal ER that involve in ER folding reaction as well as its interaction with other intracellular organelles like Golgi apparatus, plasma membrane. Therefore, understanding of transport pathways in intracellular Ca^{2+} stores plays an important role in elucidating which one is a key Ca^{2+} store for remaining stable levels of $[Ca^{2+}]_c$ in the cytoplasm of the fungi like sarcoplasmic/endoplasmic reticulum in plant and mammalian cell or vacuole in yeast.

IV. Ca^{2+}/calmodulin regulated signaling pathways in fungi

1. Calmodulin signaling pathway

Calmodulin (CAM) is considered as one of major Ca^{2+} signal tranducers of Ca^{2+} signals and is the primary EF-hand calcium-binding protein in all eukaryotic cells. CAM undergo a conformational change upon binding up to four Ca^{2+} ions released from intracellular Ca^{2+} stores and can regulate a multitude of different CAM-binding proteins that are mostly unable to bind Ca^{2+} themselves. It is now known that the calmodulin signaling pathway plays a central role in connection with multiple features that are responsible for the development of all organisms. The studies of yeast calmodulin indicated that although having four predicted EF-hand domains like the primary structure of vertebrate counterpart, *S.cerevisae* calmodulin can bind a maximum of three molecules of Ca^{2+} due to the defect of helix-loop-helix confirmation at the most C-terminal EF-hand (site IV) (Starovasnik *et al.*, 1993; Luan *et al.*, 1987). Although *S.cerevisae* calmodulin plays Ca^{2+}-independent roles for growth and is essential for cytoskeletal actin organization, endocytosis and nuclear division (Kubler *et al.*, 1994; Desrivieres *et al*., 2002), some Ca^{2+}-dependent CAM proteins such as calcineurin and the serine/threonine kinases have been identified (Zelter *et al.*, 2004). Like the general characteristics of vertebrate and plant counterparts, the calmodulin genes of filamentous fungi such as *Neurospora crassa* and *Magnaporthe oryzae* also have 4 EF-hand domains and an open reading frame encoding a predicted protein of 149 and 112 amino acids respectively, showing over 80% identity to not only the human calmodulin protein sequence but also other fungal ones. Moreover, most of fungi do contain many genes encoding Ca^{2+}/CAM binding proteins such as calcineurin and serine/threonine protein kinases involving in various intracellular calcium signaling pathways via calmodulin like HOG signaling, DNA damage response, endocytosis, exocytosis, etc. that cause many defects on fungal development and virulence (Quoc *et al*., 2008). Recent studies

of calmodulin function in various fungi by using reverse and pharmacological approaches indicated that fungal calmodulins play a crucial role in growth, infection structure development and pathenicity (Quoc *et al.*, 2008).

2. Calcineurin signaling pathway

One serine – threonine specific Ca^{2+}/CAM activated phosphatase of the protein phosphatase (PP) family that highly conserved in eukaryotes, is calcineurin consisting of a heterodimer of a catalytic subunit A (calcineurin A) and a regulatory subunit B (calcineurin B). The activation of calcineurin signaling pathway is occured from the release of the active site from an autoinhibitory domain caused by the binding between Ca^{2+}/calmodulin and C-terminal region of activated catalytic subunit required an association of the two subunits (Watanabe *et al.*, 1996; Fox and Heitman, 2002). The function of calcineurin as a novel anti-infective agent against invasive infections of the three major fungal pathogens of humans such as such as *Cryptococcus neoformans, Candida albicans* and *Aspergillus fumigatus* (reviewed in Steinbach *et al.*, 2007) indicated an important virulence determinant, cyclophilins (CYP) that are one of three groups of related enzymes including FK binding proteins and parvulins in the immunophilin family. These proteins that are highly expressed in a wide range of species from bacteria, fungi, plants and animals, have peptidyl-prolyl *cis-trans* isomerases activity accelearting protein folding by catalyzing the isomerization of peptide bonds that precede Pro residues (Fischer and Schmid, 1990). The formation of cyclophilins-CsA and FK binding proteins-FK506 complexes when using *in vitro* calcineurin inhibitors such as cyclosporine A (CsA) and FK506 respectively indicated the involvement of cyclophilins (CYP) and FK binding proteins in fungal infection-related developments and virulence in phytopathogenic fungi (Ahn *et al.*, 2007; Lee and Lee, 1998; Viaud *et al.*, 2002, 2003). However, recent studies have been focused on elucidating the genes encoding cyclophilins, FK binding proteins, catalytic calcineurin, regulatory calcineurin and other elements of the calcineurin signaling pathways in many fungi. In the model of *M.oryzae* calcineurin signaling pathway described at here (Fig. 1), the mutants of cyclophilin-encoding gene, CYP1 did not show the defect on vegetative growth and appressorium formation in contrast to the defects on condinial development including penetration peg formation, cellular turgor generation and sporulation of which may involve in the interaction between CYP1 and calcineurin or between CYP1 and other targets. However, the activity of calcineurin was inhibited by the formation of CYP1-CsA complex with the presence of cytotoxic cyclosporine A (CsA) causing the prevention of Ca^{2+}/calmodulin dependent protein

phosphatase signaling that results the defects in the regulation of appressorium morphogenesis and hyphal development (Viaud *et al.*, 2002). Like cyclosporine A (CsA), another immunosuppressive compound, FK506, can also cause the defects on infection structure formation, hypha growth and virulence in filamentous fungi through the formation of FK binding proteins-FK506 complexes (Cruz *et al.*, 2001; Odom *et al.*, 1997; Prokisch *et al.*, 1997). In *S.cerevisae*, the complex of FKBP proline rotamase (FPR1)-FK506 inactivated calcineurin protein resulting in growth inhibition indicating that FPR1 is a target for the calcineurin inhibito FK506. Although there are no any papers reporting the function of peptidyl cis-trans isomerase genes, the homologous of *S.cerevisae* FPR1/2/3/4 like genes in the genome of *Magnaporthe oryzae* should be further studied to elucidate FK506-dependent and –independent manners like their cyplophilins during fungal development as demonstrated in the model of *M.oryzae* calcineurin signaling pathways (Fig. 1). Interestingly, functional identification of each subunit in *M.oryzae* calcineurin by using RNA silencing mediated dual promoter system, pSD1, indicated that the regulatory subunit B plays a more crucial role in the pathogenicity of the fungus than does catalytic subunit A (Quoc *et al.*, 2008). The role of rugulatory subunit B involving in virulence and growth was also observed in knock-out mutants of a CNB-like gene in *N.crassa* (Koth and Free, 1998) and *C.neoformans* (Fox *et al.*, 2001). Current work focuses on other calcineurin regulators or binding partners such as *S.cerevisae* calcipressin/RCN1 and calcineurin binding protein, CBP1 that also observed in human pathogenic fungi, *Cryptococcus neoformans* (Fox and Heitman, 2005).

3. Ca^{2+}/CAM binding kinase mediated signaling pathways

There are many intracellular signaling pathways caused by Ca^{2+}/CAM binding kinase cascades indicating the key role of Ca^{2+}/CAM in the regulation of various signaling in the cytoplasm. Recent studies indicated that fungal species do contain many *S.cerevisae* homologs of Ca^{2+}/CAM dependent serine/threonine protein kinases such as calmodulin-dependent protein kinases (Cmk1,2-like genes) likely participating in stress-activated signaling pathway, Rck1,2 –like genes known as a suppressor of cell cycle check point mutations and shown to be involved in the high osmolarity glycerol (HOG) pathway in fission yeast (Bilsland *et al.*, 2000, 2004) and other protein kinases involving in DNA damage response, endocytosis, the mitotic exit network (MEN) and calcium signaling in mitochondria.

a. Ca^{2+}/calmodulin dependent protein kinase pathway

The activity of Ca^{2+}/calmodulin-dependent serine/threonine-specific protein kinases consisting of an N-terminal catalytic domain, a regulatory domain and an associate domain are primarily regulated by the Ca^{2+}/calmodulin complex. In fission yeast, two genes encoding calmodulin-dependent protein kinases, CMK1 and CMK2, exhibited highest similarity with mammalian calmodulin-dependent multifunctional protein kinase II. Although biochemical studies on the proteins expressed in bacteria showed that the CMK1 product has similar properties to the CMK2 product, the differences between *S.cerevisae* CMK1 and CMK2 indicated that CMK2 was activated more by yeast calmodulin (CAM) and autophosphorylation (Ohya *et al.*, 1991) meanwhile the mutants of CMK2 caused DNA replication checkpoint defects but not DNA damage checkpoint defects in *Schizosaccharomyces pombe* (Alemany *et al.*, 2002). In fungi, the identification and characterization of *S.cerevisae* CMK1-, CMK2 - like genes have been well documented but just shown their important role in fungal growth and development. In the model showing the predicted cellular roles of Ca^{2+}/calmodulin dependent protein kinases in *M.oryzae* causing the rice blast disease (Fig. 2), two possible orthologs of *S.cerevisae* Cmk1 and Cmk2 including MGG09912.5 and MGG00925.5 respectively, were identified (Zelter *et al.*, 2004). Of two *M.oryzae* Ca^{2+}/calmodulin dependent kinases, the RNAi mediated knock-down mutants of Cmk1 like gene (*mgg09912.5)* showed stronger defects on fungal development and virulence than did *Cmk2* mutants (*mgg00925.5)* (Quoc *et al.*, 2008) indicating the pivotal role of Cmk1 like gene that is consistent with other fungal ones such as *A.nidulans* CMKA, *N.crassa* CAMK1, *S.nodorum* CpkA (Kornstein *et al.*, 1992; Dayton and Means, 1996; Dayton *et al*., 1997; Yang *et al*., 2001; Solomon *et al.*, 2006). Moreover, the capcibility of activated Ca^{2+}/calmodulin dependent protein kinases mediated phosphorylation that possibly involving in the regulation of many different pathways such as fungal phospholipid synthesis (Tuinen *et al.*, 1984; Bartlet *et al.*, 1988; Giri and Khuller, 1998), aflatoxin production (Jayashree *et al.*, 2000) was also described in some fungi likely *Microsporum gypseum, Neurospora crassa, Aspergillus nidulans,* and *Aspergillus parasiticus*. However, it is unclear which signal of activated Ca^{2+}/calmodulin dependent protein kinases mediated phosphorylation actually involves in fungal development and virulence as well as their interaction with other intracellular signal transduction that should be further elucidated.

b. Osmotic, oxidative and nutrient stress pathways

In the fungal cell, intracellular glycerol production triggered by the high-osmolarity glycerol (HOG) response MAPK pathway plays an important role in

preventing cellular dehydration in high osmolarity conditions. Although HOG pathway is activated by two independent mechanisms, stress-activated mitogen-activated protein kinase Hog1 (MAPK Hog1) phosphorylated by the MAP kinase kinase (MAPKK) Pbs2 and translocated to the nucleus has been shown to be important for targeting a number of genes involving in glycerol synthesis and stress responses in *S.cerevisae* (Brewster *et al.*, 1993; O'Rourke and Herskowitz, 2004; Gustin *et al.*, 1998). Moreover, the activity of *S.cerevisae* Hog1p has been known to be regulated by the phosphorylation by some reported substrates such as the tyrosine phosphatases Ptp2 and Ptp3 (Wurgler-Murphy *et al.*, 1997) and Ca^{2+}/calmodulin dependent serine/theronine specific protein kinase, RCK2 that binding strongly with Hog1 at the C-termonal region and that being phosphorylated specifically at Ser519 upon hyperosmotic shock and during oxidative stress resulting in the increase of Rck2 kinase activity (Bisland-Marchesan *et al.,* 2000; Bisland *et al.*, 2004). Although the effect of Rck2 in osmoregulation seem be not involved in Hog1 because Rck2 null mutants are sensitive to many agents of oxidative stress rather than of osmotic stress (Bisland-Marchesan *et al.*, 2000), activated Rck2 kinase is dependent on the phosphorylation by activated Hog1 in *S.cerevisae* HOG signaling pathway. In addition, *S.cerevisae* Rck2 kinase also involves in increasing the activity of Zrc1 to manage Zn^{2+} homeostasis under high Zn conditions caused by the complex of Rck2-Zrc1 (Bisland *et al.*, 2004). Based on well-definition of *S.cerevisae* Rck2 kinase, one possible ortholog of *S.cerevisae* Rck2 and Rck2 kinase (MGG08547.5) was identified in the genome of rice blast fungus, *M.oryzae* (Zelter *et al.*, 2004). Although there is not published evidence demonstrating clearly Rck2 kinase pathway in fungi, RNA silencing mediated Rck2 like mutants (*mgg08547.5*) showed severe defects on growth and sporulation in *M.oryzae* (Quoc *et al.*, 2008) indicating the key role of *M.oryzae* Rck2 like gene in fungal development and virulence. Further studies will elucidate mechanistic reasons of above mutant phenotypes as well as mechanism of oxidative stress resistance and cell cycle check point mutations that well characterized in yeast in the predicted model of *M.oryzae* Rck2 pathway (Fig. 2).

In *S.cerevisae,* the complex of Snf1 kinase known as an AMP-activated serine/threonine protein kinase consists of Snf1p, Snf4p and members of the Sip1p/Sip2p/Gal83p family (Schmidt and Cartney, 2000). *S.cerevisae* SNF1 complex is activated by phosphorylation by three upstream kinases, Pak1, Tos3 and Elm1 requiring for transcription of glucose-reppressed genes in the reponse to the stress of glucose deprivation (Hardie *et al*., 1998; Jiang and Carlson, 1996; McCartney *et al.*, 2005). In phytopathogenic fungus *M.oryzae,* the closest *S.cerevisae* Tos3 homolog (MGG06421.5) known as Ca^{2+}/calmodulin dependent kinase, was identified (Zelter *et*

al., 2004). By using RNA silencing approach, the knock-down mutants of the Tos3 like gene, MGG06421.5) were fully pathogenic against the host plants despite slower growth on rich media and moderately reduced sporulation (Quoc *et al.*, 2008) indicating that MGG06421.5 is not essential gene during phosphorylation of *M.oryzae* Snf1 like gene (MGG0803.5). BLAST search indicated that at least two upstream kinases including *S.cerevisae* Pak1 (Sak1) like gene (MGG05061.5) and Tos3 like gene (MGG06421.5) exit in the genome of *M.oryzae.* Therefore, the activity of *M.oryzae Snf1* kinase could be triggered by the association of two kinases of which *M.oryzae Pak1* like gene (MGG05061.5) may be the predominant activator of *Snf1* kinase *in vivo* as demonstrated in *S.cerevisae* (McCartney *et al.*, 2005). Further experiments should be done to clarify this point and to elucidate the interaction of proteins in *M.oryzae Snf1* complex involving in fungal development and pathogenicity (Fig. 2).

c. Endocytosis and actin cytoskeleton

An additional involvement of Ca^{2+}/calmodulin dependent protein kinase has been shown to be an essential part of the main components that are required for cortical actin cytoskeleton and endocytosis. *S.cerevisae* Ca^{2+}/calmodulin dependent protein kinase End3 containing EH domain forms a complex through the interaction with other EH domain protein, Pan1p at N-terminus of End3p *in vivo* that contributes significantly to their functions involving in organization of the actin cytoskeleton and endocytosis (Tang *et al.*, 1997). Furthermore, End3p-Pan1p complex also contributes in the formation of a heterotrimeric complex, Sla1p/End3p/Pan1p by binding with the C-terminal repeats of Sla1p at the first long repeat domain of Pan1p and the N-terminal region of End3p of which all were regulated by phosphorylation by a serine/thereonine kinase Prk1p (Tang *et al.*, 2000; Zeng *et al.*, 2001). Therefore, the role of this proteins complex is required not only for normal actin cytoskeleton organization but also for normal cell wall morphogenesis due to the the exhibition of severe cell wall defect in *S.cerevisae* (Tang *et al.*, 2000). In fungi, the role of endocytosis during conidial germination of *M.grisea* was described by using live cell imaging of FM4-64 (Atkinson *et al.*, 2002). In addition, endocytosis also plays essential role for initial pathogen recoginition, mating, spore formation and germination in the corn smut fungus, *Ustilago maydis* (Fuchs *et al.*, 2006). However, the mechanisms of fungal endocytosis still remain unclear even though the proteins involving in endocytosis in *S.cerevisae* and their homologues in *N.crassa* and *U.maydis* was described (Read and Kalkman, 2003; Fuchs and Steinberg, 2005). The organization of fungal actin cytoskeleton has been known as a key determinant for the establsihment of cell polarity and the maintenance

of polarized cell growth in fungi (Torralba *et al.*, 1998; Fischer, 2007) but not genetically described which mechanism of fungal actin cytoskeleton actually involves in fungal development. One recent study of S.*cerevisae* End3 like gene (MGG06180.5) in the rice blast fungus, *Magnaporthe oryzae* indicated that the knock-down mutants of MGG06180.5 exhibited strong defects on growth and sporulation (Quoc *et al.,* 2008) suggesting *M.oryzae* End3 like gene could play pivotal role in the mechanism of *M.oryzae* endocytosis. Not like the complex of three proteins, Sla1p/Pan1p/End3p in yeast however, no homolog of *S.cerevisae* Pan1p is recognized by BLAST seach in the genome of *M.oryzae*. Therefore, it is interesting for further studies to elucidate the mechanisms of fungal endocytosis and actin cytoskeleton organization through the activity of multi proteins complexes (Fig. 2).

d. DNA damage response

Not like well-demonstration of the DNA damage response regulation in yeast, humans and other eukaryotic model systems (Caspari and Carr, 1999; Shiloh, 2001), little information has been known about its regulation in fungi until now. In *S.cerevisae,* one Ca^{2+}/calmodulin dependent protein kinase, Dun1p, containing a Forkhead-as-associated (FHA) domain and a serine/threonine kinase domain, was known as an effector kinase to regulate many downstream cellular processes in the regulation of DNA damage response through the phosphorylation of Thr-380 in the activation loop of Dun1 by another kinase, Rad53 (Chen *et al.*, 2007). In filamentous fungi, the homologues of *S.cerevisae* Dun1 like genes were identified in *M.oryzae, N.crassa* and *A.fumigatus* (Zelter *et al.*, 2004). Although the mechanisms of DNA damage regulation has been poorly understood in filamentous fungi, the essential role of *S.cerevisae* Dun1 like gene (MGG01596.5) in cell growth and virulence of the rice blast fungus, *M.oryzae,* was described by using RNA silencing mediated vector pSD1 (Quoc *et al.*, 2008), indicating that the function of DNA damage response related genes could be presumed to be conserved not only in yeast but also in fungi. Moreover, BLAST search also revealed the appearance of gene families, especially serine/threonine protein kinases like *S.cerevisae* Rad like genes in the genome of fungi indicating that the mechanisms of DNA damage response in fungi could be more complicated and different than in yeast.

e. The mitotic exit network (MEN)

In *S.cerevisae,* when the mitotic spindle moves into the neck between the mother and bud, the exit of mitosis trigerred mitotic exit network (MEN) was inhibited

by the activity of the spindle position checkpoint (SPC) contributed by many conserved protein kinases regulated by phosphorylation (Pereira and Schiebel, 2005; D'Aquino *et al.*, 2005). One of those proteins known to be localized at the cortex of the mother cell throughout most of the cell cycle and at the bud neck in late anaphase cells (Pereira and Schiebel, 2005; D'Aquino *et al.*, 2005) is *S.cerevisae* Ca^{2+}/calmodulin dependent protein kinase Kin4 (Zelter et al., 2004). An essential role of Kin4 is to prevent exit from the mitosis in cells with mispositioned nuclei by inhibiting MEN through the maintenance of Bub2/Gfa1 - and the counteraction of Cdc5 activity (Pereira and Schiebel, 2005). In addition, current studies also indicated that functional similarity of *S.cerevisae* Kin4 could be observed in two putative MEN inhibitors, Dma1 and Dma2 causing premature mitotic exit in the presence of misoriented spondles (Fraschini *et al.*, 2004). Although no reported evidence was described the precise function of Kin4 like genes in the fungal SPC, an initial mutant phenotypic analysis of Kin4 like gene (MGG01196.5) in *M.oryzae* showed its involvement in growth and appressorium formation, but seem be not in pathogenicity (Quoc *et al.*, 2008). Therefore, it will be interesting for futher studies to investigate deeply the function of *S.cerevisae* Kin4 like gene in the activity of the spindle position checkpoint (SPC) in *Magnaporthe oryzae* as well as to elucidate its interaction with other signals in fungal cell (Fig. 2).

f. Ca^{2+} dependent respiratory NADH dehydrogenase in fungi

The mechanisms of the mitochondrial respiratory chain in fungi have been described previously (see reviews in Joseph-Horne and Hollomon, 2000; Joseph-Horne *et al.*, 2001). Therefore the part of this review concentrates in only the function of Ca^{2+}/calmodulin dependent protein kinases of which encoding external NADH dehydrogenases in fungal NADH-oxidizing mechanisms. In fungal respiratory chain, one of three largest membrane-bound complexes known to play the role in electron transfer from mitochondrial NADH to ubiquinone with proton translocation across the mitochondrial inner membrane, is mitochondrial NADH dehydrogenase complex I. Beside mitochondrial oxidoreductase activity in *S.cerevisae* derives from a single matrix-oriented protein, Ndi1p (Marres *et al.*, 1991), genetic analysis of *S.cerevisae* genomic DNA indicated two *S.cerevisae* open reading frames YMR145c/NDE1 and YDL085w/NDE2 of which each encode a mitochondrial external NADH dehydrogenase (Luttik *et al.*, 1998). In contrast to *S.cerevisae* non-proton pumping NADH dehydrogenases, their homologues listed in the genome of *N.crassa, M.oryzae*, and *A.fumigatus* from the website database (www.fungalcell.org) contain Ca^{2+}- binding EF hand motif (Melo *et al.*, 1999). In *Neurospora crassa,* the activity of NDE1 protein

involving in NADPH oxidation was likely caused by conformational changes through Ca^{2+} binding resulting in the increase of enzyme/substrate affinity (Melo *et al.*, 2000). Not like *S.cerevisae* NDE1 that was known to be more important than NDE2 (Luttik *et al.*, 1998), *N.crassa* NDE2 was suggested as the key external dehydrogenase being responsible for the oxidation of cytosolic NADH and NADPH under physiological conditions due to highly deficiency in the oxidation of both NADH and NADPH throughout a large range of pH (Carneiro *et al.*, 2004). Although EF motif does not exit in the structure of *N.crassa* NDE2, the oxidation of cytosolic NADH was also stimulated in nde2 mitochondria with the presence of Ca^{2+} indicating that the effect of calcium is due to the activity of Ca^{2+} dependent protein kinase, NDE1 allowing the exogenous NADH to pass the inner mitochondrial membrane and be oxidized by this internal enzyme (Carneiro *et al.*, 2004). Furthermore, phenotypic analysis of *N.crassa* NDE1 and NDE2 showed that NDE1 played an important role in vegetative growth under standard conditions and sexual development meanwhile NDE2 is not essential either for the vegetative or the sexual phase of the life cycle of *N.crassa* (Luttik *et al*., 1998; Carneiro *et al.*, 2004). Thus, it will be of interest to investigate if the homologues of *S.cerevisae* NDE1 and NDE2 are similar in structure, function and regulation to the external NADH dehydrogenases as well as to elucidate the mechanisms for oxidation of cytosolic NADH in other pathogenic fungi as described in the figure 2.

g. Ca^{2+} regulation of mitochondrial carriers in fungi

The activity of Ca^{2+} transport from the cytosol to mitochondria was caused by either Ca^{2+} uniporter or the mechanisms mediating transport of Ca^{2+} across the mitochondrial inner membrane in which the mitochondrial carrier family (MC) exits. The Ca^{2+} binding mitochondrial carrier family that is made up of aspartate/glutamate carriers (the AGCs) and the ATP-Mg/Pi carriers, is known as a subset of MCs. In this review, we only concentrate in the role of ATP-Mg/Pi carriers known to have Ca^{2+}-binding motifs (EF hand) of which the AGCs lack in yeast and fungi in contrast to the AGCs of higher eukaryotes and human (Cavero *et al*., 2003 and see the review in Satrustegui *et al.*, 2007). In yeast, putative Ca^{2+}-binding capacity of the mitochondria localized *S.cerevisae* ATP-Mg/Pi carrier, so-called SAL1 (Chen, 2004) was activated by two well-conserved EF-hand motifs (EF1 and EF2) suggesting that Ca^{2+} binding is apparently essential for its physiological function *in vitro*. In addition, the mutants of a bi-functional isoform of the adenine nucleotide translocases (ANTs), Aac2p was compensated for its viability (V) function but not respiration (R) function by SAL1 of which expression was able to rescue the lethality of aac2Δ sal1Δ double mutants (Chen,

2004). Further findings also support for the role of *S.cerevisae* SAL1 that EF hand motifs of Sal1p is critical for Ca^{2+} sensor to promote ATP-Mg uptake in mitochondria in exchange for Pi during growth on glucose in the absence of ANT (Cavero *et al*., 2005). Although no evidence has not been reported the characterization of *S.cerevisae* SAL1 like genes in fungi until now, the identification of homologues of *S.cerevisae* SAL1 in filamentous fungi from the website database (www.fungalcell.org) will confirm whether this model is correct in fungi as well as the involvement of Ca^{2+} binding mitochondrial carriers in fungal development and virulence (Fig. 2).

V. Concluding remarks

Apparently, Ca^{2+} signaling system in excellent model yeasts has been characterized sufficiently well in contrast to little information on systemic demonstration of Ca^{2+} signals in fungi indicating $[Ca^{2+}]_{cyt}$ mediated network of different signaling pathways and various mechanisms of Ca^{2+} regulation in Ca^{2+} channels and intracellular Ca^{2+} stores. In most fungal cells, the understanding of Ca^{2+} signaling proteins could be initiated by pharmacological approaches using protein inhibitors and has been advanced genetically since the complete of fungal genomic sequences allowing identify conserved homologues of *S.cerevisea* Ca^{2+} signaling proteins in various fungi. This may be useful for elucidating their functions involving in fungal development and virulence through the phenotypic analysis of mutants caused by genetics approaches such as forward and reverse genetics.

To date, knowledge on the signaling role of Ca^{2+} with relevant proteins in fungi still remains many questions despite a considerable progress in complete understanding Ca^{2+} signaling system. Furthermore, Ca^{2+} signaling system in fungi could be more completed than in yeast due to the occurrence of many homologues of *S.cerevisae* Ca^{2+} signaling proteins. Although current studies have just described the role of fundamental Ca^{2+} signaling related genes such as phospholipase C, calmodulin, calcineurin in various species of fungi, a little information of other classes of fungal Ca^{2+} signaling proteins particularly in Ca^{2+}/calmodulin dependent protein kinases involving in the interaction between Ca^{2+} and many different kinase pathways of signal transduction, mechanisms for Ca^{2+} homeostasis at plasma membrane, Ca^{2+} stores in fungal cells are not yet investigated and still unknown. Therefore, further functional analysis of these Ca^{2+}-signaling components described in this review or in other several organism models, has been considered as a major challenge to provide important insights allowing us to understand deeply how Ca^{2+} is integrated into fungal

signaling networks in the establishment of growth and penetration structure formation during host-fungi interactions.

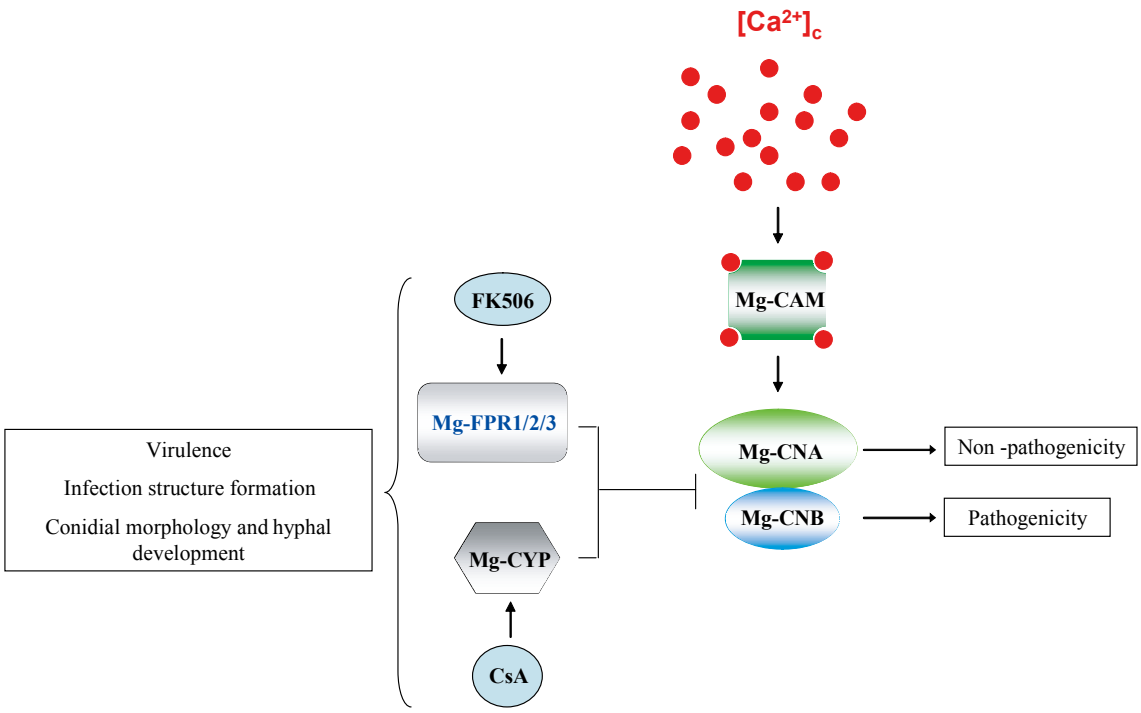


Figure 1. Calcineurin pathway in the rice blast fungus, *Magnaporthe oryzae.* The relevant phenotypes attributed to calcineurin signaling in *M.oryzae* are shown as the output of this pathway. Pathway genes shown in black color have been studied by using currently employed methods such as calcineurin inhibitors (CsA, FK506), gene disruption and RNA silencing. Genes shown in green color have not yet been studied in *M.oryzae.*

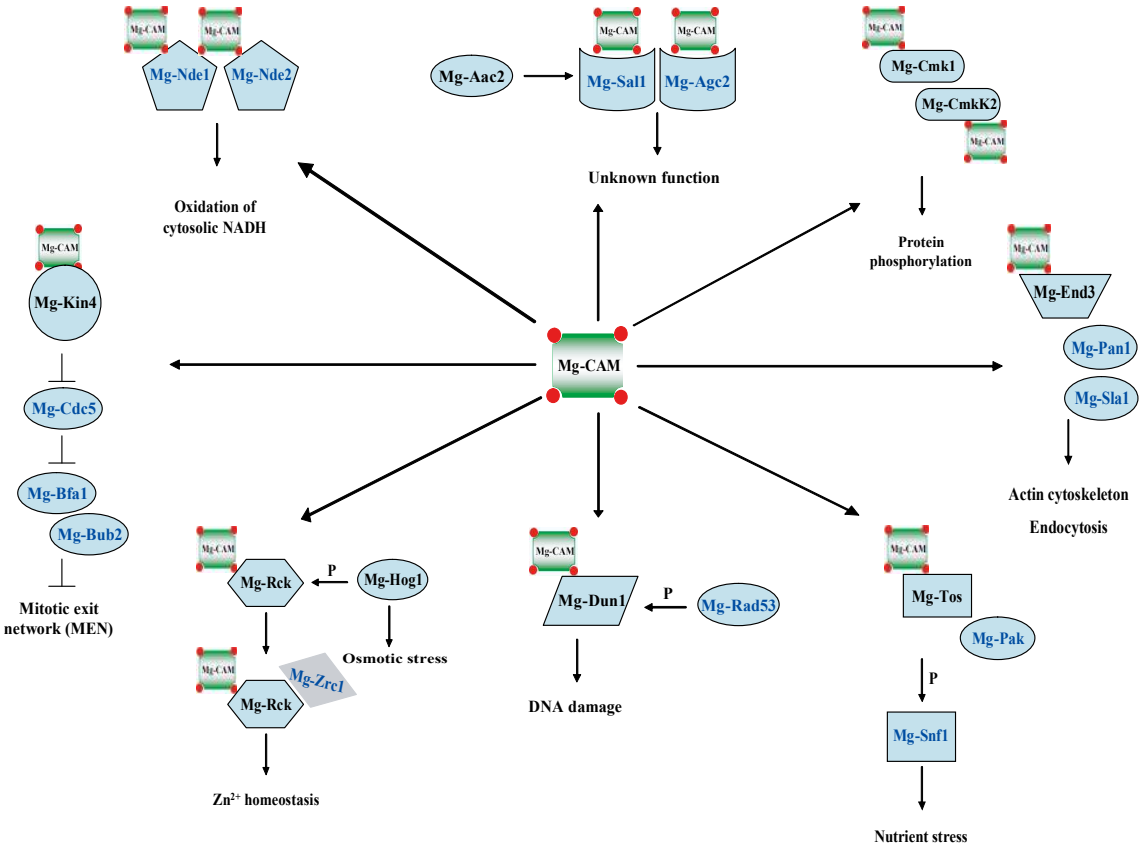


Figure 2. A comprehensive model of Ca^{2+}/calmodulin mediated network of different intracellular signaling pathways in *M.oryzae.* This predicted model was drawn by applying the information from studies in yeasts. Genes given in blue have no information on their functions involving in fungal development and virulence in *M.oryzae.*

CHAPTER V

General discussion

Until now, it is clearly known that RNA mediated gene silencing, so-called RNA silencing (Baulcombe, 1999) of which the genetical and biochemical mechanisms were conserved in most of eukaryotes such as posttranscriptional gene silencing (PTGS) in plants (Waterhouse *et al.*, 1998), quelling in the fungus *Neurospora crassa* (Ramano and Macino, 1992) and RNA interference in animals (Fire *et al.*, 1998; Hammond *et al*., 2000), is one of powerful approaches to explore gene function in post-genomic era. Many RNA silencing applications have been attempted successfully in various organisms by using different techniques such as soaking, feeding, injecting in *C. elegans*, *D. melanogaster* or plant viruses in plants for delivering dsRNA into living cells (Fire *et al.*, 1998; Tabara *et al.*, 1998; Timmons *et al*., 1998; Riuiz *et al*., 1998). Another method namely vector based dsRNA expression systems known to be able to effectively suppress target gene expression, have been developed in last decade. One of those systems that can transcribe siRNAs by RNA polymerase III promoters such as H1, U6 (Yu *et al.,* 2002; Sui *et al.*, 2002) or produce hairpin- (hp), intron hairpin- (ihp) RNAs containing sequence homologous to the target genes as an inverted repeat under the control of a strong promoter such as 35S CMV promoter (Wesley *et al.*, 2001; Waterhouse and Helliwell, 2003), has been named "single promoter system".

In fungi, single promoter system has been used effectively in a wide range of fungal species particularly in human and plant pathogenic fungi such as *Cryptococcus neoformans, Histoplasma capsulatum, Venturia inaequalis, Phytopthora infestans, Ophiostoma floccosum, O. piceae, Bipolaris oryzae* and etc (Liu *et al.*, 2001; Rappleye *et al.*, 2004; Fitzgerald *et al.*, 2004; Whisson *et al.*, 2005; Tanguay *et al.*, 2006; Moriwaki *et al.*, 2007). Recent studies indicated applicability of single promoter system that induced RNA silencing efficiently in the heterothalic fungus, *Magnaporthe oryzae* (formerly *Magnaporthe grisea)* which has been considered as the causal agent of rice blast disease and an excellent model organism for studying fungal phytopathogenicity and host-fungal interactions (Kadotani *et al*., 2003; Nakayashiki *et al.,* 2005). Herein, one of vector based dsRNA expression systems, so-called "dual promoter system" that was designed from two opposing polymerase II promoters, promises a feasible alternative tool to single promoter system with hairpin and intron hairpin RNA expressing constructs generally limited to a small or moderate scale for exploring gene function in the genome of *M.oryzae* due to two steps of oriented cloning, difficulty in sequencing, time- consuming and costly usage of the oligos for generation.

Dual promoter vectors provide new high-throughput applications for exploring gene function in ascomycete fungi.

The use of dual promoter system based silencing vectors has been described in many organisms with various degrees of success. In mammalian cells, dual promoter vectors based siRNA delivery strategy by using two different opposing human H1 and U6 polymerase III promoters or convergent U6, H1 expression cassette that drive expression of both strands of any templates cloned in between the cloning sites, can efficiently suppress the expression of not only both reporter and endogenous genes (Kaykas and Moon, 2004; Tran *et al*., 2003; Nassanian *et al.*, 2007) but also gene functions such as NF-$_K$B signaling pathway related genes (Zheng *et al*., 2003), Hepatitis C virus subgenomic replication (Korf *et al*., 2007). Although silencing efficiency of dual promoter system was showed to be a little lower that that of hpRNA-or ihpRNA-expressing RNA silencing vectors in human and plant pathogenic fungi such as *Histoplasma capsulatum*, *Magnaporthe oryzae* (Rappleye *et al*., 2004; Quoc *et al*., 2008), it is possible to overcome this drawback of dual promoter system by using co-silencing strategy in which eGFP gene fragment was used as an indicator of the silencing level in the resulting transformants (Quoc *et al.*, 2008). This simple method has been shown previously by using hpRNA-expressing vectors (Liu *et al.,* 2001; Mounya *et al.,* 2004) with many constructions used to test RNA silencing in some fungi. Similar to the results obtained when using single promoter system based simultaneous silencing of endogenous genes, the silencing level of a target gene did not always correlate with that of the eGFP gene due to the occurrence of single gene silencing. In addition, the application of dual promoter system by using co-silencing method for systemic functional analysis of calcium signaling proteins in the genome of *M.oryzae* indicated that this system, for example the pSD1 vector, requires an insert of at least 300-500 bp for efficient silencing induction and would be more useful to analyze the function of a gene family as a group than to precisely analyze the function of an individual family member (Quoc *et al.*, 2008). Therefore, the combination of this approach with other currently employed methods such as gene disruption techniques or sequence-specific mutagenesis will provide deepen insights of gene functions in the genome of not only rice blast fungus, *Magnaporthe oryzae* but also other filamentous fungi.

Understanding of calcium signaling machinery in M. oryzae infective development and pathogenicity

Based on closest homologous of calcium signaling proteins in *S. cerevisae,*

many of those proteins have been identified in the rice blast fungus, *Magnaporthe oryzae* (Zelter *et al*., 2004). In addition, some of them have been demonstrated as the core components of calcium-signaling pathway in comparison of major signaling pathways between *S. cerevisae* and *M. oryzae* (Dean *et al*., 2005). Therefore, the purpose of this study was to answer the functions of calcium signaling proteins inferred in *M. oryzae* by using RNA silencing approach. A predicted overview of key intracellular Ca^{2+} proteins involved in Ca^{2+} entry and exit from the cytosol, major signaling pathways was summarized in the figure 6 of chapter III by applying the information from studies in yeasts and mammalian cells (Quoc *et al.*, 2008). Our results indicated not only interesting novel findings on genetic aspects of Ca^{2+} signaling in filamentous fungi such as Spf1-and Neo-like Ca^{2+} pumps, calreticulin, and calpactin heavy chain that are esential for fungal pathogenicicty but also functional consistency with previous experiments of some fundamental Ca^{2+} signaling proteins such as phospholipase C, calmodulin, calcineurin, Cmk1,2-Ca^{2+}/calmodulin binding proteins, Pmr1-Ca^{2+} pump (see supplemental table 3). Furthermore, we also recognized that the infection-related developments in some mutants of Ca^{2+} signaling proteins such as phospholipase C, calcineurin catalytic subunit, *Tos3*-, *Kin4*-like kinases, and *Ena1*-like P-type ATPases did not necessarily result in loss of pathogenicity of *M.oryzae* as well as the Ca^{2+} signaling proteins were likely to be involved in multiple signaling pathways rather than a specific pathway in *M.oryzae* (Quoc *et al.*, 2008). Therefore, further studies should be carried out to elucidate a more precise function of each Ca^{2+} signaling protein, the intracellular location of each protein should be determined and measurement of intracellular free Ca^{2+} in each mutant should be analyzed.

Central role of Ca^{2+}/calmodulin at the crossroads of signaling known to be involved in fungal growth, development and virulence

Ca^{2+} that has been considered as one of important intracellular second messengers, plays a key role in numerous signal transduction pathways in eukaryotic cells. In plants, the changes of $[Ca^{2+}]_{cyt}$ concentration showed essential roles not only in plant growth and development but also plant defense reactions. For examples, $[Ca^{2+}]_{cyt}$ could stimulate exocytosis of defence-related proteins and compounds by phosphorylating Ca^{2+}-dependent kinase, synthaxin (Nuhse *et al.*, 2003) as well as involve in NO accumulation that was known to be one of the early events involved in the activation of plant defence after pathogen attacks (Wendehenne *et al.*, 2001; 2004). In addition, $[Ca^{2+}]_{cyt}$ changes also play an important role in phytoalexin accumulation

and HR-related cell death (Kurosaki *et al*., 1987; Stab and Ebel, 1987; Xu and Health, 1998; Grant *et al*., 2000). The roles of calmodulin, particularly of Ca^{2+}/CAM-binding proteins in the induction of plant defence responses and development (Heo *et al*., 1999; Ivashuta, 2005; Romeis *et al.*, 2000,2001) were described even though their mechanisms are still poorly known (Bouche *et al.*, 2005). Not like in plants, regulatory role of Ca^{2+} has been deeply studied in fungal growth, infection structures development and virulence in human and plant pathogenic fungi. Until now, it has been firmly found that Ca^{2+} and calmodulin (CAM) play an important role in hypha tip growth, hypha extension and branching, zoospore biology, sporulation, spore attachment, morphogenesis, germination, appressorium formation, toxin biosysthesis and pathogencicity in various fungi (see supplemental table 3). Interestingly, the mechanisms of relationships between Ca^{2+}/calmodulin (CAM) and other proteins containing EF-motif, Ca^{2+} -dependent protein kinases have been well-described in yeasts and mammalian cells indicating that Ca^{2+} and CAM play a central role at the crossroads of signaling such as exocytosis, endocytosis, DNA damage, osmotic, oxidative, nutrient stress response, MEN, respiratory chain and etc. involving in growth and development of organisms. However, it is still remained unanswered questions about the contribution of Ca^{2+}/CAM in the network of intracellular signaling pathways causing the effects on fungal development and virulence in the rice blast fungus, *M.oryzae* particularly and other filamentous fungi. The information of excellent models like yeasts will support for further studies to elucidate those mechanisms that was known to be more complex in fungi by knocking down or knocking out their homologous in yeasts via various techniques such as RNA silencing, gene disruption or sequence-specific mutagenesis.

Summary

In chapter II

In this chapter, we established vector based dsRNA expression system, so-called "dual promoter system" that carries two convergent opposing RNA polymerase II promoters, *Aspergillus nidulans TrpC* (PtrpC) and *gpd* (Pgpd*)*. Although silencing efficiency of pSD1 designed from two different opposing promoters, PtrpC and Pgpd*,* is lower than hpRNA-or ihpRNA RNA silencing vectors, this silencing vector was shown to work efficiently on a large scale of endogenous or exogenous genes that is generally limited in hairpin or intron hairpin silencing contructs. Furthermore, high-throughput application of pSD1 for exploring gene function in ascomycete fungi was also described in systematic functional analysis of calcium signaling proteins in the gemone of rice blast fungus, *Magnaporthe oryzae.* (Quoc *et al*., 2008). We also showed the correlation of not only between the length of inserts and silencing efficiency induced by two dual promoter silencing vectors, pSD1 and pSD2 (inverted PtrpC promoter plasmid), but also co-silencing between the indicator gene, GFP and the target in this work. This assessment will promise further applications of dual promoter system that can be a feasible alternative to existing systems for genome-wide RNAi screening in the rice blast fungus, *Magnaporthe oryzae* particularly and in other filamentous fungi generally.

In chapter III

In this chapter, we performed genome-wide functional analysis of Ca^{2+} signaling proteins in *M. oryzae* using an RNA silencing approach. The target includes 37 genes encoding fundamental Ca^{2+} transport proteins (for example. Ca^{2+}-permeable channels, -pumps and -transporters), Ca^{2+} signal mediator proteins (phospholipase C, CAM, calcineurin, calnexin) as well as other Ca^{2+} binding proteins. The gene set includes more than twenty genes whose orthologs have never been genetically examined in filamentous fungi. RNA silencing or RNA interference (RNAi) offers efficient tools for knocking-down the expression of a gene of interest in a living cell. An advantage of the RNA silencing approach is its possible applicability to essential genes, which cannot be knocked-out. Some fundamental Ca^{2+} signaling proteins such as phospholipase C, CAM, calcineurin, and the Ca^{2+}/CAM-dependent kinase (Rad53) are known to be essential genes in *S. cerevisiae* or other fungi (Allen *et al.*, 1994; Chung *et al.*, 2006; Rasmussen *et al.*, 1990, 1994). Therefore, RNA silencing may

offer a uniform platform for a systematic study of Ca^{2+} signaling proteins. To date, gene knock-down experiments by RNA silencing were mostly carried out using silencing vectors producing hairpin or intron-containing hairpin RNA (hpRNA or ihpRNA) in filamentous fungi (Liu *et al.*, 2002; Kadotani *et al.*, 2003, Fitzgerald *et al.*, 2004; Rappleye *et al*., 2004; Mouyna *et al.*, 2004; McDonald *et al.*, 2005; Tanguay *et al.*, 2006). Since construction of a hpRNA- or ihpRNA-expressing vector requires two steps of oriented cloning, its applicability is generally limited to a small or moderate scale. To overcome the limitations of hpRNA-expression vectors, we have established a dual promoter system of RNA silencing in filamentous fungi, allowing construction of silencing vectors in a high-throughput manner. The silencing vector, pSilent-Dual1 (pSD1) carries two convergent opposing RNA polymerase II promoters, *Aspergillus nidulans TrpC* (P*trpC*) and *gpd (*P*gpd*), and multi-cloning sites between the promoters. Independent transcription of a target gene from each promoter produces a pool of sense and anti-sense RNAs in the cell, which would combine together into long dsRNAs to be processed into siRNAs by Dicer. This system allowed us to identify novel Ca^{2+} signaling-related genes involved in various biological aspects of *M. oryzae* such as growth, sporulation, appressorium formation, and pathogenicity.

In chapter IV

Ca^{2+} has been known to play a central role of intracellular processes demonstrated by many reviews of Ca^{2+} signaling system in cells of various organisms from yeast, plant to humans. For the last decades and until now, the insights on intracellular functional networks of Ca^{2+} signaling proteins have been deeply demonstrated in excellent model yeasts, *Schizosaccharomyces cerevisae* and *Saccharomyces cerevisae*. However, a little information of Ca^{2+} signals was described systemically in fungi even though many studies of Ca^{2+} signaling proteins indicated their involvement in fungal development such as growth, conidia morphogenesis and virulence. Current studies on functional analysis of calcium signaling machinery in fungi indicated several unknown features distinguished from the model yeasts. This chapter will summarize the key findings on intracellular functional networks triggered by the interaction between Ca^{2+} signaling proteins and other signals in the model yeasts and their homologues in various species of fungi, particularly in phytopathogneic fungus causing rice blast, *Magnaporthe oryzae.* This will also provide our current understanding of Ca^{2+} signaling biology that involves in invasive fungal infection during plant-fungi interactions.

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Supplemental data

Supplemental Table 1: Phenotypic characterization of RNAi mutants of calcium signaling proteins in *Magnaporthe oryzae*

Gene	Mutant name	RNAi effects								
		Growth rate[a] (mm)			Mel.[b]	Sporulation[c]	Germination[d]	Appressoria[e]	Mor.[f]	Path.[g]
		3 days	6 days	9 days		(x10^5/ml)	(/100 spores)	(/100 spores)		B N C
Br48-GFP (parent strain)		14.5 ± 0.6	31.33 ± 1.34	51.05 ± 4.33	1	162.33 ± 12.50	95.00 ± 5.00	86.00 ± 5.66	N	5 5 5
Ca^{2+} permeable channels										
MGG05643.5	S2	8.67 ± 2.31*	24.06 ± 3.47*	41.44 ± 5.48	2	22.00 ± 14.17**	72.00 ± 31.19	5.5 ± 3.54**	A	4 3 2
	S4	11.05 ± 2.04*	27.66 ± 2.46	43.72 ± 6.32	2	30.00 ± 11.00**	81.66 ± 16.20	3.00 ± 1.41**		4 3 3
MGG12128.5	S1	9.73 ± 3.09	27.67 ± 4.91	41.56 ± 4.83	2	61.67 ± 10.97**	83.00 ± 11.79	31.50 ± 7.78*	N	5 4 4
	S4	10.55 ± 3.64	30.56 ± 3.37	49.33 ± 6.46	3	82.66 ± 31.07*	96.00 ± 2.00	23.00 ± 14.14*		5 5 4
MGG09828.5	S2	7.27 ± 1.85**	17.55 ± 3.73**	25.00 ± 6.39**	1	32.00 ± 9.17**	80.33 ± 15.31	31.00 ± 11.31*	A	3 2 1
Ca^{2+} pumps										
MGG04550.5	S1	10.00 ± 1.69*	26.34 ± 3.4	39.72 ± 2.26*	3	0.00 ± 0.00**	ND	ND	ND	ND
	S2	9.44 ± 1.88*	27.55 ± 3.51	41.89 ± 3.37*	2	0.00 ± 0.00**	ND	ND	ND	ND
MGG04890.5	S1	10.78 ± 2.07*	28.61 ± 5.48	43.22 ± 2.89	2	0.00 ± 0.00**	ND	ND	ND	ND
	S2	9.44 ± 1.17**	26.94 ± 4.55	40.83 ± 1.92*	1	0.00 ± 0.00**	ND	ND	ND	ND
MGG11727.5	S1	7.66 ± 0.29**	24.17 ± 1.26**	35.00 ± 1.00**	4	0.00 ± 0.00**	ND	ND	ND	ND
	S4	10.77 ± 3.03	19.38 ± 1.67**	33.44 ± 3.21**	4	0.00 ± 0.00**	ND	ND	ND	ND
MGG010730.5	S1	7.22 ± 0.58**	21.44 ± 0.92**	35.94 ± 1.78**	1	26.33 ± 8.51**	94.00 ± 2.65	11.5 ± 3.54**	A	4 3 3
	S3	9.11 ± 2.59*	21.94 ± 5.35*	34.56 ± 6.42*	2	37.00 ± 1.00**	95.00 ± 2.65	20.00 ± 9.89*		4 3 2
MGG02074.5	S3	8.7 ± 1.92**	23.77 ± 2.43**	34.67 ± 3.32**	3	25.00 ± 9.85**	96.66 ± 1.53	13.5 ± 4.95**	A	0 0 0
	S4	8.06 ± 0.77**	21.39 ± 1.84**	33.94 ± 0.48**	3	32.00 ± 6.25**	88.67 ± 7.09	8.00 ± 1.41**		0 0 0

MGG04066.5	S1	7.67 ± 0.17**	19.56 ± 3.77**	30.78 ± 2.26**	3	8.00 ± 2.65**	90.67 ± 6.81	4.0 0 ± 2.83**	A	0 0 0
	S2	7.44 ± 0.59**	21.28 ± 2.06**	32.94 ± 2.92**	3	9.00 ± 4.00**	91.67 ± 5.67	4.00 ± 1.41**		0 0 0
MGG05078.5	S2	8.5 ± 0.93**	20.89 ± 2.55**	32.78 ± 1.92**	3	21.33 ± 8.51**	83.67 ± 5.51	5.5 ± 4.95**	A	0 0 0
	S4	8.61 ± 0.51**	22.75 ± 2.71**	34.55 ± 4.14**	3	22.67 ± 2.52**	92.00 ± 4.36	19.5 ± 2.12**		0 0 0
MGG06925.5	S3	8.94 ± 1.92**	25.94 ± 3.86	40.55 ± 5.22	2	0.00 ± 0.00**	ND	ND	ND	ND
	S4	10.00± 0.93**	26.17 ± 2.73*	40.55 ± 4.6*	2	0.00 ± 0.00**	ND	ND		ND
MGG07971.5	S2	9.06 ± 2.69*	23.39 ± 2.97*	38.06 ± 5.18*	3	41.33 ± 15.01**	77.33 ± 8.33*	0.00 ± 0.00**	A	1 0 0
	S3	10.56 ± 4.88	26.72 ± 7.29	42.72 ± 9.99	3	0.00 ± 0.00**	ND	ND		ND
MGG02487.5	S2	9.66 ± 2.49**	25.17 ± 3.82*	42.11 ± 5.7	3	0.00 ± 0.00**	ND	ND	ND	ND
	S3	9.22 ± 1.44**	25.78 ± 1.97*	41.72 ± 3.01*	2	0.00 ± 0.00**	ND	ND		ND
MGG13279.5	S3	7.78 ± 3.03*	19.44 ± 2.38**	34.55 ± 5.02*	4	0.00 ± 0.00**	ND	ND	ND.	ND
	S4	6.16 ± 2.36**	16.72 ± 3.98**	29.11 ± 5.03**	3	0.00 ± 0.00**	ND	ND		ND
MGG12005.5	S4	6.83 ± 1.32**	20.61 ± 2.15**	33.88 ± 2.84**	2	27.33 ± 8.51**	6.0 ± 2.65**	0.00 ± 0.00**	A	2 0 0
Ca^{2+} transporters										
MGG11454.5	S1	8.16 ± 0.7**	33.66 ± 0.76	51.83 ± 0.29	3	37.66 ± 9.86**	94.66 ± 4.5	12.55 ± 6.36**	N	5 5 5
	S2	7.66 ± 0.57**	31.33 ± 0.28	48.33 ± 0.58	3	44.66 ± 11.15**	98.66 ± 1.52	10.00 ± 1.41**		5 5 5
MGG01193.5	S2	9.11 ± 1.77**	30.06 ± 4.88	48.5 ± 8.43	3	73.33 ± 21.22**	94.33 ± 4.04	8.5 ± 2.12**	N	5 4 4
	S3	8.17 ± 0.87**	25.72 ± 4.29	39.34 ± 7.42	2	36.00 ± 20.30**	82.33 ± 5.85	12.5 ± 4.95**		5 4 4
MGG08710.5	S2	11.28 ± 2.27	30.94 ± 2.59	48.78 ± 5.54	2	37.67 ± 16.17**	93.33 ± 1.53	6.5 ± 2.12**	A	5 5 5
	S4	8.78 ± 1.74**	28.17 ± 2.68	48.72 ± 2.79	2	76.00 ± 19.00**	99.00 ± 1.00	5.5 ± 4.95**		5 5 5
MGG04159.5	S3	9.16 ± 2.02*	25.66 ± 0.17**	42.06 ± 2.8*	2	37.33 ± 31.64**	80.33 ± 27.15	24.00 ± 5.66**	A	5 5 5
MGG01381.5	S2	11.67 ± 4.77	28.55 ± 6.09	46.05 ± 8.94	2	158.67 ± 16.29	97.66 ± 2.52	3.5 ± 2.12**	N	5 5 4
MGG01638.5	S3	8.50 ± 1.00**	19.49 ± 1.09**	30.55 ± 1.34**	3	0.00 ± 0.00**	ND	ND	ND	ND

Phospholipase C-δ										
MGG02444.5	S1	9.11 ± 0.98**	26.33 ± 0.42**	41.78 ± 3.62*	3	0.00 ± 0.00**	ND	ND	ND	ND
	S3	8.73 ± 1.37**	24.77 ± 2.67**	39.50 ± 4.16*	3	0.00 ± 0.00**	ND	ND	ND	ND
MGG05332.5	S2	10.16 ± 2.4*	28.38 ± 1.69	46.89 ± 3.15	3	15.66 ± 3.22**	99.00 ± 1.00	7.00 ± 2.83**	A	4 2 1
	S4	11.16 ± 2.74	32.22 ± 4.95	51.10 ± 7.53	4	2.33 ± 3.21**	98.00 ± 1.00	38.5 ± 10.6**		4 3 2
MGG05905.5	S1	11.61 ± 1.8	32.33 ± 4.59	51.5 ± 8.5	3	44.67 ± 9.07**	99.33 ± 0.58	72.00 ± 2.82	A	5 4 4
	S2	11.66 ± 2.84	30.22 ± 2.82	47.55 ± 3.7	3	53.66 ± 17.21**	95.00 ± 3.00	11.5 ± 2.12**		5 3 2
MGG02682.5	S1	9.66 ± 2.08**	25.16 ± 3.65	43.55 ± 4.38	2	0.00 ± 0.00**	ND	ND	ND	ND
Calmodulin										
MGG06884.5	S2	10.17 ± 2.09*	28.17 ± 1.45*	48.11 ± 1.23	2	9.00 ± 3.61	95.33 ± 1.53	8.5 ± 6.36**	A	5 3 3
	S3	9.61 ± 1.99*	18.83 ± 1.26**	32.33 ± 2.08**	3	24.00 ± 7.00**	92.33 ± 7.37	9.00 ± 4.24**		2 0 0
Calcineurin (catalytic)										
MGG07456.5	S2	8.02 ± 1.8**	22.61 ± 1.88**	41.11 ± 2.11*	2	24.00 ± 7.21**	92.33 ± 4.16	33.00 ± 4.24**	N	5 5 5
	S4	7.27 ± 2.57**	21.38 ± 2.51**	40.56 ± 2.16*	2	39.00 ± 10.44**	94.00 ± 3.61	47.00 ± 4.24*		5 5 5
Calcineurin (regulatory)										
MGG06933.5	S3	8.67 ± 1.76**	20.11 ± 1.34**	34.55 ± 3.41**	4	7.00 ± 3.00**	56.33 ± 7.37**	7.00 ± 1.41**	A	1 0 0
	S4	8.16 ± 1.29**	20.99 ± 1.85**	33.71 ± 2.08**	4	0.00 ± 0.00**	ND	ND		ND
Ca^{2+} and CAM binding proteins										
MGG00925.5	S1	11.5 ± 0.5**	18.16 ± 0.28**	29.83 ± 0.28**	3	0.00 ± 0.00**	ND	ND	A	ND
	S2	12.33 ± 0.7**	25.00 ± 1.00**	40.83 ± 0.28**	2	9.33 ± 5.03**	92.00 ± 7.00	8.5 ± 0.7**		3 2 2
MGG09912.5	S1	11.78 ± 2.56	23.05 ± 1.23**	36.55 ± 2.08**	2	0.00 ± 0.00**	ND	ND	ND	ND
	S4	7.22 ± 1.68**	12.5 ± 2.18**	20.00 ± 3.04**	4	0.00 ± 0.00**	ND	ND		ND
MGG06421.5	S2	9.11 ± 2.7*	24.99 ± 4.51	40.72 ± 5.11	3	37.67 ± 5.86**	93.66 ± 10.11	89.00 ± 7. 07	N	5 5 5

MGG08547.5	S1	10.25 ± 2.3*	25.81 ± 1.98*	41.08 ± 0.71*	1	0.00 ± 0.00**	ND	ND	ND	ND
	S4	8.25 ± 3.69*	24.47 ± 2.11**	40.22 ± 2.50*	1	0.00 ± 0.00**	ND	ND		ND
MGG01196.5	S4	8.06 ± 1.86**	23.19 ± 2.02**	39.83 ± 4.48*	3	211 ± 19.69	98.00 ± 2.65	11.5 ± 0.71**	N	4 4 4
MGG01596.5	S1	10.44 ± 0.9**	26.61 ± 1.99*	42.66 ± 3.75	3	0.00 ± 0.00**	ND	ND	ND	ND
	S3	7.72 ± 0.78**	20.89 ± 1.42**	34.48 ± 2.75**	2	0.00 ± 0.00**	ND	ND		ND
MGG06180.5	S1	9.5 ± 0.33**	24.61 ± 2.22**	39.00 ± 4.92*	1	0.00 ± 0.00**	ND	ND	ND	ND
	S3	9.00 ± 0.72**	25.28 ± 3.19*	41.00 ± 5.93	1	0.00 ± 0.00**	ND	ND		ND
Calreticulin/Calnexin										
MGG01607.5	S2	15.00 ± 4.35	26.88 ± 3.09	42.33 ± 3.32	3	11.66 ± 1.52**	43.00 ± 8.18**	00.00 ± 0.00	A	0 0 0
	S3	12.00 ± 3.04	21.27 ± 2.41**	33.33 ± 2.46**	3	0.00 ± 0.00**	ND	ND		ND
Calpactin heavy chain										
MGG06847.5	S1	13.39 ± 4.55	22.94 ± 3.14*	36.78 ± 5.16*	5	0.00 ± 0.00**	ND	ND	A	ND
	S2	12.5 ± 3.5	22.38 ± 2.37**	36.38 ± 3.69*	4	31.00 ± 10.14**	74.67 ± 12.09	0.00 ± 0.00**		0 0 0

Phenotypic defects observed in knock-down mutants were indicated by superscript notation on the MG numbers. a, growth rate; b, pigmentation; c, sporulation; d, conidial germination; e, appresorium formation; f, conidial morphology; g, pathogenicity. B, Barely cv. Nakaizumi; N, Wheat cv. Norin 4; C, Wheat cv. Chinese Spring; ND, not determined.; N, normality; A, abnormality; * ($P<0.05$) and ** ($P<0.01$). Based on the melanin color of transformants observed on PDA media, they were categorized into five classes from white colonies (completely silenced phenotype) corresponding to level 5 to dark colonies (non-silenced phenotype) corresponding to level 1.

Supplemental Table 2. Sets of gene-specific primers used in this study

Primer designation	DNA-Sequence	Target
PtrpC-ClaI-R	5'-TCAT'CGATGCTTGGGTAGAATAGGTAAG-3'	*trpC* promoter
PtrpC-SalI-F	5'-GGCCG'TCGACGACGTTAACTGATATTGAAG-3'	
Pgpd-SacI-F	5'-TTAGAGCTCGCCCCGAAGTGGAAAGGCT-3'	*gpd* promoter
Pgpd-NotI-R	5'-TAGCGGCCGCAAATAAAGGTTCTTGGATGG-3'	
GFP_{412} -HindIII-F	5'-TGAAGCTTCCACCTACGGCAAGCTGACCC -3'	GFP gene
GFP_{412} -ClaI-R	5'-CTGATCGATCCGTCCTCGATGTTGTGGCGG-3'	
MG02444-F	5' ACCATCCCTTCCCAGTCGCTTCCGC 3'	Phospholipase C-δ
MG02444-R	5' GGAAGTGCTGGATCTCGCGGAACTC 3'	
MG05332-F	5' GGCCCAAGGATCAGATCGCCACTTT 3'	
MG05332-R	5' GGCGGCTGTTTGGGCTCGGTTTTGC 3'	
MG05905-F	5' CGAGGAGGAGTACAACACGCCGCTG 3'	
MG05905-R	5' CTGCCGGCCGCATCTCCAGTGACAT 3'	
MG02682-F	5' CGCGCAGAGGGATTGGACTTTGAGC 3'	
MG02682-R	5' GGGTCAGGGTAAGCTCGCATCAGGA 3'	
MG01607-F	5' ACTACCCCCTACGTGATCATGTTCG 3'	Calreticulin = Calnexin
MG01607-R	5' AGGGACCACAGCCAGAAACCTCGCC 3'	
MG06847-F	5' ATGCGGACGGGCTCCGCAAGGCGAT 3'	Calpactin heavy chain
MG06847-R	5' GCCCTCGGTTGCATCGTACAGGGTC 3'	
MG06884-F	5' GCGAGATCTATGATGGCCAGGAAAATGAA 3'	Calmodulin
MG06884-R	5'GCGGGTACCAGTCAATGCGTCCGTCACC 3'	
MG06933-F	5' GCGAGATCTAATGATTGCCATCTTCGACG 3'	Calcineurin (regulatory)
MG06933-R	5' GCGGGTACCCTAGCGTATGCTTATGCTG 3'	
MG07456-F	5' GCGAGATCTATTGACCGATTCAGGGAACC 3'	Calcineurin (catalytic)
MG07456-R	5' GCGGGTACCAGAGTCTGCACTTCTTATC 3'	
MG011454-F	5' CCGCTCATCCTACGTCCGGGCTCCTCACC 3'	Ca^{2+} transporters
MG011454-R	5' TCAGTGCCCTCCTCATACATGTTGAACCAG 3'	
MG01193-F	5' GAGTGAGCAGTTTCGACATCCCCGG 3'	
MG01193-R	5' CGTTCATGGTGAATATGACCGGCCC 3'	
MG08710-F	5' CTCGGACTCTTCGGGGCGTTCACGC 3'	
MG08710-R	5' TGGGGAGTGTCGGTCGCAAAGCTCG 3'	
MG04159-F	5' ATAGTGCATGTCAGCACGGCACCAG 3'	
MG04159-R	5' CCCAGCATTGAGGCCAGGGGGATAA 3'	
MG01381-F	5' CGCGCGCTCCGCTGGTGTTACTTCG 3'	
MG01381-R	5' AGATGTGGAGTCGTCGCAGAGCCGT 3'	
MG01638-F	5' CTCAAGGGGACCGCCCGACGTTATC 3'	
MG01638-R	5' CCACCACCTAACCGGCTTGGCACAC 3'	
MG05643-F	5' CGTCGAGATACCCGCAGCAACCACC 3'	Ca^{2+} permeable channels
MG05643-R	5' CTGGAATGTCGGTGGAGGAGGCGCT 3'	
MG12128-F	5' GCGAGATCTTTGAGAATAGCAGTTGCATG 3'	
MG12128-R	5' GCGGGTACCCTACATCAAGGCTAAACCGA 3'	
MG09828-F	5' GCGAAGCTTCTCGACTATGCAGACGACCA 3'	
MG09828-R	5' GCGCTCGAGCTACTCCTCATCTGCACCGT 3'	
MG00925-F	5' AATCAGACGCAGTGGACCTGATACG 3'	Ca^{2+} and CAM binding proteins
MG00925-R	5' GACGAACGGGTGTTGAAGCGCCTCG 3'	
MG09912-F	5' GCAAAGCGCCCGATTCGGATCTGGT 3'	
MG09912-R	5' GGTCGCGCGATCGTCTGGGTTGGCC 3'	
MG06421-F	5' GGGAACTCCTCGGAGAGCGCTGGAG 3'	
MG06421-R	5' CGTTGCTGTCCAAGCTACTCTCAGC 3'	
MG08547-F	5' GAAGCAGCCGAAGCCGAAGCAGCCT 3'	
MG08547-R	5' TTGAGGTTGTTGAGGATGGCTGGCG 3'	
MG01196-F	5' CCAGGCACAGTTGGCTGAGTGAGTA 3'	

MG01196-R	5’ AAGGCTGGTTGCGGTGGCTGCTTGG 3’	
MG01596-F	5’ ATCCACCGCGCAGCCAAAAACCCCG 3’	
MG01596-R	5’ CCCATACGCCCTCCTTCACCTCGTC 3’	
MG06180-F	5’ CTGGGCCTACGATCAACAAAGACGC 3’	
MG06180-R	5’ TGTTGTTGCCTCGACCTCAGATGCG 3’	
MG04550-F	5’ GCGAAGCTTAGGCCGATATTGGTGTCGCA 3’	Ca^{2+} pumps
MG04550-R	5’ GCGCTCGAGATAACAGGCGCGCTGATGTA 3’	
MG04890-F	5’ TCCAAAAGCCTCAGCCAGCCGGTCA 3’	
MG04890-R	5’ AACCACACCAGCGCGTTTGCAGTCC 3’	
MG11727-F	5’ GCGAGATCTGGATGGACCTCCTGCTCAAT 3’	
MG11727-R	5’ GCGGGTACCACAGCTTGGCTGTAATTGCT 3’	
MG10730-F	5’ CGCCATCGGTCGTTTCCTTGGCGTG 3’	
MG10730-R	5’ GGGCGGGTCGTAGATGCCAATGAGG 3’	
MG02074-F	5’ GTAACACCCAATACTCGCCCCAGTG 3’	
MG02074-R	5’ GCCGTTGGCAGGCTGCTGGCGCAGG 3’	
MG04066-F	5’ CCAGGAGCCGTAGTGAGGACCAGCA 3’	
MG04066-R	5’ GCCTGTCGTCCCTCCTTGCCTACGA 3’	
MG05078-F	5’ CCGCAAAGAGACCACCCCTGCCATC 3’	
MG05078-R	5’ CGAAACATGGAGCGGCGGAGCGACT 3’	
MG06925-F	5’ CTGCTTCTTGGCGGGGACTGCATCG 3’	
MG06925-R	5’ CCGCAGGGGGTTTAGCAATGGACGG 3’	
MG07971-F	5’ CCAAGCAAGCCGACAAGCCAAAGCC 3’	
MG07971-R	5’ AAGTCTCGGTACGCCATCGCCACAG 3’	
MG0 2487-F	5’ CCAGACAAGCTCGCCTATCTCGCCG 3’	
MG02487- R	5’ CGTCCTCTCCGGTAGCCTGTTGTCC 3’	
MG13279-F	5’ GGTCAGGGAGACGATCGACCAAGAG 3’	
MG13279-R	5’ GACCCTGTGATGCAAGTTCGTCGGC 3’	
MG12005-F	5’ CAGGCGAGGATTTGGTGGTTGAGGG 3’	
MG12005-R	5’ CTCGCTGTCGGTGGTGAGTTGCTTG 3’	

Protein class	Protein name	Organism	Method	Mutant phenotypes	References
	Plc1	*S. cerevisae*	Gene deletion	Reduced growth rates in media containing non-glucose carbon sources	web
	Caplc1 *Caplc2* *Caplc3*	*C. albicans*	Gene disruption	Defects in hypha formation and not essential to virulence	Kunze *et al.*, 2005
	Ncplc1	*N. crassa*	RIP	Defects in growth and branching	Gavric *et al.*, 2007
	Cplc1	*C. parasitica*	Gene disruption	Slow growth and produced colonies characterized by little aerial mycelia and deep orange in color, reduced virulence and defect in colony morphology	Chung *et al.*, 2006
Phospholipase C	*Mg02444* *Mg05332* *Mg05905* *Mg02682*	*M.oryzae*	RNA silencing	Retarded growth, blocked melanin, reduced sporulation and appressorium formation, conidial abnormality and essential for virulence	In this study
	Unknown	*C. gloeosporioides*	PLC inhibitor, Neomycin	Inhibited appresorium formation by approximately 55% at 10 μM	Kim *et al.*, 1998, Uhm *et al.*, 2002
		C. miyabeanus		Inhibited appressorium formation by approximately 64% at 100 μM	Ahn and Suh, 2007
		C. coccodes and *C.dematium*		Inhibited conidial germination and appressorium formation at 1 mM but can observe at 0.1mM in *C.dematium*	Ahn *et al.,* 2003

Calreticulin/ Calnexin	*Cne1*	*S. cerevisae*	Gene deletion	Viable, increase of cell-surface expression of ste2-3p, increase in secretion of heterologously expressed mammalian alpha 1-antitrypsin. ~30% decrease in beta-1,6-glucan upon disruption of CNE1, reduced fitness in rich medium (YPD)	web
	ClxA	*A. niger*	Gene disruption	Not an essential gene	Wang *et al.*, 2003
	Mg01607	*M. oryzae*	RNA silencing	Blocked melanin, reduced sporulation and not an essential gene	In this study
Calpactin heavy chain (annexin)	*AnxC3.1*	*A. niger*	Gene deletion	Not an essential gene	Khalaj *et al.*, 2004
	MG06847	*M.oryzae*	RNA silencing	Retarded growth, blocked melanin, reduced sporulation and appressorium formation, conidial abnormality and essential for virulence	In this study

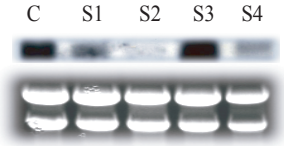
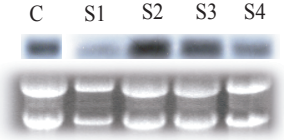
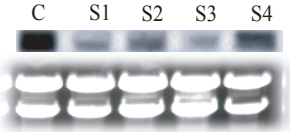
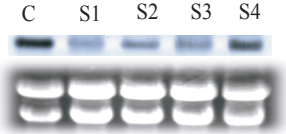
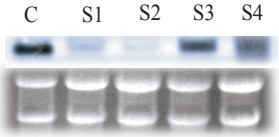
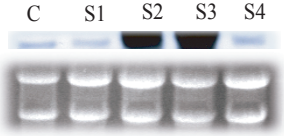
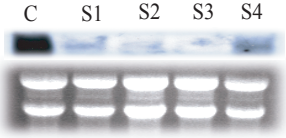
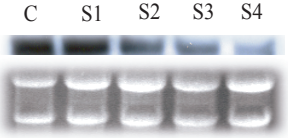
	Cmd1p	*S.cerevisae*	Gene deletion	Inviable	web
	AnCAM	*A. nidulans*	Gene disruption	Lethality	Rasmussen *et al.*, 1990
	MG06884	*M.oryzae*	RNA silencing	Retarded growth, blocked melanin, reduced sporulation and appressorium formation, conidial abnormality and essential for virulence	In this study
	Unknown	*C. gloeosporioides*	Calmodulin		Kim *et al.*, 1998
		C. miyabeanus	antagonists:		Uhm *et al.*, 2002
		C. coccodes and	W7, phenoxy	Inhibited appressorium	Ahn and Suh, 2007
		C.dematium	benzamine,	formation/germination) vegetative	Ahn *et al.*, 2003
		C. trifolii	chloropromazi	growth	Warwar and
		N. crassa	ne, TFP,		Dickman, 1996
Calmodulin		*B. emersonii*	R-24578,48-80		Rao *et al.*, 1997
		M.oryzae			Simao and Gomes,
		Z. radicans			201
		Erysiphe pisi		Inhibited *E.pisi* colony development on pea leaves	Lee and Lee, 1998
		C.albicans		Block germ tube formation and morphogenesis, hypha formation.	Liu and Kolattukudy, 1999 Magalhaes *et al.*,
	CamPb	*P. brasiliensis*		Inhibited the mycelium to yeast transition shown to be essential for establishment of infection	1991 Singh *et al.*, 2001 Roy and Datta,
		C. nicotianae		Markedly reduced cercosporin production for fungal pathogenesis	1987 Carvalho *et al.*, 2003 Chung, 2003

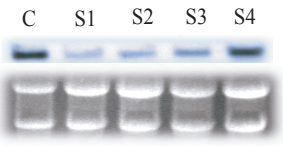
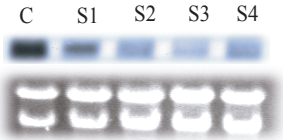
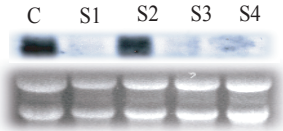
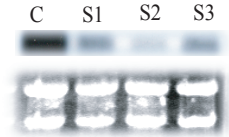
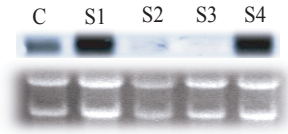
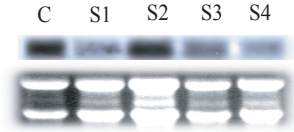
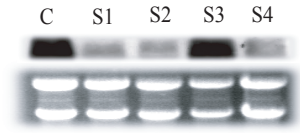
	Cna1p, Cna2p *Ppb+*	*S.cerevisae* *S. pombe*	Gene deletion	Inviable Morphological and development abnormalities (cytokinesis and cell shape defects)	web Yoshida *et al.*, 1994
	CnaA	*A. fumigatus* *A. nidulans* *A. oryzae*	Gene disruption	Hypha appeared more compact, more dense and extremely blunted, defects in growth extension, conidia (smooth conidial surface), essential for virulence, inhibited cell cycle progression and cell growth.	da Silva Ferreira *et al.*, 2007 Juvvadi *et al*., 2001 Rasmussen *et al.*, 1990 Steinbach *et al*., 2006
Calcineurin A (catalytic)	*Cmp1*	*C.albicans*	Gene deletion	Inhibited growth in 300 mM $CaCl_2$, essential for growth at 37^0C, virulence, hypersentivity to Na^+, Li^+, Mn^{2+} and alkaline pH	Bader *et al*., 2003
	Cna1	*C. neoformans*	Gene disruption Calcineurin inhibitors: CsA and FK506	Temperature, CO_2, pH sensitive in conditional growth in vitro, prevent pathogenicity in vivo, prevent mating haploid fruiting and basidiospore production	Cruz *et al*., 2001 Odom *et al*., 1997
	Cna1	*N.crassa*	Antisense technology Calcineurin inhibitors: CsA and FK506	Defects in hypha growth, morphology	Prokisch *et al*.,1997
	MG07456	*M.oryzae*	RNA silencing	Retarded growth, reduced sporulation and appressorium formation	In this study

	Cnb1	*S.cerevisae*	Gene deletion	Null mutant is viable, Li^+,Na^+, NaCl, pH8 sensitive	web
	Cnb1	*N.crassa*	Insertional mutagenesis	Defect in vegetative growth	Kothe and Free, 1998
	Cnb	*C. neoformans*	Gene disruption	Mating defect, essential for virulence and antifungal drug action	Cruz *et al*., 2001 Fox *et al*., 2001
Calcineurin B (regulatory)	*Cnb1*	*C. albicans*	Calcineurin inihbitor	Viable and markedly hypersentative to uconazole or membrane perturbation with SDS (survival during membrane stress)	Cruz *et al.*, 2002
	MG06933	*M.oryzae*	RNA silencing	Retarded growth, blocked melanin, reduced sporulation and appressorium formation, conidial abnormality and essential for virulence	In this study
Calcineurin	Unknown	*C. miyabeanus* *M.oryzae* *B. cinerea*	Calcineurin inhibitor:Cyclosporin A	Inhibited infection formation and appressorium formation	Ahn and Suh, 2007 Lee and Lee, 1998 Viaud *et al.*, 2002 Viaud *et al.*, 2004

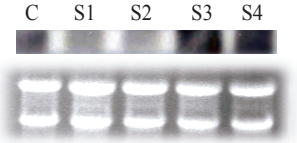
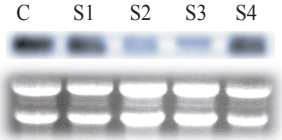
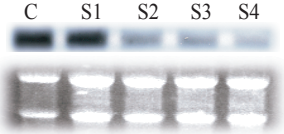
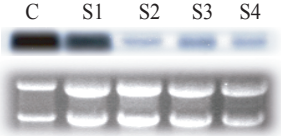
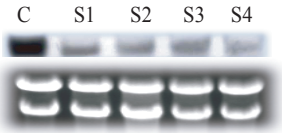
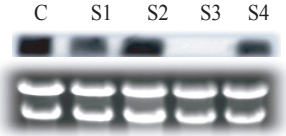
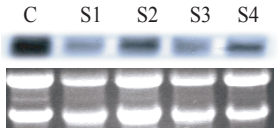
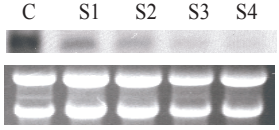
	Cmk1p, Cmk2p	*S. cerevisae*	Gene deletion	Null mutant is viable, exhibits slow rate of spore germination	Web
	CmkA, *CmkB, CmkC*	*A. nidulans*	Gene disruption	Defects in growth, nuclear division, cell cycle regulation and germination	Nathakumar *et al*., 1996 Joseph and Means, 2002 Kornstein *et al,* 1992 Dayton and Means, 1996 Dayton *et al.*, 1997
$Ca^{2+}$and CAM binding					
	Camk1	*N.crassa*	Gene disruption	Growth defect	Yang *et al.*, 2001
	CpkA,*CpkB, CpkC*	*S. nodorum*	Gene disruption	Defects in growth and sporulation	Solomon *et al.*, 2006
	MG00925 *MG09912*	*M. oryzae*	RNA silencing	Retarded growth, blocked melanin, reduced sporulation and appressorium formation, conidial abnormality and essential for virulence	In this study
	Unknown	*M. gypseum*	CAMPK inhibitor:KN62	Inhibition in the incorporation of labeled acetate into total phospholipids	Giri and Khuller, 1999

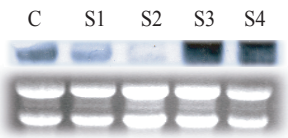
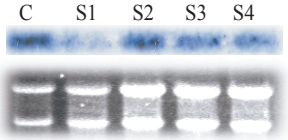
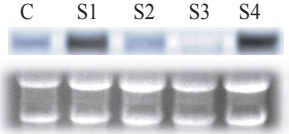
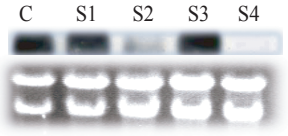
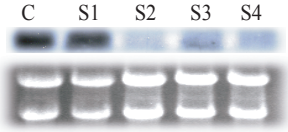
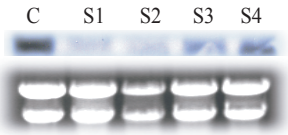
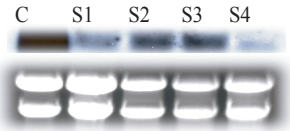
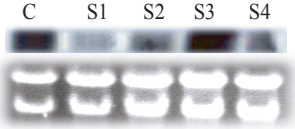
	Pmr1p	*S. cerevisae*	Gene deletion	growth defect on a non-fermentable (respiratory) carbon source, defective protein sorting and processing, failure to concentrate Ca2+ or Mn2+ within the Golgi secretory pathway, Ca^{2+} and Mn^{2+} sensitive	web
	Klpmr1	*K. lactis*	Gene disruption	Defective cell wall morphogenesis	Uccelletti *et al*., 1999
	Ylpmr1	*Y. lipolytica*	Gene disruption	Growth defect	Park *et al*., 1998
Ca^{2+} pump	*Pppmr1*	*P. pastoris*	Gene disruption	Growth defect	Dux and Inan, 2006
	Afpmr1	*A. fumigatus*	Gene disruption	Defective growth phenotype	Soriani *et al*., 2005
	PmrA	*A. niger*	Gene disruption	Growth retardation	Yang *et al.*, 2001
	MG04550 *MG02.852* *MG09892* *MG010730*	*M. oryzae*	RNA silencing	Retarded growth, blocked melanin, reduced sporulation and appressorium formation, conidial abnormality and essential for virulence	In this study

Class of protein	Name	Closest homologs in *S.cerevisae*	Northern analysis
Calcium permeable channel	MGG05643.5	Cch1p	
	MGG12128.5	Mid1p	
	MGG09828.5	Yvc1p	
Calcium pump	MGG04550.5	Pmr1p	
	MGG04890.5	Pmc1p	
	MGG11727.5	Pmr1p	
	MGG010730.5	Ena2p Ena5p	
	MGG02074.5	Ena1p Ena2p	

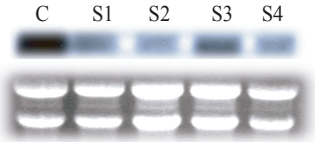
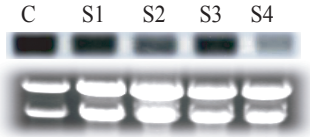
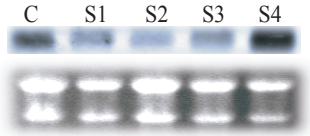
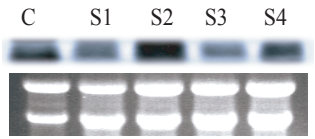
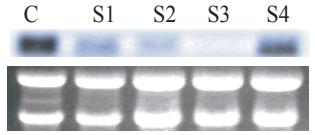
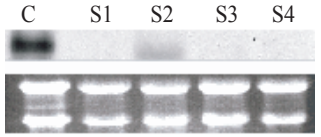
Class of protein	Name	Closest homologs in *S.cerevisae*	Northern analysis
	MGG04066.5	Neo1p	
	MGG05078.5	Ena2p	
	MGG06925.5	YOR291W	
Calcium pump	MGG07971.5	Pmc1p	
	MGG02487.5	Pmc1p	
	MGG13279.5	Ena2p	
	MGG12005.5	Spf1p	

Supplemental Figure 1-1. Northern blot analysis of RNAi mutants of calcium signaling genes in *Magnaporthe oryzae.*
C, Br48-GFP (parent strain); S1-S4, transformants with a pSD1-based silencing construct

Class of protein	Name	Closest homologs in *S.cerevisae*	Northern analysis
Calcium exchangers	MGG11454.5	Vcx1p	
	MGG01193.5		
	MGG08710.5		
	MGG04159.5		
	MGG01381.5	YNL321W	
	MGG01638.5	Ecm27p	
Phospholipase C	MGG02444.5	Plc1p	
	MGG05332.5		

Class of protein	Name	Closest homologs in *S.cerevisae*	Northern analysis
Phospholipase C	MGG05905.5	Plc1p	
	MGG02682.5		
Calmodulin	MGG06884.5	Cmd1p	
Calcineurin (catalytic)	MGG07456.5	Cna1p, Cna2p	
Calcineurin (regulatory)	MGG06933.5	Cnb1p	
Calcium and CAM binding	MGG00925.5	Cmk1p, Cmk2p	
	MGG09912.5	Cmk1p, Cmk2p	
	MGG06421.5	Paksp	

Supplemental Figure 1-2. Northern blot analysis of RNAi mutants of calcium signaling genes in *Magnaporthe oryzae.*
C, Br48-GFP (parent strain); S1-S4, transformants with a pSD1-based silencing construct

Class of protein	Name	Closest homologs in *S.cerevisae*	Northern analysis
Calcium and CAM binding	MGG08547.5	Rck1p, Rck2p	
	MGG01196.5	Kin4p	
	MGG01596.5	Dun1p	
	MGG06180.5	End3p	
Calreticulin - calnexin	MGG01607.5	Cne1p	
Calpactin heavy chain	MGG06847.5	No hit	

Supplemental Figure 1-3. Northern blot analysis of RNAi mutants of calcium signaling genes in *Magnaporthe oryzae*.
C, Br48-GFP (parent strain); S1-S4, transformants with a pSD1-based silencing construct