GENETIC ANALYSIS OF CALCIUM SIGNALING GENES IN *NEUROSPORA CRASSA*

A

Thesis submitted to partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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April 2014

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DECLARATION

I do hereby declare that the content embodied in this thesis entitled **"Genetic Analysis of Calcium Signaling Genes in *Neurospora crassa*"** is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, for the award of degree of Doctor of Philosophy, under the supervision of **Dr. Ranjan Tamuli**. The research work presented in this thesis is original and has not been submitted in part or full for any degree or diploma to any other institute or university to the best of my knowledge and belief.

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CERTIFICATE

It is to certify that the research work in this thesis entitled **"Genetic Analysis of Calcium Signaling Genes in *Neurospora crassa*"** has been carried out at the Indian Institute of Technology Guwahati, by **Mr. Ravi Kumar** (Roll No. 09610618), for the award of degree of Doctor of Philosophy in Biotechnology, under my supervision. The outcome of the research work presented in this thesis is original and has not been submitted in part or full for any degree or diploma to any other university or institute.

November 2013

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List of Abbreviations

bar	ignite resistance gene
BLAST	basic local alignment search tool for proteins
BROAD	Broad Institute at MIT
bp	base pairs
CCD	conserved domain database
cDNA	complementary DNA
cm	centimeter
DAG	diacylglycerole
dH_2O	distilled water
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
EtOH	ethanol
FGSC	Fungal Genetics Stock Center
g	gram
gDNA	genomic DNA
GPCR	G-protein-coupled receptor
h	hour
H_2O_2	hydrogen peroxide
hph	hygromycin B resistance gene
HygB	hygromycine B
hyg^R	hygromycine resistance
kb	kilo base pairs
kDA	kilo Dalton
KO	knock-out
kV	kilo volts

LB	Luria Bertani
M	molar
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase (MAP2K)
MAPKKK	mitogen-activated protein kinase kinase kinase (MAP3K)
mat a	mating type locus a
mat A	mating type locus A
Mb	mega base pairs
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
MSUD	meiotic silencing by unpaired DNA
MT	mitochondria
N. crassa	*Neurospora crassa*
NCBI	National Centre for Biotechnology Information
ng	nanogram
NIH	National Institutes of Health
nm	nanometer
nt	nucleotide
OD_{600}	optical density at 600 nm
ORF	open reading frame
PCR	polymerase chain reaction
pH	potentia hydrogenica
PKC	protein kinase C
RIP	repeat-induced point mutation
RNA	ribonucleic acid
RNas	ribonuclease
rpm	revolutions per minute

SCM	synthetic crossing medium
s	second
TB	terrific both
UV light	ultra-violet light
V	volt
v/v	volume per volume
VM	Vogel's medium
w/v	weight per volume
wt	wild type
Ω	Ohm
µg	microgram
µm	micrometer

Acknowledgements

This thesis arose in part out of years of research that has been done since I came to Tamuli group. During this journey, I have worked with a great number of people whose contribution in assorted ways to the research and the making of the thesis deserve special mention. It is a pleasure to convey my gratitude to them all in my humble acknowledgment.

At the onset I would like to record my gratitude to my supervisor, **Dr. Ranjan Tamuli**, for his keen supervision and guidance from the very first day of my research that has enabled me to develop an understanding of Neurospora genetics. I am also indebted to him for patiently going through the various versions of my thesis and for his critical comments.

I am grateful to Kevin McCluskey, Fungal Genetics Stock Centre (FGSC), University of Missouri, for providing all the calcium/calmodulin-dependent kinases (Ca^{2+}/CaMKs) and wild-isolated strains free of charge for my study. This thesis would not have been possible without their valuable support.

I thank Department of Biotechnology, Government of India, for partial financial support and Ministry of human resource Development, Government of India.

I would like to thanks Dr. Kasbeskar's lab, CCMB, Hyderabad for providing *Sad-1* strain and Southern analysis facility.

Besides my advisor, I would like to thank all the DPPC members from department of biotechnology and physics in IIT Guwahati, especially to Dr. Venkata Dasu Veeranki (HOD), Dr. Bithiah Grace Jaganathan and Dr. Bosanta R Boruah for their constructive comments on my work.

A special thanks goes to Digar Singh for PhD students in the IIT Guwahati, I like to thank him for being the first one who support and encourage me during entire journey of PhD. I like to thank Rekha Deka, Upasana Sharma, Ravi Gedela, Vijya Laxmi, Ananya Berman,

Dhruv Aditya Shrivastva, Kumar Jagdish, Durga, Dibakar, Samaya, Rimmle and Mayurakhi, an exceptionally talented student, for her constructive and encouraging support for expeiments.

A special thanks to Digar, Rohitash and Himanghsu for their wonderful company and sharing all our nice conversations from hostel and enjoy the course work.

I convey special acknowledgement to Swati, without her support and encouragement in IIT Guwahati have been colourless.

Lastly, I offer my regards to all of those who supported me in any respect during the completion of my work and my journey.

Ravi Kumar

IIT Guwahati 2014

Synopsis

Calcium (Ca^{2+}) is a ubiquitous second messenger molecule that plays a versatile role in intracellular signaling and responsible for controlling numerous cellular processes in eukaryotes (Gadd 1994; Berridge et al. 1998; Shaw and Hoch 2001; Sander et al. 2002; Davies and Terhzaz 2009). Binding of Ca^{2+} changes proteins confirmation and charges, which are the two universal signaling tools of signal transduction, and thus govern protein function (Clapham 2007). The incredible versatility of Ca^{2+}-signaling ranges from processes such as fertilization, development and differentiation and several other cellular activities (Berridge et al. 1998). In filamentous fungi, Ca^{2+}-signaling plays an important role in a range of processes such as growth, hyphal tip branching, infection structure differentiation, secretion, sexual development, sporulation, thermotolerance and oxidative stress (Ohya et al. 1991; Pausch et al. 1991; Iida et al. 1995; Moser et al. 1996; Shaw and Hoch 2001; Valle-Aviles et al. 2007; Tamuli et al. 2011, 2013; Deka et al. 2011; Rodriguez-Caban et al. 2011). The Ca^{2+}-signaling machinery in the filamentous fungus *Neurospora crassa* is unique and relatively more complex than other eukaryotic organisms. The Ca^{2+}-signaling system in *N. crassa* is consisting of 48 Ca^{2+}-signaling proteins and this system appears to be significantly different from plant and animal cell, especially in relation to second messenger systems responsible for Ca^{2+}-release from internal stores (Galagan et al. 2003; Borkovich et al. 2004; Zelter et al. 2004). *N. crassa* lacks recognizable inositol-1,4,5-trisphosphate (InsP3) receptors, which mediated Ca^{2+}-release from intracellular stores in plant and animal cells (Galagan et al . 2003; Borkovich et al. 2004). Moreover, key components of Ca^{2+}-release mechanism in plant and animal cells such as ADP ribosyl and ryanodine receptor proteins are absent in *N. crassa*. However, knowledge about the main components involves in any one of the Ca^{2+}-mediated signal response pathway is still unclear in *N. crassa* or in any of the related fungus.

The Ca^{2+}-signaling proteins of *N. crassa* can be grouped into seven different types. *N. crassa* has three Ca^{2+} channel proteins, nine Ca^{2+}-and cation-ATPases, six recognizable Ca^{2+}/H^{+} exchangers, two novel putative Ca^{2+}/Na^{+} exchangers, four novel phospholipase C-δ subtype (PLC-δ) proteins, 23 Ca^{2+} and/or calmodulin (CaM) binding proteins and one CaM

(Galagan et al. 2003; Borkovich et al. 2004; Zelter et al. 2004). These proteins effectively coordinate to maintain Ca^{2+} homeostasis in *N. crassa* and respond to change in intracellular free $[Ca^{2+}]_c$ through a Ca^{2+} signaling cascade. In *N. crassa*, $[Ca^{2+}]_c$ is maintained at very low level ~0.1 to 0.2 µM (Miller et al. 1990; Silverman-Gavrila and Lew 2001). The change in $[Ca^{2+}]_c$ induce calcium/calmodulin-dependent kinases (Ca^{2+}/CaMKs), which are serine/threonine (Ser/Thr) protein kinases, and modulate diverse cellular response through signal transduction (Chin and Means 2000; Hook and Means 2001; Sorderling and Stull 2001; Zelter et al. 2004). The general domain organization of Ca^{2+}/CaMKs includes a highly conserved N-terminal catalytic domain and a C-terminal regulatory region consisting of overlapping autoinhibitory and Ca^{2+}/CaM binding domains (Swulius and Waxham 2008; Tamuli et al. 2011). Binding of Ca^{2+}/CaM activates Ca^{2+}/CaMKs by removing the autoinhibitory domain that allows the catalytic pocket to access the substrate (Hook and Means 2001). The Ca^{2+}/CaMK enzyme are classified into two groups based on their substrate specificity, one group has broad substrate specificity and the other group has narrow substrate specificity. Members of the broad substrate specificity group have the ability to phosphorylate many different proteins and include monomeric enzymes such as CaMKK, CaMKI and IV besides the multimeric enzyme CaMKII (Hook and Means 2001; Swulius and Waxham 2008). The narrow substrate specificity group includes dedicated kinases such as phosphorylase kinase, myosin-light chain kinase (MLCK) and CaMKIII (Hook and Means 2001; Swulius and Waxham 2008). Fungal homologues of the Ca^{2+}/CaMKs have been identified in *Aspergillus nidulans* (Dayton et al. 1996; Joseph and Means 2000), *Colletotrichum gloeosporioides* (Kim et al. 1998), *Saccheromyces cerevisiae* (Ohya et al. 1991; Pausch et al. 1991), *Schizosaccharomyces pombe* (Rasmussen 2000) and *Sporothrix schenckii* (Valle-Aviles et al. 2007; Rodriguez-Caban et al. 2011). However, little is known about the cellular roles of Ca^{2+}/CaMKs in *N. crassa*. In this work, I have studied cellular roles and genetic interactions of four Ca^{2+}/CaMKs, NCU09123, NCU02283, NCU06177 and NCU09212 that are annotated as CAMK-1, CAMK-2, CAMK-3 and CAMK-4, respectively, in *N. crassa*. I found that these kinases regulate multiple cellular functions such as growth, thermotolerance, survival in oxidative stress and fertility in *N. crassa*.

Chapter 1 describes briefly about the Ca^{2+} signaling pathway in *N. crassa* and other related fungi. In addition, this Chapter also describes the life cycle of *N. crassa* and meiotic silencing. Meiotic silencing is a defense mechanism that operates during the sexual phase (Shiu et al. 2001). Furthermore, this Chapter also contains brief description of our current understanding about thermotolerance and oxidative stress in fungi.

Chapter 2 describes the strains, media, growth, maintenance and crosses of *N. crassa*. The wild-type and Ca^{2+}/CaMKs knockout mutant strains were obtained from the Fungal Genetic Stock Center (FGSC), University of Missouri, Kansas City, MO 64110, Missouri, USA. Media and procedures for growth, maintenance and crosses were essentially as described previously by Davis and De Serres (1970). The knockout mutants were generated by using a high throughput gene knockout procedure, using the *hph* selectable marker that encodes hygromycin B phosphotransferase and confers resistance to hygromycin (Colot et al. 2006). PCR, RT-PCR, Southern hybridization and other molecular biology experiments were performed by using the standard protocols essentially as described by Sambrook and Russell (2001) or manufacturers' protocols.

Chapter 3 describes the role of Ca^{2+}/CaMKs in sexual development of *N. crassa*. Crosses homozygous for Δ*camk-1::hph* and Δ*camk-2::hph* mutants display an intermediate and barren phenotype, respectively. However, another two Ca^{2+}/CaMKs mutants, Δ*camk-3::hph* and Δ*camk-4::hph* were fertile, in homozygous crosses and produced thousands of progeny ascospores. I have extended this to another 16 Ca^{2+}-signaling mutants and all these mutants showed fertile phenotype in homozygous crosses. Crosses homozygous for the Δ*camk-1* mutant produced normal looking perithecia, only a few asci were recovered from the perithecia formed in a cross and displayed an intermediate phenotype (produced few hundred progeny ascospores). Additionally, crosses homozygous for the Δ*camk-2::hph* mutant displayed a barren phenotype (produced very few progeny ascospores). However, Δ*camk-1::hph* and Δ*camk-2::hph* mutant are found to mate successfully with wild-type and produced thousands of progeny ascospores. To exclude the possibility that a direct contact between mutant and wild-type allows complementation of the mutant, I performed crosses using Δ*camk-1::hph* and Δ*camk-2::hph* mutant strains both as male and female parents; however,

such crosses were fully fertile. Therefore, the phenotypes of the Δ*camk-1::hph* and Δ*camk-2::hph* homozygous crosses were consistent even when any of the mating type was used as either male or female parent. These results suggest that out of the four Ca^{2+}/CaMKs, only *camk-1* and *camk-2* play a role in sexual development in *N. crassa* in a recessive manner.

The gene structure, domain organization and sequence analysis of *camk-1*, *camk*-2, *camk-3* and *camk-4* confirmed the presence of conserved CaMK signature motifs. In a phylogenetic analysis, Ca^{2+}/CaMKs from *N. crassa* were clustered with broad substrate specificity kinases. I have also analyzed the 5'-flanking genomic regions (~2 kb) of the Ca^{2+}/CaMKs from *N. crassa* in order to predict the putative regulatory elements involved in transcription regulation. All the four Ca^{2+}/CaMKs of *N. crassa* possess TATA less promoter with GC rich sequences near the transcription start sites (TSS) and contain putative sites for regulatory elements, some of which are GATA, activator of nitrogen regulated genes, pheromone response element, activator of stress genes, pH responsive regulators and Dde box. These regulatory sequences could further support the involvement of *N. crassa* Ca^{2+}/CaMKs in specific cell functions.

In Chapter 4, I describe complementation analysis of Δ*camk-1::hph* and Δ*camk-2::hph* mutants. For complementation analysis, fragment carrying the *camk-1* and *camk-2* were PCR amplified from the wild-type and cloned into the *SmaI* site of plasmid vector pBARGEM7-1 that resulted in pRK-1 and pRK-2 constructs and transformed into the Δ*camk-1::hph* and Δ*camk-2::hph* recipients, respectively. Initial transformants were crossed with the opposite mating type strain of the corresponding mutants to isolate homokaryotic strains. Crosses homozygous for the homokaryotic transformants for pRK-1 and pRK-2 constructs are fully fertile. These results suggest that *camk-1* and *camk-2* genes are essential for full fertility in *N. crassa*. In addition, the *camk-2* product is necessary during the meiosis of the fertilized zygote. The crosses homozygous for *Δcamk-2::hph* mutant display a more severe phenotype than the *Δcamk-1::hph* mutant. To determine whether the *camk-2* product is necessary before karyogamy or during the meiosis of the fertilized zygote, I tested the effect of meiotic silencing of *camk-2*. Meiotic silencing is an RNAi based post-transcriptional gene silencing process triggered by unpaired DNA sequences homologous chromosomes during meiosis

(Shiu et al. 2001). To test the effect of meiotic silencing of the *camk-2*, I performed crosses involving *camk-2*::*bar* strains possessing an ectopic copy of the *camk-2* tagged with the *bar* selective marker. The *camk-2*::*bar* strain crossed with the wild-type has resulted in a barren phenotype. However, the cross between the *camk-2*::*bar* strain with *Sad-1*, a semi-dominant suppressor of meiotic silencing, is fully fertile. Moreover, fertile phenotype is also observed in the crosses involving homozygous *camk-2*::*bar* strains. These results support the hypothesis that product of the *camk-2* during meiosis is necessary for full fertility in *N. crassa*.

In Chapter 5, I described phenotype of the double mutants involving Δ*camk-1::hph,* Δ*camk-2::hph*, Δ*camk-3::hph* and Δ*camk-4::hph* to understand the genetic interaction of these Ca^{2+}/CaMKs. I have generated *Δcamk-1::hph Δcamk-2::hph*, *Δcamk-4::hph Δcamk-2::hph* and *Δcamk-3::hph Δcamk-2::hph* double mutants and tested their phenotype. Crosses homozygous for *Δcamk-1::hph Δcamk-2::hph*, *Δcamk-4::hph Δcamk-2::hph* and *Δcamk-3::hph Δcamk-2::hph* double mutants display barren phenotype like the *Δcamk-2::hph* homozygous crosses. In addition, ascospores produced in barren crosses display lower survival percentage than the wild-type. Furthermore, the *Δcamk-1::hph Δcamk-2::hph* double mutant shows a severe growth defect, suggesting a synthetic interaction of these two Ca^{2+}/CaMKs. The average growth rates of the wild-type and Ca^{2+}/CaMKs mutants follow the order Δ*camk-2::hph* ≅ *wild-type* ≅ Δ*camk-3::hph* ≅ Δ*camk-1::hph* ≅ Δ*camk-4::hph* > Δ*camk-3::hph* Δ*camk-2::hph* ≅ Δ*camk-4::hph* Δ*camk-2::hph* > Δ*camk-1::hph* Δ*camk-2::hph*. The Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-3::hph* Δ*camk-2::hph* double mutants grow slowly than both the parental single mutants and the wild-type strains. In addition, Δ*camk-1::hph* Δ*camk-2::hph*, Δ*camk-4::hph* Δ*camk-2::hph*, and Δ*camk-3::hph* Δ*camk-2::hph* double mutant strains display reduced aerial hyphae. I also analyzed if the disruption of the Ca^{2+}/CaM binding could have any phenotypic effect using the calmodulin (CaM) antagonist trifluoperazine (TFP). Interestingly, the growth rates showed three distinct patterns on the medium supplemented with the CaM antagonist TFP (100 µM); wild-type and Δ*camk-2::hph* were comparatively less inhibited, Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-1::hph* Δ*camk-2::hph* were severely inhibited, whereas inhibition of the Δ*camk-1::hph*, Δ*camk-3::hph*, Δ*camk-4::hph* and Δ*camk-3::hph* Δ*camk-2::hph* were intermediate. The CaM antagonist TFP

severely inhibits growth of the Δ*camk-4::hph* Δ*camk-2::hph* mutant, which otherwise showed a relatively higher growth rates. The synthetic phenotype of the Δ*camk-4::hph* Δ*camk-2::hph* mutant in exposure to TFP treatment suggest that these two Ca^{2+}/CaMKs play an important role in growth and activated upon Ca^{2+}/CaM binding. These results consistently suggest genetic interaction of *camk-2* with *camk-1*, *camk-3*, and *camk*-4.

I have also tested if the Ca^{2+}/CaMKs in *N. crassa* play any role in thermotolerance and survival in oxidative stress. The *Δcamk-1::hph*, *Δcamk-3::hph*, *Δcamk-4::hph*, *Δcamk-1::hph Δcamk-2::hph*, *Δcamk-4::hph Δcamk-2::hph* and *Δcamk-3::hph Δcamk-2::hph* mutants show a decreased survival percentage both in uninduced and induced thermotolerance conditions than the wild-type and *Δcamk-2::hph* mutant strain. The survival percentage on exposure to 52°C from 44°C for induced thermotolerance follow the order *Δcamk-2::hph* > wild-type > *Δcamk-3::hph* > *Δcamk-3::hph Δcamk-2::hph* > *Δcamk-1::hph Δcamk-2::hph* > *Δcamk-1::hph* > *Δcamk-4::hph Δcamk-2::hph* > *Δcamk-4::hph*. In addition, on exposure to 52°C from 30°C for uninduced thermotolerance, the survival percentage follow the order wild-type > *Δcamk-2::hph* > *Δcamk-3::hph* > *Δcamk-1::hph Δcamk-2::hph* > *Δcamk-4::hph Δcamk-2::hph* > *Δcamk-3::hph Δcamk-2::hph* > *Δcamk-1::hph* > *Δcamk-4::hph*. The *Δcamk-2::hph* mutant shows lower survival percentage in uninduced but higher survival percentage in induced condition. Therefore, *camk-2* could be a mediator of cell-death triggered by heat during induced condition. Moreover, survival percentage in H_2O_2-induced oxidative stress follow the order *Δcamk-2::hph* > wild-type > *Δcamk-1::hph Δcamk-2::hph* > *Δcamk-1::hph* > *Δcamk-4::hph Δcamk-2::hph* > *Δcamk-4::hph* > *Δcamk-3::hph* > *Δcamk-3::hph Δcamk-2::hph*. The *Δcamk-2::hph* mutant shows an increased survival percentage than the wild-type. The *Δcamk-3::hph Δcamk-2::hph* double mutant has shown a severe sensitivity to H_2O_2 stress. These results consistently suggest genetic interactions of *camk-2* with *camk-1, camk-3,* and *camk-4*.

Additional results, overall summary of this thesis works, and the scope for further studies have been discussed in last section of the thesis.

Dedicated

to

My Mothers

Chapter 1

An introduction to *Neurospora crassa* and intracellular calcium signaling

1.1 The biology of *Neurospora crassa*

Neurospora has been known to science since the nineteenth century as a causative organism of fungal infestation in French bakeries (Perkins and Davis 2000). *Neurospora crassa*, has been established as a eukaryotic model organism in 1920s and since then it has become an experimental system (Sun 1964; Davis and De Serres 1970; Perkins and Davis 2000). *N. crassa* has been extensively used in diverse field of biological research, such as genetics, biochemistry, and molecular biology etc. (Galagan et al. 2003).

The filamentous fungus *N. crassa* is a multicellular haploid organism. Its genome size of ~43 Mb, divided into seven linkage groups (LG I-VII, size ranges from 4 to 10.3 Mb each), and contains 10,082 protein-coding genes (Figure 1.1; Davis and De Serres 1970; Perkins and Davis 2000; Galagan et al. 2003). As a heterotroph organism, *N. crassa* can use a number of carbon sources such as acetate, succinate, glycerol, glucose, and other monosaccharide and a number of oligo and polysaccharides. Similarly, *N. crassa* can use nitrate, nitrite, ammonium and amino acids as a nitrogen sources. In addition to carbon and nitrogen, *N. crassa* requires only a few simple salts, trace elements and a single vitamin, biotin, for vigorous growth (Sun 1964; Davis and De Serres 1970; Burnett 1975; Perkins and Davis 2000).

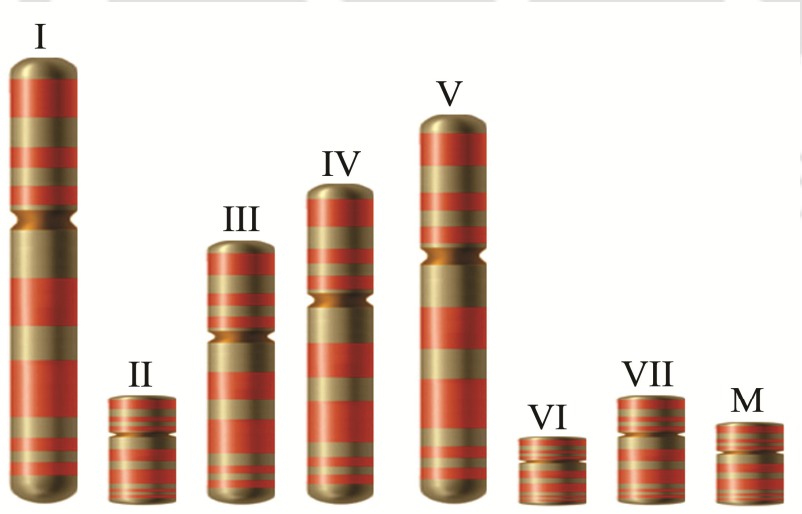


Figure 1.1 Assembly of chromosomes in *Neurospora crassa*. The mapped genomic regions are separated into seven chromosomes according to their linkage groups (LG I to LG VII) and mitochondria (M). Major synteny blocks are represented.

1.2 The life cycle of *Neurospora crassa*

The vegetative system of *N. crassa* is composed of multinucleate branched filaments or hyphae. The hyphae are segmented by incomplete cross-walls or septa that allow cytoplasm to flow along the hyphae usually in direction of the growth, carrying nuclei, mitochondria and other inclusions for some distance. In the vegetative phase of *N. crassa*, considerable hyphal anastomosis takes place within and between strains. A hyphal system is also called a mycelium. *N. crassa* has three forms of spores; two asexual spores known as macroconidia and microconidia, and one sexual ascospore (Sun 1964; Davis and De Serres 1970; Burnett 1975) and produced early during the vegetative growth. Macroconidia are multinucleate, with an average nuclear number of 2.5 in most media. Macroconidia are used to inoculate vegetative cultures and they serve also as the fertilizing (male) parent in sexual crosses. Microconidia are formed late in growth by a process quite distinct from macro conidial formation. On the other hand, ascospores are formed at the end of the sexual process; they can survive for a long period in dormant stage at which genotypes may be isolated in unambiguously pure form (Davis and De Serres 1970).

N. crassa is a heterothallic filamentous fungus that contains two nonswitching mating type, *A* and *a* in a sexual phase in its life cycle (Figure 1.2). Sexual development in *N. crassa* is induced by limiting nitrogen and carbon sources. In response to nutrient starvation, either of the mating type of *N. crassa* can form the female sexual structure called protoperithecium. Special receptor hyphae, called trichogyne, emanate from the protoperithecium. Fusion plasmogamy of trichogynes with a male element of the opposite mating-type during fertilization initiates the development of the perithecium, the multicellular sexual apparatus, and results in plasmogamy. After, plasmogamy, the male and the female derived nuclei coexist in a heterokaryotic tissue and divide mitotically until they are sorted into dikaryotic tissue, in which each cell compartment contains only one nucleus of each mating type (Raju 1980, 1992; Springer 1993). In the dikaryotic tissue, nuclei of opposite mating type do not fuse immediately, they undergo a synchronous mitosis to isolate two nuclei in the tip of a specialized hook-shaped cell structure called the crozier. Here the nuclei fuse to form a diploid zygote nucleus during karyogamy. The resulting diploid zygote nucleus immediately undergoes the two meiotic divisions and a postmeiotic mitosis to yield eight sexual spores

called ascospores within an ascus in an order that reflects their lineage. Ascospores are then forcefully ejected from the perithecium and ascospores must be subjected to a heat shock (~64ºC for 60 min in a typical laboratory condition) to activate and produce vegetative mycelia. This allows crosses to be analyzed at any time after they are mature, and the activation treatment kills parental cells and other contaminants at the same time. The two mating types segregate in a 1:1 ratio among the ascospores, as two alleles of a single gene (Davis and De Serres 1970; Perkins and Davis 2000).

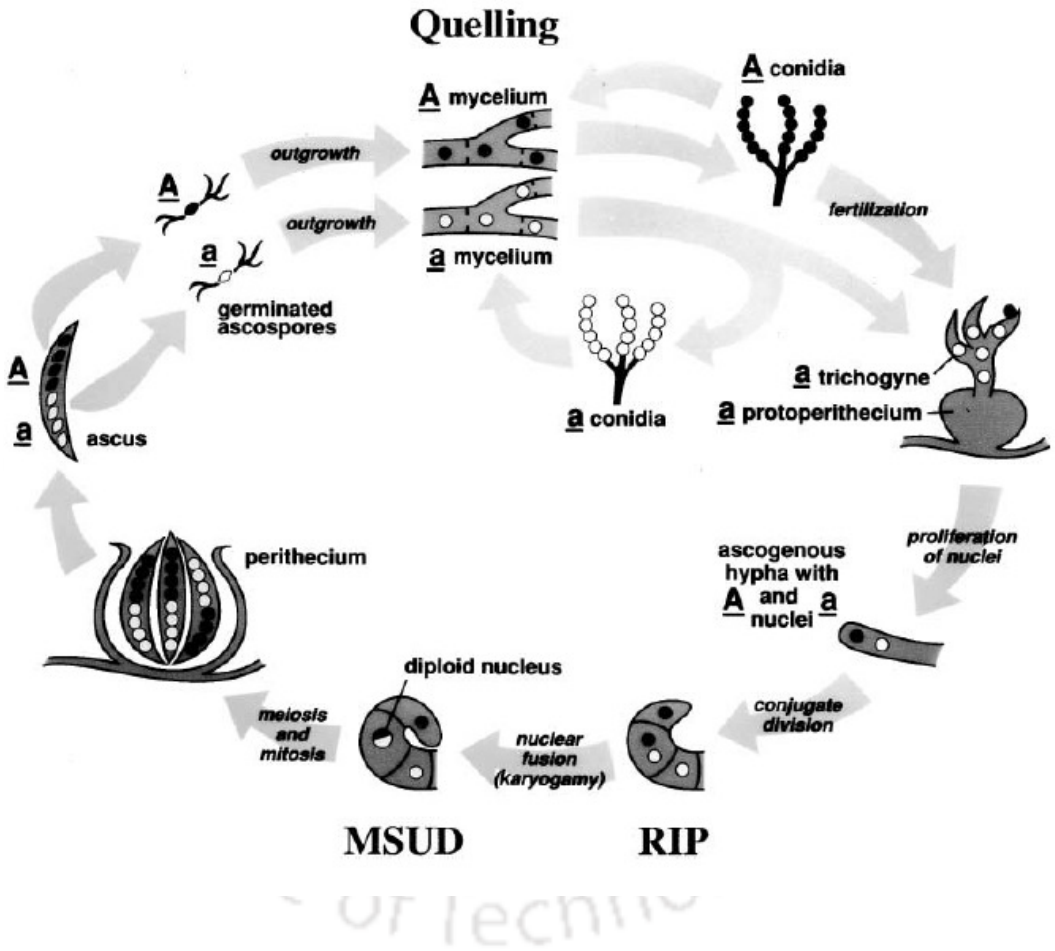


Figure 1.2 The Life cycle of *N. crassa*. Indicated are the sites for quelling, RIP (repeat-induced point mutation), and MSUD (meiotic silencing by unpaired DNA). Mating occurs only between strains of *mat A* and *mat a*. After fertilization, the haploid nuclei proliferate in the premeiotic ascogenous tissue, which gives rise to asci within the developing ascocarp (perithecium). In the young ascus, the haploid nuclei derived from the *mat A* and *mat a* parents fuse. The diploid zygote nucleus immediately undergoes the two meiotic divisions

and a postmeiotic mitosis in the common cytoplasm of the ascus. The resulting eight haploid nuclei are then sequestered into eight spindle-shaped (American football-shaped) ascospores, which are held in linear order in the narrow ascus. Maturing ascospores become multinucleate and pigmented. About 200 asci are produced in each perithecium. Adopted from Shiu et al. (2001).

1.3 Gene silencing mechanisms that operates during the life cycle of *Neurospora crassa*

In *N. crassa*, three gene silencing mechanisms have been discovered, these are quelling, repeat-induced point mutation (RIP) and meiotic silencing by unpaired DNA (MSUD). These gene silencing mechanism are believed to be involved to control the amplification of unwanted sequences such as transposons and viruses. Quelling may occur in somatic cells during vegetative life, either as direct or indirect consequence of the presence of abnormal numbers of DNA copies similar to co-suppression in plants and RNA interference in animals and quelling in *N. crassa* (Dalmay et al. 2000). Another silencing process called RIP occurs before karyogamy defense genome-wide scan for duplicated sequences, followed by extensive introduction of C to T mutations in any such sequence. Unlike quelling, RIP is irreversible. Tandemly repeated rDNA sequences within the nucleolus organizer are escaped from RIP, while DNA sequences encoding 5S RNA and tRNA are below the size threshold of the RIP machinery. One plausible role of RIP is the inactivation of transposons that have already been duplicated at least once (Selker 1990, 1997).

Another RNAi-based gene silencing mechanism in *N. crassa* is meiotic silencing that operates after karyogamy (Shiu et al. 2001). Meiotic silencing is similar to quelling but only occurs during meiosis (Aramayo and Metzenberg 1996; Shiu et al. 2001; Shiu and Metzenberg 2002). DNA unpaired in the first meiotic prophase, results in both self-silencing and trans silencing of all DNA homologous to it, whether paired or not. Even single-copy DNA sequence that is present in one parent but not in the other will be silenced. The assay that lead to discovery of the meiotic silencing utilized, an extra copy of *ascospore maturation-1* (*asm-1*; Aramayo and Metzenberg 1996) is placed at an ectopic location in one parent but not the other, all copies of this gene (paired or unpaired) are silenced during sexual development resulting in the production of white and inviable ascospores (Shiu et al. 2001).

Meiotic silencing appears to be a robust mechanism and additional genes have been successfully used as reporting markers for its activity. These include *actin* (*act*$^{+}$) and *β-tubulin* (*bml*^{R}), whose unpairing result in the abortion of most asci and *Round spore* (*r*$^{+}$), whose unpairing leads to the production of round ascospores (instead of spindle-shaped ones).

Meiotic silencing acts on homologous genes that are not paired during prophase I of meiosis. So far five different proteins involve in meiotic silencing have been identified and all of them localize in the perinuclear region. The proteins that are found involved in meiotic silencing are SAD-1, an RNA-directed RNA polymerase (RdRP); DCL-1, a Dicer-like RNase III enzyme (also called SMS-3); SMS-2, an Argonaute-family protein; SAD-2, a protein regulating SAD-1 localization; and QIP, an exonuclease (Shiu and Metzenberg 2002; Lee et al. 2003; Shiu et al. 2006; Kelly and Aramayo 2007; Alexander et al. 2008; Lee et al. 2010; Xiao et al. 2010). SAD-1 appears to be involved in the production of a single-stranded aberrant RNA from an unpaired segment. This RNA is exported to the perinuclear region where it acts as a template for the SAD-1 mediated double-stranded RNA (dsRNA) synthesis. DCL-1 then processes the dsRNA into small interfering RNA (siRNA), which guides SMS-2 to identify and slice complementary mRNA. In current working model of meiotic silencing, SAD-2 is a protein that interacts with SAD-1 and helps transport it to the perinuclear region, whereas QIP is an exonuclease that removes the passenger strand of a siRNA duplex (Figure 1.3). The unpaired regions with homology to the reporter transcript could trigger the meiotic silencing of the reporter gene and efficiency of silencing increases as the size and homology of the unpaired region increase.

Like quelling, RIP and MSUD probably exerts surveillance over proliferated invasive elements during stages when all well-behaved genes should be haploid. Meiotic silencing could be effective against sequences that are not established in homologous positions of both parents, i.e., that are not diploid when they should be. This silencing mechanism might be important in holding down the genetic load attributable to transposable elements that move during meiosis (Shiu et al. 2001).

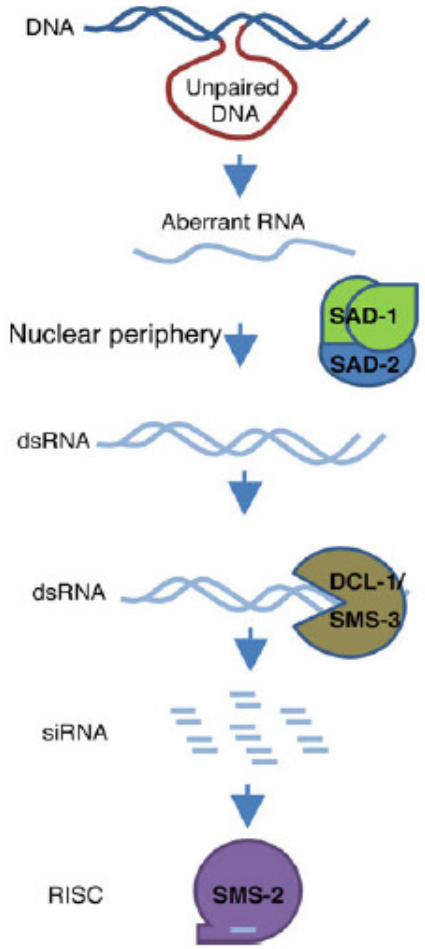


Figure 1.3 Current model of meiotic silencing in *N. crassa*. During the first meiotic prophase, an unpaired DNA triggers the transcription of aberrant RNAs from an unpaired DNA region. The RdRP, SAD-1converts aberrant RNA into dsRNAs, which are processed by DCL-1 into small RNAs, and small RNAs are then loaded onto a RISC complex with the Argonaute SMS-2 as the core component. The activation of the SMS-2 complex results in the silencing of homologous RNA. SAD-2 may function in the pathway by recruiting SAD-1 to its proper location for its function. Adopted form Liande et al. (2010).

1.4 Intracellular signaling in *Neurospora crassa*

Filamentous fungi are able to grow in more diverse environments than yeast, therefore, it is expected that this group of organisms should posses an extensive array of signaling capabilities. Analysis of genes implicated in signal transduction and environmental responses demonstrate that *N. crassa* possesses class of sensing molecules not found in the yeast. The expansion of upstream signaling proteins is often coupled with a conserved core of downstream components, suggesting the presence of extensive new signaling interactions. The intracellular signaling pathways in *N. crassa* are involved mitogen-activated protein kinase (MAPK), histidine kinase (HK), response regulator (RR), G-protein-coupled receptors (GPCRs) and calcium signaling (Ca^{2+} signaling).

In *N. crassa*, MAPK pathways integrate signal from multiple pathways including two-component signaling systems (Gustin et al. 1998). MAPK pathways consist of three serine/threonine protein kinases (MAPKKK, MAPKK, and MAPK) that act sequentially, resulting in phosphorylation of target proteins that regulate transcription, the cell cycle, or other cellular processes (Posas et al. 1996; Paul et al. 1997; Chang and Karin 2001). MAPK modules are regulated by a wide variety of signaling proteins in other fungi, including GPCRs, PAKs, histidine phosphorelays, and Cdc42p (Posas et al. 1996; Paul et al. 1997; Chang and Karin 2001). In *N. crassa* nine MAPK proteins, correspond to those found in *S. pombe* and *S. cerevisiae* have been identified, (Figure 1.4).

The intracellular signaling system consists of two basic components such as HK and RR (Figure 1.4). The HK and RR is autophosphorylated in an ATP-dependent manner on a conserved histidine residue and this autophosphorylation activity is regulated by an environmental signal sensed by the kinase. In contrast, *N. crassa* has a significantly expanded complement of 11 HK, as compared with one in *S. cerevisiae* and three in *S. pombe*. This number of HK suggests a larger role cell of HK in signaling and environmental response in *N. crassa*.

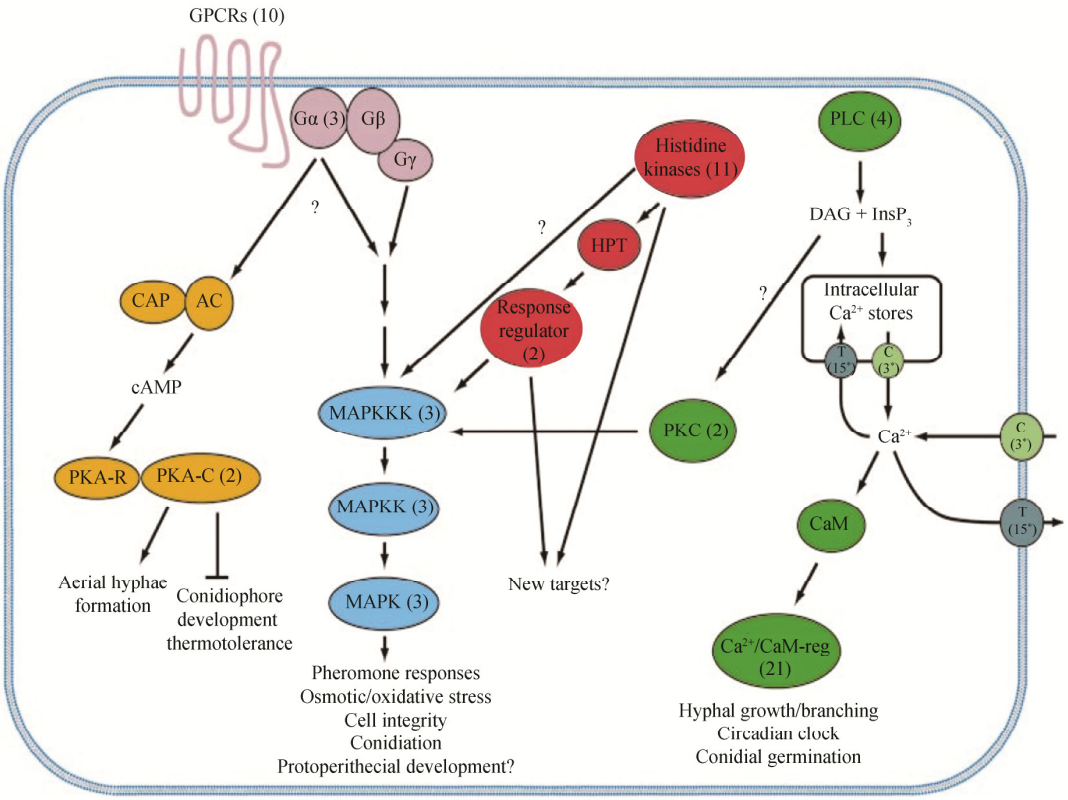


Figure 1.4 Overview of major intracellular signaling pathways in *N. crassa*. The numbers identified for each gene are in parenthesis. An asterisk indicates that the location in the plasma membrane and/or organelle membranes is not determined. AC, adenylyl cyclase; C, Ca^{2+} channel proteins; CaM, calmodulin; Ca^{2+}/CaM-reg, calcium- and calmodulin-regulated protein; CAP, cyclase-associated protein; DAG, diacylglycerol; GPCR, G-protein-coupled receptor; Gα, G protein α–subunit; Gβ, G protein β-subunit; Gγ, G protein γ-subunit; HPT, histidine-containing phosphotransfer domain protein; MAPK, MAP kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; PKA-C, protein kinase A catalytic subunit; PKA-R, protein kinase A regulatory subunit; PLC, phospholipase C; PKC, protein kinase C; T, Ca^{2+} transport protein (P-type Ca^{2+} ATPase, H^+/Ca^{2+} exchanger, or Na^+/Ca^{2+} exchanger). Adopted from Galagan et al. (2003).

The G-protein coupled receptors (GPCRs) in eukaryotes detect a wide range of environmental stimuli (Figure 1.4; Galagan et al. 2003). GPCRs regulate the activity of second messengers through their interaction with heterotrimeric G proteins (Neer 1995). A heterotrimeric G-protein consists of Gα, Gβ, and Gγ subunit. In *N. crassa*, G-protein-coupled receptor (GPR-1; Krystofova and Borkovich 2006), *guanine nucleotide alpha-1* (*gna-1*; Turner and Borkovich 1993; Ivey et al. 1996, 1999; Yang and Borkovich 1999; Borkovich et al. 2004), and *guanine nucleotide beta-1* (*gnb-1*; Yang et al. 2002; Borkovich et al. 2004) are required for female sexual development. In the next section I describe about the Ca^{2+} signaling.

1.5 Calcium signaling

Calcium ion (Ca^{2+}) is a ubiquitous second messenger molecule that plays an important role in intracellular signaling in eukaryotic organisms (Gadd 1994; Berridge et al. 1998; Shaw and Hoch 2001; Sander et al. 2002; Davies and Terhzaz 2009). Binding of Ca^{2+} changes protein confirmation and change, which are the two universal signaling tools of signal transduction, and thus Ca^{2+} regulates protein functions (Clapham 2007). Therefore, Ca^{2+} plays a central role as an intracellular signal in biological systems (Gadd et al. 1994; Sanders et al. 2002; Berridge et al. 1998; Shaw and Hoch 2001; Davies and Terhzaz 2009). The resting level of cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) is very low (typically 50-100 nM), which is maintained by active Ca^{2+}-pumps and Ca^{2+}-transporter proteins, and the Ca^{2+}-buffering capacity of the cytoplasm. However, $[Ca^{2+}]_c$ becomes an intracellular signal when its concentration is transiently increased due to activation of Ca^{2+}-permeable channels thereby allowing Ca^{2+} to flow down a concentration gradient into the cytoplasm from Ca^{2+}-storage organelles or from the external medium. Following increase of $[Ca^{2+}]_c$, it recovers to the original resting level by increased activity of Ca^{2+}-pumps and Ca^{2+}-transporters. Cells use different combination of Ca^{2+}-channels, Ca^{2+}-pumps, Ca^{2+}-transporter and other Ca^{2+} signaling proteins to produce Ca^{2+} signals with different dynamic spatial and temporal characteristics (Berridge et al. 1998).

The Ca^{2+} signaling regulates numerous cell processes such as fertilization, development and differentiation, and cell death (Berridge et al. 1998). In filamentous fungi, Ca^{2+} signaling is known to play an important role in a number of cell processes such as Ca^{2+}

stress tolerance, cell cycle progression, circadian clock, cytoskeletal organization, growth, hyphal tip branching, infection structure differentiation, secretion, sporulation, sexual development, ultraviolet (UV), thermotolerance and oxidative stress survival (Ohya et al. 1991; Gadd 1994; Shaw and Hoch 2001; Valle-Aviles et al. 2007; Deka et al. 2011; Rodriguez-Caban et al. 2011; Tamuli et al. 2011). In the filamentous fungus *N. crassa*, hyphal growths require a tip-high intracellular [Ca^{2+}] gradient and Ca^{2+} is involved in the branching signal (Reissig and Kinney 1983; Silverman-Gavrila and Lew 2002). However, detailed knowledge about the main components involved in any one Ca^{2+}-mediated signal responses pathway is still not clear for *N. crassa* or any other filamentous fungus (Galagan et al. 2003; Borkovich et al. 2004).

1.5.1 Distribution of calcium in *Neurospora crassa* organelles

In *N. crassa*, most of a cell Ca^{2+} is sequestered in vacuoles (Bowman et al. 2011). The vacuolar uptake of Ca^{2+} is responsible for sequestering excess and hazardous amounts of free Ca^{2+} from cytosol in *N. crassa* (Cornelius and Nakashima 1987). In vitro experiment demonstrated that Ca^{2+} uptake by vacuoles is decreased in absence of $MgSO_4$, indicating that Mg^{2+} has a role in vacuolar uptake of Ca^{2+}. In *N. crassa*, >90% of cell Ca^{2+} is sequestered in vacuoles with a significant amount in an insoluble form, mitochondria contain about 4% of cellular Ca^{2+}, and the remaining cells Ca^{2+} is distributed in other organelles such as endoplasmic reticulum, Golgi, nuclei, plasma membranes and various transport vesicles (Bowman et al. 2011).

1.5.2 Measurement of intracellular calcium concentrations

The cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) can be measured using different methods such as Ca^{2+}-sensitive fluorescent probes (Caswell 1979; Schmid and Harold 1988), microelectrodes (Miller et al. 1990; Silverman-Gavrila and Lew 2003) and aequorin (Nelson et al. 2004). The Ca^{2+}-selective microelectrode technique was used to determine the mean estimate of $[Ca^{2+}]_c$ in *N. crassa*, which is 92 ± 15 nM, and this value remains unchanged in the external pH range 5.8-8.4 (Miller et al. 1990).

In another method, Ca^{2+}-sensitive dye is used to measure the $[Ca^{2+}]_c$. Chlortetracycline (CTC), forms a fluorescent complex with Ca^{2+} (Schmid and Harold 1988) and therefore can be used to determine the gradient of intracellular Ca^{2+}. The *N. crassa* generally grown on medium supplemented with CTC 100 µM (dissolved in 0.1% dimethyl sulfoxide) and fluorescence of the CTC incorporated to *N. crassa* can be studied using a fluorescent microscope (CTC: 400-440 nm excitation filter, 460 nm beam splitter and 470 nm emission filter). Although, CTC fluorescence is affected by membrane density and Mg^{+} ions, the relative CTC fluorescence is much greater for membrane-associated Ca^{2+} than Mg^{+} (Hallett et al. 1972) and the distribution of intracellular membrane can be assessed with N-phenyl-1-napthylamine fluorescence (NPN: 380/9 nm excitation filter, 405 nm beam splitter and 470 nm emission filter; Schmid and Harold 1988).

Ca^{2+}-selective microelectrodes can be made using micropipettes containing a sensor mixture (Tsien and Rink 1980; Miller et al. 1990). The sensor mixture is essentially Ca^{2+}-sensitive fluorescent dyes such as fluo-3 and Fura Red. The *N. crassa* hyphae of size 50-100 µm long and 12-15 µm diameter are impaled at some distance behind the tip and the $[Ca^{2+}]_c$ is then imaged and quantified using dual-dyes ratio imaging (Silverman-Gavrila and Lew 2003). In an alternative method, the growing edge of a *N. crassa* colony is cultured in dark for 18-20 h in the presence of two different dyes, Ca^{2+}-sensitive fluo-3 and Ca^{2+}-insensitive SNARF-1 (Levina et al. 1995). Only a few growing hyphae accumulated the dyes and these hyphae are then rinsed with Vogel's medium (pH 4.2) and observed under a confocal microscope (Levina et al. 1995).

In a recently developed methodology, codon-optimized aequorin has been successfully used for Ca^{2+} measurement in living *N. crassa*. Aequorin is a Ca^{2+}-binding photoprotein isolated from the jellyfish, *Aequorea victoria* (Charbonneau et al. 1985; Inouye et al. 1985). Aequorin is composed of a ~22 kDs apo-aequorin (the apoprotein), the coelenterazine (the luciferin) and bound oxygen (Kendall and Badminton 1998; Nelson et al. 2004). On Ca^{2+} binding to aequorin (aequorin has three EF-hand Ca^{2+}-binding sites), a conformational change converts aequorin into an oxygenase (luciferase) that catalyses the oxidation of coelenteramide (Kendall and Badminton 1998). The excited coelenteramide remains non-

covalently bound to apo-aequorin and releases the energy as blue light (λ_{max} = 470 nm) to reverse back to ground state (Shimomura and Johnson 1975; Kendall and Badminton 1998). Therefore, the amount of luminescence is dependent upon the concentration of free Ca^{2+}, and aequorin can be used to investigate intracellular $[Ca^{2+}]_c$ in a variety of systems including the living organism (Nelson et al. 2004).

1.5.3 Calcium signaling system in *Neurospora crassa*

The Ca^{2+} signaling system in *N. crassa* is complex and consisting of 48 Ca^{2+} signaling proteins (Figure 1.5; Table 1.1). The Ca^{2+} signaling system of *N. crassa* is significantly different from plant and animal cells, mainly in relation to second messenger systems responsible for Ca^{2+}-release from internal stores (Galagan et al. 2003; Borkovich et al. 2004; Zelter et al. 2004). Recognizable inositol-1,4,5-trisphosphate (InsP3) receptors, which mediate Ca^{2+}-release from intracellular stores in plant and animal cells have not been identified in *N. crassa* (Galagan et al. 2003; Borkovich et al. 2004). However, InsP3 is present within *N. crassa* hyphae and physiological evidence suggested that InsP3-activated Ca^{2+} channel activity support a role in Ca^{2+} signaling (Silverman et al. 2001; Cornelius et al. 1989). In addition, *N. crassa* also lacks other key components of Ca^{2+} release mechanisms in plant and animal cells such as ADP ribosyl cyclase and ryanodine receptor proteins (Galagan et al. 2003). Besides, extracellular Ca^{2+}-sensing receptor protein, as reported in animal cells for sensing changes in the extracellular concentration of Ca^{2+} is absent in *N. crassa* (Brown et al. 1993; Borkovich et al. 2004). Interestingly, both Ca^{2+}/Na^+ and Ca^{2+}/H^+ exchangers are present in fungi, although, animal posses only Ca^{2+}/Na^+ exchangers and plant contain only Ca^{2+}/H^+ exchangers (Borkovich et al. 2004).

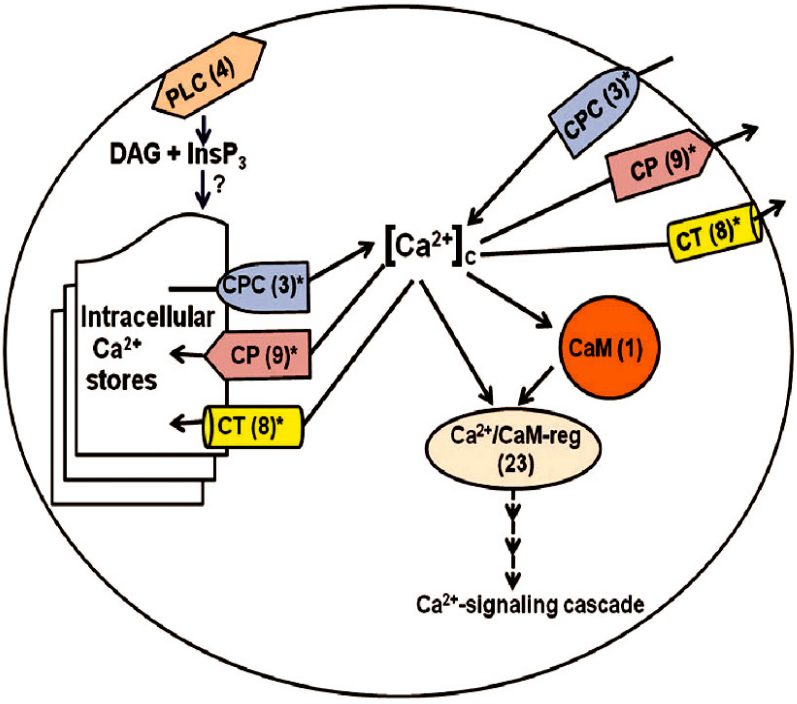


Figure 1.5 Overview of major intracellular Ca^{2+} signaling process in *N. crassa*. The Ca^{2+} signaling proteins, CPC: Ca^{2+}-permeable channel, CP: Ca^{2+}-ATPase, CT: Ca^{2+}/H^{+}-transporter, PLC: phospholipase C, CaM: calmodulin, Ca^{2+}/CaM reg: Ca^{2+} and/or calmodulin regulated are shown with respect to their cellular location (* indicated that location has not been determined) and total protein numbers (indicated in parenthesis). Adopted from Tamuli et al. (2013).

The Kozak sequences has been determined using bioinformatics analysis, the consensus is C43 (A35/C35) G41C45C52 (A68/G20) (C43/A27/T18) C54**A100T100G**100G54C48 (Table 1.1; Tamuli et al. 2013). This consensus is similar to the Kozak sequence described for *N. crassa* (C57NNNC77A81 (A44/C43) 'T'3**A99T100G99**G51C53; Bruchez et al. 1993), with few exceptions. The start codon (ATG) is 100% conserved within the Kozak sequence of all the 48 Ca^{2+} signaling genes. In addition, prevalence of stop codons TAA, TAG and TGA are, respectively, ~47% (23/48), ~29% (14/48) and ~22% (11/48).

[a]**Table 1.1 Calcium signaling proteins in *Neurospora crassa***

S. no.	NCU no.	Name	Type of protein	Best overall[b] (*e*-value; organism; protein name; accession number)
1.	02762.7		Ca^{2+} permeable channel	0; *Verticillium dahliae* (cch1); EGY18507.1
2.	06703.7		Ca^{2+} permeable channel	2e-97; *Paracoccidioides brasiliensis* (MID1); EEH23338.1
3.	11680.7[c]		Ca^{2+} permeable channel	0; *Ajellomyces dermatitidis* (Yvc1); EGE78766.1
4.	03305.7	NCA1	Ca^{2+}-ATPase	0; *Trichophyton tonsurans* (SCA-1); EGD96734.1
5.	04736.7	NCA2	Ca^{2+}-ATPase	0; *Magnaporthe oryzae* (Plasma membrane calcium-transporting ATPase 3); EHA56671.1
6.	05154.7	NCA3	Ca^{2+}-ATPase	0; *Glomerella graminicola* (Calcium-translocating P-type ATPase); EFQ29373.1
7.	03292.7	PMR1	Ca^{2+}-ATPase	0; *Uncinocarpus reesii* (PMR1); XP_002541437.1
8.	08147.7	PH-7	Ca^{2+}-ATPase	0; *Glomerella graminicola* (Potassium/sodium efflux P-type ATPase); EFQ36596.1
9.	04898.7		Ca^{2+}-ATPase	0; *Cordyceps militaris* (Cation-transporting ATPase 4); EGX91104.1
10.	03818.7		Ca^{2+}-ATPase	0; *Verticillium dahliae* (Neo1p); EGY18069.1
11.	07966.7		Cation-ATPase	0; *Trichophyton tonsurans* (Cta3p); EGD97988.1
12.	10143.7[d]		Cation-ATPase	0; *Cordyceps militaris* (ATPase

				type 13A2); EGX92563.1
13.	07075.7	CAX	Ca^{2+}/H^{+} exchanger	0; *Glomerella graminicola* (Calcium/proton exchanger); EFQ30300.1
14.	00916.7		Ca^{2+}/H^{+} exchanger	2e-176; *Aspergillus fumigates* (Membrane bound cation transporter); XP_001481534.1
15.	00795.7		Ca^{2+}/H^{+} exchanger	1e-149; *Aspergillus niger* (Membrane bound cation transporter); XP_001400827.2
16.	06366.7		Ca^{2+}/H^{+} exchanger	0; *Sclerotinia sclerotiorum* (Ca^{2+}/H^{+} antiporter); XP_001589752.1
17.	07711.7		Ca^{2+}/H^{+} exchanger	4e-160; *Trichophyton tonsurans* (Vacuolar calcium ion transporter/H^{+} exchanger); EGD98067.1
18.	05360.7		Ca^{2+}/H^{+} exchanger	0; *Metarhizium anisopliase* (Calcium permease); EFY95914.1
19.	02826.7		Ca^{2+}/ Na^{+} exchanger	0; *Verticillium albo-atrum* (Sodium/calcium exchanger protein); XP_003004985.1
20.	08490.7		Ca^{2+}/ Na^{+} exchanger	1e-83; *Aspergillus niger* (Sodium/calcium transporter); XP_001397155.1
21.	01266.7		Phospholipase C	0; *Sordaria macrospora* (Phosphoinositide-specific phospholipase C); XP_003348116.1
22.	06245.7	PLC-1	Phospholipase C	0; *Glomerella graminicola*

				(Phosphatidylinositol-specific phospholipase C); EFQ28596.1
23.	11415.7[e]		Phospholipase C	0; *Glomerella graminicola* (Phosphatidylinositol-specific phospholipase C); EFQ31595.1
24.	02175.7		Phospholipase C	3e-125; *Botryotinia fuckeliana* (BcPLC2); CCD34776.1
25.	04120.7	CaM	Calmodulin	1e-103; *Gibberella zeae* (CaM); XP_382067.1
26.	03804.7	CNA-1	Calcineurin catalytic subunit	0; *Sordaria macrospora* (Serine/threonine-protein phosphatase 2B catalytic subunit protein); XP_003352213.1
27.	03833.7	CNB-1	Calcineurin regulatory subunit /variant	2e-119; *Trichoderma reesei* (Calcineurin, beta subunit); EGR44907.1
28.	09265.7		Calnexin	0; *Sordaria macrospora* (cnx1); XP_003347545.1
29.	05225.7[f]		Ca^{2+} and/or CaM binding protein	0; *Magnaporthe oryzae* (Mitochondrial NADH dehydrogenase); EHA47323.1
30.	02115.7		Ca^{2+} and/or CaM binding protein	0; *Magnaporthe oryzae* (EF hand domain-containing protein); EHA48778.1
31.	01564.7		Ca^{2+} and/or CaM binding protein	0; *Magnaporthe oryzae* (Calcium dependent mitochondrial carrier protein); EHA48778.1
32.	06948.7		Ca^{2+} and/or CaM binding protein	2e-54; *Mycosphaerella graminicola* (Calcium ion binding, calmodulin); EGP88834.1

33.	04379.7	NCS-1	Ca^{2+} and/or CaM binding protein	4e-126; *Grosmannia clavigera* (Neuronal calcium sensor 1); EFX03580.1
34.	02738.7	PEF-1	Ca^{2+} and/or CaM binding protein	2e-130; *Verticillium dahliae* (Peflin); EGY21808.1
35.	09871.7		Ca^{2+} and/or CaM binding protein	4e-33; *Verticillium dahliae* (Centrin-3); EGY16271.1
36.	01241.7		Ca^{2+} and/or CaM binding protein	0; *Trichoderma reesei* (Mitochondrial carrier protein); EGR44893.1
37.	06347.7		Ca^{2+} and/or CaM binding protein	0; *Sordaria macrospora* (Actin cytoskeleton-regulatory complex protein); XP_003350109.1
38.	06617.7	CAMK-3	Ca^{2+} and/or CaM binding protein	7e-93; *Verticillium albo-atrum* (Myosin regulatory light chain cdc4); XP_003009631.1
39.	03750.7		Ca^{2+} and/or CaM binding protein	8e-74; *Botryotinia fuckeliana* (Calmodulin); XP_001560827.1
40.	08980.7	NDE-1	Ca^{2+} and/or CaM binding protein	0; *Grosmannia clavigera* (Alternative NADH-dehydrogenase); EFX03867.1
41.	02283.7	CAMK-2	Ca^{2+} and/or CaM binding protein	0; *Sordaria macrospora* (Calcium/calmodulin-dependent protein kinase type I); XP_003344498.1
42.	09123.7	CAMK-1	Ca^{2+} and/or CaM binding protein	0; *Sporothrix schenckii* (Calcium/calmodulin-dependent kinase); AAV80434.1
43.	02814.7	PRD-4	Ca^{2+} and/or CaM binding protein	0; *Grosmannia clavigera* (Serine/threonine-protein kinase chk2); EFX01629.1

44.	09212.7	CAMK-4	Ca^{2+} and/or CaM binding protein	0; *Verticillium dahliae* (Serine/threonine-protein kinase srk1); EGY15110.1
45.	06650.7		Ca^{2+} and/or CaM binding protein	3e-61; *Nectria haematococca* (Phospholipase A2); XP_003042542.1
46.	02411.7		Ca^{2+} and/or CaM binding protein	0; *Glomerella graminicola* (Microtubule associated protein); EFQ31793.1
47.	06177.7		Ca^{2+} and/or CaM binding protein	0; *Magnaporthe grisea* (CMKK2); ACM41720.1
48.	04265.7		Ca^{2+} and/or CaM binding protein	7e-85; *Bacillus megaterium* (Beta-fructosidase FruA); AEN90524.1

[a]Table references Tamuli et al. (2013).

[b]BLASTP search (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al. 1997, 2005) with the default parameters for each of the 48 Ca^{2+}-signaling proteins against the non-redundant protein sequence databases at the NCBI has been indicated the respective best overall match in other organisms.

[c]Split from NCU07605.1.

[d]Split from NCU01437.1.

[e]Split from NCU09655.1; [f]NCU05225.5 (was indicated as NCU08980.1 in Borkovich et al. 2004).

1.5.4 Types of calcium signaling proteins in *Neurospora crassa*

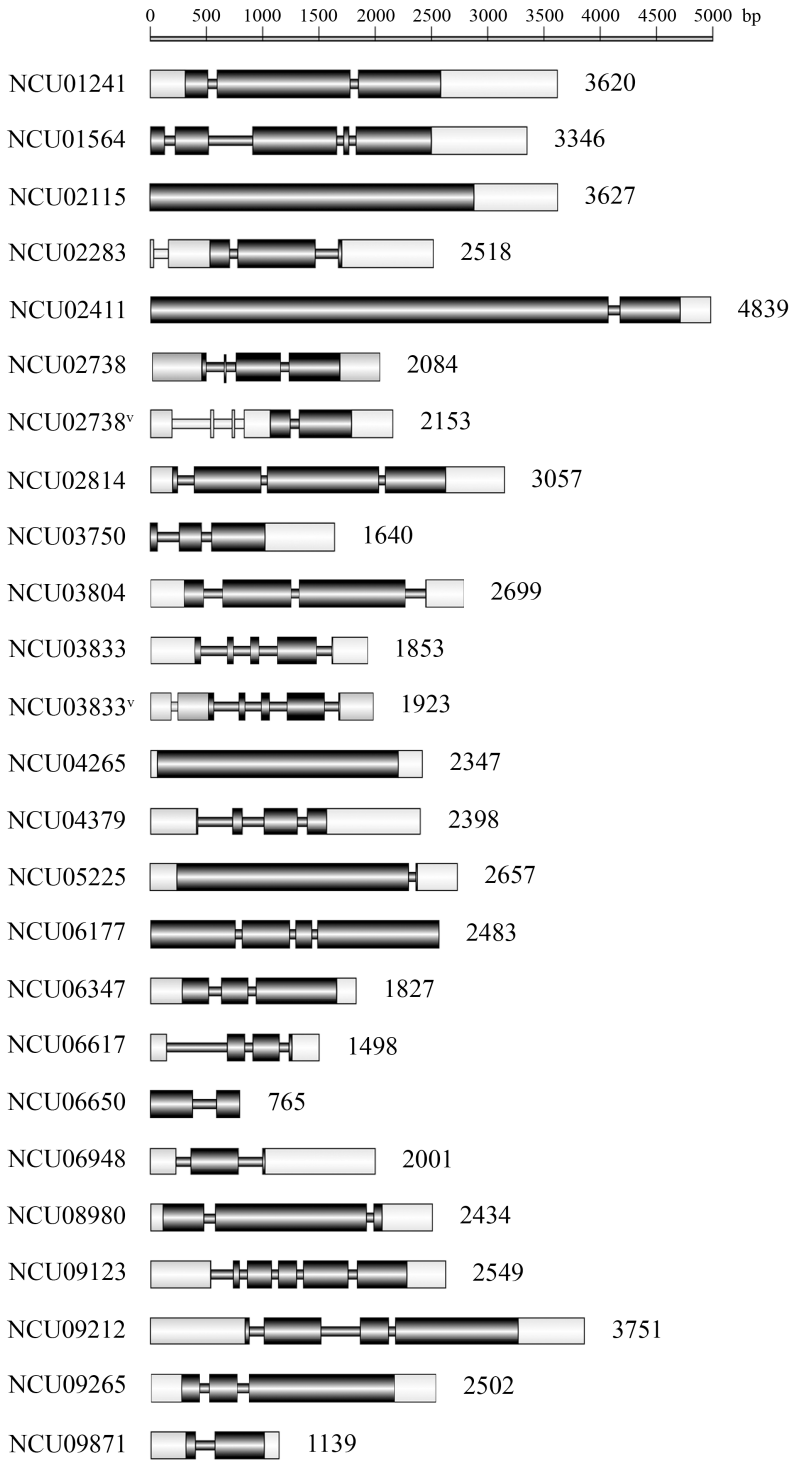
The Ca^{2+} signaling proteins of *N. crassa* can be grouped into seven different types that include three Ca^{2+} channel proteins, nine Ca^{2+}/cation-ATPases, six recognizable Ca^{2+}/H^{+} exchangers, two novel putative Ca^{2+}/Na^{+} exchangers, four novel phospholipase C-δ subtype (PLC- δ) proteins, 23 Ca^{2+} and/or calmodulin (CaM) binding proteins and one CaM (Galagan et al. 2003; Borkovich et al. 2004; Zelter et al. 2004; Tamuli et al. 2013). *N. crassa* use these proteins to effectively maintain Ca^{2+} homeostasis and to respond any change in $[Ca^{2+}]_c$ (Figure 1.5; Tamuli et al. 2013).

To understand the Ca^{2+} signaling mechanisms in *N. crassa*, several knockout mutants of Ca^{2+}-signaling genes have been generated by the *N. crassa* gene knockout project (http://www.datmouth.edu/~neurosporagenome/knockouts_completed.html) and made available through the Fungal Genetic Stock Center (FGSC), Kansas city, Missouri, USA. The knockout mutants have been generated essentially using a high-throughput gene knockout procedure (Colot et al. 2006). So far, homokaryotic knockout mutants are available only for 31 Ca^{2+}-signaling genes, heterokaryotic knockout mutants are available for 12 Ca^{2+} signaling genes and no knockout mutants are available for five genes, suggesting that for these 5 genes either the high-throughput knockout procedure did not work or they are essential for viability.

1.5.5 Calcium and/or calmodulin binding proteins

N. crassa genome analysis has identified 23 genes encoding for Ca^{2+} and/or CaM binding proteins, however, very few of these proteins have been studied (Figure 1.6A-B). One of the studied Ca^{2+} and/or CaM binding proteins is the Ca^{2+}/calmodulin-dependent Ser/Thr phosphatase called calcineurin, which is contains of a catalytic subunit, calcineurin A (CNA) and a regulatory subunit, calcineurin B (CNB) (Higuchi et al. 1991; Kothe and Free 1998). Calcineurin is involved in regulation of hyphal tip growth and branching in *N. crassa* (Prokisch et al. 1997). Another Ca^{2+} and/or CaM binding proteins, the Ca^{2+}/calmodulin-dependent kinase-1 (CAMK-1) is involved in regulating growth and the circadian clock (Yang et al. 2001). The cell functions of the Ca^{2+}/CaMKs discussed in the following part.

(A)


0 500 1000 1500 2000 2500 3000 3500 4000 4500 5000 bp
NCU01241 3620
NCU01564 3346
NCU02115 3627
NCU02283 2518
NCU02411 4839
NCU02738 2084
NCU02738v 2153
NCU02814 3057
NCU03750 1640
NCU03804 2699
NCU03833 1853
NCU03833v 1923
NCU04265 2347
NCU04379 2398
NCU05225 2657
NCU06177 2483
NCU06347 1827
NCU06617 1498
NCU06650 765
NCU06948 2001
NCU08980 2434
NCU09123 2549
NCU09212 3751
NCU09265 2502
NCU09871 1139

(B)

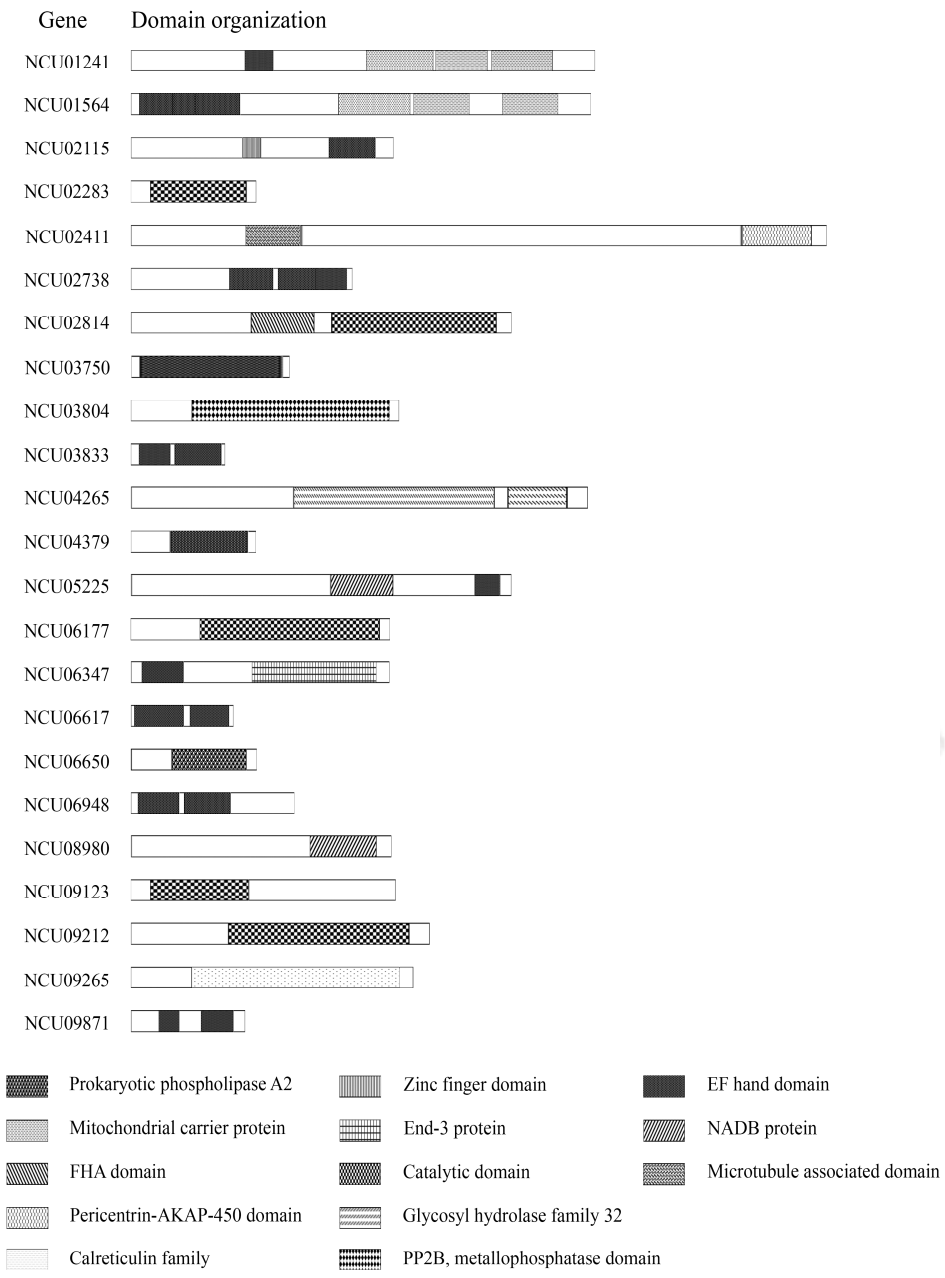


Figure 1.6 Gene structure of calcium and/or calmodulin and organization of conserved domain in the protein. **(A)** Genomic organization showing the exons (black bar) in the ORF, introns (black line) and UTRs (light gray bar). NCU02738^{v} and NCU03833^{v} are the predicted variant transcript of NCU02738 and NCU03833, respectively. **(B)** Conserved domain organization of the Ca^{2+} and/or CaM binding proteins as revealed by the NCBI CDD search. Adopted from Tamuli et al. (2013).

1.5.6 Current knowledge and function of the calcium/calmodulin-dependent kinases

1.5.6.1 Overview of calcium/calmodulin-dependent kinases

The calcium/calmodulin-dependent kinases (Ca^{2+}/CaMKs) are Ser/Thr protein kinases and they can regulate diverse cell functions in responses to increase in intracellular Ca^{2+} concentration (Wilmann et al. 2000; Hook and Means 2001; Mellstrom and Naranjo 2001). The basic domain organization of Ca^{2+}/CaMKs consist of a highly conserved N-terminal catalytic domain and a C-terminal regulatory region consisting of overlapping autoinhibitory and Ca^{2+}/CaM binding domains (Swulius and Waxham 2008; Tamuli et al. 2011). The autoinhibitory domain interacts with the catalytic domain and prevents binding of the true substrate, thereby, maintains the kinase in an inactive conformation (Okuno et al. 1994; Braun and Schulman 1995). The kinase is activated upon binding of Ca^{2+}/CaM to the CaM-binding domain that allows binding of the substrate to Ca^{2+}/CaMKs (Braun and Schulman 1995; Hook and Means 2001). Based on their substrate specificity, Ca^{2+}/CaMKs are classified into two groups; one group has broad substrate specificity and the other group has narrow substrate specificity. Members of the broad substrate specificity group have the ability to phosphorylate many different proteins and include monomeric enzymes such as CaMKK, CaMKI and IV and the multimeric enzyme CaMKII (Hook and Means 2001; Swulius and Waxham 2008). The narrow substrate specificity group are dedicated kinases such as phosphorylase kinase, myosin-light chain kinase (MLCK) and CaMKIII (Hook and Means 2001; Swulius and Waxham 2008). The cell functions of Ca^{2+}/CaMKs have been intensively investigated using mammalian systems, particularly in regulation of neuronal development and plasticity (Sorderling and Stull 2001; Colbran 2004; Elgersma et al. 2004; Griffth 2004; Schulman 2004), however, little is known about their functions in fungi. Fungal homologues of the Ca^{2+}/CaMKs have been identified in *S. cerevisiae* (Ohya et al. 1991; Pausch et al. 1991; Iida et al. 1995; Moser et al. 1996), *A. nidulans* (Kornstein et al. 1992; Dayton and Means 1996; Joseph and Means 2000, 2002), *S. pombe* (Rasmussen 2000), *Colletotrichum gloeosporioides* (Kim et al.1998), *Sporothrix schenckii* (Valle-Aviles et al. 2007; Rodriguez-Caban et al. 2011) and *N. crassa* (Yang et al. 2001; Deka et al. 2011; Tamuli et al. 2011; Kumar and Tamuli 2013). In *N. crassa*, four Ca^{2+}/CaMKs, CAMK-1, CAMK-2, CAMK-3 and CAMK-4 have been identified (Galagan et al. 2003; Borkovich et al. 2004; Tamuli et al. 2011).

1.5.6.2 Spore germination and thermotolerance

In *S. cerevisiae*, *CMK1* and *CMK2* genes encode two Ca^{2+}/CaMKII isozymes (Ohya et al. 1991; Pausch et al. 1991). The CMK1 and CMK2 proteins are 60% identical and 90% similar. CMK2 kinase becomes a constitutively active protein kinase upon autophosphorylation in vitro, while CMK1 kinase does not (Ohya et al. 1991). Deletion of *CMK2*, or both *CMK1* and *CMK2*, is not lethal, although loss of *CMK2* result in a slow rate of spore germination. Moreover, both the *Δcmk1* and the *Δcmk1 Δcmk2* cell losses the induced thermotolerance faster than the wild type and *Δcmk2* cells, indicating that the acquisition of induced thermotolerance depends on CaMKII (Pausch et al. 1991).

Another Ca^{2+}/CaMKII in *S. cerevisiae* is encoded by the gene *CLK1* (Melcher et al. 1996). The Clk1 protein also contains an N-terminal kinase domain, and a C-terminal basic segment resembling known CaM-binding sites. The protein Clk1 shares 38 and 37% identity in amino acid sequence with rat CaM kinase α and yeast CaM kinase *CMK2*, respectively (Melcher et al. 1996). The Clk1 protein is located in the cytosol and excluded from the nucleus. The *clk1Δ* mutant is viable, interestingly, elevated expression of Clk1 inhibit growth. In addition, overexpression of the C-terminally truncated Clk1 also inhibit growth, however, overproduction of catalytically inactive Clk1 had no effect of growth, suggesting that the C terminus is a negative regulatory domain (Melcher et al. 1996).

1.5.6.3 Hyphal growth and cell cycle progression

In *A. nidulans*, three Ca^{2+}/CaMKs, CMKA, CMKB and CMKC have been reported (Kornstein et al. 1992; Joseph et al. 2002). The CMKA protein shares 29% identity to that of the α-subunit of rat brain Ca^{2+}/CaMKII, 40 and 44% identity with the *S. cerevisiae* Ca^{2+}/CaMKs CMK1 and CMK2, respectively (Pausch et al. 1991; Kornstein et al. 1992). The other two *A. nidulans*, Ca^{2+}/CaMKs, CMKB and CMKC share high sequence identity with mammalian Ca^{2+}/CaMKs, rat CaMKI/IV and rat CaMKKα/β, respectively (Joseph et al. 2000). Disruption of either *cmk*A or *cmk*B is lethal, however, disruption of *cmk*C is non-lethal (Joseph et al. 2000; Joseph et al. 2002). The CMKA is shown required for hyphal growth and progression of the nuclear division cycle (Joseph et al. 2002). Both CMKB and CMKC are required for the proper temporal activation of $NIMX^{cdc2}$ (the mitotic Cdk1 in *A.*

nidulans) that is necessary for nuclear division (Joseph et al. 2000). Furthermore, CMKB and CMKA are essential for progression through G_1 and G_2, respectively, while CMKC is important for G_1 progression (Joseph et al. 2000, 2002).

In *S. pombe*, the *cmk1* gene encodes a homologue of CaMK-I (Rasmussen 2000). The CMK1 protein shows significant homology to mammalian CaMK-I. The levels of *cmk1* mRNA are shown regulated in a cell cycle-dependent manner, peaking at or near the G_1/S boundary (Rasmussen 2000). In *S. pombe*, overexpression of CMK1 caused no obvious effect on growth and division; however, expression of the CMK1-T192D mutant (T192D mimics phosphorylation of Thr-192), resulted in hyperactivation of CMK1 activity in the presence of CaM and causes cell cycle arrest as well as morphological defects (Rasmussen 2000). The corresponding residue of the Thr-192 is conserved in mammalian CaMK-I and its phosphorylation by the CaM-kinase kinase (CaM-KK) results in a hyperactivate CaMK-I (Yang et al. 2001; Haribabu et al. 1995). Both CaMK-I and CaM-KK enzymes are members of a mammalian CaMK cascade that regulates several transcription factors (Tokumitsu et al. 1995; Soderling 1999). These results suggested that *cmk1* may be important in cell cycle progression, and a CaMK cascade may be present in *S. pombe* (Rasmussen 2000).

1.5.6.4 Infection structure differentiation

Hard-surface contact in *C. gloeosporioides* induces Ca^{2+}/CaM signaling that might be necessary for the conidia to respond to host signals by germination and differentiation into appressoria required for infection of the host (Kim et al. 1998). A putative CaMK, *C. gloeosporioides* (CgCMK) was cloned from the transcripts of conidia germinated on glass surface imitating the hard waxy leaf surface (Kim et al. 1998). In *C. gloeosporioides* the response to host wax and ethylene, the host ripening hormone, requires the contact of conidia with a hard surface (Flaishman et al. 1994; Means et al. 2000). Inhibition of CaMK, using KN93 (Flaishman et al. 1995), found to interferes with germination and appressorium formation effectively in the early phase of hard-surface contact. In addition, KN93 also results in formation of the appressoria with unusual morphological features and much less melanization. An intermediate in melanin synthesis, scytalone (Mamiya et al. 1993), reversed the inhibition of melanization by KN93, however, scytalone did not restore appressorium

formation in *C. gloeosporioides*. These results suggest that CgCMK affects germination and appressorium formation during the early phase of hard-surface contact and inhibit melanization prior to the formation of scytalone (Kim et al. 1998).

Ca^{2+}/CaMK signaling pathway also plays an important role in dimorphic transition in some fungus. During the dimorphic transition of *S. schenckiii*, the etiological agent of sporotrichosis (a subcutaneous lymphatic mycosis; Travassos et al. 1980; Kubo et al. 1983; Valle-Aviles et al. 2007; Rodriguez-Caban et al. 2011). The *sscmk1*, Ca^{2+}/CaMK gene in *S. schenckii,* was predicted to encode SSCMK1 that might regulate dimorphism and thermotolerance in this fungus (Valle-Aviles et al. 2007; Rodriguez-Caban et al. 2011). The application of CaM inhibitor W-7, and Ca^{2+}/CaMK inhibitors such as KN-62 and lavendustin C, stimulate the yeast from of the *sscmk1* to mycelium transition and inhibit re-entry into the budding cycle by yeast cells. Therefore, Ca^{2+}/CaM dependent signaling pathway plays a role in the expression of the yeast morphology in *S. schenckii* (Valle-Aviles et al. 2007; Rodriguez-Caban et al. 2011).

1.5.6.5 Regulation of circadian clock

In *N. crassa*, a Ca^{2+}/CaMK (CAMK-1) specifically phosphorylates the circadian clock protein FREQUENCY (FRQ; Yang et al. 2001). The *camk-1* null mutant shows modest phase delay, a small period lengthening, and light-induced phase shifting of the circadian conidiation rhythm (Yang et al. 2001). The *camk-1* null strain grows slowly immediately after germination from ascospores, indicating that CAMK-1 plays an important role in growth and development of *N. crassa*; however, this slow growth phenotype is transient, which indicates redundancy of the kinase (Yang et al. 2001).

1.6 Objectives of this study

The Ca^{2+}/CaMKs are Ser/Thr protein kinases that sense the change in $[Ca^{2+}]_c$. The cellular roles of Ca^{2+}/CaMKs have been already investigated using mammalian system, particularly in regulation of neuronal development and plasticity. However, little is known about their cellular role of Ca^{2+}/CaMKs in *N. crassa* or any other filamentous fungus is general. In this work, I have studied cellular roles and genetic interaction of four Ca^{2+}/CaMKs of *N. crassa*.

Specifically my objectives were as follow:

1. To study the role of Ca^{2+}/CaMKs in *N. crassa*,

2. To understand the mechanism of selected Ca^{2+}/CaMKs genes in sexual development, and

3. Studies on genetic interactions of selected Ca^{2+}/CaMKs.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Source of chemicals and materials used

Agarose, amino acids, calcium chloride, ethidium bromide, sodium dodecyl sulphate (SDS), cetyltrimethylammonium bromide (CTAB), sorbitol, sorbose, fructose, sucrose, hygromycin B, basta and biotin, were procured from the Sigma-Aldrich, USA and Himedia Laboratories, Mumbai. Bacto-agar, Bacto-tryptone, and Yeast Extract were from, Sisco Research Laboratories (SRL), Mumbai. Plastic, glasswares and test tube from Tarsons, Kolkata, Laxbro, Pune and Borosil, Ahemedabad. Sephadex G-50 was from Pharmacia Biotech, Sweden. All restriction enzymes, Klenow polymerase, DNA size markers, were from New England Biolabs (NEB) Inc., USA. PCRs were performed using custom oligonucleotide primers and *Taq* DNA polymerase purchased from Integrated DNA Technologies (IDT), USA and Metabion, Germany. TripleMaster PCR system for high fidelity and long range PCR was procured from Finnzymes, Finland. Plasmid extraction and DNA purification kit were from Qiagen Inc., USA. Whatman filter paper from Whatman International Ltd., USA. X-ray films were obtained from Konica Corporation, Japan, and radiolabeled nucleotides were procured from BRIT, Hyderabad, India. All other chemicals were purchased from local manufacturers and were of analytical grade.

2.1.2 *Neurospora crassa* strains used in this study

***74-OR23-1 A* (FGSC 987) and *OR8-1 a* (FGSC 988)**

These are standard wild type *N. crassa* laboratory strains. These were obtained from the Fungal Genetics Stock Center (FGSC), University of Missouri, Kansas City, MO 64110, Missouri, USA. These strains will henceforth be referred to as *OR A* and *OR a* respectively.

ΔNCU09123.2 *A* (Δ*camk-1::hph*; FGSC 12547) and ΔNCU09123.2 *a* (Δ*camk-1::hph*; FGSC 12548)

The ΔNCU09123.2 *A* (Δ*camk-1::hph*; FGSC 12547) and ΔNCU09123.2 *a* (Δ*camk-1::hph*; FGSC 12548)) strains were generated by high-throughput gene knockout procedure, in these strains the calcium/calmodulin-dependent kinase 1 ORF was replaced by the *hph* cassette, that confers resistance to hygromycin-B (Colot et al. 2006). They were obtained from the FGSC.

ΔNCU02283.2 *a* (Δ*camk-2::hph*; FGSC 12448) and ΔNCU02283.2 *A* (Δ*camk-2::hph*; FGSC 12449)

The ΔNCU02283.2 *a* (Δ*camk-2::hph*; FGSC 12448) and ΔNCU02283.2 *A* (Δ*camk-2::hph*; FGSC 12449) strains were generated by high-throughput gene knockout procedure, in these strains the calcium/calmodulin-dependent kinase 2 ORF replaced by the *hph* cassette, that confers resistance to hygromycin-B (Colot et al. 2006). They were obtained from the FGSC.

ΔNCU06177.2 *A* (Δ*camk-3::hph*; FGSC 11536) and ΔNCU06177.2 *a* (Δ*camk-3::hph*; FGSC 11537)

The ΔNCU06177.2 *A* (Δ*camk-3::hph*; FGSC 11536) and ΔNCU06177.2 *a* (Δ*camk-3::hph*; FGSC 11537) strains were generated by high-throughput gene knockout procedure, in these strains the calcium/calmodulin-dependent kinase 3 ORF replaced by the *hph* cassette, that confers resistance to hygromycin-B (Colot et al. 2006). They were obtained from the FGSC.

ΔNCU09212.2 *a* (Δ*camk-4::hph*; FGSC 11545)

The ΔNCU09212.2 *a* (Δ*camk-4::hph*; FGSC 11545) strain was generated by high-throughput gene knockout procedure, the calcium/calmodulin-dependent kinase 4 ORF was replaced by the *hph* cassette, that confers resistance to hygromycin-B (Colot et al. 2006). This strain obtained from the FGSC.

***Sad-1 a* (FGSC 8740) and *Sad-1 A* (FGSC 8741)**

The strains *Sad-1 a* (FGSC 8740) and *Sad-1 A* (FGSC 8741), a semidominant suppressor of meiotic silencing, gifted by Dr. D.P. Kasbeskar from CCMB Hyderabad and that were originally obtained from Dr. Robert L. Metzenberg, Department of Biology, California State University, Northridge, CA 91330 (Shiu et al. 2001).

Strains generated in this study

The strains Δ*camk-4::hph A*, Δ*camk-1::hph camk-1::bar* (3) *a*, Δ*camk-1::hph camk-1:: bar* (7) *a*, Δ*camk-1::hph camk-1:: bar* (12) *A*, Δ*camk-1::hph* (23) *a*, Δ*camk-1::hph* (35) *A*, Δ*camk-2::hph camk-2:: bar* (15) *A*, Δ*camk-2::hph camk-2:: bar* (19) *A*, Δ*camk-2::hph camk-2:: bar* (21) *a*, Δ*camk-2::hph* (16) *a*, Δ*camk-2::hph* (10) *A*, *camk-2:: bar* (4) *a*, *camk-2:: bar* (29) *A*,

Δ*camk-1::hph* Δ*camk-2::hph* (3) *A*, Δ*camk-1::hph* Δ*camk-2::hph* (6) *a*, Δ*camk-4::hph* Δ*camk-2::hph* (19) *A*, Δ*camk-4::hph* Δ*camk-2::hph* (23) *a*, Δ*camk-3::hph* Δ*camk-2::hph* (4) and Δ*camk-3::hph* Δ*camk-2::hph* (18) *A* were generated in this study.

2.1.3 Bacterial strains used in this study

***Escherichia coli* DH5-α**

The Escherichia coli DH5-α, genotype *SupE44 ΔlacU169 (ϕ80lacZ ΔM15) hsdR17 recA1 end A1 gyrA96 thi-1 relA1*, is a recombination-deficient suppressing strain used for plating and growth of plasmids. The ϕ80lacZ ΔM15 permits α-complementation with the amino terminus of β-galactosidase encoded in pUC vectors. It was used for all routine transformations, plasmid isolation and selection of recombinants.

2.1.4 Plasmid vectors used in this study

pBARGEM7-1

The pBARGEM7-1 is a high copy number plasmid vector of 4.5 kb in size (Pall and Brunelli 1993) contains the 2.7 kb DNA fragment of the *bar* selectable marker that confers resistance to basta under control of the *A. nidulans trpC* promoter. Insertion of markers into these polylinkers will, in most cases, disrupt the function of the lacZα gene which can be monitored by blue-white screening in medium containing X-gal using such *E. coli* host strains.

2.1.5 Bacterial media, antibiotics and commonly used solutions

Commonly used solutions were prepared essentially as described by Sambrook et al. (2001).

1. LB (Luria Bertani) broth: 10 g bacto-tryptone, 10 g sodium chloride and 5 g bacto-yeast extract dissolved in 900 ml deionized H_2O, and adjusted the pH 7.0 with 5 N NaOH (~0.2 ml). Then the volume of the solution was adjusted to 1 liter with deionized H_2O and sterilized by autoclaving.
2. LB agar: LB solidified with 1.5% agar.
3. SOB medium: 20 g bacto-tryptone, 5 g bacto-yeast extract and 0.5 g NaCl, dissolved in 900 ml deionized H_2O, and added 10 ml of a 250 mM solution of KCl (this solution is made by dissolving 1.86 g of KCl in 100 ml of deionized H_2O and adjust the pH 7.0 with 5 N NaOH). Then the volume of the solution was adjusted to 1 liter with deionized H_2O and

sterilized by autoclaving. Just before use, 5 ml of sterile solution of 2 M $MgCl_2$ was added. Then made by dissolving 19 g of $MgCl_2$ in 90 ml of deionized H_2O was added. Then the volume of the solution was adjusted to 100 ml with deionized H_2O and sterilized by autoclaving.

4. SOC medium: SOC medium is identical to SOB medium, except that it contains 20 mM glucose added in 900 ml deionized H_2O. After the SOB medium was autoclaved, allowed it to cool to 60°C or less. Then added 20 ml of a sterile 1 M solution of glucose (made by dissolving 18 g of glucose in 90 ml of deionized H_2O). After the glucose has dissolved completely, the volume of the solution adjusted to 100 ml with deionized H_2O and sterilized by passing it through a 0.22 µm filter.
5. TB (Terrific Broth): Added 12 g bacto-tryptone, 24 g bacto-yeast extract and 4 ml glycerol in 900 ml deionized H_2O. Dissolved the solution by shaking and then sterilized by autoclaving. Allowed the solution cool to 60°C or less, and then added 100 ml of a sterile solution of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4. This solution was made by dissolving 2.31 g of KH_2PO_4 and 12.54 g of K_2HPO_4 in 90 ml of deionized H_2O. After complete dissolving of the salts, adjusted the volume of the solution to 100 ml with deionized H_2O and sterilized by autoclaving.
6. Ampicillin: A 1000X stock solution of 100 mg/ml ampicillin was made in sterile double-distilled H_2O and stored in aliquots of 150 µl at -20°C.
7. Hygromycin B: A stock solution of 120 mg/ml was made in sterile H_2O and stored at -20°C.
8. basta: A stock solution of 100 mg/ml was made in sterile H_2O and stored at -20°C.
9. IPTG (isopropyl-β-D-thiogalactopyranoside): A 20% solution of IPTG was made by dissolving 2 g of IPTG in 8 ml of distilled H_2O. The volume of the solution was adjusted to 10 ml with H_2O and sterilized by passing it through a 0.22 µm disposable filter. The solution was dispenced into 1 ml aliquots and stored them at -20°C.
10. X-gal 2% w/v (5 bromo-4-chloro-3-indolyl-β-D-galactoside): A 2% stock solution was made by dissolving X-gal in dimethylformamide at a concentration of 20 mg/ml solution in a glass or polypropylene tube. Wrapped the tube containing the solution with aluminum foil to prevent damage by light and store at -20°C.
11. 1XTAE: 0.04 M Tris-acetate, 0.001 M EDTA.

12. 1X TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0).
13. 1X SSC: 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0).
14. Lysis buffer for Neurospora genomic DNA isolation: 10 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 10 mM EDTA, 1% SDS and 1% CTAB.
15. Lysis buffer for plasmid miniprep: 0.1 N NaOH, 0.01 M EDTA (pH 8.0), and 1% SDS.
16. Phenol:Chloroform:Isoamyl mixture (25:24:1): Phenol was prepared by the equilibration of liquefied and distilled phenol containing 0.1% hydroxyquinoline with 0.1 M Tris-HCl pH 8.0. It was mixed in equal volume with chloroform: isoamyl alcohol (24:1) and stored in a dark bottle, at 4°C.
17. Ethidium bromide (10 mg/ml): 1 g of ethidium bromide to 100 ml of distilled H_2O.
18. Gel-loading Buffer (Type III): Dissolved 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in sterile H_2O, stored at 4°C.
19. 0.5 M EDTA (pH 8.0): Dissolved 186.1g of disodium ethylenediaminetetra-acetate.2 H_2O in 800 ml of distilled H_2O, adjusted pH to 8.0 by NaOH, adjusted the volume to 1 liter with distilled H_2O and sterilized by autoclaving.
20. 1 M $MgCl_2$: Dissolved 203.3 g of $MgCl_2.6H_2O$ in 800 ml of distilled H_2O, adjusted the volume to 1 liter with distilled H_2O and sterilized by autoclaving.
21. 1 M $MgSO_4$: Dissolved 12 g of $MgSO_4$ in a final volume of 100 ml of H_2O. Sterilize by autoclaving or filtration and stored at room temperature.
22. 5 M LiCl: Dissolve 21.2 g of LiCl in a final volume of 90 ml of H_2O. Adjusted the volume of the solution to 100 ml with H_2O. Sterilized the solution by passing it through a 0.22 µm filter or by autoclaving and stored the solution at 4°C.
23. 2.5 M $CaCl_2$: Dissolved 11 g of $CaCl_2.6H_2O$ in a final volume of 20 ml of distilled H_2O. Sterilized the solution by passing it through a 0.22 µm filter and stored in 1 ml aliquots at 4°C.
24. 4 M KCl: Dissolved 10 g of solid KCl in H_2O, sterilized by autoclaving and store at room temperature.
25. 5 M NaCl: Dissolved 292.2 g of NaCl in 800 ml of distilled H_2O, adjusted the volume to 1 liter with distilled H_2O and sterilized by autoclaving. Stored the NaCl solution at room temperature.

26. 10 N NaOH: To 800 ml H_2O, slowly added 400 g of NaOH pellets, with continuous stirring. As an added precaution, the beaker was placed on ice. When the pellets were dissolved completely, adjusted the volume to 1 liter with H_2O. Stored the solution in a plastic container at room temperature.
27. 10% (v/v) Glycerol: Diluted 1 volume of absolute glycerol in 9 volume of sterile H_2O. Sterilized the solution by passing it through a prerinsed 0.22 µm filter and stored in 200 ml aliquots at 4°C.
28. Lysozyme: Dissolved the solid lysozyme at a concentration of 10 ml in 10 mM Tris-Cl (pH 8.0) immediately before use.
29. RNAase A: Dissolved pancreatic RNAase (DNAase free) at a concentration of 10 mg/ml in 10 mM Tris.Cl (pH 7.5), 15 mM NaCl and stored at -20°C.
30. 20% Sodium dodecyl sulfate (SDS): Dissolved 200 g of SDS in 900 ml of distilled water, adjusted pH to 7.2 by adding few drops of concentrated HCl, volume was then adjusted to 1 liter with distilled H_2O.
31. 20XSSC: Dissolved 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of distilled H_2O, adjusted the volume to 1 liter with distilled H_2O and sterilized by autoclaving.
32. Alkaline Lysis Solution I (plasmid preparation): This solution contains 50 mM glucose, 25 mM Tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0) prepared from standard stocks in batches of ~100 ml, the solution was sterilized by autoclaving and stored at 4°C.
33. Alkaline Lysis Solution II (plasmid preparation): 0.2 N NaOH (freshly diluted from a 10 N stock), 1% (w/v) SDS. The solution II was prepared fresh and stored at room temperature.
34. Alkaline Lysis Solution III (plasmid preparation): This solution contains 60 ml of 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml distilled H_2O. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. The solution was stored at 4°C and transferred it to an ice bucket just before use.
35. 0.5 M Sodium-phosphate buffer: 70.98 g of Sodium-phosphate was dissolved for 1 liter, adjusted pH to 7.2 with ~4 ml of 85% H_3PO_4 (orthophosphoric acid).
36. 1 M Tris: Dissolved 121.1 g of Tris base in 800 ml of distilled H_2O, adjusted pH to the desired value by adding concentrated HCl (for pH 7.4, 7.6, and 8.0, ~ 70 ml, 60 ml and 42

ml of concentrated HCl was added, respectively), adjusted the volume to 1 liter with distilled H_2O and sterilized by autoclaving.

2.1.6 Solutions for growth, maintenance and crossing of *Neurospora crassa* strains

Media for culturing *N. crassa* were prepared essentially as described in Davis and De Serres (1970).

Biotin solution

5 mg of biotin was dissolved in 100 ml of 50% (v/v) ethanol, stored at 4 ^{0}C.

Trace element solution

Trace element solution was prepared by adding the following compounds successively, with stirring to 95 ml of distilled H_2O.

Citric acid.$1H_2O$	5.00 g
$ZnSO_4.7H_2O$	5.00 g
Fe $(NH_4)_2 (SO_4)_2.6H_2O$	1.00 g
$CuSO_4.5H_2O$	0.25 g
$MnSO_4.1H_2O$	0.05 g
H_3BO_3	0.05 g
$Na_2MoO_4.2H_20$	0.05 g

The final volume was adjusted to 100 ml with H_2O; 1 ml of chloroform was added as a preservative and stored at room temperature.

Synthetic Media

Vogel's medium N (Vogel 1964) prepared as a 50X-strength solution as follows:

To 750 ml of distilled H_2O, the following ingredients were added in order, dissolving each one prior to addition of the next.

Na_3 citrate.$5H_2O$	150 g
KH_2PO_4	250 g
NH_4NO_3	100 g

$MgSO_4.7H_2O$	10 g
$CaCl_2.2H_2O$ (predissolved in 20 ml H_2O)	5 g
Biotin solution	5 ml
Trace element solution	5 ml

The volume of the solution was adjusted to one liter and 3 ml of chloroform was added as a preservative.

Vogel's-glucose medium (VGM)

Vogel's medium N	1X
Glucose	1.5% (w/v)

Vogel's-glucose agar medium (VGM agar)

Vogel's medium N	1X
Glucose	1.5% (w/v)
Agar	2.0% (w/v)

4X Synthetic crossing medium (SCM)

4X strength synthetic crossing medium were prepared by adding the following compounds with stirring to 500 ml of H_2O and sterilized by autoclaving.

KNO_3	2.0 g
K_2HPO_4	1.4 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	1.0 g
$CaCl_2$	0.2 g
NaCl	0.2 g
Biotin solution	0.2 ml
Trace element solution	0.2 ml

Synthetic crossing medium agar (SCM agar)

SCM	1X
Glucose	1.5% (w/v)
Agar	2.0% (w/v)

Sterilized by autoclaving.

10X FGS ("Sorbose")

Sorbose	20% (w/v)
Fructose	0.5% (w/v)
Glucose	0.5% (w/v)

Sterilized by autoclaving.

Vogel's-sorbose agar medium

FGS	1X
Vogel's medium N	1X
Agar	2.0% (w/v)

Sterilized by autoclaving.

Top agar

FGS	1X
Vogel's medium N	1X
Agar	2.8% (w/v)

Sterilized by autoclaving.

Solutions of supplements (http://www.fgsc.net/methods/stanford.html)

1. L-arginine: 1.25 ml per 100 ml medium from a stock of 40 mg/ml in distilled H_2O.
2. L-histidine HCl: 2 ml per 100 ml medium from a stock of 25 mg/ml in distilled H_2O.
3. L-leucine: 4 ml per 100 ml medium from a stock of 5 mg/ml in distilled H_2O.

Sterilization

All glassware and plasticware were sterilized by autoclaving at a steam pressure of 15 psi at 120°C for 20 min. Solutions were prepared in double-distilled H_2O and generally sterilized by autoclaving. Heat-sensitive solutions were sterilized by filtering through a sterile 0.45 µm nitrocellulose filter (Millipore, USA).

2.2 Methods

2.2.1 Strain maintenance

Bench-stocks of *N. crassa* were maintained in glass tubes as conidial cultures on solid media (Vogel's minimal media supplemented with 2% glucose as carbon source). After 4-5 days of inoculation, conidia were used to inoculate other slants, flasks, race tubes and liquid cultures (as per the requirement). Conidia production generally stops after 7 -10 days, but they usually stay viable at room temperature for a year or longer. However, older cultures take longer to germinate than young ones. When use frequently cultures were kept at 4°C in a closed cabinet.

2.2.2 Media supplements

Metabolic mutants like *his*$^-$ or *leu*$^-$ require additional supplements for growth. Mutant selection or maintenance requires the addition of selective agents to the growth media. For routine laboratory work, it was convenient to maintain concentrated stock solutions of supplements that can be easily added to the media. To avoid autoclaving, supplement stock solutions were filter sterilized and stored at -20°C and added after autoclaving.

2.2.3 Stock management

About 7-10 days old cultures on slants (same as for bench-stocks) were closed with cotton plugs, sealed with Parafilm and frozen at –20°C. Conidia prepared in this way can survive at least a few years.

2.2.4 Lab practice

All *N. crassa* transfers, inoculations, plantings, etc. were performed on a UV sterilized Laminar flow work bench, taking care to work near a Bunsen burner. All transfers were done in a dust-free room. Decontamination of the work-space was accomplished with 70% absolute ethanol. All used cultures, contaminated glassware or media were decontaminating by autoclaving.

2.2.5 Conidial suspensions-inoculum

Liquid cultures were inoculated with conidial suspensions, such that the final concentration was between 0.1 and 1 x 10^6 conidia per 1 ml of media. Suspensions for inoculation (inoculum) were made from conidia grown in 250 ml Erlenmeyer flasks with 25-50 ml solid Vogel's medium for 6-8 days or more to yield sufficient amounts of conidia. Inocula were prepared by adding 50-100 ml sterile H_2O into the flask cultures, reclosing the flasks, swirling and shaking for 20 to 40 min. To prevent contamination care was taken so that airborne conidia had settled before opening the flask. Conidia were then suspended in sterile H_2O, and filtered through cheesecloth layers. Aliquots of conidial suspension were stored at -20°C. Conidial suspensions stored in this way have slightly lower germination capability and stable for at least 1 year.

2.2.6 Liquid and solid cultures

Liquid cultures were used to grow mycelium mass for evaluation of molecular components. Mycelia were grown in liquid medium by inoculating conidial suspension or agar plug of mycelium into the Vogel's medium. The cultures were then incubated at 30°C with shaking at 150-200 rpm for 2 to 3 days. Vigorous shaking (200 rpm) prevents formation of clumps and improves aeration. In solid cultures in 55 mm petri dishes containing Vogel's agar medium by inoculating agar plug of mycelium were then incubated at 30°C for 2 to 3 days, *Neurospora* forms a conidium on the surface. The culture were harvested with vacuum filtration, dried with blotting, and cut into small piece then lyophilized. Convert the fine powder forms with a pestle mortar the powdered mycelium used for further studies.

2.2.7 Growth rate experiments

Growth was initially measured by placing either conidia or a plug of agar containing mycelium in the center of a petri dish containing Vogel's medium and colony diameter was measured every 2-3 h to obtain linear rates of diameter increase over a period of 28 h. Strains that show lower growth rate on petri dish were further analyzed by using standard race tube assay (Ryan et al. 1943). A race tube is a hollow glass tube (40 or 60 cm long, 1.2 to 1.4 cm in diameter) bent at the ends. The race tube was partially filled with a Vogel's medium and inoculated with either conidia or mycelial plug at one end and incubated at 30°C for 3 days. The growth of strains were determined by measuring the mycelial extension rates at every 12 h intervals in race tube and growth rates calculated as cm h^{-1}. When the mycelial front reached nearly the end of the race tubes, the tubes were scanned (600 dpi resolution).

2.2.8 Thermotolerance and oxidative stress analysis

Protocols for thermotolerance and oxidative stress analysis were essentially as described previously (Yang and Borkovich 1999; Michan et al. 2003). To measure the thermotolerance, five day-old conidia were inoculated into liquid Vogel's glucose media at a concentration of 1 X 10^6 cells/ml and germinated for 2 h with shaking at 200 rpm in dark at 30°C. These germlings were then divided into two equal parts; one part was held at 30°C (uninduced) and the other part at 44°C (induced) for 30 min, and both parts were then given a 52°C lethal heat shock for 20 min (Yang and Borkovich 1999). Percent survival was obtained by dividing the number of viable colonies on plates subjected to heat treatment at 30°C or 44°C by the number of viable colonies on plates held at 30°C throughout the experiment (control).

Similarly, to assay oxidative stress induced by H_2O_2, ~1 X 10^6 cells/ml conidia were inoculated on plates containing Vogel's medium with 2% bacto agar and incubated at 30°C for 2 days. Plates were then divided in two sets, one set was treated with 10 mM H_2O_2 (final concentration) for 10 min, washed with sterile water and incubated for additional 2 days at 30°C. The other set was incubated without any H_2O_2 treatment. Percent survival was scored by dividing the number of colonies obtained after H_2O_2 treatment by the number on a plate containing cells that were not exposed to H_2O_2, and multiplying by 100.

2.2.9 Crosses, fertility and ascospore collection

Crosses between *N. crassa* strains were conducted using standard techniques. Crosses were performed by confrontation between opposite mating type mycelia inoculated as plugs on synthetic crossing medium (SCM) in 55 mm petri dishes. The crosses were incubated at 22°C in a low temperature incubator (usually BOD incubator) for 3 to 4 weeks, by which time ascospores were shot to the lid of the petri dish. Strains were also crossed to opposite mating type as either the female (protoperithecial) or male (fertilizing) parent to detect mating-specific defects. Dilute conidial suspensions of the male parent were applied directly to 7-day-old SCM cultures of opposite mating type of strains containing fully differentiated protoperithecia. The formation of protoperithecia and development of perithecia were examined microscopically by using a stereomicroscope and images were photographed with digital camera.

Ascospores productivity of the crosses was scored under a dissection microscope by examining the completed crop of ascospores ejected to the lid of the petri dish. Based on the total ascospores produced in a plate culture, the phenotypes of the crosses were scored as fertile (thousands of progeny ascospores were produced), barren (exceptionally few progeny ascospores were produced), or intermediate (few hundred progeny ascospores were produced).

Generally ascospores began to be shot within 16-18 days in *N. crassa* crosses. Ascospores were harvested by washing the lids with ~1 ml of sterile H_2O. They are then plated on sorbose agar plate, and picked. Picking was done under dissection microscope by cutting out a small block of agar below the ascospore and transferring it to a slant with minimal or selective media and ascospores were activated by heat shock at 64°C for 1 h in a shaking water bath.

2.2.10 Assay for meiotic silencing

The meiotic silencing was assayed using the crosses involving tester strains in which an extra copy of gene was introduced ectopically. The ectopic copy remains unpaired in meiosis in crosses of the testers with the wild-type strain of opposite mating type and induces the synthesis of small interfering RNA (RNAi) that silences it as well as its paired native homologs via a gene silencing process called meiotic silencing (Shiu et al. 2001; Hammond et al. 2013; Nagasowjanya et al. 2013). In homozygous tester (*tester A* × *tester a*) crosses or in crosses of the testers with the *Sad-1* (suppressor of meiotic silencing), meiotic silencing does not occur and the asci develop normally (Shiu et al. 2001; Nagasowjanya et al. 2013).

2.2.11 Transformation of the *Neurospora crassa* strain by electroporation

This protocol is based on the method described by Margolin et al. (1997, 2000). The recipient strain was incubated in six to eight 250 ml conical flasks containing Vogel's glucose agar medium and incubated at 30°C for about a week. The conidia were harvested in sterile H_2O and separated from the mycelium by passing the suspension through cheesecloth attached to a 250 ml conical flask (a 250 ml flask with attached cheesecloth was sterilized beforehand). The conidial suspension was taken in a 30 ml Corex tube and centrifuged at 800 rpm for 6 min in a Sorvall HB-4 rotor in 4°C. The conidial mass was washed twice in 30 ml of sterile H_2O and resuspended in 1 M sorbitol at a concentration of 3 X 10^9 per ml. About 40 µl of the conidial suspension was mixed with the pRK-1 and pRK-2 constructs (the selectable DNA) and the mixture was placed in a pre-chilled electroporation cuvette (0.2 cm gap; BioRad Laboratories, Hercules, CA). Electroporation was done in the Gene Pulser Xcell™ Total System (*BIO-RAD*, Hercules, CA) and conditions for electroporation were capacitance 25 µF, voltage 1.5 kV and resistance 600 Ω. The time constant varied from 13 to 15 ms. Immediately after the pulse, ~600 µl of chilled 1 M sorbitol was added, kept on ice for 5 min and the transformant conidial suspension was mixed with top agar and plated on a Vogel's-sorbose agar plate supplemented with the appropriate antibiotic. Usually about 20-30 transformants were obtained per plate and were 'pickable' under a dissection microscope after 2 days. A control transformation was done without adding selectable DNA to eliminate the possibility of any contamination.

2.2.12 Scoring for antibiotic resistance

Antibiotics resistance was scored on medium containing sorbose and supplemented with the antibiotic. The antibiotics used were hygromycin B (220 µg/ml from 120 mg/ml stock in H_2O) or basta (400 µg/ml from 100 mg/ml stock in H_2O) for selection of progeny containing *hph* cassette or *bar* selectable marker, respectively. Aqueous solution of trifluoperazine (100 µM) was added to the Vogel's medium for CaM-inhibition studies.

2.2.13 Preparation of ultracompetent cells

Ultracompetent cells were prepared essentially as described by Inoue et al. (1990). A single colony of *E. coli* DH5-α maintained on a fresh LB agar plate was inoculated in 5 ml of LB and incubated at 37°C at 200 rpm for 16 h. The 1 ml of this overnight culture was inoculated into 100 ml of LB medium and incubated at 18°C at 200 rpm, till the OD_{600} reached 0.750. The culture was chilled on ice and centrifuged at 1,500 x g for 15 min at 4°C to pellet the cells. The cells were then resuspended in 32 ml of ice cold Transformation Buffer (TB: 10 mM PIPES pH 6.7, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$) and incubated on ice for 10 min. The centrifugation process was repeated and the cells were resuspended in 8 ml of TB containing 7% DMSO. Cell suspension was distributed into 100 µl aliquots, frozen in liquid nitrogen and stored at -70°C. Ultracompetent cells were used for cloning experiments involving ligation of DNA fragments.

2.2.14 Transformation of ultracompetent *E. coli* cells by heat shock

Competent cells were removed from the -80°C freezer and thawed on ice. Ligated DNA sample (~6 µl) was added to the competent cells and mixed gently. The cells were incubated on ice for 30 min, following which they were subjected to heat shock at 42°C for 90 sec. After the heat shock ~600 µl of LB was added to the cells and the tube was incubated at 37°C with shaking at 250 rpm for 1 h. The cells were then socked for 30 min at room temperature and plated on 85 mm LB agar plates containing 50 µg/ml of ampicillin. The plates were incubated at 37°C for overnight and transformants were screened by colony PCR.

2.2.15 Isolation of nucleic acids

(i) Small-scale isolation of plasmid DNA

Small-scale miniprep of plasmid was made by boiling lysis method (Sambrook et al. 2001). Briefly, overnight bacterial culture (~5 ml of with antibiotic was centrifuged) for 2 min, dispensed the pellet in ~100 µl distilled H_2O, mixed with 100 µl of lysis buffer for bacteria and boiled for 2 min. Then 50 µl of 1 M $MgCl_2$ was added, kept on ice for 2 min, centrifuged for 2 min, added 50 µl of alkaline lysis solution III, kept on ice for 2 min, centrifuged for 10 min, collected supernatant in a fresh microcentrifuge tube and mixed with ~0.6 volume of isopropanol, kept on ice for 5 min, centrifuged for 10 min, washed the pellet with 70% ethanol, air- dried and dissolved in sterile H_2O. All centrifugation steps were carried out at 12,000 rpm at 4°C.

(ii) Large scale isolation of plasmid DNA

Large-scale of plasmid maxiprep was made by alkaline lysis method (Sambrook et al. 2001). ~5 ml of rich medium (LB or Terrific Broth) containing the appropriate an ampicillin antibiotic supplement. A single colony of transformed bacteria was inoculated in LB or Terrific broth on contains antibiotic in starter culture that was used to inoculation of ~500 ml from single colony. Incubate on the culture at the appropriate temperature with vigorous shaking until the bacteria late log phase (OD_{600} = ~0.6) and incubate the culture for 12 h at 37°C with vigorous shaking. An aliquot (1-2 ml) of the bacterial culture was removed to a fresh microfuge tube and stored it at 4°C. Harvested the remaining bacterial cells from the 500 ml culture by centrifugation at 4100 rpm for 15 min at 4°C. The supernatant was discarded and resuspend the pellet in 18 ml of alkaline lysis solution I. Then add 2 ml of a freshly prepared solution of 10 mg/ml lysozyme. Then 40 ml of freshly prepared alkaline lysis solution II was added and closed the top of the centrifuge bottle and mix the contents thoroughly by gently inverting the bottle several times. Incubated the bottle for 5-10 min at room temperature and 20 ml of ice-cold alkaline lysis solution III was added. The top of the centrifuge bottle was then closed and mixed the contents gently but well by swirling the bottle several times (until two distinguishable liquid phases were disappeared). The bottle was placed on ice for 10 min and centrifuged the bacterial lysate at 11,000 rpm for 30 min at 4°C in a medium speed centrifuge. At the end of the centrifugation step, the supernatant was

transferred into fresh sterile 50 ml centrifuge tube and ~0.6 volume of isopropanol was added. Mixed the contents well and stored the bottle for 10 min at room temperature and centrifuged at 8000 rpm for 15 min at room temperature to recover the precipitated nucleic acids. The supernatant was discarded carefully, and inverted the open bottle on a paper towel to allow the last drops of supernatant to drain away. Rinsed the pellet and the walls of the bottle with 70% ethanol at room temperature. The ethanol was drain off completely and the centrifuge tube was kept open on a pad of paper towels for a few min at room temperature. Finally, dissolved the dried pellet of nucleic acids in 3 ml of TE (pH 8.0) and purify the crude plasmid DNA.

(iii) *Neurospora crassa* genomic DNA isolation

The strain of interest was grown in liquid Vogel's-glucose medium at 30°C for 3 to 5 days. The mycelial mass was harvested by vacuum filtration and lyophilized. The dried mycelia were ground with glass beads (0.2 µm in diameter) using a mortar and a pestle to fine powder. Approximately 150 mg of the powdered mycelia was taken in a 1.5 ml microfuge tube and 1 ml of lysis buffer added to it. Complete mixing of the mycelia and lysis buffer was achieved using a pipette tip. The tube was incubated at 65°C for 30 min, followed by centrifugation at 15,000 rpm for 10 min. All centrifugations were carried out at 25°C. The supernatant was taken in a fresh microfuge tube and 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) mixture was added to it. The tube was rotated in a cell mixer for 15 min. and centrifuged at 15,000 rpm for 10 min. The aqueous phase was carefully removed and the phenol:chloroform:isoamyl alcohol treatment repeated. The aqueous phase was taken in a fresh microfuge tube and washed with 600 µl of chloroform to remove the last traces of phenol. The aqueous phase was taken in a fresh tube and genomic DNA precipitated by adding 1.5 volumes of absolute ethanol. The tube was gently inverted a few times and the genomic DNA pelleted by centrifuging the tube briefly at 15,000 rpm. The supernatant was discarded and the pellet was washed with 70% ethanol. The tube was centrifuged for 2 min at 15,000 rpm and the supernatant was completely removed. The genomic DNA pellet was allowed to dry at room temperature for 15 min and finally dissolved in about 50 µl of TE (pH 8.0).

2.2.16 Isolation of sterols and analysis by UV spectrophotometry

The *N. crassa* strain of interest was grown in liquid Vogel's-glucose medium at 30°C for 3 to 5 days. The mycelial mass was harvested by vacuum filtration and lyophilized. The dried mycelia were ground with glass beads (0.2 mm in diameter) using a mortar and a pestle to fine powder. About 50 mg of the powdered mycelia was taken in a 1.5 ml microfuge tube and 750 µl each of methanol and chloroform was added to the tube and kept on a rotary shaker overnight. The mycelial mass was removed by centrifugation at 12,000 rpm for 10 min. The chloroform-methanol extract which contains the lipids was washed once with 0.9 % NaCl and twice with 2 M KCl to remove the saponifiable lipids. The aqueous and organic phases were separated by centrifugation and the bottom organic phase was transferred to a fresh tube. This step separates the saponifiable lipids from non-saponifiable lipids like sterols. The organic phase, which contains the sterols was air-dried and the sterols were dissolved in about 20 ml of chloroform. This sample was diluted 1: 200 in ethanol and its UV absorption spectrum (200 to 300 nm) was recorded in a UV-Vis spectrophotometer.

2.2.17 PCR

Polymerase chain-reaction (PCR) was used to amplify specific DNA sequences from genomic DNA or cDNA for molecular cloning purposes and genotyping of *N. crassa* gene deletion strains. All PCRs were performed in the BIO-RAD (Life science Instruments, France) and using one of the following DNA polymerases, Thermo Scientific *Taq* DNA Polymerase (Cat. no. EP0401, Thermo Scientific, USA) and Phusion High-Fidelity DNA Polymerase (Cat. no. F-530S, Thermo Scientific, USA).

The PCR conditions were varies with respect to the product size and annealing temperature of the primers. Ordinarily, the reaction conditions used were a 3 min denaturation at 94°C followed by 30 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 60°C, and 1 min elongation at 72°C. The total extension was 72°C for 5 min. For high fidelity PCR, protocol was followed as instructed by the manufacture of the TripleMaster PCR system, Thermo Scientific, USA.

2.2.18 Digestion of DNA with restriction endonuclease

An aliquot of 500 ng to 2 µg of DNA was digested with 5 units of restriction endonuclease per µg of DNA in a final volume of 50 µl. The reaction was carried out for 3 h using suitable buffers and assay conditions specified by the manufacturers. The enzyme was heat-inactivated by heating the digested samples at 65°C for 10 min. The digested DNA fragments were analyzed by agarose gel electrophoresis. Digestion with two enzymes was done after consulting the enzyme compatibility chart present in the New England Biolabs (NEB).

2.2.19 Agarose gel electrophoresis

The DNA samples were suspended in water containing one-sixth the volume of 6X DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol). Depending upon the size of the fragments to be resolved, the samples were loaded on 0.7% to 1.5% agarose gels cast on 1X TAE containing 0.5 µg/ml ethidium bromide. Electrophoresis was carried out in 1X TAE at 5 V/cm. Standard DNA size markers were run alongside for estimation of DNA fragment sizes. The ethidium bromide stained DNA samples were visualized on a UV transilluminator, Syngene, UK.

2.2.20 Purification of DNA fragments from agarose gels

PCR-amplified DNA required for the preparation of probes or for transformation was purified from agarose gels using the Qiaquick Gel Extraction Kit (Qiagen, CA). The sample containing DNA was resolved on a 1% high purity agarose gel. The DNA bands were visualized on a UV transilluminator and the DNA band to be eluted was excised and transferred to a weighed 1.5 ml centrifuge tube. DNA from the gel slice was eluted according to the instructions provided by the manufacturer.

2.2.21 Quantitation of nucleic acids

The concentration of nucleic acids was estimated by measuring the OD at 260 nm (Sambrook et al. 2001). The following empirical relationships were used to calculate the concentrations. The OD of 1 corresponds to approximately 50 µg/ml of double-stranded DNA, 40 µg/ml of RNA and 33 µg/ml of single-stranded oligos. The purity of nucleic acids was estimated by calculating the OD_{260}: OD_{280} ratio. The pure DNA has OD_{260}/OD_{280} of around 1.8.

2.2.22 Preparation of radiolabeled DNA probes by random primer labeling

Double-stranded DNA was radiolabeled using random primers as described by Feinberg and Vogelstein (1983) using a multiprime labelling kit. About 50-100 ng of double-stranded DNA was denatured in a volume of 20 µl by boiling for 5 min and quick chilling on ice. This was followed by the sequential addition of 5 µl of random primers solution, 5 µl of 10X reaction buffer, 4 µl each of dCTP, dGTP, dTTP, 40 µ Ci of α-[32P]-dATP and 2 units of Klenow enzyme. The reaction volume was made up to 50 µl and the reaction was carried out at 37°C for 1 h. The enzyme was inactivated at 75°C for 10 min and the probe was separated from the unincorporated nucleotides by Sephadex G-50 spun column chromatography (described below), denatured in boiling water for 5 min and added to the prehybridization solutions.

2.2.23 Sephadex G-50 column chromatography for purification of radiolabeled probe

Sephadex gel filtration column chromatography was employed to separate out unincorporated radionucleotides from DNA solutions. A sterile 1 ml disposable plastic syringe was plugged with sterile glass wool and filled with the Sephadex G-50 slurry previously equilibrated with TE pH 8.0. The column was packed by centrifugation at 1000 rpm for 5 min in a Sorvall HB-4 rotor at room temperature. Then 50 µl of the DNA solution to be purified was loaded and the column was centrifuged at 1000 rpm for 5 min at room temperature to elute the purified DNA.

2.2.24 Southern hybridization

The DNA samples to be hybridized were digested with appropriate restriction enzymes and resolved on 0.8% to 1.5% agarose gels alongside DNA markers. The gel was stained with ethidium bromide, photographed and the DNA was transferred to Hybond N^+ membrane by vacuum blotting in a vacuum blotter apparatus (made by the CCMB in-house facility, Hyderabad) for 10 min in 0.25 N HCl followed by 1 h in 0.4 N NaOH. The blot was then rinsed in 2X SSC and air-dried. Prehybridization was carried out at 65°C for 2 h in 20 ml of 0.5 M sodium phosphate and 7% SDS. The blot was hybridized at 65°C for 12 h by the addition of denatured radiolabeled probe (prepared by random primer labeling as described above). The blot was washed in 100 ml of 2X SSC, 0.5% SDS for 20 min at room

temperature, in 1X SSC, 0.5% SDS for 20 min at 65°C and 0.5X SSC, 0.5% SDS for 20 min at 65°C. The hybridized blot was exposed to X-ray film at -70°C for 6 to 12 h, developed or exposed to Fujifilm imaging plate, BAS-IP MS 2025 for ~1 h and developed using Fujifilm FLA-3000, Fuji Photo Film Co. Ltd., Japan.

2.2.25 Extraction of RNA from *Neurospora crassa*

TRIzol® reagent was used (Invitrogen, CA) to extract total RNA from *N. crassa*. About 500 mg of fungal hyphae was snap frozen in liquid nitrogen and homogenised after the addition of 1 ml TRIzol® Reagent. Following homogenization, insoluble material was removed from the homogenate by centrifugation at 12,000 rpm for 10 min at 4°C. The resulting pellet contained extracellular membranes, polysaccharides and high molecular weight DNA, while the supernatant contained RNA.

The homogenised sample was then incubated at room temperature for 5 min to allow the dissociation of the nucleoprotein complexes and 0.2 ml of chloroform was then added to the tube. The tube was shaken vigorously by hand for 15 s and then incubated at room temperature for 3 min. The sample was once again centrifuged at 12,000 rpm for 15 min at 4°C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

The aqueous phase was transferred to a fresh tube and the RNA precipitated by the addition of 0.5 ml isopropyl alcohol. The sample was incubated at room temperature for 10 min, and centrifuged at 12,000 rpm for 10 min at 4°C. After decanting the supernatant, the RNA pellet was washed once with 75% ethanol, vortexed and once again centrifuged at 12,000 rpm for 5 min at 4°C. At the end of the procedure the RNA pellet was briefly air-dried, and the RNA dissolved in RNase-free water by passing the solution a few times through a pipette tip, incubating for 10 min at 55 to 60°C and stored at -80°C.

2.2.26 Reverse Transcription PCR (RT-PCR)

In RT PCR, total RNA is first reverse transcribed into its DNA complement using the enzyme reverse transcriptase and then specific genes amplified by PCR. The total RNA isolated from *N. crassa* was first reverse transcribed using oligo-dT primers and the Platinum® *Taq* DNA Polymerase. The reagent concentrations and thermo cycler conditions was followed as instructed by the manufacture of the ThermoScript™ RT-PCR (Thermo Scientific, USA). The RT-PCR products were analysed by 1.2% agarose gel electrophoresis.

2.3 Database and software used

2.3.1 BLAST

This was used for aligning nucleotide and amino acid sequences and for searching sequences databases. This is available at http://www.ncbi.nlm.nih.gov/BLAST/

2.3.2 ClustalW

This software was used for multiple alignments of DNA and protein sequences. It is available at http://www.ebi.ac.uk/clustalw/

2.3.3 Conserved domain database

Conserved domain database (CDD) is a protein annotation resource that consists of a collection of well-annotated multiple sequence alignment models for ancient domains and full-length proteins. This is available at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

2.3.4 ExPasy translate tool

This was used for translating DNA sequences to protein sequences. It is available at http://expasy.org/tools/dna.html.

2.3.5 GeneDoc 2.7.0

The GeneDoc is used for full featured multiple sequence alignment editor, analyzer and shading.

2.3.6 Maps sites for restriction enzymes

This was used for restriction analysis of DNA sequences. It is available at http://www.restrictionmapper.org/

2.3.7 MatInspector

MatInspector is a software tool that utilizes a large library of matrix descriptions for transcription factor binding sites to locate matches in DNA sequences. It is available at http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html

2.3.8 *Neurospora crassa* database

Genome resources for *Neurospora* is available at http://www.broad.mit.edu/annotation/genome/neurospora/Home.html.
The web site for Fungal Genetics Stock Center is http://www.fgsc.net/

2.3.9 Primer3

This was used for analysis of the secondary structure of oligonucleotide primers. It is available at http://frodo.wi.mit.edu/

2.3.10 Site for reverse-complement

To convert a DNA sequence into reverse-complement counterpart, sequence manipulation suite (SMS) software package was used. It is available at http://www.bioinformatics.vg/sms/rev_comp.html

Chapter 3

Role of calcium/calmodulin-dependent kinases (Ca^{2+}/CaMKs) in sexual development of *Neurospora crassa*

3.1 Introduction

Analysis of the *N. crassa* sequence has identified four calcium/calmodulin-dependent kinases (Ca^{2+}/CaMKs), *camk-1*, *camk-2*,*camk-3*, and *camk-4*, encoded by the genes NCU09123, NCU02283, NCU06177 and NCU09212, respectively (Galagan et al. 2003; Borkovich et al. 2004). All Ca^{2+} signaling knockout mutants were generated using a high-throughput gene knockout procedure developed by the *Neurospora* genome project (http://www.dartmouth.edu/~neurosporagenome/proj_overview.html; Colot et al. 2006) and obtained from the Fungal Genetic Stock Center (FGSC). The Δ*camk-1::hph*, Δ*camk-2::hph*, Δ*camk-3::hph* and Δ*camk-4::hph* knockout mutants were generated by deleting the respective ORF using a gene deletion cassette tagged with *hph* that encodes a *hygromycin B phsphotransferase* (*hph*) and confers resistance to hygromycin.

The *N. crassa* Ca^{2+}/CaMKs show sequence similarity to *C. gloeosporioides* Ca^{2+}/CaMK called CgCMK that might be involved in germination and appressorium formation (Kim et al. 1998). In *N. crassa*, cell to cell communication plays an important role during the mating of nonswitching mating type, *A* and *a*, in sexual phase of its life cycle (Nelson and Metzenberg 1992; Raju 1992; Nelson 1996). It undergoes a complex sexual developmental process to form protoperithecia when subject to nitrogen starvation, light and low temperature (Nelson and Metzenberg 1992; Nelson 1996). Specialized receptive hyphae called trichogynes are extended from the protoperithecia and fuse with the fertilizing cell of the opposite mating type. After fertilization, protoperithecia develop into perithecia where multiple asci, each contain eight ordered ascospores, are formed (Raju 1992). Apart from few genes such as *female and male fertility-1* (*fmf-1*; Johnson 1979), *sexual development* (*sdv*; Nelson and Metzenberg 1992), *Ascospore maturation-1* (*Asm-1*; Aramayo and Metzenberg 1996), *ascus development* (*asd-1*; Nelson et al. 1997), *plenty of it-2* (*poi-2*; Kim and Nelson 2005) and *quelling defective-2 interacting protein* (*qip-2*; Maiti et al. 2007), the detailed mechanism of sexual development in *N. crassa* is not yet known. Besides, knowledge about the role of Ca^{2+}/CaMKs in sexual development process of *N. crassa* is still unclear.

In this Chapter, I describe the role of Ca^{2+}/CaMKs in sexual development of *N. crassa* using the available knockout mutants. I found that out of four Ca^{2+}/CaMKs, *camk-1* and *camk-2* genes play a recessive role in sexual development of *N. crassa*.

3.2 Results

3.2.1 Construction of knockout mutants of Ca^{2+}/CaMKs

The gene disruption cassette contain (in the order of 5' to 3') ~1-1.3 kb of 5' sequence flanking the ORF to be deleted, the selectable marker *hph* (the gene for *hygromycin B phsphotransferase*; Gritz and Davies 1983) driven by the *trpC* promoter (Staben et al. 1989) and flanked by the engineered *MmeI* restriction site and ~1-1.3 kb of 3' flanking sequence of the gene target ORF (Figure 3.1). These three fragments along with a gapped yeast vector were assembled in yeast using the endogenous homologous recombination system and the final linear deletion cassette was PCR amplified from the assembled construct by using the specific primer pairs. The *MmeI* restriction sites were designed into the junction between the flanks and the *hph* cassette to allow the adjacent gene specific 20 bp sequences to serve as molecular bar code. Digestion of genomic DNA by *MmeI* (which cuts 20 bp downstream of its recognition sequences) followed by ligation mediated PCR will create products that can be amplified. The deletion cassette was transformed into *Δmus-51* or *Δmus-52* strains by electroporation in order to increase the frequency of homologous integration.

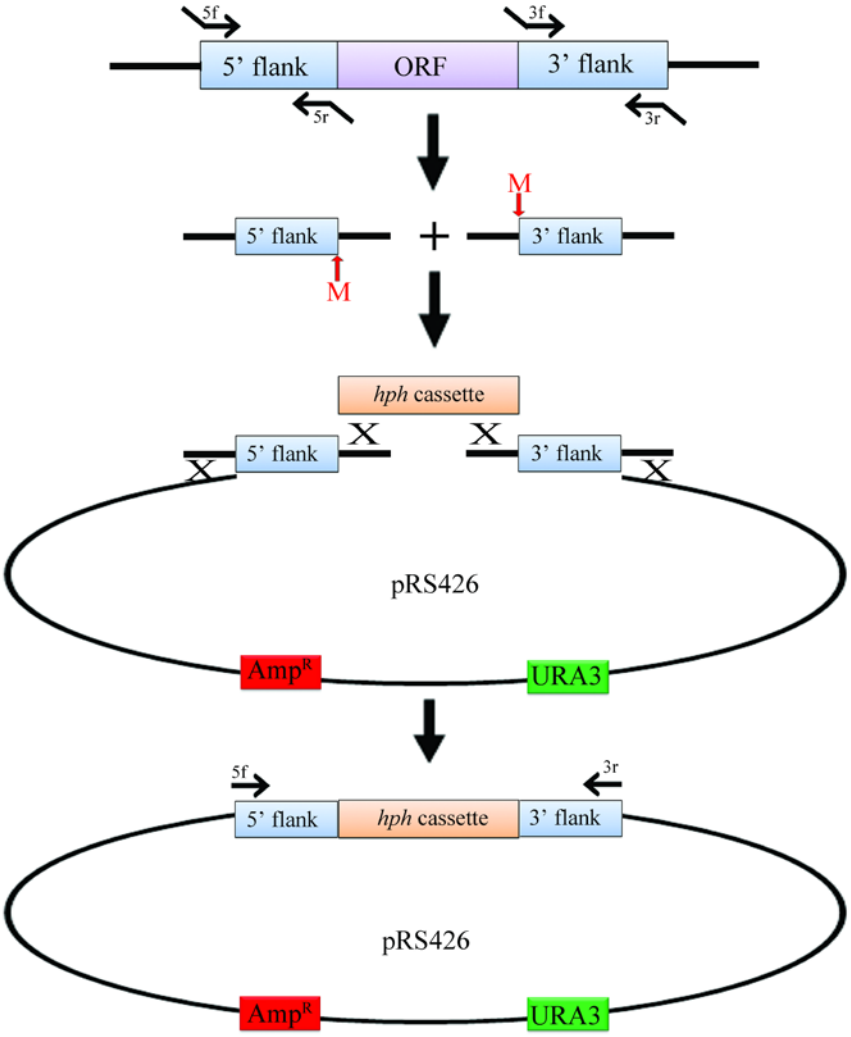


Figure 3.1 Strategy to generate deletion constructs for Ca^{2+}/CaMKs. The 5' and 3'-flanks of the target Ca^{2+}/CaMK were amplified separately from wild-type with primers 5f with 5r and 3f with 3r. Primers 5r and 3f were designed to incorporate *MmeI* site (M) and contained 5' tails homologous to the *hph* cassette, whereas, tails of 5f and 3r were homologous to the pRS426 vector. The *hph* cassette was separate PCR amplified from the pCSN44 vector using specific primer pairs (Staben et al. 1989; Colot et al. 2006). The two flanks were cotransformed into yeast along with the *hph* cassette and gapped yeast shuttle vector (pRS426) to generate the circular construct through homologous recombination. The final linear deletion cassette was PCR amplified from the pooled yeast DNA with primers 5f and 3r. The *hph* is transcribed in the antisense direction relative to the direction of transcription of the target gene (adapted from Colot et al. 2006).

All deletion strains of Ca^{2+}/CaMKs were generated by the NIH *Neurospora* Genome Project and confirmed using Southern analysis (http://borkovichlims.ucr.edu/southern/;information regarding the knockout mutants of all Ca^{2+}/CaMKs are available in the FGSC, http://www.dartmouth.edu/~neurosporagenome/proj_overview.html; Figure A.1 in appendix; Colot et al. 2006). I had obtained *Δcamk-1::hph* (FGSC 12547 A, FGSC 12548 a), *Δcamk-2::hph* (FGSC 12448 a, FGSC 12449 A), *Δcamk-3::hph* (FGSC 11536 A, FGSC 11537 a) and *Δcamk-4::hph* (FGSC 11545 a) from the FGSC. I have generated the four *Δcamk-4::hph A* mutant strains from a cross of *Δcamk-4::hph a* with *OR A*. Six hygromycin resistant progenies out of 20 progenies isolated from a *Δcamk-4::hph a* X *OR A* were screened by PCR to verify the knockout mutant allele and crossed with *OR a* (to determine the mating type). From this screening, four *Δcamk-4::hph A* mutant strains were identified (Figure 3.2; Table 3.1).

Table 3.1 Primers used for confirmation of knockout mutants of Ca^{2+}/CaMKs by PCR analysis

S. no.	Primer	Sequence (5'→3')	Reference
1.	1NCU09123F	GATGTAGCTGAAGTTGGTGG	This study
2.	5NCU02283F	AGGAGAAGTCTGAGAAGAGG	This study
3.	1NCU06177F	GAGTAGATGACCATGGTTGG	This study
4.	1NCU09212F	CGTATTCAACTCCAGGTAGC	This study
5.	5HPHR	ATCCACTTAACGTTACTGAAATC	Deka et al. 2011

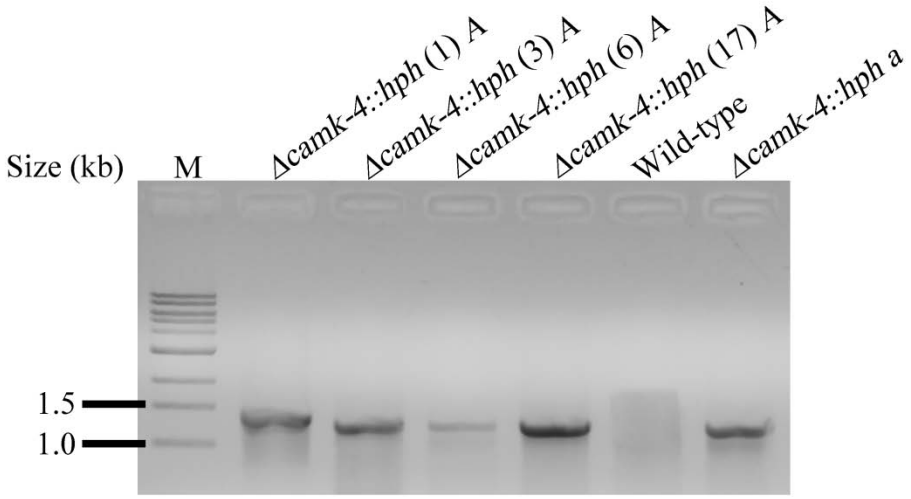
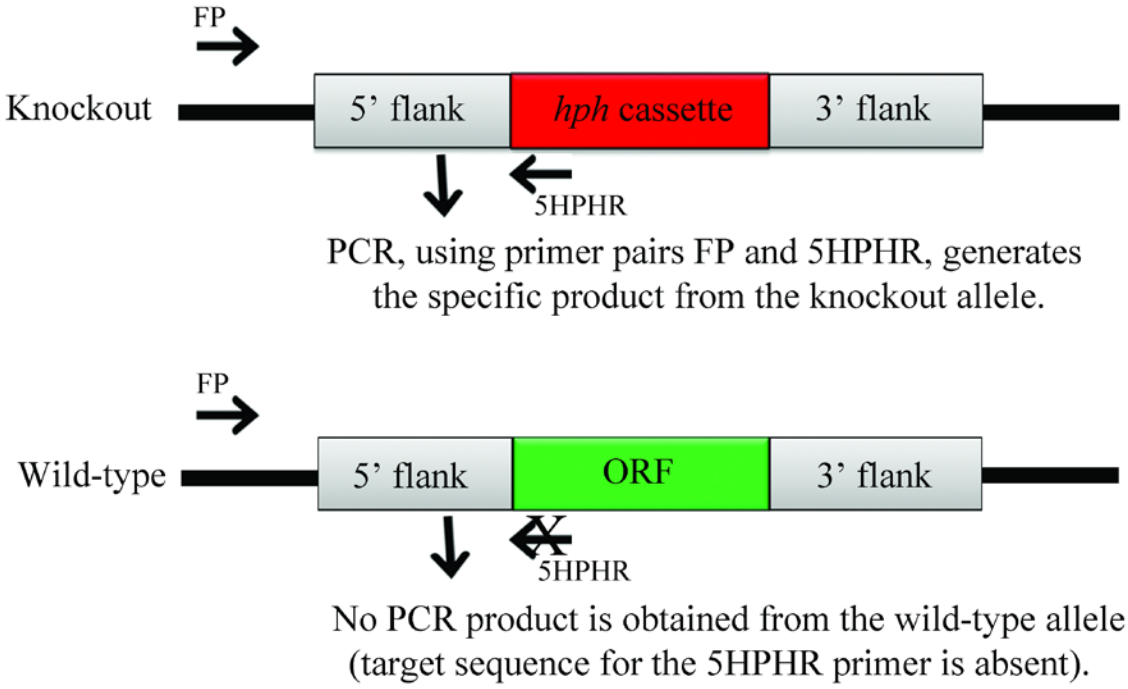


Figure 3.2 Verification of Δ*camk-4::hph A* mutant strains by PCR analysis. The amplification of PCR product of size ~1.2 kb, indicated presence of the Δ*camk-4::hph* mutant allele in the homokaryotic progenies. The PCR products were visualized in a 1% agarose gel; M: 1 kb DNA ladder.

3.2.2 Verification knockout mutants of Ca^{2+}/CaMKs by PCR analysis

The *N. crassa* knockout mutants *Δcamk-1::hph*, *Δcamk-2::hph*, *Δcamk-3::hph* and *Δcamk-4::hph* were generated using a high-throughput gene knockout procedure (Colot et al. 2006; Figure 3.3A) and obtained from the FGSC. In order to verify the knockout mutants of Ca^{2+}/CaMK, PCR analysis was performed using the specific forward primers (1NCU09123F, 5NCU02283F, 1NCU06177F and 1NCU09212F, respectively, for the upstream 5' flanks of the *camk-1*, *camk-2*, *camk-3* and *camk-4* genes) to generate a knockout allele specific PCR product and the specific reverse primer (5HPHR) that is specific for the *hph* cassette (Table 3.1). The PCR analysis verified the presence of the knockout allele in the respective mutants of Ca^{2+}/CaMKs (Figure 3.3B).

(A)



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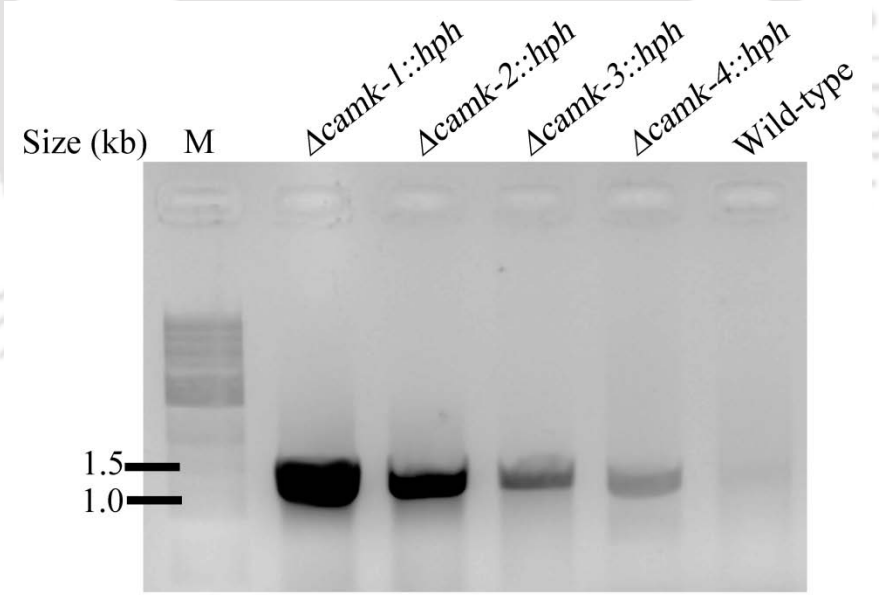


Figure 3.3 Verification of knockout mutants of Ca^{2+}/CaMKs. (A) Strategy for the PCR analysis to verify the knockout mutants of Ca^{2+}/CaMKs. The forward primer (FP) is specific for upstream sequence of the target knockout allele and 5HPHR primer is specific to the *hph* cassette. Therefore, using these two primer pairs, knockout allele specific PCR product is obtained from the knockout mutants; however, no PCR product is obtained from the wild-type

ORF. **(B)** The Δ*camk-1::hph*, Δ*camk-2::hph*, Δ*camk-3::hph* and Δ*camk-4::hph* mutants were verified using the forward primers 1NCU09123F, 5NCU02283F, 1NCU06177F and 1NCU09212F specific for the upstream of the 5' flank of *camk-1*, *camk-2*, *camk-3* and *camk-4* genes, respectively, along with the common reverse primer 5HPHR that is specific for the *hph* cassette. Amplification of PCR products of size ~1.3, 1.2, 1.2 and 1.2 kb, verified presence of the Δ*camk-1::hph*, Δ*camk-2::hph* and Δ*camk-3::hph* knockout alleles, respectively; PCR products were visualized in a 1% agarose gel; M: 1 kb DNA ladder.

3.2.3 The *camk-1* and *camk-2* genes are involved in sexual development

Sexual development in *N. crassa* is activated by nitrogen starvation that induces a type of hyphal aggregation leading to generation of specialized female sexual organs called protoperithecia (Nelson and Metzenberg 1992; Raju 1992; Nelson 1996). To test if Ca^{2+} signaling genes are involved in sexual development of *N. crassa*, I screened knockout mutants of 20 Ca^{2+} signaling genes to determine their role in fertility (Table 3.2). I found that two knockout mutants of Ca^{2+}/CaMKs, Δ*camk-1::hph* and Δ*camk-2::hph*, displayed a fertility defect in homozygous crosses and produced less ascospores. Crosses homozygous for Δ*camk-1::hph* mutant strain produced normal looking perithecia, only a few asci were recovered from all perithecia from one petri dish and displayed an intermediate (produced few hundred progeny ascospores; Table 3.2, entry 2) phenotype. Additionally, crosses homozygous for Δ*camk-2::hph* mutant strain displayed a barren (produced exceptionally few progeny ascospores; Table 3.2, entry 3) phenotype. Crosses heterozygous for Δ*camk-1::hph* and Δ*camk-2::hph* mutant strains were fertile (Table 3.2, entries 4-7). In addition, crosses homozygous for another 18 knockout mutants of Ca^{2+} signaling genes, including the mutants of two other Ca^{2+}/CaMKs mutants Δ*camk-3::hph* and Δ*camk-4::hph*, were fertile like the wild-type (produced thousands of progeny ascospores; Table 3.2, entries 8-25). The heterozygous crosses involving the wild-type and the knockout mutants of these 18 Ca^{2+} signaling genes were also fully fertile (data not shown). These results suggested that *camk-1* and *camk-2* genes play a role in sexual development in *N. crassa*.

Table 3.2 Phenotypes of crosses involving knockout mutants of Ca^{2+} signaling genes

S. no.	Cross	Ascospores produced	Phenotype
1.	*OR a* X *OR A*	abundant	fertile
2.	Δ*camk-1::hph a* X Δ*camk-1::hph A*	few hundred (~500)	intermediate
3.	Δ*camk-2::hph a* X Δ*camk-2::hph A*	very few (<200)	barren
4.	Δ*camk-1::hph a* X *OR A*	abundant	fertile
5.	Δ*camk-1::hph A* X *OR a*	abundant	fertile
6.	Δ*camk-2::hph a* X *OR A*	abundant	fertile
7.	Δ*camk-2::hph A* X *OR a*	abundant	fertile
8.	Δ*camk-3::hph a* X Δ*camk-3::hph A*	abundant	fertile
9.	Δ*camk-4::hph a* X Δ*camk-4::hph A*	abundant	fertile
10.	Δ*NCU02814.2::hph a* X Δ*NCU02814.2::hph A*	abundant	fertile
11.	Δ*NCU06650.2::hph a* X Δ*NCU06650.2::hph A*	abundant	fertile
12.	Δ*NCU07075.2::hph a* X Δ*NCU07075.2::hph A*	abundant	fertile
13.	Δ*NCU08147.2::hph a* X Δ*NCU08147.2::hph A*	abundant	fertile
14.	Δ*NCU09655.2::hph a* X Δ*NCU09655.2::hph A*	abundant	fertile
15.	Δ*NCU04379.2 ::hph a* X Δ*NCU04379.2::hph A*	abundant	fertile
16.	Δ*NCU05255.2::hph a* X Δ*NCU05255.2::hph A*	abundant	fertile
17.	Δ*NCU06366.2::hph a* X Δ*NCU06366.2::hph A*	abundant	fertile
18.	Δ*NCU07966.2::hph a* X Δ*NCU07966.2::hph A*	abundant	fertile
19.	Δ*NCU02826.2::hph a* X Δ*NCU02826.2::hph A*	abundant	fertile
20.	Δ*NCU06948.2::hph a* X Δ*NCU06948.2::hph A*	abundant	fertile
21.	Δ*NCU00916.2::hph a* X Δ*NCU00916.2::hph A*	abundant	fertile
22.	Δ*NCU06703.2::hph a* X Δ*NCU06703.2::hph A*	abundant	fertile
23.	Δ*NCU00795.2::hph a* X Δ*NCU00795.2::hph A*	abundant	fertile
24.	Δ*NCU05154.2::hph a* X Δ*NCU05154.2::hph A*	abundant	fertile
25.	Δ*NCU05164.2::hph a* X Δ*NCU05164.2::hph A*	abundant	fertile

3.2.4 The Δ*camk-1::hph* and Δ*camk-2::hph* mutants showed a fertility defect even when strains of any of the opposite mating type were used as either male or female parent

Two Ca^{2+}/CaMKs, *camk-1* and *camk-2* were found to play a role in sexual development as evident by the fertility defect of the homozygous crosses. However, crosses heterozygous for both Δ*camk-1::hph* and Δ*camk-2::hph* mutant mate successfully with wild-type and produced thousands of ascospores (Figure 3.4A-C; Table 3.3, entries 1-4, 7-10). To exclude the possibility that a direct contact between mutant and wild type allows complementation of the mutant, I performed crosses involving Δ*camk-1::hph* and Δ*camk-2::hph* mutant strains as male or female parents. The Δ*camk-1::hph* and Δ*camk-2::hph* female parents were grown individually on SCM and incubated at 22°C for 6-7 days to allow formation of protoperithecia that were fertilized with the male parent of the opposite mating type. The formation of protoperithecia, perithecia development and ascospores production were examined microscopically. All such crosses showed same fertility defect, and therefore, phenotypes of the Δ*camk-1::hph* and Δ*camk-2::hph* homozygous crosses were consistent even when any of the mating type strain was used as either male or female parent (Figure 3.4A-C; Table 3.3, entries 5-6, 11-12). These results suggested that both *camk-1* and *camk-2* genes play a recessive role in sexual development of *N. crassa*.

Table 3.3 Phenotype of crosses involving Ca^{2+}/CaMK mutants as male or female parents

S. no.	Female parent	Male parent	Ascospores produced	Phenotype
1.	Δ*camk-1::hph a*	*OR A*	abundant	fertile
2.	Δ*camk-1:hph A*	*OR a*	abundant	fertile
3.	*OR a*	Δ*camk-1:hph A*	abundant	fertile
4.	*OR A*	Δ*camk-1::hph a*	abundant	fertile
5.	Δ*camk-1:hph A*	Δ*camk-1::hph a*	few hundred (~500)	intermediate
6.	Δ*camk-1::hph a*	Δ*camk-1:hph A*	few hundred (~500)	intermediate
7.	Δ*camk-2::hph a*	*OR A*	abundant	fertile
8.	Δ*camk-2::hph A*	*OR a*	abundant	fertile
9.	*OR a*	Δ*camk-2::hph A*	abundant	fertile
10.	*OR A*	Δ*camk-2::hph a*	abundant	fertile

11.	Δ*camk-2::hph a*	Δ*camk-2::hph A*	very few (<200)	barren
12.	Δ*camk-2::hph A*	Δ*camk-2::hph a*	very few (<200)	barren

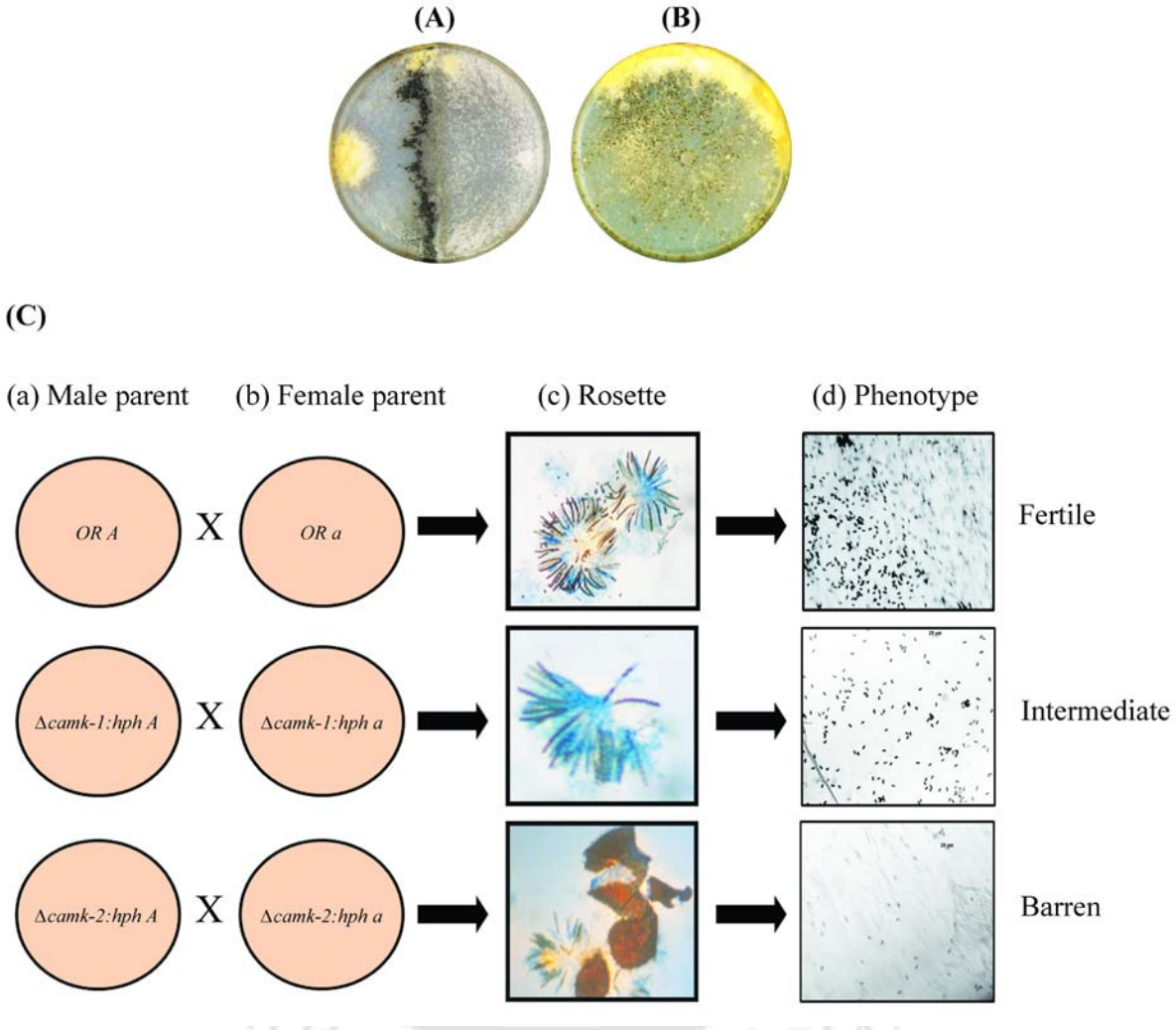


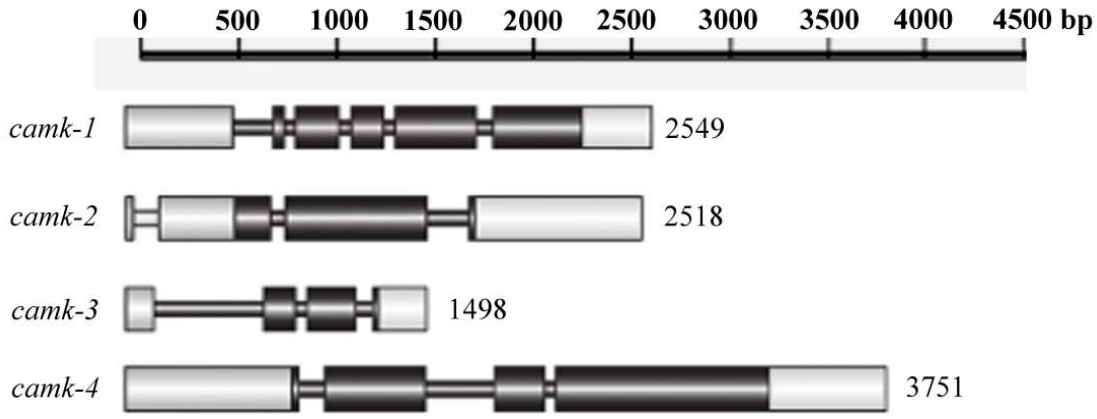
Figure 3.4 Phenotype of crosses involving Δ*camk-1::hph* and Δ*camk-2::hph* mutants. (A) In mating type co-confrontation crosses (strains of opposite mating type were co-inoculated in petri dish containing SCM), perithecia developed abundantly in the contact zone (the black line in the center). A representative picture of a typical cross is shown. **(B)** A representative picture of a fertile cross showing the ejected ascospores. **(C)** The *OR A*, Δ*camk-1::hph A*, and Δ*camk-2::hph A* strains were used as male parent (a) that were fertilized with the opposite mating type using as the female parent (b). A representative picture of a rosette from each type of the cross is also shown (c). Ascospores, ejected on the lid, were harvested with ~1 ml

sterile H_2O and placed on a glass slide with equal proportion for a representative picture to show the relative ascospores produced in the cross (i.e. the fertility of the cross; d).

3.2.5 The *Neurospora crassa* Ca^{2+}/CaMKs contain conserved signature motifs and belong to the cluster of clades comprising of broad substrate specificity kinases

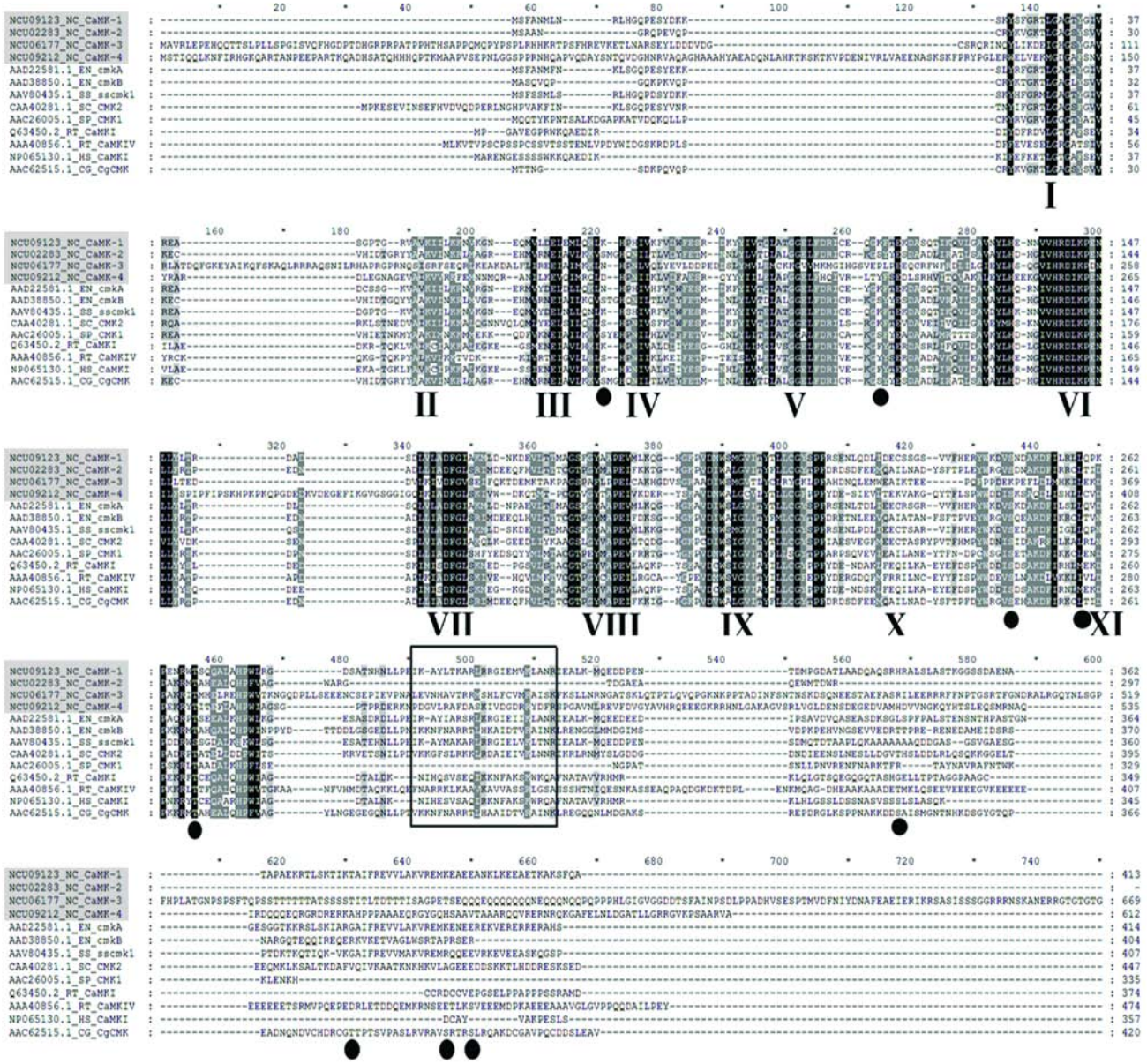
The four Ca^{2+}/CaMKs, NCU09123 (accession number XP_958895), NCU02283 (accession number XP_959927), NCU06177 (accession number XP_962989) and NCU09212 (accession number XP_959156) have been annotated as *camk-1*, *camk-2*, *camk-3* and *camk-4*, respectively, in the version 7 of *N. crassa* genome database (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html). The Ca^{2+}/CaMKs genes share a common core structure consisting of exons, introns and untranslated regions (Figure 3.5A). The general domain organization of Ca^{2+}/CaMKs includes a highly conserved N-terminal catalytic domain and a C-terminal regulatory region consisting of overlapping autoinhibitory and Ca^{2+}/CaM binding domains (Figure 3.5B). Ca^{2+}/CaMKs in *N. crassa* also possess 11 conserved kinase domains, one putative CaM binding domain and nine putative phosphorylation sites, like the putative CaM kinase CgCMK of *C. gloeosporioides* (Kim et al. 1998; Deka et al. 2011; Tamuli et al. 2011; Figure 3.5C). A phylogenetic analysis using the sequence of Ca^{2+}/CaMKs homologues from fungi to mammals revealed that all four Ca^{2+}/CaMKs from *N. crassa* are distinct from the clades comprising of narrow substrate kinases and form different closet clades with their homologues. The *N. crassa* kinase *camk-1* was clustered with CaMKs from fungi, *camk-2* was clustered with mammalian and fungal CaMKs, *camk-3* was clustered with CaMKKs from fungi, and *camk-4* was clustered with serine/threonine-protein kinases from fungi (Figure 3.5D). I had also analyzed the 5'-flanking genomic regions (~2 kb) of the Ca^{2+}/CaMKs from *N. crassa* in order to predict the putative regulatory elements involved in transcription. All the four Ca^{2+}/CaMKs contain TATA less promoter with GC rich sequences near the transcription start site and contain putative sites for regulatory elements, some of which are GATA, activator of nitrogen regulated genes, pheromone response element, activator of stress genes, pH responsive regulators and Dde box (Figure 3.5E). These regulatory sequences could indicate regulation of the Ca^{2+}/CaMKs in *N. crassa*.

(A)



(B)

(C)



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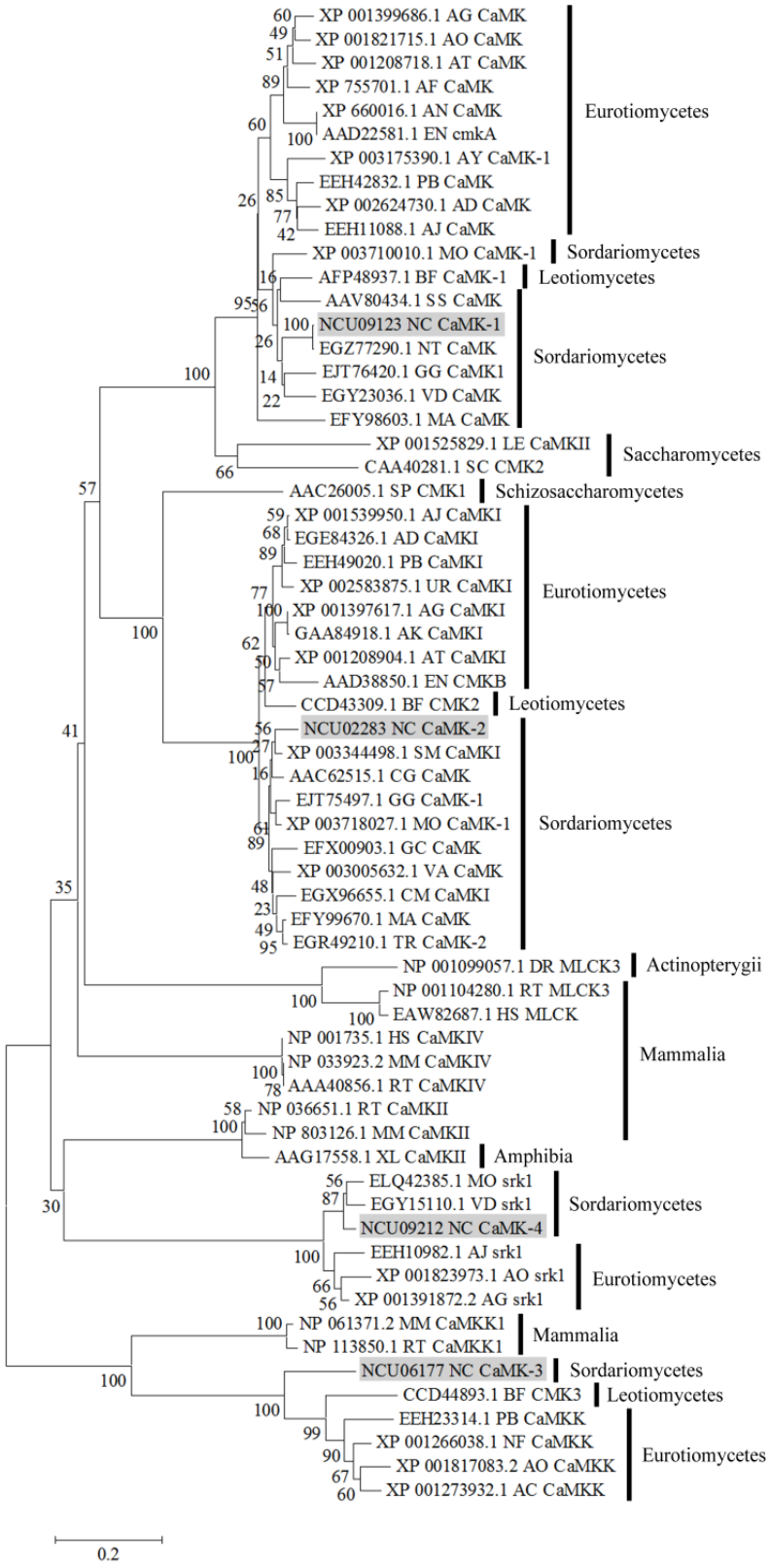
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EEH11088.1 AJ CaMK
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XP 003710010.1 MO CaMK-1
Sordariomycetes
AFP48937.1 BF CaMK-1
Leotiomycetes
AAV80434.1 SS CaMK
NCU09123 NC CaMK-1
EGZ77290.1 NT CaMK
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EGY23036.1 VD CaMK
EFY98603.1 MA CaMK
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Mammalia
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Amphibia
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Sordariomycetes
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Eurotiomycetes
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NP 113850.1 RT CaMKK1
Mammalia
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Leotiomycetes
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Eurotiomycetes
0.2

(E)

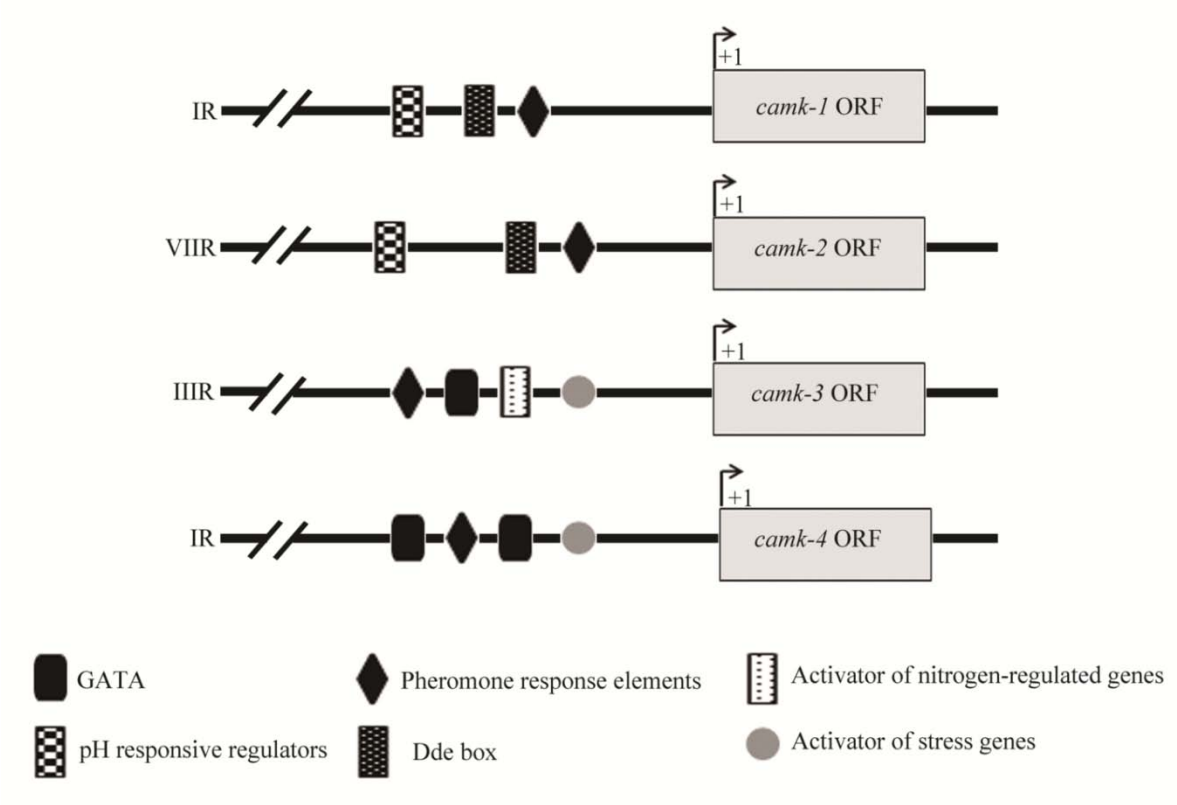


Figure 3.5 Sequence analysis of Ca^{2+}/CaMKs.

(A) Gene structure of the Ca^{2+}/CaMKs of *N. crassa* showing exons (black bar), introns (black line) and UTRs (light grey bar).

(B) General domain organization of Ca^{2+}/CaMKs include an N-terminal kinase domain (red) followed by an autoinhibitory (yellow) and overlapping CaM-binding domain (blue).

(C) Sequence alignment of Ca^{2+}/CaMK homologues from fungi to human. The Ca^{2+}/CaMKs contain 11 (I to XI) conserved kinase domains, one putative CaM binding domain and nine putative phosphorylation sites. The box indicates the putative CaM-binding domain and solid circles below the CG sequences indicate potential autophosphorylation sites containing $^{R}{}_{K}XX^{T}{}_{S}$ consensus phosphorylation site for CaMKs (Kim et al.1998). Conserved amino acids are indicated in black (100%), dark gray (>80%) and light gray (>60%). The sequences used are CG, *Colletotrichum gloeosporioides*; EN, *Emericella nidulans*; HS, *Homo sapiens*; NC, *Neurospora crassa*; RT, *Rattus norvegicus*; SC, *Saccharomyces cerevisiae*; SP, *Schizosaccharomyces pombe*; SS, *Sporothrix schenckii*.

(D) Phylogenetic analysis Ca^{2+}/CaMKs sequences using the minimum-evolution (ME) method, 500 bootstrap replications (bootstrap values are indicated in the point at nodes) as test of phylogeny and the software MEGA5.1. The sequences used are AC, *Aspergillus clavatus*; AD, *Ajellomyces dermatitidis*; AF, *Aspergillus fumigatus*; AG, *Aspergillus niger*; AJ, *Ajellomyces capsulatus*; AK, *Aspergillus kawachii*; AN, *Aspergillus nidulans*; AO, *Aspergillus oryzae*; AT, *Aspergillus terreus*; AY, *Arthroderma gypseum*; BF, *Botryotinia fuckeliana*; CG, *Colletotrichum gloeosporioides*; CM, *Cordyceps militaris*; DR, *Danio rerio*; EN, *Emericella nidulans*; GC, *Grosmannia clavigera*; GG, *Gaeumannomyces graminis var.tritici*; HS, *Homo sapiens*; LE, *Lodderomyces elongisporus*; MA, *Metarhizium anisopliae*; MM, *Mus musculus*; MO, *Magnaporthe oryzae*; NC, *Neurospora crassa*; NF, *Neosartorya fischeri*; NT, *Neurospora tetrasperma*; PB, *Paracoccidioides brasiliensis*; RT, *Rattus norvegicus*; SC, *Saccharomyces cerevisiae*; SM, *Sordaria macrospora*; SP, *Schizosaccharomyces pombe*; SS, *Sporothrix schenckii*; TR, *Trichoderma reesei*; UR, *Uncinocarpus reesii*; VA, *Verticillium albo-atrum*; VD, *Verticillium dahliae*; XL, *Xenopus laevis.* The Ca^{2+}/CaMKs of *N. crassa* (highlighted in the tree) are described by the annotation number as available in *N. crassa* sequence release version 7 at the Broad Institute and the NCBI accession number for others, followed by the accession number, organism, kinase name and phylum. Bar at the bottom of the ME tree indicates scale of genetic distance.

(E) The predicted regulatory elements in the promoter sequences of Ca^{2+}/CaMKs from *N. crassa*. The ORF of *camk-1*, *camk-2*, *camk-3* and *camk-4* genes are shown in their respective chromosomal location along with their immediate 5'- flanking sequence (~2 kb) used for the analysis of regulatory sequences. Some of the regulatory sequences identified for each gene are shown. The +1 transcription start site (TSS) is indicated using arrows.

3.3 Discussion

Crosses homozygous for Δ*camk-1::hph* and Δ*camk-2::hph* mutants, respectively, showed an intermediate (produced few hundred progeny ascospores) and a barren (produced exceptionally few progeny ascospores) phenotype (Table 3.2, entries 2-3). However, crosses homozygous for Δ*camk-3::hph* and Δ*camk-4::hph* mutants were fully fertile (Table 3.2, entries 8-9). Therefore, out of the four Ca^{2+}/CaMKs, only *camk-1* and *camk-2* were found to play a role in sexual development in *N. crassa*. The phenotypes of the *Δcamk-1::hph* and *Δcamk-2::hph* homozygous crosses were consistent even when any of the mating type was used as either male or female parent (Figure 3.4A-C; Table 3.3, entries 5-6, 11-12). Thus, I found that out of the four Ca^{2+}/CaMKs of *N. crassa*, only *camk-1* and *camk-2* play a role in sexual development in a recessive manner. Moreover, *camk-1* and *camk-2* genes were expressed under normal condition during the vegetative growth of *N. crassa* (Figure A.2 in appendix). Additionally, the Ca^{2+}/CaMKs of *N. crassa* possess conserved signature motifs; they were clustered with the broad substrate specificity kinases and their promoter region contains consensus sequence for regulatory elements (Figure 3.5A-E).

The Ca^{2+}/CaMKs were also found important for sexual development in other organisms. In *N. crassa*, few sexual development genes were identified previously such as *female and male fertility-1* (*fmf-1*; Johnson 1979) arrest the perithecial development when crossed either a male or a female parent and *sexual development* (*sdv*; Nelson and Metzenberg 1992). The Ca^{2+}/CaMKI homologue in *C. gloeosporioides*, CgCMK, might be involved in germination and appressorium induction (Kim et al. 1998). The CAMK-1 homologue in *S. schenckii*, *sscmk1*, might be regulating dimorphism (Valle-Aviles et al. 2007). In *X. laevis*, CaMKIx, activated Ca^{2+}/CaMKI was found to phosphorylate various proteins including synapsin I, histones, and myelin basic protein in the late stages of embryogenesis (Kinoshita et al. 2004). In addition, activation of Ca^{2+} channels leads to Ca^{2+} influx that is an essential step in initiation of acrosome reaction during fertilization in human, and abnormal activity was detected in the Ca^{2+}-permeable channels from infertile human sperm membrane (Goodwin et al. 1997; Ma and Shi 1999). Binding of Ca^{2+}/CaM activates Ca^{2+}/CaMKs by removing the autoinhibitory domain that allows the catalytic pocket to access the substrate (Hook and Means 2001). Ca^{2+}/CaMKs are classified into two groups based on their substrate

specificity, one group has broad substrate specificity and the other group has narrow substrate specificity. Members of the broad substrate specificity group have the ability to phosphorylate many different proteins and include monomeric enzymes such as CaMKK, CaMKI and IV besides the multimeric enzyme CaMKII (Hook and Means 2001; Swulius and Waxham 2008). The narrow substrate specificity group includes dedicated kinases such as phosphorylase kinase, myosin-light chain kinase (MLCK) and CaMKIII (Hook and Means 2001; Swulius and Waxham 2008).

This is the first report demonstrating using a genetic approach involvement of *camk-1* and *camk-2* genes in sexual development in *N. crassa* (Deka et al. 2011; Tamuli et al. 2011, 2013; Kumar and Tamuli 2013, under review). Further studies on the *camk-1* and *camk-2* genes will be discussed in the next Chapter.

Chapter 4

Essential role of *camk-1* and *camk-2* genes in fertility and the requirement of *camk-2* during meiosis

4.1 Introduction

In the previous Chapter, I found that the *camk-1* and *camk-2* genes play a recessive role in sexual development of *N. crassa*. In *N. crassa*, sexual development is triggered by nitrogen limitation and fusion of *A* and *a* nuclei result in formation of a dikaryon in structures called ascogenous hyphae. The ascogenous tissue is microscopic, and therefore, biochemistry of sexual development process is difficult. In this Chapter, I describe complementation analysis that demonstrated the essential role of *camk-1* and *camk-2* genes in sexual development of *N. crassa*.

In the sexual phase of *N. crassa*, the fertilized dikaryon undergoes meiosis in a complex developmental structure called perithecium, where zygotic cells undergo meiosis in an unsynchronized manner. The zygote, or ascus mother cell, is the only diploid cell known in *N. crassa*. The zygote undergoes two rounds of meiosis followed by a post-meiotic mitotic division resulting in eight ordered nuclei, which undergo cellularization. On completion of cellularization, melanized ascospores are ejected from the perithecium (Raju 1980, 1992).

In *N. crassa*, an RNAi-based gene silencing mechanism called meiotic silencing operates during meiosis. If any unpaired DNA is detected during prophase I of meiosis, meiotic silencing mechanism will silence the expression of the unpaired DNA and all homologous copies (Shiu et al. 2001). I have tested the effect of meiotic silencing of *camk-2* gene in sexual development of *N. crassa*.

The complementation analysis of the Δ*camk-1::hph* and Δ*camk-2::hph* mutants have shown that *camk-1* and *camk-2* genes are essential for full fertility in *N. crassa*. In addition, the *camk-2* product is found essential during meiosis of the fertilized zygote for full fertility.

4.2 Results

4.2.1 Cloning of *camk-1* and *camk-2* genes

A fragment of size ~4693 bp carrying the *camk-1* gene was PCR amplified from the wild-type using the primer pairs 1NCU09123F and 2NCU09123R (Figure 4.2A, C; Table 4.1). Similarly, another fragment of size ~4157 bp carrying the *camk-2* gene was PCR amplified from the wild-type using the primer pairs 5NCU02283F and 6NCU02283R (Figure 4.2B-C; Table 4.1). PCRs were performed using custom oliogonucleotide primers (Metabion, Germany and IDT, USA), Phusion High-Fidelity PCR Kit (Finzymes, Finland) and PCR fragments were purified using QIAquick Gel Extraction Kit (QIAGEN, CA) according to the manufacturers protocol. The purified *camk-1* and *camk-2* fragments were cloned into the *SmaI* site of the plasmid vector pBARGEM7-1 (FGSC 19; Pall and Brunelli 1993) that resulted in pRK-1 and pRK-2 constructs, respectively (Figure 4.1). The pRK-1 and pRK-2 plasmid constructs were transformed into the *E. coli* DH5α (Hanahan 1983) competent cells, plasmid were then isolated and the correct insertion of *camk-1* and *camk-2* genes were verified using the *XhoI* and *ScaI* restriction digestion (Figure 4.2D-E).

Table 4.1 Primers used for cloning of *camk-1* and *camk-2* genes

S. no.	Primer	Sequence (5'→3')	Reference
1.	1NCU09123F	GATGTAGCTGAAGTTGGTGG	This study
2.	2NCU09123R	ATATGTACCCCGACCTGCTC	This study
3.	5NCU02283F	AGGAGAAGTCTGAGAAGAGG	This study
4.	6NCU02283R	TCCAGGACTGATGTGTATGC	This study

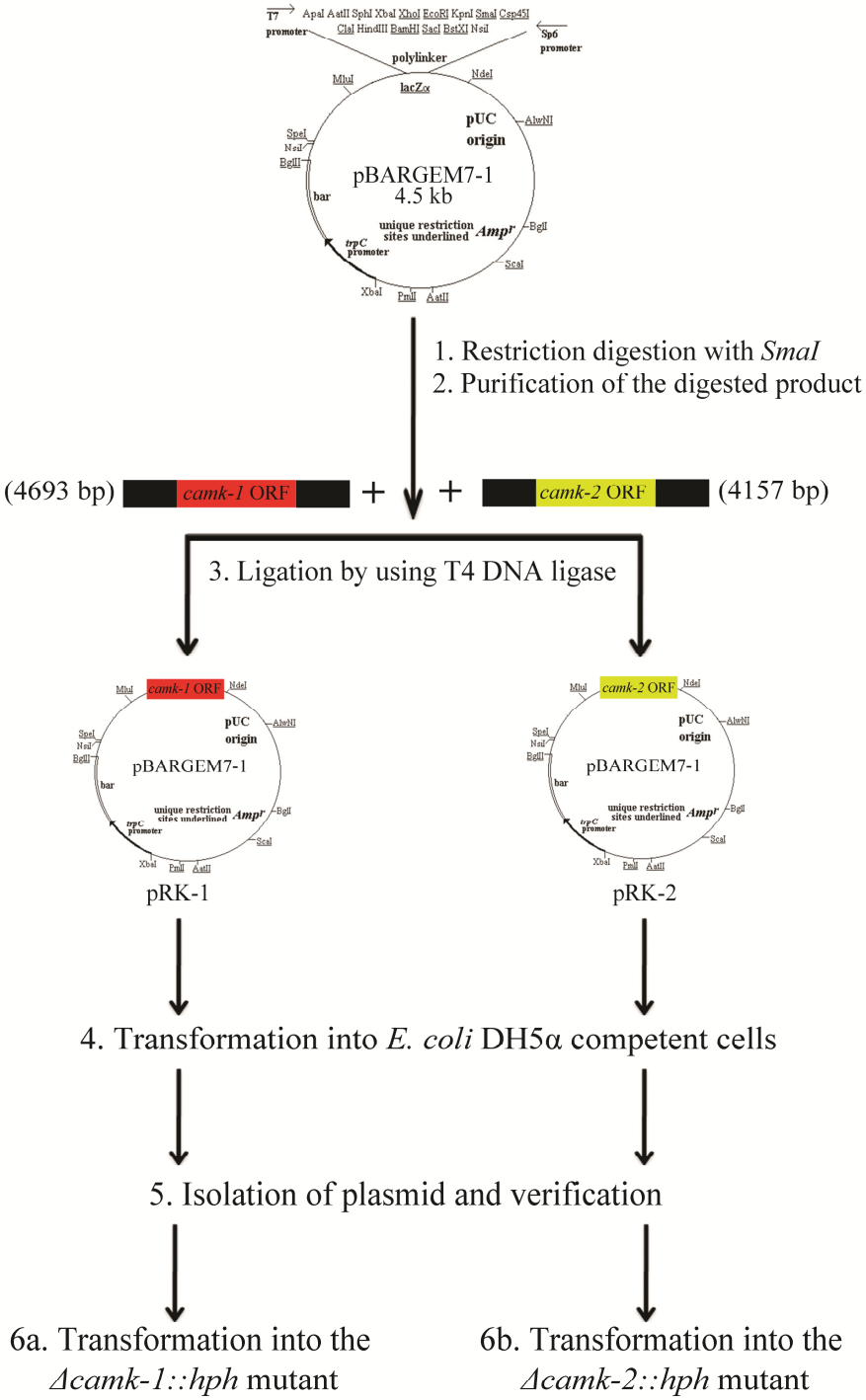
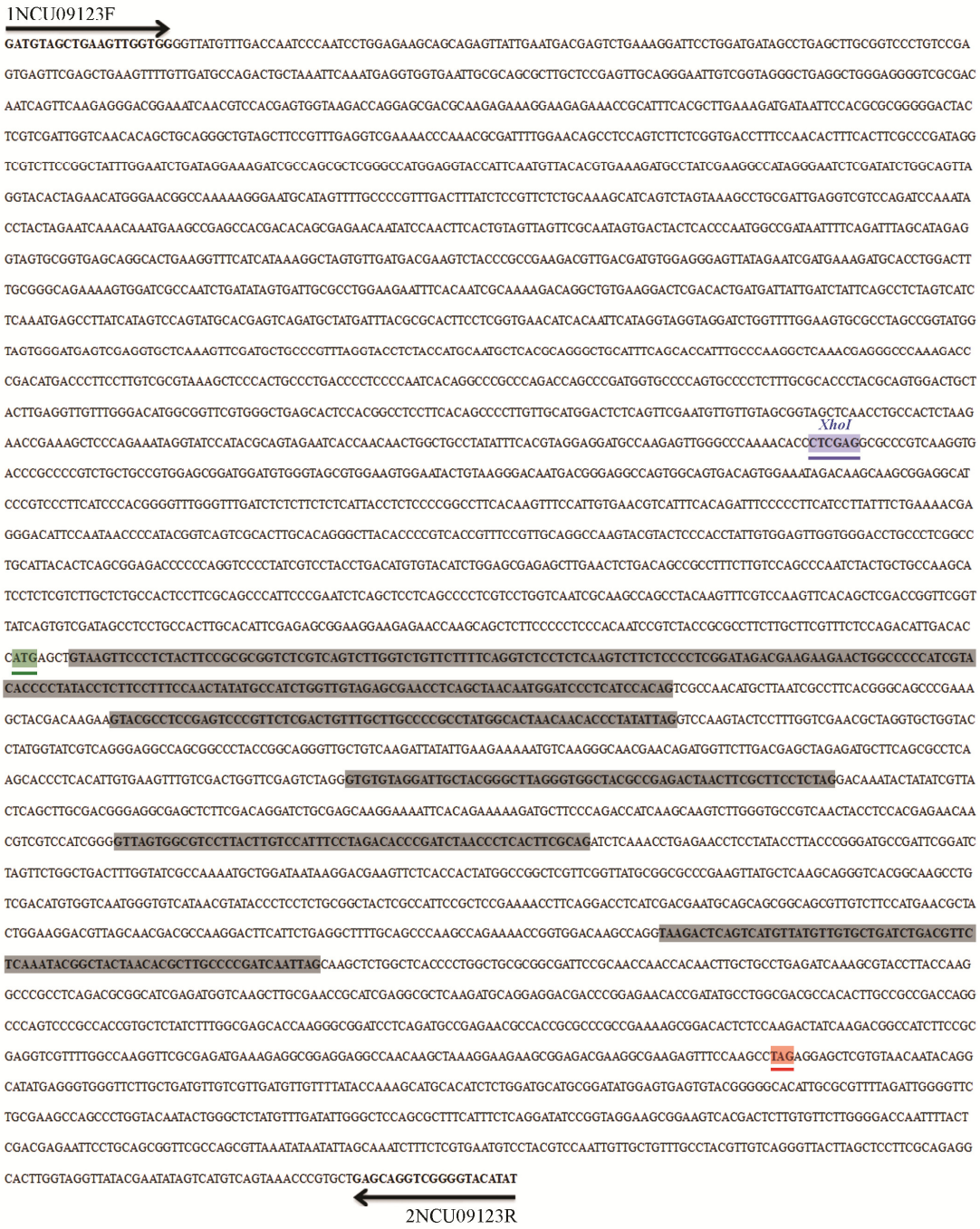


Figure 4.1 Schematics of pRK-1 and pRK-2 constructs. Cloning of the ~4693 and 4157 bp fragments carrying *camk-1* and *camk-2* genes into the *SmaI* site of the pBARGEM7-1 plasmid vector that contains *bar* selectable marker, lacZα for β-galactosidase and *amp* for bacterial selection. The pRK-1 and pRK-2 constructs were transformed, respectively, into the *Δcamk-1::hph* and *Δcamk-2::hph* mutants.

(A)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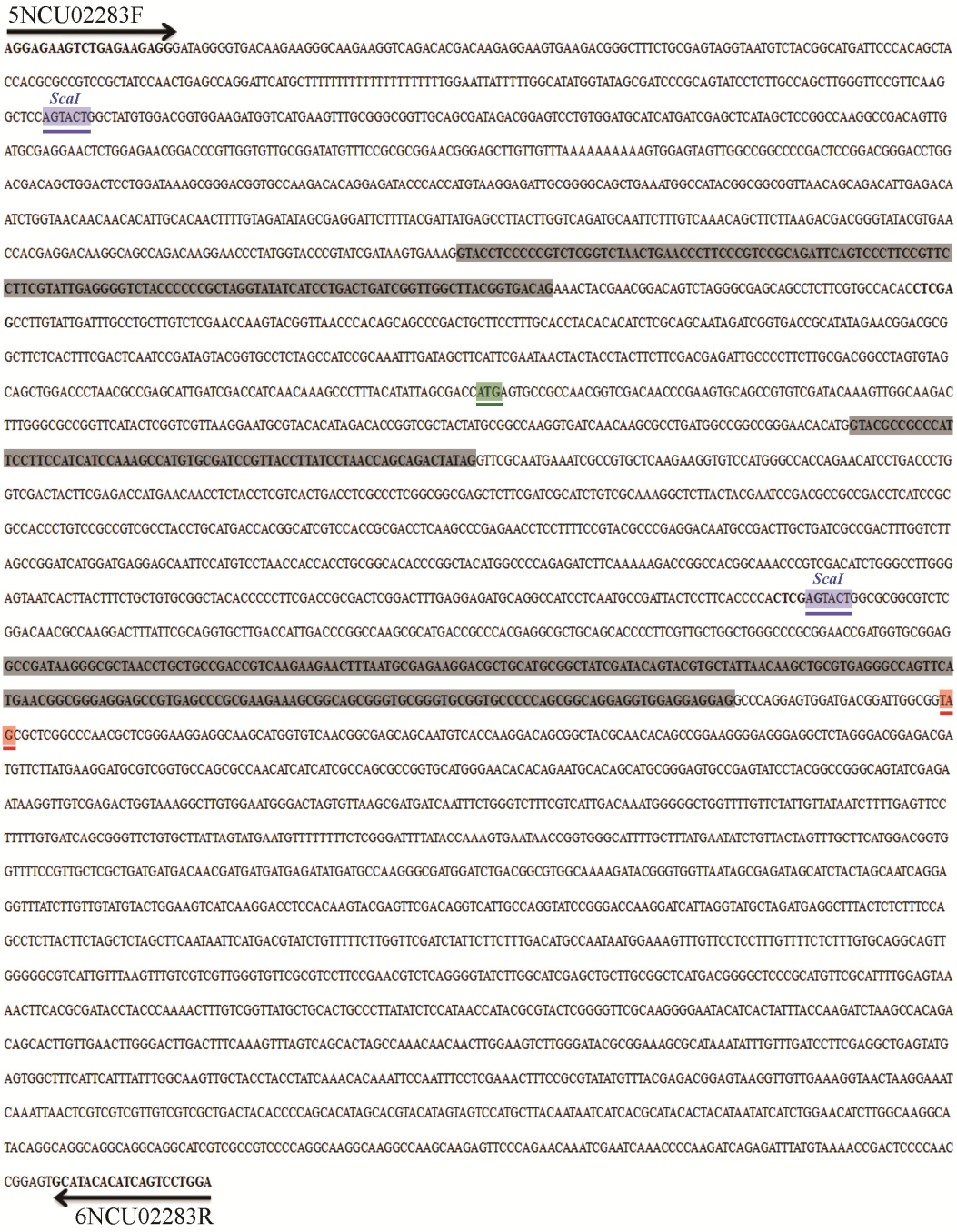
2NCU09123R
XhoI

(B)



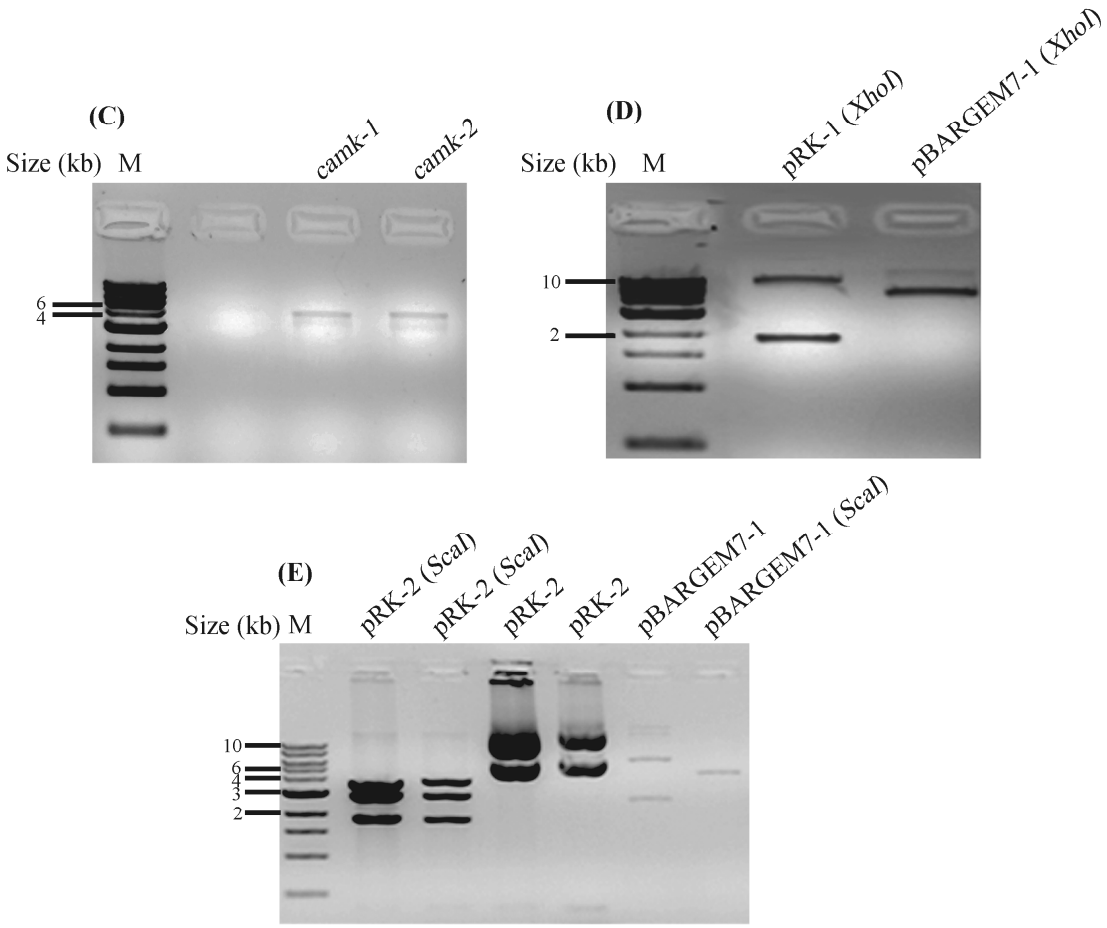


Figure 4.2 Cloning of *camk-1* and *camk-2* genes. **(A)** The sequence of the ~4693 bp fragment carrying the *camk-1* gene is shown. The primer pairs 1NCU09123F and 2NCU09123R are underlined and arrow head indicates the 5'→3' direction. The start (ATG) and stop (TAG) codons are underlined and highlighted in green and red colors, respectively. The exons between the start and stop codons are highlighted in gray. The *XhoI* restriction site in the fragment is highlighted in blue and underlined. **(B)** The sequence of the ~4157 bp fragment carrying the *camk-2* gene is shown. The primer pairs 5NCU02283F and 6NCU02283R are underlined and arrow head indicates the 5'→3' direction. The start (ATG) and stop (TAG) codons are underlined and highlighted in green and red colors, respectively. The exons between the start and stop codons are highlighted in gray. The *ScaI* restriction site in the fragment is highlighted in blue and underlined. **(C)** Agarose gel electrophoresis of PCR products of the *camk-1* (~4693 bp) and the *camk-2* (~4157 bp) fragments from the wild type. **(D)** Confirmation of the pRK-1 construct generated by cloning of the *camk-1* insert into the

SmaI site of the pBARGEM7-1 vector. The pRK-1 construct was digested with *XhoI* enzyme that resulted in two fragments of size ~7.4 and 1.7 kb, respectively. As a control, the pBARGEM7-1 plasmid vector was digested with *XhoI* that linearized the plasmid of size 4.5 kb. (**E)** Conformation of the pRK-2 construct generated by cloning of the *camk-2* insert into the *SmaI* site of the pBARGEM7-1 vector. The pRK-2 construct digested with *ScaI* enzyme that resulted in three fragments of size ~1.8, 2.9 and 3.8 kb, respectively, the uncut pRK-2 construct, uncut pBARGEM7-1 control plasmid vector and the *ScaI* linearized pBARGEM7-1 vector of size 4.5 kb were visualized in a 1% agarose gel; M: 1 kb DNA ladder.

4.2.2 Transformation of pRK-1 and pRK-2 constructs into the Δ*camk-1:: hph* and Δ*camk-2::hph* mutant strains

The pRK-1 and pRK-2 constructs were transformed into the *Δcamk-1::hph A* and *Δcamk-2::hph A* recipient knockout mutant strains by electroporation. Initial transformants were selected on sorbose plate supplemented with antibiotics basta (400 µg/ml). For each of the pRK-1 and pRK-2 construct, eight initial heterokaryotic transformants were obtained. Genomic DNA of these heterokaryotic transformants were isolated and presence of the correct transgene was verified by PCR using gene specific primer pairs 5NCU09123F and 6NCU09123R, and 2NCU02283F and 3NCU02283R, respectively, for the *camk-1* and the *camk-2* transgenes (Figure 4.3A-B; Table 4.2).

Table 4.2 Primers used for confirmation of Δ*camk-1:: hph* and Δ*camk-2::hph* heterokaryotic transformant strains

S. no.	Primer	Sequence (5'→3')	Reference
1.	5NCU09123F	CGCCAACATGCTTAATCGCC	This study
2.	6NCU09123R	TAGTCTTGGAGAGTGTCCGC	This study
3.	2NCU02283F	AGGAGAAGTCTGAGAAGAGG	This study
4.	3NCU02283R	GCAGCCGTGTCGATACAAAG	This study

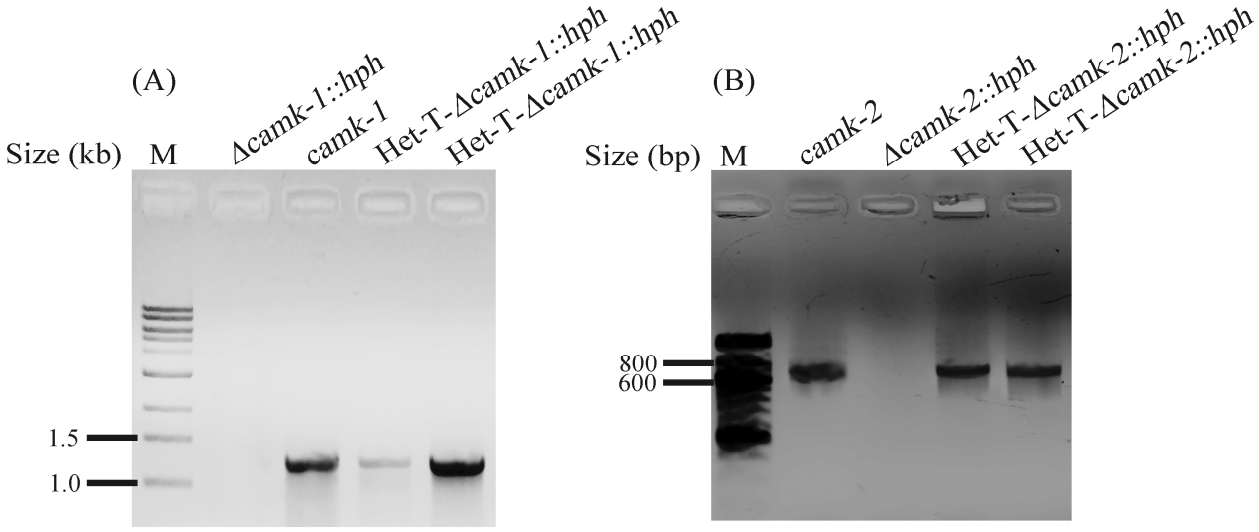


Figure 4.3 Verification of the Het-T-Δ*camk-1::hph* and Het-T-Δ*camk-2::hph* heterokaryotic transformants by PCR. **(A)** The PCR product of size ~1116 bp, obtained using the primer pairs 5NCU09123F and 6NCU09123R, indicated presence of the *camk-1* gene in the Het-T-Δ*camk-1::hph* transformants. PCR products were visualized in a 1% agarose gel; M: 1 kb DNA ladder (NEB). **(B)** The PCR products of size ~751 bp, obtained using primer pairs 2NCU02283F and 3NCU02283R, indicated presence of the *camk-2* gene in the Het-T-*Δcamk-2::hph* heterokaryotic transformants. PCR products were visualized in a 1% agarose gel; M: 100 bp DNA ladder.

4.2.3 Isolation of Δ*camk-1::hph camk-1:: bar* and Δ*camk-2::hph camk-2:: bar* homokaryotic transformants

The Het-T-*Δcamk-1::hph A* and Het-T-*Δcamk-2::hph A* heterokaryotic transformants were crossed, respectively, with the Δ*camk-1::hph a* and Δ*camk-2::hph a* mutant strains. Crosses were harvested after about 25 days, ascospores were germinated by heat shock and the Δ*camk-1::hph camk-1:: bar* and Δ*camk-2::hph camk-2:: bar* homokaryotic strains were isolated based on their resistance to basta (400 µg/ml). Furthermore, genomic DNA from the basta resistant progenies were isolated presence of the *camk-1* or *camk-2* transgenes were verified by PCR analysis using primer pairs 5NCU09123F and 6NCU09123R or 2NCU02283F and 3NCU02283R, respectively (Figure 4.4; Table 4.2). Three homokaryotic

transformants each for Δ*camk-1::hph camk-1:: bar* and Δ*camk-2::hph camk-2:: bar* were identified and used for further studies.

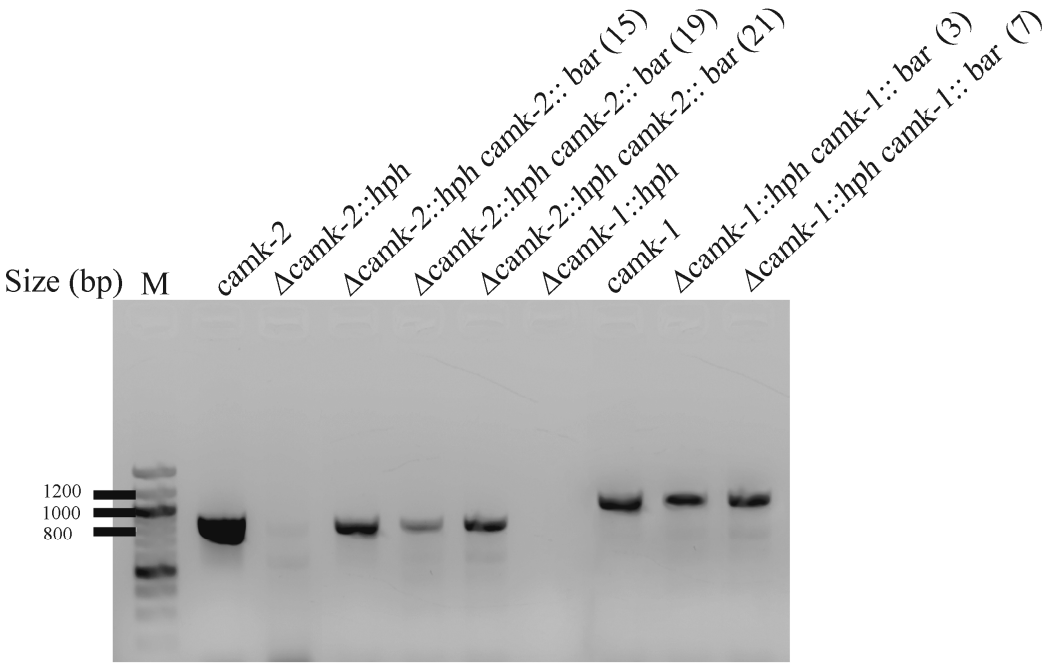


Figure 4.4 Verification of Δ*camk-1::hph camk-1::bar* and Δ*camk-2::hph camk-2::bar* homokaryotic transformants by PCR analysis. PCR using primer pairs 5NCU09123F and 6NCU09123R produced *camk-1* amplicon of size 1116 bp. Similarly, PCR using primer pairs 2NCU02283F and 3NCU02283R produced *camk-2* amplicon of size 751 bp. PCR products were visualized in a 1% agarose gel; M: 1 bp DNA ladder.

4.2.4 The *camk-1* and *camk-2* genes are essential for full fertility in *Neurospora crassa*

The phenotype of crosses involving Δ*camk-1::hph camk-1:: bar* and Δ*camk-2::hph camk-2:: bar* homokaryotic transformant strains were examined. The crosses homozygous for the Δ*camk-1::hph camk-1:: bar* and the Δ*camk-2::hph camk-2:: bar* were fully fertile and produced thousands of ascospores, suggesting that both *camk-1::bar* and *camk-2::bar* transgenes complement the respective mutants (Table 4.3, entries 8-9, 16-17). In addition, crosses heterozygous for the Δ*camk-1::hph camk-1:: bar* homokaryotic transformant strains with wild-type and Δ*camk-1::hph* were fully fertile and produced thousands of ascospores

(Table 4.3, entries 2-7). Similarly, crosses heterozygous for the Δ*camk-2::hph camk-2:: bar* homokaryotic transformant strains with wild-type and Δ*camk-2::hph* were also fully fertile and produced thousands of ascospores (Table 4.3, entries 10-15). Therefore, the *camk-1* and *camk-2* genes are essential for full fertility in *N. crassa*.

Table 4.3 Complementation analysis of the *camk-1* and *camk-2* genes in *N. crassa*

S. no.	Cross	Ascospores produced	Phenotype
1.	*OR a* X *OR A*	abundant	fertile
2.	Δ*camk-1::hph camk-1::bar* (3) *a* X *OR A*	abundant	fertile
3.	Δ*camk-1::hph camk-1::bar* (7) *a* X *OR A*	abundant	fertile
4.	Δ*camk-1::hph camk-1:: bar* (12) *A* X *OR a*	abundant	fertile
5.	Δ*camk-1::hph camk-1::bar* (3) *a* X Δ*camk-1::hph* (35) *A*	abundant	fertile
6.	Δ*camk-1::hph camk-1::bar* (7) *a* X Δ*camk-1::hph* (35) *A*	abundant	fertile
7.	Δ*camk-1::hph camk-1::bar* (12) *A* X Δ*camk-1::hph* (23) *a*	abundant	fertile
8.	Δ*camk-1::hph camk-1::bar* (3) *a* X Δ*camk-1::hph camk-1::bar* (12) *A*	thousands	fertile
9.	Δ*camk-1::hph camk-1::bar* (7) *a* X Δ*camk-1::hph camk-1::bar* (12) *A*	thousands	fertile
10.	Δ*camk-2::hph camk-2::bar* (15) *A* X *OR a*	thousands	fertile
11.	Δ*camk-2::hph camk-2::bar* (19) *A* X *OR a*	thousands	fertile
12.	Δ*camk-2::hph camk-2::bar* (21) *a* X *OR A*	thousands	fertile
13.	Δ*camk-2::hph camk-2::bar* (15) *A* X Δ*camk-2::hph* (16) *a*	abundant	fertile
14.	Δ*camk-2::hph camk-2: bar* (19) *A* X Δ*camk-2::hph* (16) *a*	abundant	fertile
15.	Δ*camk-2::hph camk-2::bar* (21) *a* X Δ*camk-2::hph* (10) *A*	abundant	fertile
16.	Δ*camk-2::hph camk-2::bar* (15) *A* X Δ*camk-2::hph camk-2::bar* (21) *a*	abundant	fertile
17.	Δ*camk-2::hph camk-2::bar* (19) *A* X Δ*camk-2::hph camk-2::bar* (21) *a*	abundant	fertile

4.2.5 Requirement of the *camk-2* during meiosis in *Neurospora crassa*

The phenotypes of the crosses homozygous for the *Δcamk-2::hph* were more severe than the *Δcamk-1::hph*. To determine whether the *camk-2* product is necessary before karyogamy or during the meiosis, the effect of meiotic silencing of *camk-2* gene was tested. Meiotic silencing is an RNAi- based post-transcriptional gene silencing process triggered by unpaired DNA between homologous chromosomes during meiosis (Shiu et al. 2001; Hammond et al. 2013). To test the effect of meiotic silencing of the *camk-2* gene, I performed crosses involving *camk-2::bar* strain that contained an ectopic copy of the *camk-2* tagged with the *bar* selective marker. The phenotype of the *camk-2::bar* crossed with the wild-type displayed a barren (produced few ascospores; Figure 4.5; Table 4.4, entries 2-3) phenotype. Microscopic analysis of perithecia from these crosses showed that they produced less number of asci as compared to the control crosses involving wild-type strain (Figure 4.5). When *camk-2::bar* strain was crossed with the *Sad-1*, a semidominant suppressor of meiotic silencing (Shiu et al. 2001), full fertility was restored and thousands of ascospores were produced (Figure 4.5; Table 4.4, entries 4-5). The homozygous *Sad-1* control cross was sterile (Table 4.4, entry 1). Moreover, fertile phenotype was also observed when crosses were made between *camk-2::bar* strains of opposite mating type (Figure 4.5; Table 4.4, entry 6). These results suggested that meiotic silencing of the *camk-2* gene results in a barren phenotype, and therefore, the product of *camk-2* could be essential during meiosis for full fertility of *N. crassa*.

Table 4.4 Meiotic silencing of the *camk-2* gene

S. no.	Cross	Ascospores produced	Phenotype	Meiotic silencing (Yes/No)
1.	*Sad-1 a* X *Sad-1 A*	no	sterile	No
2.	*camk-2::bar* (4) *a* X *OR A*	very few	barren	Yes
3.	*camk-2::bar* (29) *A* X *OR a*	very few	barren	Yes
4.	*camk-2::bar* (4) *a* X *Sad-1 A*	abundant	fertile	No
5.	*camk-2::bar* (29) *A* X *Sad-1 a*	abundant	fertile	No
6.	*camk-2::bar* (4) *a* X *camk-2::bar* (29) *A*	abundant	fertile	No

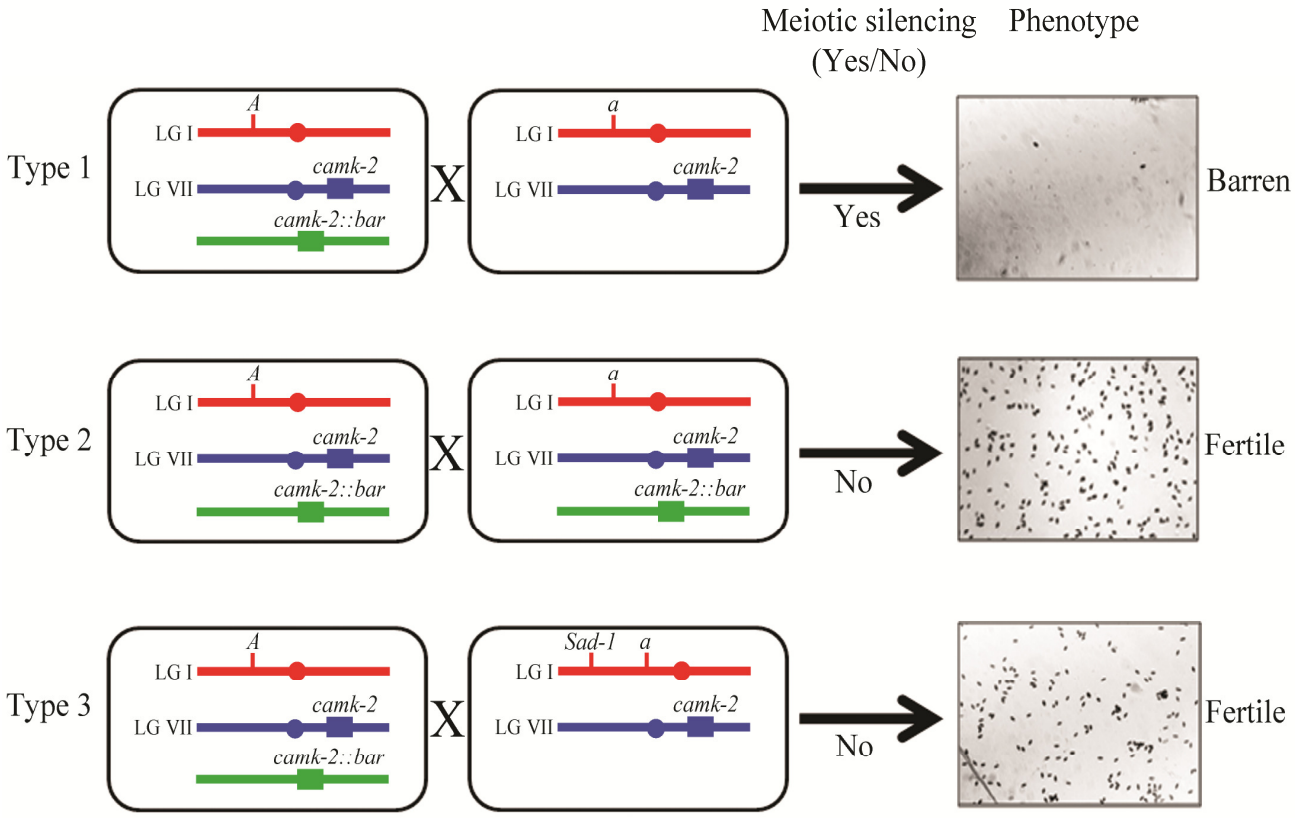


Figure 4.5 Meiotic silencing assay of the *camk-2* gene. Type 1 involves cross of *camk-2::bar* strain with the wild-type of opposite mating type. Type 2 is a homozygous cross of *camk-2::bar* strains of opposite mating type. Type 3 involves cross of *camk-2::bar* strain with *Sad-1* of opposite mating type. The phenotype of the Type 1 cross was barren due to meiotic silencing of *camk-2* (a copy of the *camk-2* was unpaired). However, meiotic silencing was not occurred in both Type 2 (all copies of the *camk-2* were properly paired in the homozygous cross) and Type 3 (due to presence of the *Sad-1* allele, the semi-dominant suppressor of meiotic silencing) crosses; therefore, these crosses were fully fertile. Ascospores ejected on the lid of the petri dish of the respective cross was harvested with ~1 ml of sterile H_2O, a sample of equal volume was placed on a microscopic slide, observed under a dissection microscope and photographed using a camera (representative pictures show the relative ascospores produced in each type of the cross).

4.3 Discussion

The complementation analysis of the Δ*camk-1::hph* and Δ*camk-2::hph* mutants showed that *camk-1* and *camk-2* genes are necessary for full fertility in *N. crassa* (Table 4.3, entries 8-9, 16-17). Moreover, to determine whether the *camk-2* product is necessary before karyogamy or during the meiosis, the effect of meiotic silencing of *camk-2* gene was tested. The crosses involving an unpaired copy of the *camk-2* showed a barren phenotype (Figure 4.5; Table 4.4, entries 2-3), which was suppressed by the semidominant suppressor of meiotic silencing *Sad-1* (Table 4.4, entries 4-5). Moreover, meiotic silencing was also absent in the *camk-2::bar* homozygous crosses and showed fertile phenotype (Table 4.4, entry 6). These results suggested that the *camk-2* product is essential during meiosis for full fertility in *N. crassa*.

The key to the sexual development process in heterothallic fungi *N. crassa* is communication between cells of opposite mating type (Kronstad and Staben 1997). *N. crassa* undergoes a complex sexual developmental process to form protoperithecia in response to nitrogen starvation, light and low temperature (Nelson and Metzenberg 1992; Nelson 1996). Specialized receptive hyphae, called trichogynes, are extended from the protoperithecia and fuse with the fertilizing cell of the opposite mating type. The resulting diploid zygote nucleus immediately undergoes the two meiotic divisions (meiosis I and II) and a postmeiotic mitosis. The resulting eight haploid nuclei are then sequestered into eight ascospores, which held in linear order (Raju 1992). Apart from few sexual development (*sdv*) and pheromone related genes, little is known about sexual developmental process in *N. crassa* (Johnson 1978; Nelson and Metzenberg 1992). Meiotic silencing is triggered by unpaired DNA between homologous chromosomes that silence the unpaired DNA including the paired homologous (Shiu et al. 2001). The fertility defect of strain lacking *camk-1* and *camk-2* suggested the requirement of these two Ca^{2+}/CaMKs during sexual development. It is possible that both CAMK-1 and CAMK-2 phosphorylates the downstream target protein necessary for normal sexual development.

In this study, two Ca^{2+}/CaMKs *camk-1* and *camk-2* have been shown essential for full fertility in *N. crassa*. In addition, *camk-2* is required during meiosis for full fertility in *N. crassa*. Genetic interaction of Ca^{2+}/CaMKs in *N. crassa* will be discussed in the next Chapter.

Chapter 5

Studies on genetic interaction of *camk-1*, *camk-2*, *camk-3* and *camk-4* genes

5.1 Introduction

In the previous Chapter, I described the role of Ca^{2+}/CaMKs, CAMK-1 and CAMK-2 in sexual development. *N. crassa* also possesses two other Ca^{2+}/CaMKs known as CAMK-3 and CAMK-4. These Ca^{2+}/CaMKs share significant sequence similarities (Figure 3.5C). In addition, CAMK-1 can specifically phosphorylate the circadian clock protein in *N. crassa* (Yang et al. 2001). Disruption of the *camk-1* gene resulted in a modest phase delay, a small period lengthening, and light-induced phase shifting of the circadian conidiation rhythm (Yang et al. 2001). Immediately after germination from ascospores, the *camk-1* null mutant was grown slowly indicating that CAMK-1 plays an important role in growth and development of *N. crassa*. The slow growth phenotype of the Δ*camk-1* mutant is transient, which indicates redundancy of the kinases (Yang et al. 2001). The CAMK-1 protein is 71% similar to CMKA of *A. nidulans* and 53% similar to CaMK-II of *S. cerevisiae*. CMKA is required for hyphal growth and progression of the nuclear division cycle *A. nidulans* (Dayton et al. 1996). On the other hand, CAMKII is required for spore germination, growth and acquisition of thermotolerance in *S. cerevisiae* (Ohya et al. 1991; Pausch et al. 1991; Iida et al. 1995). However, studies on genetic interaction, which could provide information regarding epistatic relationships of Ca^{2+}/CaMKs has remained elusive.

In this Chapter, I described phenotype of the double mutants generated by crossing the single mutants of Δ*camk-1::hph*, Δ*camk-2::hph*, Δ*camk-3::hph* and Δ*camk-4::hph* strains to understand the genetic interaction of Ca^{2+}/CaMKs in *N. crassa*.

5.2 Results

5.2.1 Construction and PCR verification of Δ*camk-1::hph* Δ*camk-2::hph*, Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-3::hph* Δ*camk-2::hph* double mutant strains

The Δ*camk-1::hph* Δ*camk-2::hph*, Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-3::hph* Δ*camk-2::hph* double mutants were generated by crossing the single mutant strains of the opposite mating type (Figure 5.1A). Generally, ascospores began to shot within 16-18 days and phenotypes of the crosses were scored under a dissection microscope after 25 days (i.e., when ascospore production reached the plateau), by examining the completed crop of ascospores ejected to the lid of the petri dish. Ascospores were harvested by washing the lids with ~1 ml sterile H_2O and heat shocked to germinate on a sorbose plate. The well germinated individual progeny strains were transferred to plate containing Vogel's glucose medium and screened for hygromycin resistance (hyg^R). The hyg^R progenies were further screened by PCR to identify the double mutants. The forward primers 1NCU09123F, 5NCU02283F, 1NCU06177F and 1NCU09212F that are specific for upstream of the 5'flank of the genes *camk*-1, *camk-2*, *camk-3*, and *camk-4*, respectively, were used with the common reverse primer 5HPHR that is specific for the *hph* cassette used to generate the knockout mutants (Figure 5.1B; Table 5.1). Finally, I have generated two strains each for Δ*camk-1::hph* Δ*camk-2::hph*, Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-3::hph* Δ*camk-2::hph* double mutants and used these strains for further studies.

Table 5.1 Primer used for verification of double knockout mutant strains

S. no.	Primer	Sequence (5'→3')	Reference
1.	5HPHR	ATCCACTTAACGTTACTGAAATC	Deka et al. 2011
2.	1NCU09123F	GATGTAGCTGAAGTTGGTGG	This study
3.	5NCU02283F	AGGAGAAGTCTGAGAAGAGG	This study
4.	1NCU06177F	GAGTAGATGACCATGGTTGG	This study
5.	1NCU09212F	CGTATTCAACTCCAGGTAGC	This study

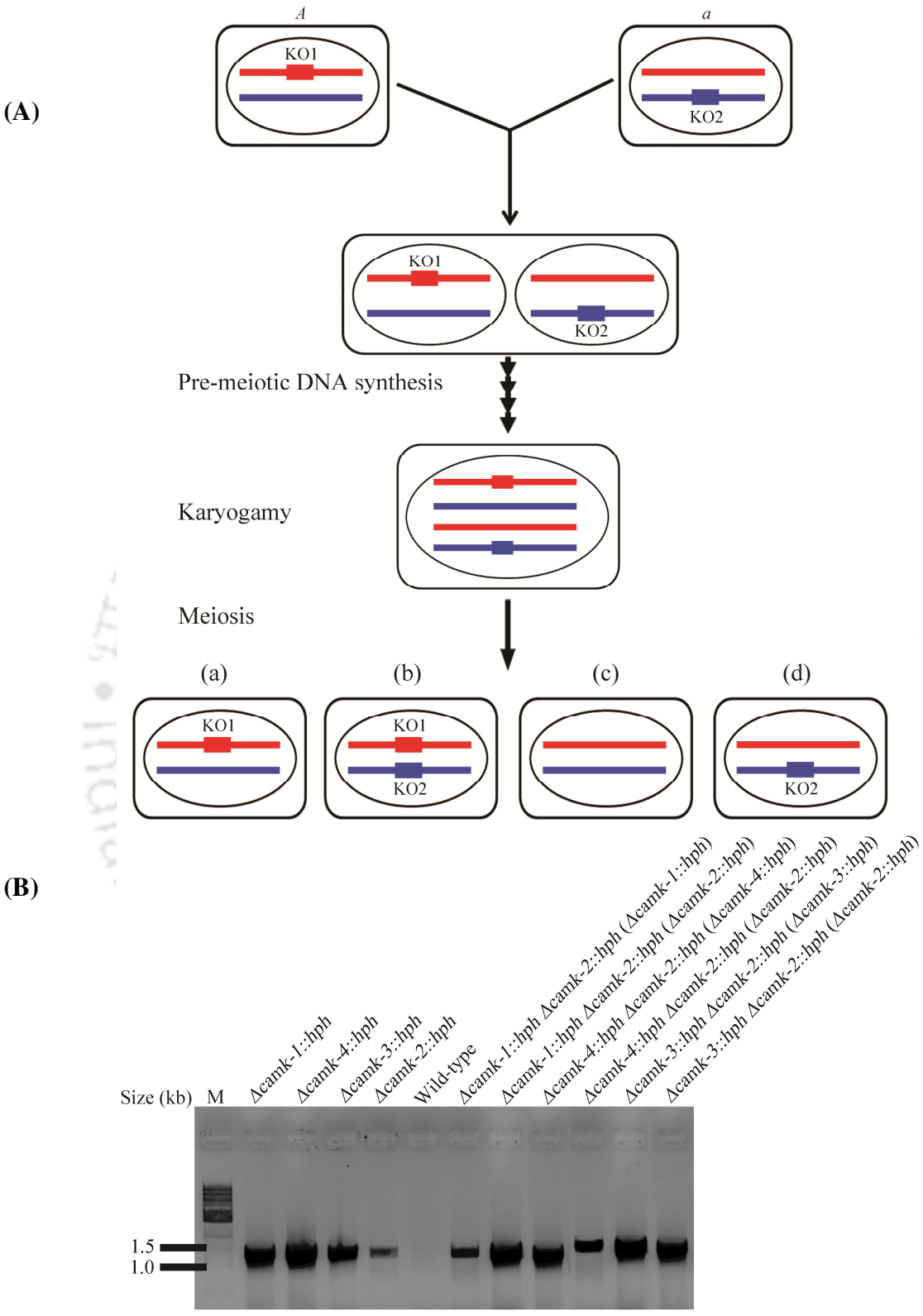


Figure 5.1 Construction of double knockout mutants. **(A)** Strategy to generate the double knockout mutants. The *A* and *a* mating type strains contain a single knockout mutant allele

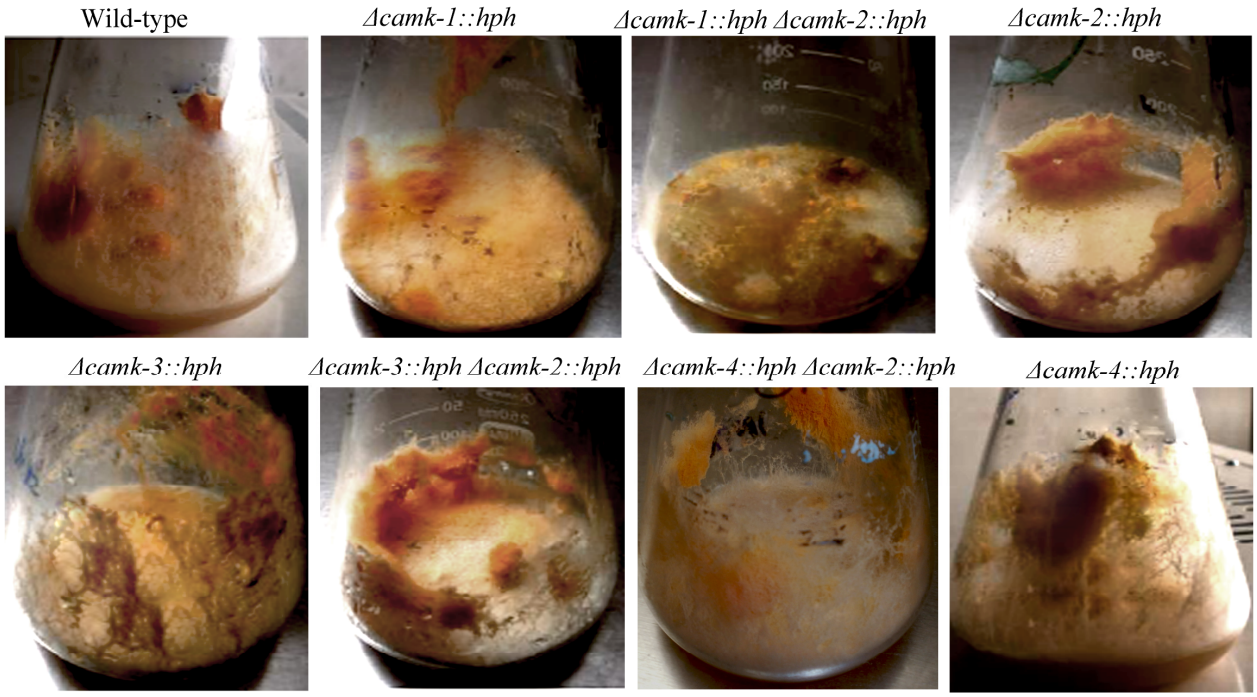
designated as KO1 and KO2, respectively. After fertilization, karyogamy and meiosis event, four types of progeny strains; (a) mutant bearing the KO1 allele, (b) double mutant bearing KO1 and KO2 allele, (c) wild-type, and (d) mutant bearing KO2 allele (1:1:1:1 segregation ratio) are produced. For simplicity, only the relevant linkage groups are shown and assumed that the KO1 and KO2 alleles are unlinked. Red and blue boxes illustrate haploid nucleus containing the KO1 and KO2 mutant alleles. **(B)** PCR analysis of the Ca^{2+}/CaMK double knockout mutants**.** The *Δcamk-1::hph Δcamk-2::hph*, *Δcamk-4::hph Δcamk-2::hph* and *Δcamk-3::hph Δcamk-2::hph* double mutants were verified by using the forward primers 1NCU09123F, 1NCU09212F, 1NCU06177F, and 5NCU02283F specific for the upstream region of the genes *camk-1*, *camk-2, camk-3*, and *camk-4*, respectively, along with the common reverse primer 5HPHR that is specific for the *hph* cassette used to generate the knockout mutants (Table 5.1). Amplification of PCR products of size ~1.3, 1.2, 1.2, and 1.2 kb, indicate presence of the *camk-1*, *camk-2*, *camk-3* and *camk-4* knockout alleles, respectively. PCR products were visualized in a 1% agarose gel; M: 1 kb DNA ladder.

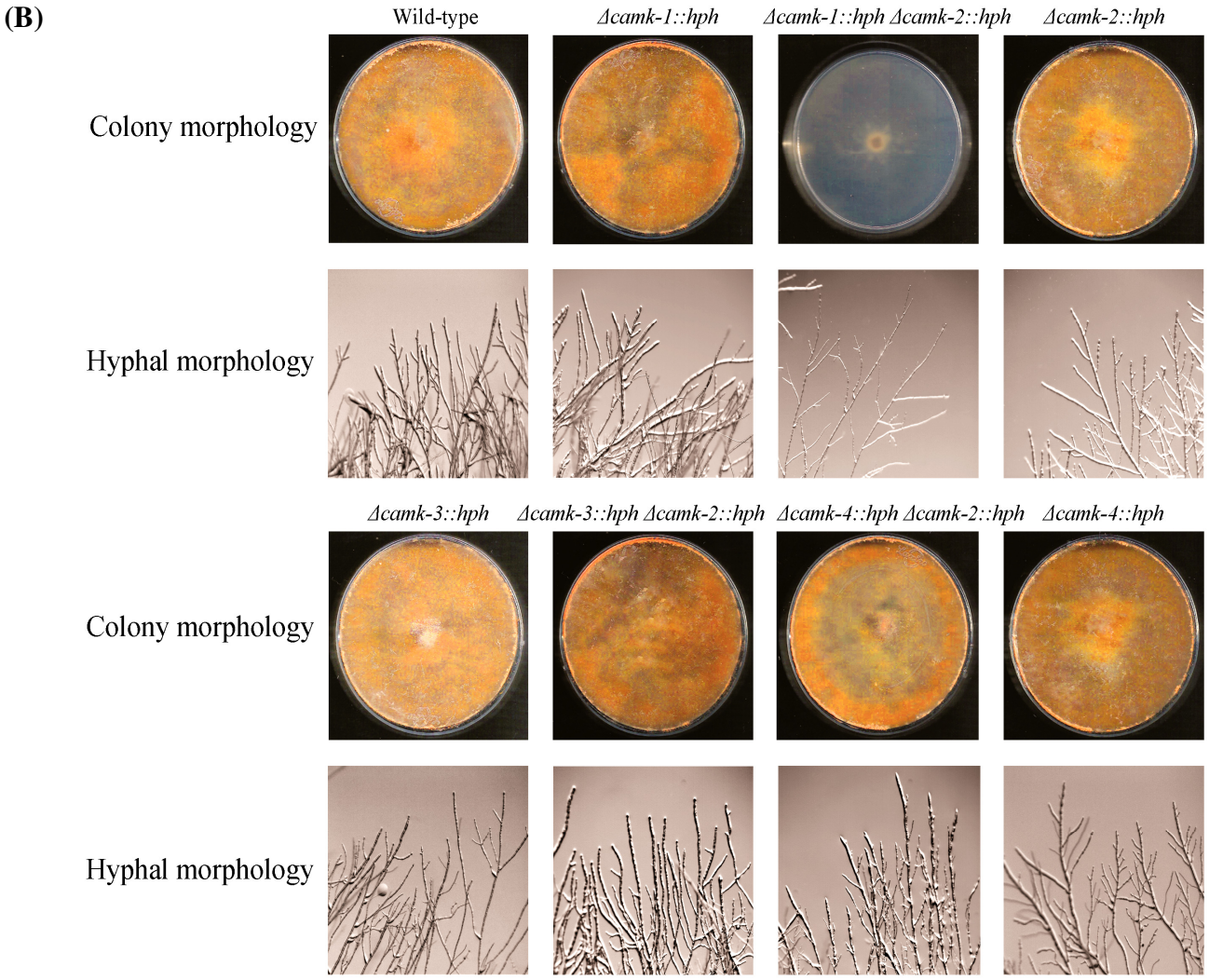
5.2.2 The *Δcamk-1::hph Δcamk-2::hph* double mutant showed distinct colony morphology and growth defect

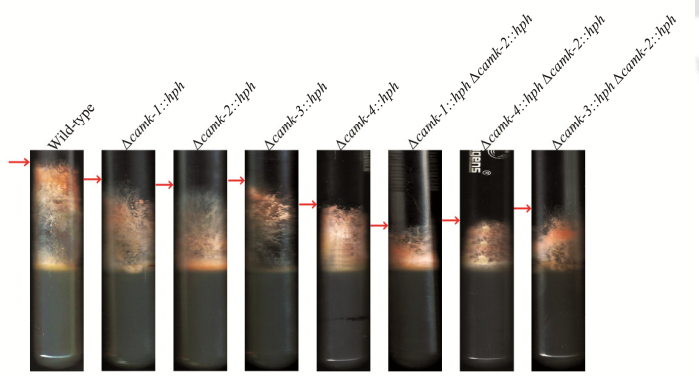
I studied growth phenotypes of the double mutants of Ca^{2+}/CaMKs and found that the *Δcamk-1::hph Δcamk-2::hph* double mutant stably showed a distinct colony morphology and severe growth defect (Figure 5.2A-B). The *Δcamk-1::hph Δcamk-2::hph* double mutant displayed a matty-type colony with severely reduced aerial hyphae (Figure 5.2A-B). The *Δcamk-1::hph Δcamk-2::hph* double mutant also produced very few conidia (asexual spores) when cultured in way that result in abundant conidia production from the wild-type, for example, when grown in agar slant or in Erlenmeyer flasks. However, the *Δcamk-1::hph*, *Δcamk-2::hph*, *Δcamk-3::hph*, *Δcamk-4::hph*, *Δcamk-4::hph Δcamk-2::hph* and *Δcamk-3::hph Δcamk-2::hph* mutant strains displayed colony morphology similar to the wild-type (Figure 5.2A). We found distance between hyphal branching point apparently shorter in *Δcamk-3::hph* and longer in *Δcamk-4::hph* mutant strains (Figure 5.2B). In addition, growth of the aerial hyphae was severely reduced in *Δcamk-1::hph Δcamk-2::hph*, *Δcamk-4::hph Δcamk-2::hph*, and *Δcamk-3::hph Δcamk-2::hph* double mutants (Figure 5.2C-D; Table 5.2). These results

suggested for a synthetic interaction between *camk-1* and *camk-2*; *camk-2* and *camk-3* and *camk-2* and *camk-4* in *N. crassa*.

(A)




(B)
Wild-type
Δcamk-1::hph
Δcamk-1::hph Δcamk-2::hph
Δcamk-2::hph
Colony morphology
Hyphal morphology
Δcamk-3::hph
Δcamk-3::hph Δcamk-2::hph
Δcamk-4::hph Δcamk-2::hph
Δcamk-4::hph
Colony morphology
Hyphal morphology


(C)
Wild-type
Δcamk-1::hph
Δcamk-2::hph
Δcamk-3::hph
Δcamk-4::hph
Δcamk-1::hph Δcamk-2::hph
Δcamk-4::hph Δcamk-2::hph
Δcamk-3::hph Δcamk-2::hph

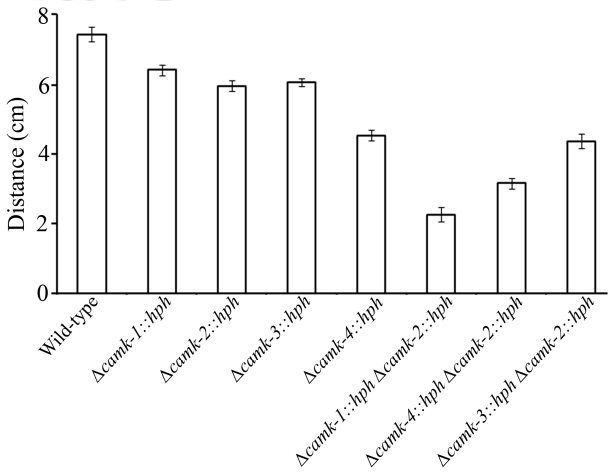

(D)
Distance (cm)
0
2
4
6
8
Wild-type
Δcamk-1::hph
Δcamk-2::hph
Δcamk-3::hph
Δcamk-4::hph
Δcamk-1::hph Δcamk-2::hph
Δcamk-4::hph Δcamk-2::hph
Δcamk-3::hph Δcamk-2::hph

Table 5.2 Aerial hyphae height of wild-type and Ca^{2+}/CaMK mutants

S. no.	Strain	Average height of aerial hyphae (cm; n = 3)
1.	Wild-type	7.433 ± 0.208
2.	Δ*camk-1::hph*	6.433 ± 0.152
3.	Δ*camk-2::hph*	5.966 ± 0.152
4.	Δ*camk-3::hph*	6.066 ± 0.115
5.	Δ*camk-4::hph*	4.533 ± 0.152
6.	*Δcamk-1::hph Δcamk-2::hph*	2.266 ± 0.208
7.	*Δcamk-4::hph Δcamk-2::hph*	3.166 ± 0.152
8.	*Δcamk-3::hph Δcamk-2::hph*	4.366 ± 0.208

Figure 5.2 Phenotype of the mutants of Ca^{2+}/CaMKs in *N. crassa*. (A) Colony morphology of the indicated strains grown in 250 ml Erlenmeyer flasks for 10 days at 30°C. The *Δcamk-1::hph Δcamk-2::hph* double mutant showed a distinct colony morphology (matty-type growth). **(B)** Colony and hyphal morphology of the indicated strains incubated at 30°C for 3 days in 55 mm petri plates containing Vogel's medium. The *Δcamk-1::hph Δcamk-2::hph* double mutant showed a severe growth defect. **(C-D)** Aerial hyphae heights of all the tested mutant strains were reduced in comparison to the wild-type. The edges of aerial hyphae were indicated by arrows. Error bars indicate the standard errors calculated from the data for three independent experiments.

5.2.3 The Δ*camk-1* Δ*camk-2* double mutant displayed slow mycelial extension rate

I measured the mycelial extension rates of the mutants by using the standard race tubes to extend the period of growth of the strains up to 72 h. The Δ*camk-1::hph* Δ*camk-2::hph* double mutant displayed a slow mycelial extension rate than the wild-type, Δ*camk-1::hph*, Δ*camk-2::hph*, Δ*camk-3::hph*, Δ*camk-4::hph*, Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-3::hph* Δ*camk-2::hph* mutant strains. The average mycelial extension rates followed the order Δ*camk-2::hph* ≅ *wild-type* ≅ Δ*camk-3::hph* ≅ Δ*camk-4::hph* ≅ Δ*camk-1::hph* > Δ*camk-3::hph* Δ*camk-2::hph* ≅ Δ*camk-4::hph* Δ*camk-2::hph* > Δ*camk-1::hph* Δ*camk-2::hph* (Figure 5.3;

Table 5.3). Thus, Δ*camk-1::hph* Δ*camk-2::hph* double mutant showed a severe growth defect. One possible explanation for the severity of the Δ*camk-1::hph* Δ*camk-2::hph* double mutant could be that these two genes function in parallel pathways in an essential or very important process. In addition, the *Δcamk-4::hph Δcamk-2::hph* and *Δcamk-3::hph Δcamk-2::hph* double mutants also showed a slow growth phenotype than both the single mutants and the wild-type (Figure 5.3; Table 5.3). Therefore, lack of any of the individual Ca^{2+}/CaMK did not show any significant effect on the growth phenotype; however, double mutants of Ca^{2+}/CaMKs showed a slow growth phenotype. These results suggested that genetic interaction of the *camk-1*, *camk-2*, *camk-3* and *camk-4* genes play an important role in growth of *N. crassa*.

Table 5.3 Mycelial extension rate of wild-type and knockout mutants of Ca^{2+}/CaMKs

S. no.	Strain	Average mycelial extension rate (cm/h)
1.	Wild-type	0.332 ± 0.344
2.	Δ*camk-1::hph*	0.311 ± 0.328
3.	Δ*camk-2::hph*	0.336 ± 0.922
4.	Δ*camk-3::hph*	0.328 ± 0.556
5.	Δ*camk-4::hph*	0.312 ± 0.425
6.	Δ*camk-1::hph* Δ*camk-2::hph*	0.030 ± 0.156
7.	Δ*camk-4::hph* Δ*camk-2::hph*	0.249 ± 0.453
8.	Δ*camk-3::hph* Δ*camk-2::hph*	0.275 ± 0.336

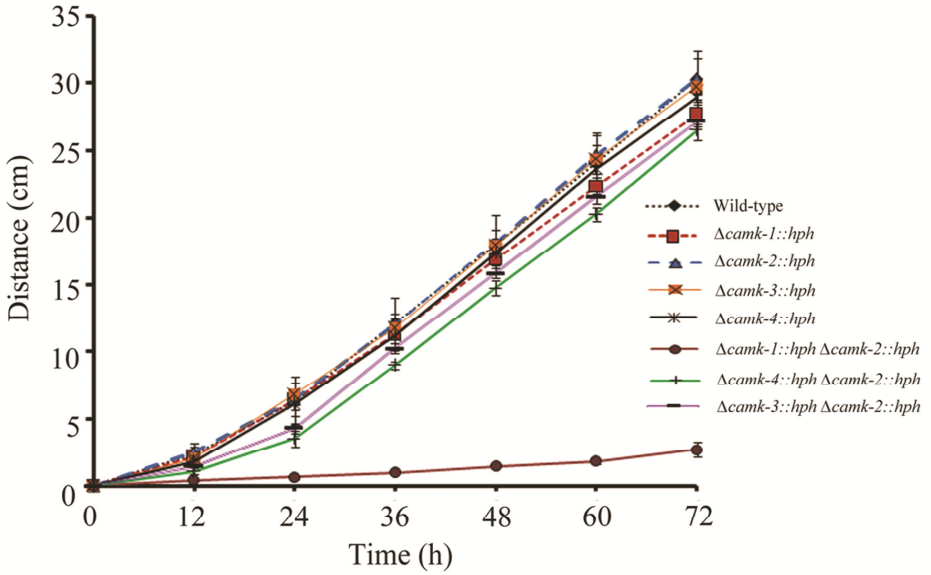


Figure 5.3 Mycelial extension rates of wild-type and knockout mutants of Ca^{2+}/CaMKs. Rates of mycelial extension (apical growth) of the wild-type, Δ*camk-1::hph*, Δ*camk-2::hph*, Δ*camk-3::hph*, Δ*camk-4::hph*, Δ*camk-1::hph* Δ*camk-2::hph*, Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-3::hph* Δ*camk-2::hph* strains were measured using race tubes. Error bars indicate the standard errors calculated from the data for three independent experiments.

5.2.4 Effect of calmodulin (CaM) antagonist trifluoperazine (TFP)

The Ca^{2+}/CaMKs possess a putative CaM binding domain and binding of Ca^{2+}/CaM is essential for activation of these kinases (Hook and Means 2001). I used the calmodulin (CaM) antagonist trifluoperazine (TFP) to test if disruption of the CaM could have any phenotypic effect using the calmodulin (CaM) antagonist trifluoperazine (TFP) in single and double mutants of Ca^{2+}/CaMKs. Interestingly, on addition of the TFP in growth medium, the mycelial extension rates showed three distinct patterns of inhibition; wild-type and Δ*camk-2::hph* were comparatively less inhibited, the Δ*camk-1::hph*, Δ*camk-3::hph*, Δ*camk-4::hph* and Δ*camk-3::hph* Δ*camk-2::hph* show intermediate inhibition, and Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-1::hph* Δ*camk-2::hph* were severely inhibited (Figure 5.4; Table 5.4). Therefore, TFP severely inhibited growth of the Δ*camk-4::hph* Δ*camk-2::hph* mutant, which showed a relatively higher mycelial extension rates otherwise. The synthetic phenotype of the Δ*camk-4::hph* Δ*camk-2::hph* mutant in exposure to TFP treatment suggested that these two Ca^{2+}/CaMKs could be activated upon Ca^{2+}/CaM binding and play an important role in

growth. These results consistently suggested for genetic interactions of *camk-2* with *camk-1, camk-3,* and *camk-4* genes.

Table 5.4 Mycelial extension rate of wild-type and knockout mutants of Ca^{2+}/CaMKs on medium supplemented with TFP

S. no.	Strain	Average mycelial extension rate (cm/h) in TFP (100 µM)
1.	Wild-type	0.049 ± 0.011
2.	Δ*camk-1::hph*	0.032 ± 0.041
3.	Δ*camk-2::hph*	0.046 ± 0.078
4.	Δ*camk-3::hph*	0.033 ± 0.047
5.	Δ*camk-4::hph*	0.036 ± 0.049
6.	Δ*camk-1::hph* Δ*camk-2::hph*	0.015 ± 0.055
7.	Δ*camk-4::hph* Δ*camk-2::hph*	0.018 ± 0.032
8.	Δ*camk-3::hph* Δ*camk-2::hph*	0.033 ± 0.055

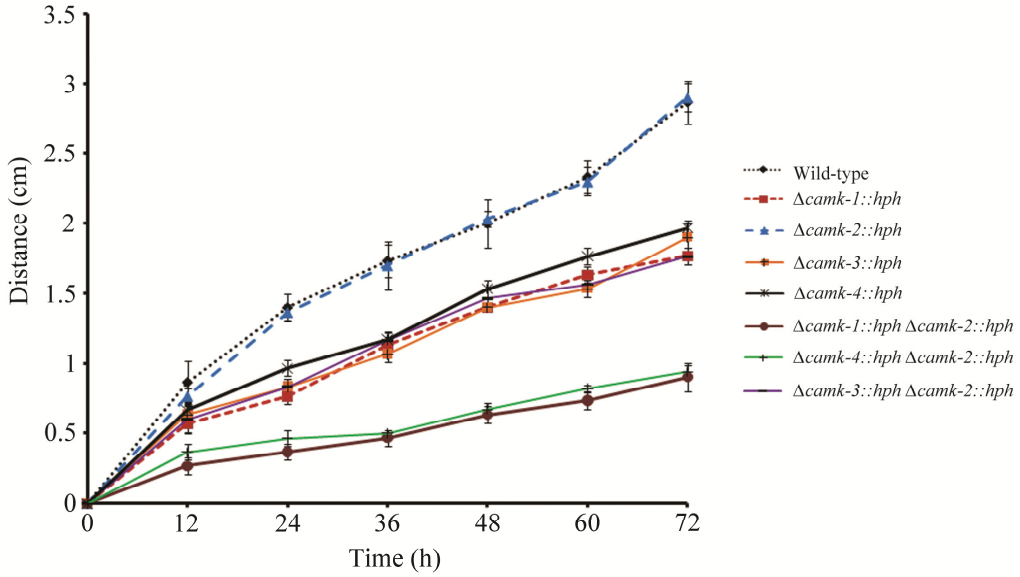


Figure 5.4 Effect of CaM antagonist TFP on growth of the *N. crassa* strains. Mycelial extension (growth) rates were measured using race tube containing medium supplemented with 100 µM TFP. Error bars indicate the standard errors calculated from the data for three independent experiments.

5.2.5 Role of Ca^{2+}/CaMKs in acquiring thermotolerance

I have also tested if the Ca^{2+}/CaMKs in *N. crassa* play any role in acquiring thermotolerance. The *Δcamk-1::hph*, *Δcamk-3::hph, Δcamk-4::hph, Δcamk-1::hph Δcamk-2::hph, Δcamk-4::hph Δcamk-2::hph* and *Δcamk-3::hph Δcamk-2::hph* mutants showed a decreased thermotolerance (using the protocol as described in Chapter 2) both in uninduced and induced conditions than the wild-type and *Δcamk-2::hph* mutant strain. The survival percentage on exposure to 52°C from 44°C for induced thermotolerance followed the order *Δcamk-2::hph* > wild-type > *Δcamk-3::hph* > *Δcamk-3::hph Δcamk-2::hph* > *Δcamk-1::hph Δcamk-2::hph* > *Δcamk-1::hph* > *Δcamk-4::hph Δcamk-2::hph* > *Δcamk-4::hph* (Figure 5.5; Table 5.5). In addition, on exposure to 52°C from 30°C for uninduced thermotolerance, the survival percentage followed the order wild-type > *Δcamk-2::hph* > *Δcamk-3::hph* > *Δcamk-1::hph Δcamk-2::hph* > *Δcamk-4::hph Δcamk-2::hph* > *Δcamk-3::hph Δcamk-2::hph* > *Δcamk-1::hph* > *Δcamk-4::hph* (Figure 5.5; Table 5.5). The *Δcamk-2::hph* mutant showed lower survival percentage in uninduced but higher survival percentage in induced condition. Therefore, *camk-2* could be a mediator of cell-death triggered by heat shock during induced condition.

Table 5.5 Survival percentage of wild-type and knockout mutants of Ca^{2+}/CaMKs in acquiring thermotolerance

S. no.	Strain	% Survival	
		Induced	Uninduced
1.	Wild-type	5.880 ± 0.383	1.697 ± 0.260
2.	Δ*camk-1::hph*	0.414 ± 0.193	0.166 ± 0.190
3.	Δ*camk-2::hph*	10.73 ± 1.036	0.975 ± 0.387
4.	Δ*camk-3::hph*	1.005 ± 0.910	0.305 ± 0.310
5.	Δ*camk-4::hph*	0.293 ± 0.192	0.126 ± 0.125
6.	Δ*camk-1::hph* Δ*camk-2::hph*	0.525 ± 0.227	0.263 ± 0.086
7.	Δ*camk-4::hph* Δ*camk-2::hph*	0.470 ± 0.385	0.247 ± 0.230
8.	Δ*camk-3::hph* Δ*camk-2::hph*	0.674 ± 0.539	0.232 ± 0.213

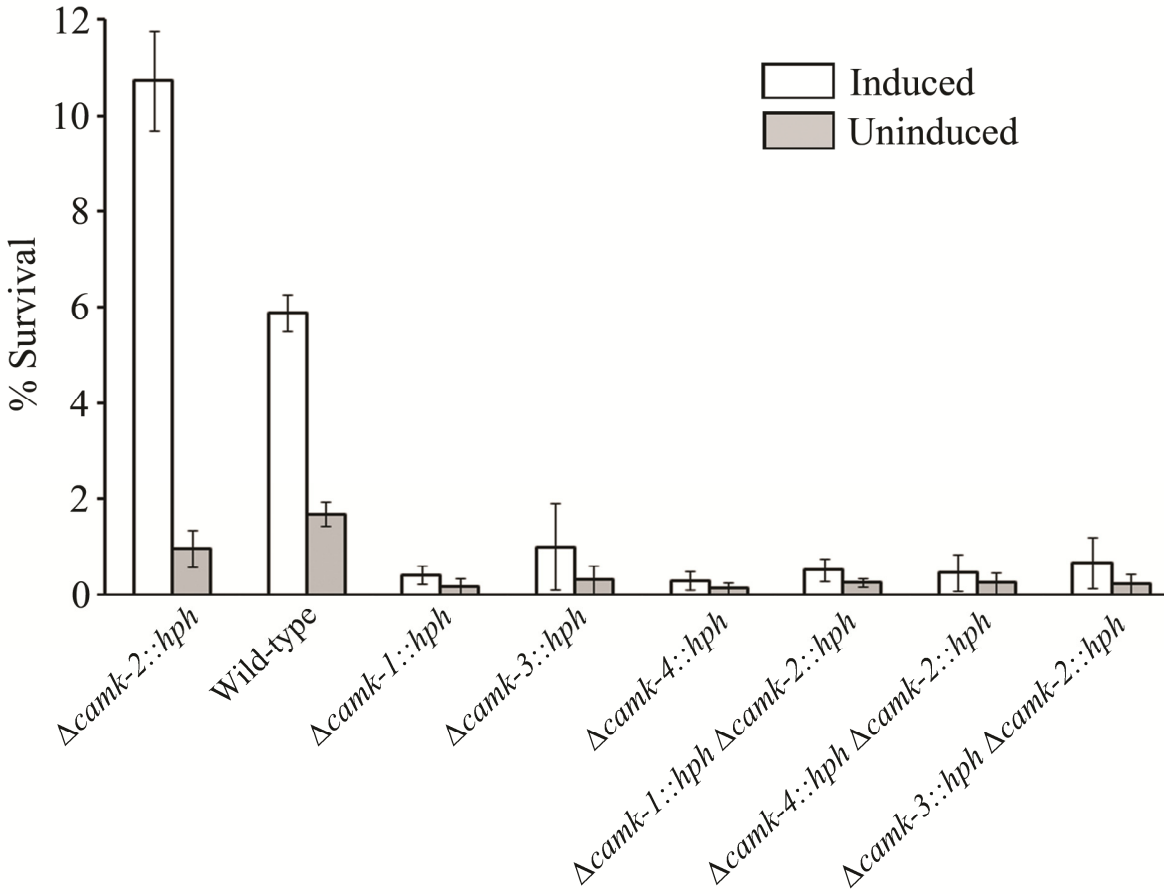


Figure 5.5 Thermotolerance of knockout mutants of Ca^{2+}/CaMKs. Viability of 2 h old germlings in exposure to 52°C lethal temperature with (induced; white boxes) or without (uninduced; gray boxes) pre-exposure to a sublethal heat shock temperature at 44°C. Percent survival was obtained by dividing the number of viable colonies after heat treatment by the number on the 30°C control plate and multiplying by 100. Error bars indicate the standard errors calculated from the data for three independent experiments.

5.2.6 Role of Ca^{2+}/CaMKs on survival in exposure to oxidative stress

To test, if Ca^{2+}/CaMKs play any role in survival in exposure to H_2O_2-mediated oxidative stress, I determined the viability of the *N. crassa* Ca^{2+}/CaMKs mutants in exposure to H_2O_2-mediated oxidative stress. The survival percentage followed the order Δ*camk-2::hph* > wild-type > Δ*camk-3::hph* ≅ Δ*camk-1::hph* Δ*camk-2::hph* > Δ*camk-1::hph* ≅ Δ*camk-4::hph* ≅ Δ*camk-4::hph* Δ*camk-2::hph* > Δ*camk-3::hph* Δ*camk-2::hph* (Figure 5.6; Table 5.6). The survival percentage was more in the Δ*camk-2::hph* mutant than the wild-type. However, Δ*camk-1::hph*, Δ*camk-3::hph*, Δ*camk-4::hph*, Δ*camk-1::hph* Δ*camk-2::hph* and Δ*camk-4::hph* Δ*camk-2::hph* mutants showed lower survival than the wild-type (Figure 5.6; Table 5.6). Interestingly, the Δ*camk-3::hph* Δ*camk-2::hph* double mutant was severely sensitive to 10 mM H_2O_2 stress, suggesting a novel synthetic interaction between *camk-3* and *camk-2*.

Table 5.6 Survival percentage of wild-type and knockout mutants of Ca^{2+}/CaMKs in exposure to oxidative stress

S. no.	Strain	% Survival (10 mM H_2O_2)
1.	Wild-type	20.55 ± 0.220
2.	Δ*camk-1::hph*	14.15 ± 0.665
3.	Δ*camk-2::hph*	25.84 ± 0.780
4.	Δ*camk-3::hph*	14.72 ± 1.002
5.	Δ*camk-4::hph*	13.85 ± 0.580
6.	Δ*camk-1::hph* Δ*camk-2::hph*	14.25 ± 2.081
7.	Δ*camk-4::hph* Δ*camk-2::hph*	13.51 ± 1.078
8.	Δ*camk-3::hph* Δ*camk-2::hph*	1.975 ± 0.441

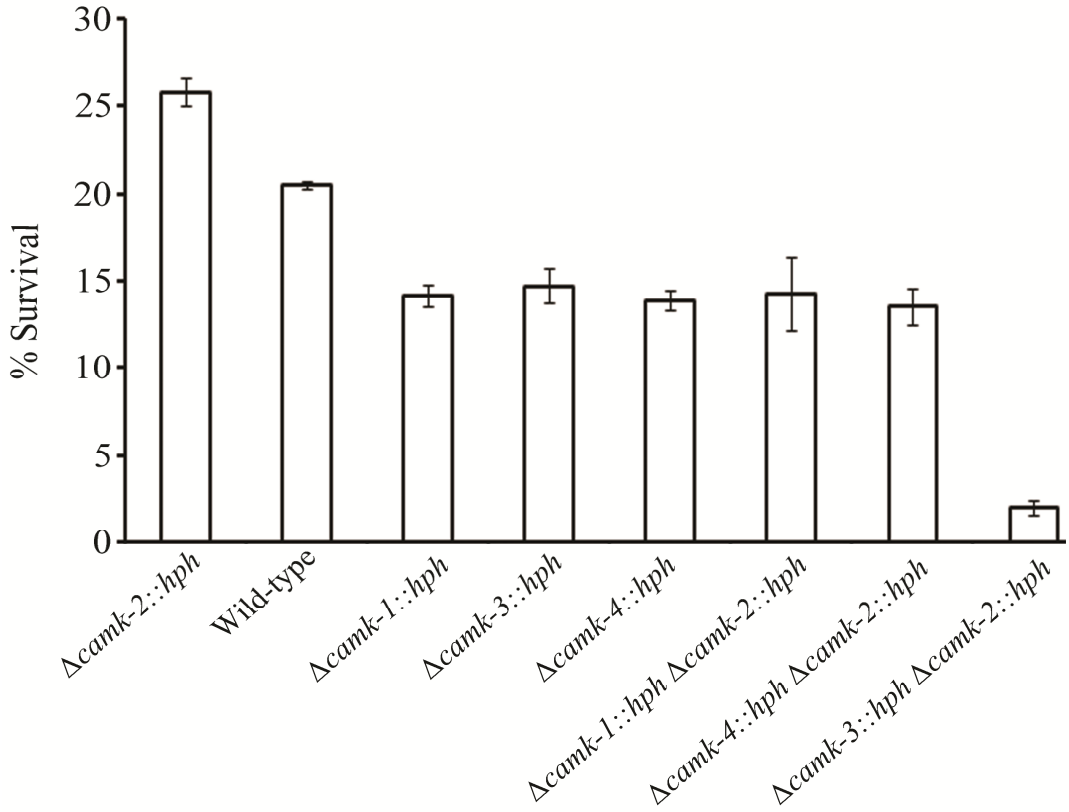


Figure 5.6 H_2O_2 sensitivity assay. Two days old colonies (genotype indicated below the bar) were given 10 mM H_2O_2 treatment for 10 min, washed and incubated at 30°C for 2 days. The survival percentage was scored by the number of colonies obtained after H_2O_2 treatment by the number on a plate containing cells that were not exposed to H_2O_2 and multiplying by 100. Error bars indicate the standard errors calculated from the data for three independent experiments.

5.2.7 Viability of ascospores were less in crosses heterozygous for double mutants of Ca^{2+}/CaMKs

I also tested the fertility phenotype of the *Δcamk-1::hph Δcamk-2::hph*, *Δcamk-4::hph Δcamk-2::hph* and *Δcamk-3::hph Δcamk-2::hph* double mutants. Crosses homozygous for *Δcamk-1::hph Δcamk-2::hph*, *Δcamk-4::hph Δcamk-2::hph* and *Δcamk-3::hph Δcamk-2::hph* double mutants displayed barren phenotype like the *Δcamk-2::hph* homozygous crosses (Table 5.7, entries 10, 15, 20). In addition, I determined viability of ascospores from some of these crosses and found that viability of the ascospores produced in barren crosses had lower survival percentage than the wild-type (Figure 5.7). Unexpectedly, crosses homozygous for *Δcamk-2::hph* but heterozygous for either *Δcamk-1::hph*, *Δcamk-3::hph* or *Δcamk-4::hph* were fully fertile (Table 5.7, entries 6-9, 11-14, 16-19). I had also determined the genotype of some progenies from such crosses with unexpected phenotype and I found that the segregation ratio of parental genotypes in f_1 progenies was ~1:1. These results indicated that *camk-2* play an important role in fertility and ascospores viability.

Table 5.7 Phenotypes of crosses involving knockout mutants of Ca^{2+}/CaMKs

S. no.	Cross	Ascospores produced	Phenotype
1.	*OR a* X *OR A*	abundant	fertile
2.	Δ*camk-1::hph a* X Δ*camk-1::hph A*	few hundred (~500)	intermediate
3.	Δ*camk-2::hph a* X Δ*camk-2::hph A*	very few (<200)	barren
4.	Δ*camk-3::hph a* X Δ*camk-3::hph A*	abundant	fertile
5.	Δ*camk-4::hph a* X Δ*camk-4::hph A*	abundant	fertile
6.	Δ*camk-1::hph* Δ*camk-2::hph* (6) *a* X Δ*camk-1::hph A*	abundant	fertile
7.	Δ*camk-1::hph* Δ*camk-2::hph* (3) *A* X Δ*camk-1::hph a*	abundant	fertile
8.	Δ*camk-1::hph* Δ*camk-2::hph* (6) *a* X Δ*camk-2::hph A*	abundant	fertile
9.	Δ*camk-1::hph* Δ*camk-2::hph* (3) *A* X Δ*camk-2::hph a*	abundant	fertile

10.	Δ*camk-1::hph* Δ*camk-2::hph* (6) *a* X Δ*camk-1::hph* Δ*camk-2::hph* (3) *A*	very few (<100)	barren
11.	Δ*camk-4::hph* Δ*camk-2::hph* (19) *A* X Δ*camk-4::hph a*	abundant	fertile
12.	Δ*camk-4::hph* Δ*camk-2::hph* (23) *a* X Δ*camk-4::hph A*	abundant	fertile
13.	Δ*camk-4::hph* Δ*camk-2::hph* (19) *A* X Δ*camk-2::hph a*	abundant	fertile
14.	Δ*camk-4::hph* Δ*camk-2::hph* (23) *a* X Δ*camk-2::hph A*	abundant	fertile
15.	Δ*camk-4::hph* Δ*camk-2::hph* (19) *A* X Δ*camk-4::hph* Δ*camk-2::hph* (23) *a*	very few (~53)	barren
16.	Δ*camk-3::hph* Δ*camk-2::hph* (4) *a* X Δ*camk-3::hph A*	abundant	fertile
17.	Δ*camk-3::hph* Δ*camk-2::hph* (18) *A* X Δ*camk-3::hph a*	abundant	fertile
18.	Δ*camk-3::hph* Δ*camk-2::hph* (4) *a* X Δ*camk-2::hph A*	abundant	fertile
19.	Δ*camk-3::hph* Δ*camk-2::hph* (18) *A* X Δ*camk-2::hph a*	abundant	fertile
20.	Δ*camk-3::hph* Δ*camk-2::hph* (4) *a* X Δ*camk-3::hph* Δ*camk-2::hph* (18) *A*	very few (~36)	barren

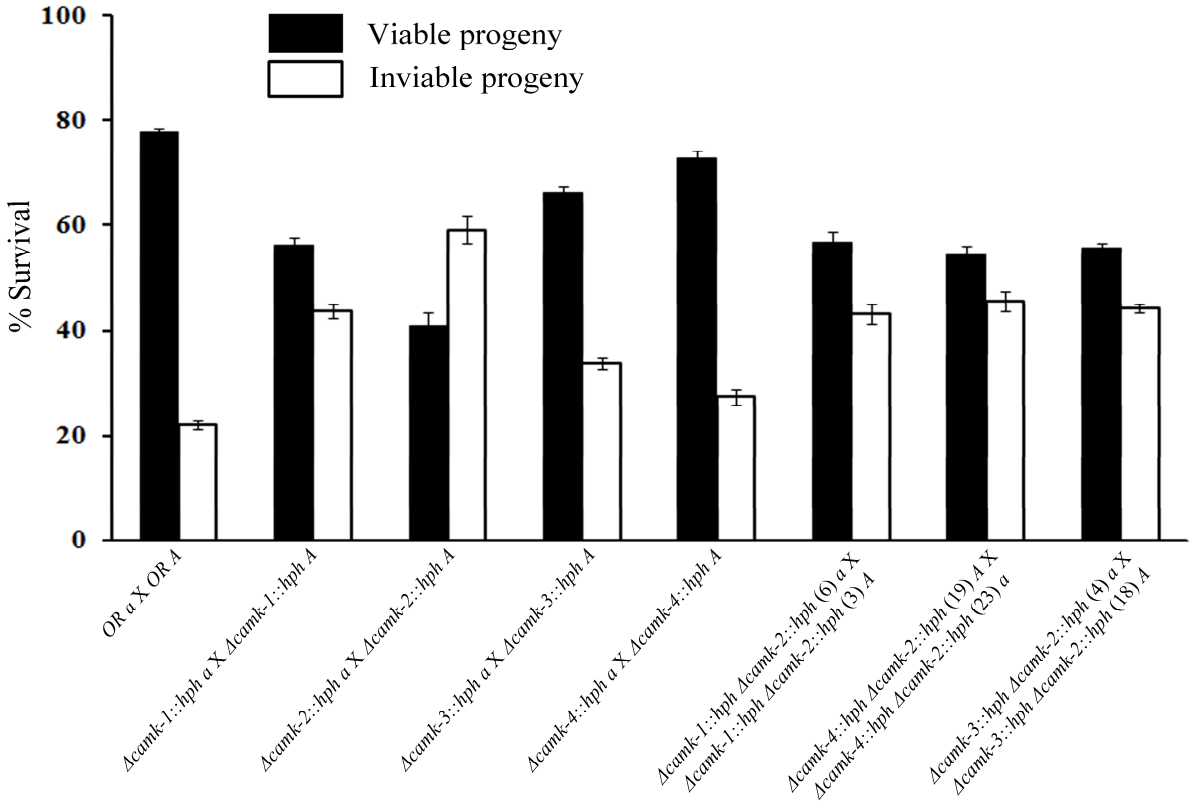


Figure 5.7 Viability of ascospores produced in crosses involving mutants of Ca^{2+}/CaMKs. Eight-month old ascospores from various crosses (indicated below the bar) were germinated on sorbose containing medium and exposed to heat sock at 64°C and incubated at 30°C for overnight. The plates were then observed under a dissection microscope and the germinated as well ungerminated (ungerminated ascospores were once again confirmed after 36 h by observing the same plate under the dissection microscope) ascospores were counted to calculate total number of ascospores plated. The viability of ascospores was determined by dividing the number of ascospores germinated by the total number of ascospores plated and multiplying by 100. Error bars indicate the standard errors calculated from the data for three independent experiments.

5.3 Discussion

Calcium (Ca^{2+}) plays an important role in intracellular signaling process in eukaryotes including fungi (Gadd 1994; Shaw and Hoch 2001; Sanders et al. 2002). The Ca^{2+} signaling process primarily involves transient increase in $[Ca^{2+}]_c$ that induce a number of effector proteins including Ca^{2+}/CaMKs (Chin and Means 2000; Hook and Means 2001; Sorderling and Stull 2001; Zelter et al. 2004). To understand the genetic interaction of Ca^{2+}/CaMKs, I have generated double knockout mutants of Ca^{2+}/CaMKs in *N. crassa* and studied their phenotypes. The *Δcamk-1::hph Δcamk-2::hph* double mutant showed a severe morphological defect and reduced mycelial extension rate, suggesting a synthetic interaction of these two Ca^{2+}/CaMKs (Figure 5.2A-B; Figure 5.3; Table 5.3). The Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-3::hph* Δ*camk-2::hph* double mutants displayed lower growth rates than their parental single mutants and the wild-type (Figure 5.3; Table 5.3). In addition, Δ*camk-1::hph* Δ*camk-2::hph*, Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-3::hph* Δ*camk-2::hph* double mutant strains displayed reduced aerial hyphae (Figure 5.2C; Table 5.2). Moreover, on Vogel's glucose medium supplemented with the CaM antagonist TFP (100 µM), the growth of the wild-type and Δ*camk-2::hph* were comparatively less inhibited, Δ*camk-1::hph*, Δ*camk-3::hph*, Δ*camk-4::hph* and Δ*camk-3::hph* Δ*camk-2::hph* were intermediate, and Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-1::hph* Δ*camk-2::hph* were severely inhibited (Figure 5.4; Table 5.4). I also tested if the Ca^{2+}/CaMKs in *N. crassa* play any role in thermotolerance and survival in oxidative stress. The *Δcamk-1::hph*, *Δcamk-3::hph*, *Δcamk-4::hph*, *Δcamk-1::hph Δcamk-2::hph*, *Δcamk-4::hph Δcamk-2::hph* and *Δcamk-3::hph Δcamk-2::hph* mutants showed a decreased survival percentage both in uninduced and induced thermotolerance conditions than the wild-type and *Δcamk-2::hph* mutant strains (Figure 5.5; Table 5.5). Moreover, survival percentage in H_2O_2-induced oxidative stress followed the order *Δcamk-2::hph* > wild-type > *Δcamk-1::hph Δcamk-2::hph* > *Δcamk-1::hph* > *Δcamk-4::hph Δcamk-2::hph* > *Δcamk-4::hph* > *Δcamk-3::hph* > *Δcamk-3::hph Δcamk-2::hph* (Figure 5.6; Table 5.6). The *Δcamk-2::hph* mutant showed an increased survival percentage than the wild-type in response to H_2O_2-induced oxidative stress (Figure 5.6; Table 5.6). The *Δcamk-3::hph Δcamk-2::hph* double mutant displayed severe sensitivity to H_2O_2 stress.

The *camk-1* null mutant of *N. crassa* transiently show a slow growth phenotype immediately after germination from ascospores, indicating that CAMK-1 has important but redundant role in growth and development (Yang et al. 2001). The Ca^{2+}/CaM possess a putative CaM-binding domain, and binding of Ca^{2+}/CaM is essential for the activation of these kinases (Hook and Means 2001). The regulatory functions of calmodulin-dependent protein phosphorylation during conidial germination and hyphal growth in *N. crassa* (Praveen Rao et al. 1997*)*. In *S. cerevisiae*, CaMKII was found necessary for acquisition of induced thermotolerance (Iida et al. 1995). In another study, CaMKs in human T lymphocytes appeared to have a role in H_2O_2-induced phosphorylation of the inhibitor of κB (IκB), indicating CaMKs as the potential therapeutic targets to minimize activation of the transcription factor NF-κB induced by oxidative stress (Howe et al. 2002). It was found that in *N. crassa*, germinating spores have a higher survival percentage to the lethal shock at 52°C on pre-exposure to 44°C and this thermal protection is meditated by the heat shock proteins that are synthesized optimally at 44°C (Plesofsky-Vig and Brambl 1985). In *S. cerevisiae*, the acquisition of induced thermotolerance depends on CaMKII, encoded by *cmk1* and *cmk2* (Iida et al. 1995). The *Δcmk1* and the *Δcmk1 Δcmk2* mutants were found slightly hypersensitive and hypersensitive, respectively, to lethal heat-shock, and showed significantly lower levels of induced thermotolerance than those of wild-type and *Δcmk2* mutant (Iida et al. 1995). H_2O_2 is a deleterious agent that generates harmful and potent free •OH radicals, via the Haber-Weiss reaction, and lipid peroxides derived from H_2O_2 could damage the membrane (Haber and Weiss 1934; Halliwell and Gutteridge 1984). In addition, H_2O_2 at highest concentration induced the signs of apoptosis in Jurkat cells (Howe et al. 2002).

Crosses homozygous for the Δ*camk-1::hph* Δ*camk-2::hph*, Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-3::hph* Δ*camk-2::hph* mutants displayed barren phenotype like the Δ*camk-2::hph* homozygous crosses (Table 5.7). In addition, viability of ascospores from some of these barren crosses was lowered than the wild-type (Figure 5.7). These results consistently suggested genetic interactions of *camk-2* with *camk-1*, *camk-3*, and *camk-4*. The Ca^{2+}/CaMKs were also found important for sexual development in other organisms. The Ca^{2+}/CaMKI homologue in *C. gloeosporioides*, CgCMK, might be involved in germination

and appressorium induction (Kim et al. 1998). In *X. laevis*, CaMKIx, activated Ca^{2+}/CaMKI was found to phosphorylate various proteins including synapsin I, histones, and myelin basic protein in the late stages of embryogenesis (Kinoshita et al. 2004). In addition, activation of Ca^{2+} channels leads to Ca^{2+} influx that is an essential step in initiation of acrosome reaction during fertilization in human, and abnormal activity was detected in the Ca^{2+} -permeable channels from infertile human sperm membrane (Goodwin et al. 1997; Ma and Shi 1999).

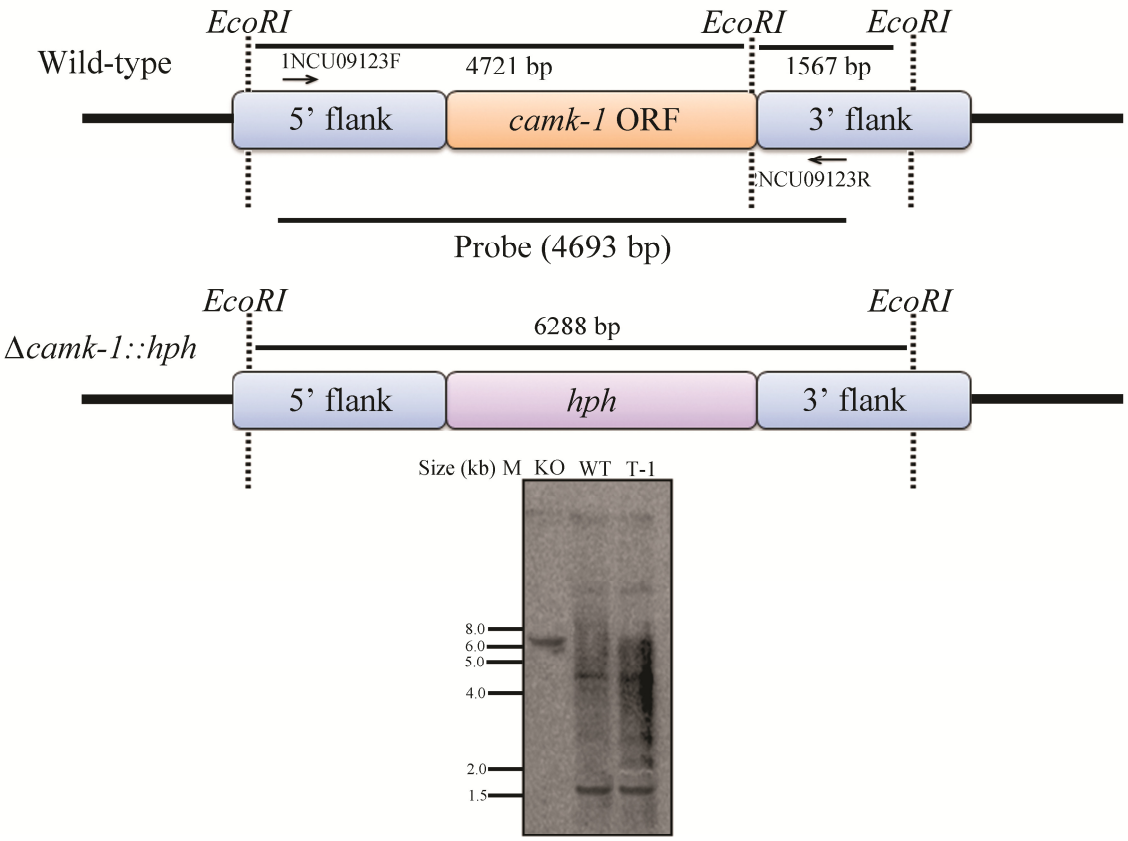
Thus, the genetic interaction studies suggested that complex interactions of *camk-1*, *camk-2*, *camk-3* and *camk-4* genes regulate growth, thermotolerance, survival in oxidative stress and sexual development in *N. crassa*.

Appendix

A.1 Southern analysis of Δ*camk-1::hph* and Δ*camk-2::hph* transformants

Confirmation of the Δ*camk-1::hph* and Δ*camk-2::hph* mutants and transformants by Southern analysis. Briefly, genomic DNA of the Δ*camk-1::hph* (KO), wild-type (WT) and Δ*camk-1::hph* transformant (T-1) were digested with *EcoRI* and probed with a 4693 bp *camk-1* fragment obtained by PCR from the wild-type genomic DNA as template using primers 1NCU09123F and 2NCU09123R (Figure A.2A; Table 4.1). The *EcoRI* has three cut sites within the probe region that resulted in two fragments of size 4721 and 1567 bp in the wild-type, the knockout has one cut that resulted in one fragment of size 6288 bp (http://borkovichlims.ucr.edu/southern/enzymes.py?gene=09123); the 4721 and 1567 bp fragments specific for the wild-type is restored in the Δ*camk-1::hph* (T-1) transformant. This result confirmed non homologous integration of *camk-1* transgene in the Δ*camk-1::hph* transformant (T-1).

(A1.A)



Similarly, genomic DNA of the Δ*camk-2::hph* (KO), wild-type (WT) and Δ*camk-2::hph* transformant (T-2) was digested with *XhoI* and probed with a 4157 bp *camk-2* fragment obtained by PCR with the primer pairs 5NCU02283F and 6NCU02283R (Figure A.2B; Table 4.1) using the wild-type genomic DNA as template. The *XhoI* has three cut sites within the probe region that resulted in two fragments of size 1118 and 6136 bp in the wild-type, the knockout has one cut that resulted in one fragment of size 7254 bp (http://borkovichlims.ucr.edu/southern/enzymes.py?gene=02283); the 1118 and 6136 bp fragments specific for the wild-type is restored in the Δ*camk-2::hph* (T-2) transformant. This result confirmed non homologous integration of *camk-2* transgene in the Δ*camk-2::hph* transformant (T-2).

(A.2B)

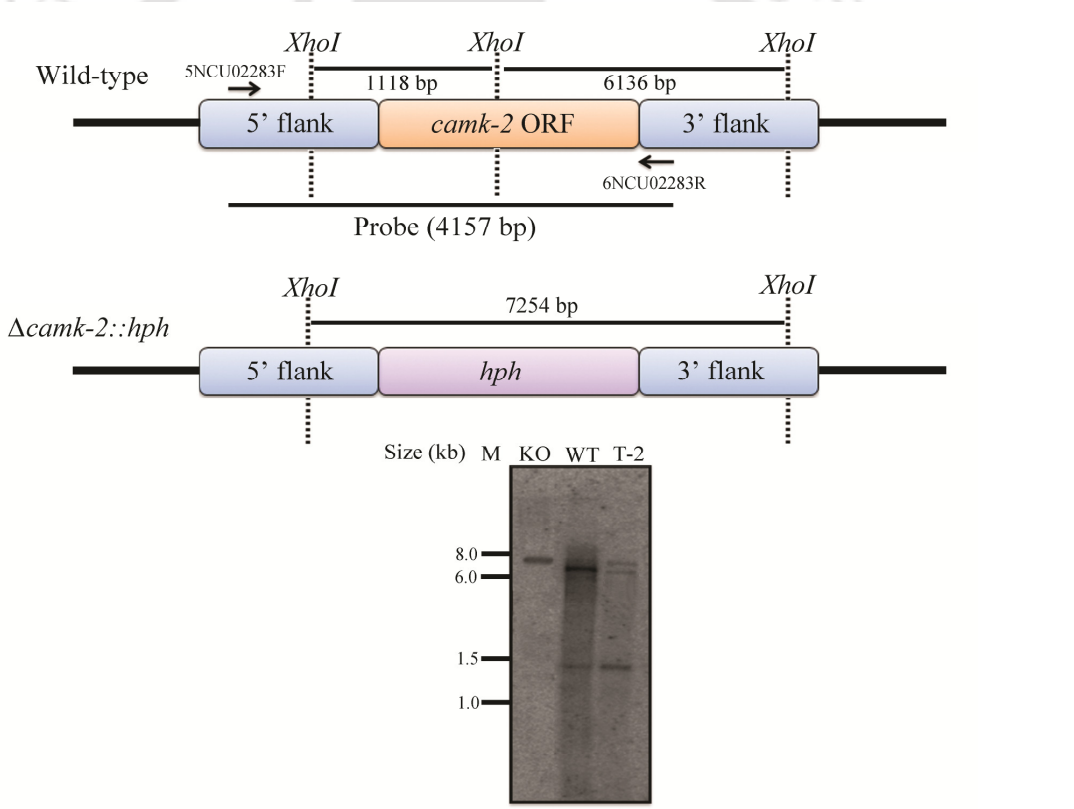


Figure A.1 Southern analysis of Δ*camk-1::hph* and Δ*camk-2::hph* transformants. Schematics of designing the *camk-1* and *camk-2* probes to hybridizing the respective Southern blot are shown. **(A.2A)** A 4693 bp probe was PCR amplified from the wild-type using primers 1NCU09123F and 2NCU09123R (top). Genomic DNA from the knockout (KO), wild-type (WT) and Δ*camk-1::hph* (T-1) were digested with *EcoRI* and resolved in a 1%

agarose gel, and transferred into a nitrocellulose membrane and probed with the 4693 bp fragment (shown above). Presence of both the wild-type and knockout allele specific fragments confirmed non homologous integration of *camk-1* transgene in the Δ*camk-1::hph* transformant (T-1). **(A.2B)** A 4157 bp probe was PCR amplified from the wild-type using primers 5NCU02283F and 6NCU02283R (top). Genomic DNA from the knockout (KO), wild-type (WT) and Δ*camk-2::hph* (T-2) were digested with *XhoI* and resolved in a 1% agarose gel, and transferred into a nitrocellulose membrane and probed with the 4157 bp fragment (shown above). Presence of both the wild-type and knockout allele specific fragments confirmed non homologous integration of *camk-2* transgene in the Δ*camk-2::hph* transformant (T-2). The Southern blots were developed using Fujifilm FLA-3000.

A.2 Verification of *camk-1* and *camk-2* expression by RT-PCR analysis

Reverse transcription-PCR (RT-PCR) was used to verify that the *camk-1* and *camk-2* genes were expressed in wild-type but not in their mutant strains. RNA extractions were performed using the TRIzol method (Invitrogen, CA). RT-PCR was performed using 1 µg total RNA and Verso Kit according to manufacture protocol (Thermo scientific, CA). To verify the *camk-1* expression, primer pairs 5NCU09123F and 6NCU09123R were used to amplify a *camk-1* fragment of 1116 bp (Figure A.2, Table A1). Similarly, to verify the *camk-2* expression, primer pairs 3NCU02283F and 4NCU02283R were used to amplify a *camk-2* fragment of 751 bp (Figure A.2, Table A1). The RT-PCR analysis showed that the *camk-1* and *camk-2* genes expressed in the wild-type. The *β-tubulin* control was expressed in the all strains (data not shown).

Table A1 Primers used for confirmation of *camk-1* and *camk-2* expression by RT-PCR analysis

S. no.	Primer	Sequence (5'→3')	Reference
1.	5NCU09123F	CGCCAACATGCTTAATCGCC	This study
2.	6NCU02283R	TAGTCTTGGAGAGTGTCCGC	This study
3.	3NCU02283F	GCAGCCGTGTCGATACAAAG	This study
4.	4NCU02283R	GTCAATGGTCAAGCACCTGC	This study

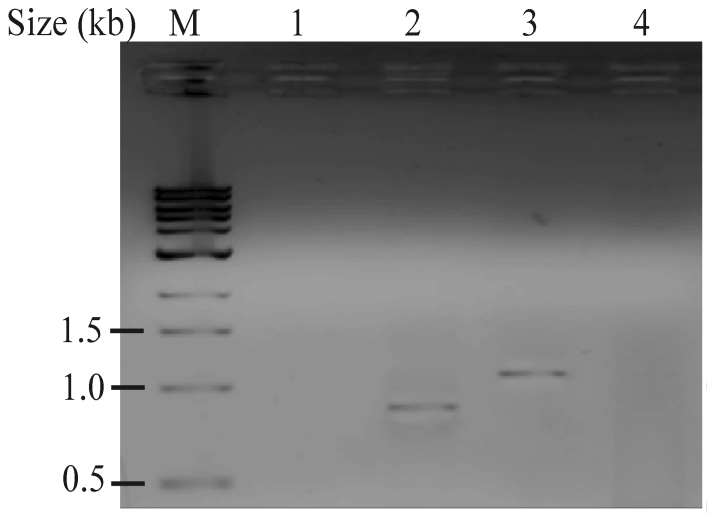


Figure A.2 Verification of *camk-1* and *camk-2* expressions by RT-PCR analysis. The amplification of PCR product of size ~1116 and 751 bp, indicate expression of *camk-1* and *camk-2* genes, respectively. The PCR products were visualized in a 1.2% agarose gel; Lane 1: Δ*camk-2::hph* (no amplification, *camk-2* is absent), Lane 2: *camk-2* is expressed from the wild-type, Lane 3: *camk-1* is expressed from the wild-type and Lane 4: Δ*camk-1::hph* (no amplification, *camk-1* is absent), M: 1 kb DNA ladder (NEB).

Conclusions and Future direction

In this work, I studied Ca^{2+} signaling genes, using available knockout mutants, for their role in sexual development of *N. crassa*. This study revealed that the Ca^{2+}/CaMKs, CAMK-1 and CAMK-2 play a recessive role in sexual development of *N. crassa*. In addition, meiotic silencing studies revealed that the product of *camk-2* during meiosis is essential for full fertility in *N. crassa*. This is the first report demonstrating the requirement of these two Ca^{2+}/CaMKs for full fertility in *N. crassa*. Additionally, the *N. crassa* Ca^{2+}/CaMK proteins possess conserved signature motif and all the four kinase proteins were clustered in different clades comprising of broad substrate specificity kinases in a phylogenetic analysis. Besides, binding site for several regulatory elements were predicted using bioinformatics tools in the promoter region of the *N. crassa* Ca^{2+}/CaMKs.

I have generated Δ*camk-1::hph* Δ*camk-2::hph*, Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-3::hph* Δ*camk-2::hph* double mutants and used to study their phenotypes for understanding the cell function that are affected by genetic interaction of Ca^{2+}/CaMKs in *N. crassa*. The *Δcamk-1::hph Δcamk-2::hph* double mutant showed a severe morphological defect and reduced mycelial extension rate, suggesting a synthetic interaction of these two Ca^{2+}/CaMKs. The Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-3::hph* Δ*camk-2::hph* double mutants displayed lower growth rates than their parental single mutants and the wild-type. In addition, Δ*camk-1::hph* Δ*camk-2::hph*, Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-3::hph* Δ*camk-2::hph* double mutant strains displayed reduced aerial hyphae. Moreover, supplemented with the CaM antagonist TFP (100 µM) the growth of the wild-type and Δ*camk-2::hph* were comparatively less inhibited, Δ*camk-1::hph*, Δ*camk-3::hph*, Δ*camk-4::hph* and Δ*camk-3::hph* Δ*camk-2::hph* were intermediate, and Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-1::hph* Δ*camk-2::hph* were severely inhibited. The *Δcamk-2::hph* mutant showed an increased survival percentage than the wild-type in response to induce thermotolerance and H_2O_2-induced oxidative stress. Crosses homozygous for the Δ*camk-1::hph* Δ*camk-2::hph*, Δ*camk-4::hph* Δ*camk-2::hph* and Δ*camk-3::hph* Δ*camk-2::hph* mutants displayed barren phenotype like the Δ*camk-2::hph* homozygous crosses. Additionally, viability of ascospores from some of these barren crosses was lowered than the wild-type. Thus, the genetic

interaction studies had revealed that *camk-1*, *camk-2*, *camk-3* and *camk-4* genes are involved in growth, thermotolerance, survival in oxidative stress and sexual development in *N. crassa*.

Notwithstanding the importance of Ca^{2+} as a central player of intracellular signaling system, our understanding of Ca^{2+} signaling mechanism in *N. crassa* has remains elusive. However, recent availability of the genome sequences and the knockout mutants has made it possible to gain detailed information about the molecular components of *N. crassa* Ca^{2+}/CaMKs genes. Future work will enable us to determine the molecular mechanism of Ca^{2+}/CaMKs target using technique such as FRET, yeast-two hybrid system etc. In addition, studies using techniques such as X-ray crystallography, NMR, etc. will help us in understanding the structure function relationship of the relationship of the Ca^{2+}/CaMKs in *N. crassa*. Therefore, detailed information about the mechanism of Ca^{2+}/CaMKs function is expected to emerge in the near future.

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PUBLICATIONS:

1. **Kumar R** and Tamuli R (2014). Calcium/calmodulin-dependent kinases are involved in growth, thermotolerance, oxidative stress survival, and fertility in *Neurospora crassa.* Archive of Microbio. 196: 295-305.
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1. Tamuli R, **Kumar R**, Srivastava DA and Deka R (2013). Calcium Signaling. In: Kasbekar DP, McCluskey K (Eds) Neurospora: Genomics and Molecular Biology. Horizon Press, United Kingdom. ISBN: 978-1-908230-12-6. (http://www.horizonpress.com/neurospora).

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2. Deka R, **Kumar R** and Tamuli R (2010). Calcium signaling genes in *Neurospora crassa*. Neurospora 2010 Meeting, Asilomar, USA, 8-11 April.

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