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International Journal of Applied Sciences and Biotechnology

A Rapid Publishing Journal

ISSN 2091-2609

Indexing and Abstracting

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CODEN (Chemical Abstract Services, USA): IJASKD

Vol-3(4) December, 2015

Available online at:

http://www.ijasbt.org

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SEM-Biotech Publishing

Impact factor*: **1.422**
Scientific Journal Impact factor#: **3.419**
Index Copernicus Value: **6.02**
IBI Factor 2015**: **4.19**

*Impact factor is issued by Universal Impact Factor. Kindly note that this is not the IF of Journal Citation Report (JCR).
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R. Gedela and R.Tamuli (2015) Int J Appl Sci Biotechnol, Vol 3(4): 668-679
DOI: 10.3126/ijasbt.v3i4.13976

ISSN (Online): 2091-2609
DOI Prefix: 10.3126/ijasbt

International Journal of Applied Sciences and Biotechnology (IJASBT)

Research Article

A ROLE OF CALCIUM SIGNALING GENES IN HETEROKARYON INCOMPATIBILITY IN *NEUROSPORA CRASSA*

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Abstract

We have studied the Ca^{2+}-signaling knockout mutants for their role in mating-type-associated heterokaryon incompatibility in *Neurospora crassa*. The found results showed on heterokaryons homokaryosis for ΔNCU05225, ΔNCU06366, ΔNCU06650, ΔNCU07075, and ΔNCU07966 Ca^{2+}-signaling knockout mutants (Neurospora crassa unit number, NCU) displayed heterokaryon *het* compatibility; however heterokaryons heterokaryosis for ΔNCU05225, ΔNCU063665, ΔNCU06650, ΔNCU07075, and ΔNCU07966 mutants displayed *het* incompatibility like the wild-type control. In addition to that Two Ca^{2+}-signaling knockout mutants ΔNCU02283, and ΔNCU09655 were tested for mating-type-associated heterokaryon incompatibility; these results showed, heterokaryons homokaryosis and heterokaryons heterokaryosis for ΔNCU02283, ΔNCU09655 mutants displayed *het* incompatibility. Cell death and hyphal compartmentation due to mating type associated incompatibility were confirmed by uptake of vital dye Evan's blue. Thus, these results of NCU05225, NCU06366, NCU06650, NCU07075, and NCU07966 Ca^{2+}-signaling gene products could play a role in mating-type-associated heterokaryon incompatibility in *N. crassa*. In this article, we are reporting initially screened Ca^{2+}-signaling gene deletion mutants of these five acts as recessive suppressors of mating type associated vegetative incompatibility in *N. crassa.*

Keywords: Ca^{+2}-signaling genes; Neurospora crassa unit number (NCU); heterokaryon incompatibility; *Neurospora crassa*; non-self-recognition; vegetative incompatibility.

Introduction

A cell containing different nuclear types in the same cytoplasm is called heterokaryon. Heterokaryon incompatibility refers to a condition when two nuclei of different genotypes cannot co-exist together in the same cytoplasm (Fig.1) (Staben and Yanofsky, 1990; Glass *et al.*, 1988, Debets and Griffiths, 1998; Worrall, 1997, Caten, 1972; Debets *et al.*, 1994; Van Diepeningen *et al.*, 1998). In *N. crassa*, both sexual and vegetative heterokaryon systems are present, sexual recognition is restricted by the mating type locus, whereas vegetative *het* incompatibility is genetically control by particular loci termed as *het* (for heterokaryon incompatibility) or *vic* (vegetative heterokaryon incompatibility)(Glass *et al.*, 1988; Ferreira, *et al.*,1998). When two fungal individuals of distinct *het* alleles come together, the consequential heterokaryotic cells are quickly damaged or strictly inhibited in their growth (Glass, 2006 and Marek *et al.*, 2003; Saupe, 2000) (Fig.1). This is analogous to histocompatibility system in invertebrates and major histocompatibility (MHC) in mammals (Xiang, 2002; Glass and Kaneko, 2003; Xiang, 2004). *N. crassa* has two mating (*mat*) types *mat A* and *mat a*. The coexistence of *mat A* and *mat a* during the vegetative phase is lethal; however, coexistence of both mating types is necessary for the sexual development (Saupe, 2000; Garnjobst *et al.*, 1956, Wu, 2001; Sarkar *et al.*, 2002). These Two mating (*mat*) types *mat A* and *mat a*, alleles are differ in sequences although they occupy the same loci in different strains, and termed as 'idiomorph' (Glass *et al.*, 1988). The *A* idiomorph is 5301 base pairs that consists of *mat A-1,mat A-2* and *mat A-3* genes; in contrast, *a* idiomorph is 3235 base pair and contains only one ORF, *mat a-1*. The total number of *het* loci in *N. crassa* is 11, five of them the mating locus, *het-c*, *-d*, *-e*, and *i*, they have been recognized using enforced heterokaryons between nearly isogenic strains. Among them, three have been characterized at the molecular level, i.e. the mating type *mat* (*mat A* and *mat a)* locus, *het-c* and *het-6* (Glass *et al.*, 1988; Xiang, 2002; Glass and Kaneko, 2003; Xiang, 2004; Saupe, 2000; Wu, 2001; Sarkar *et al.*, 2002).

A non-self-recognition process called heterokaryon incompatibility (het) that operates during the vegetative and sexual phases of the filamentous in *N. crassa* (Glass *et al.*, 1988; Ferreira *et al.*, 1998; Garnjobst, *et al.*, 1956).

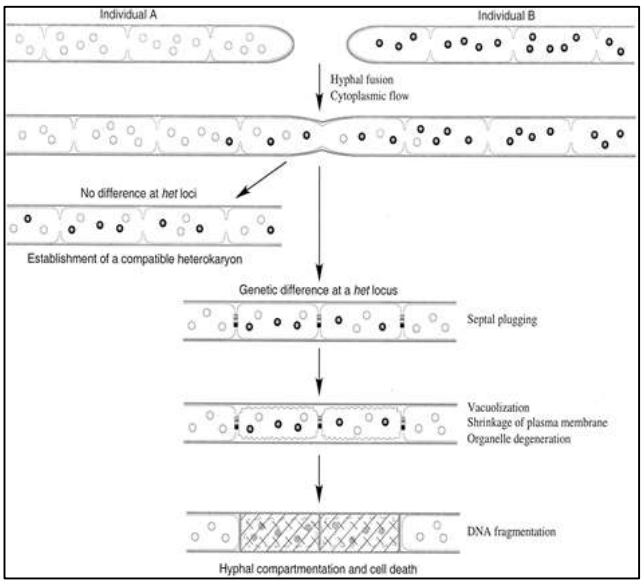


Fig. 1: Mechanism of heterokaryon incompatibility. Adapted from Gale Wichmann *et al.*, 2008.

Heterokaryon Incompatibility in Other Filamentous Fungi

The fungal mating types which are tremendously dissimilar from each other, and do not show homology between strains of the opposite sex (as opposed to the allelic relationship in most polymorphic systems). The variance between dissimilar allelic forms of a *het* gene is generally widespread, but single-amino-acid differences can be sufficient to trigger incompatibility (Newmeyer, 1970; Glass *et al.*, 1988; Deleu, 1993). The *mat A*-1 gene, encode a polypeptide of 288 amino acids region containing amino acids 90-104 has significant similarity to the MAT α1 polypeptide of *Saccharomyces cerevisiae* (Glass *et al.*, 1990), this is required for the expression of the *het* incompatibility and sexual functions. The *mat a-1* encodes a 382 amino acids residues MAT a-1 polypeptide consisting of an HMG domain, and a separate region from amino acid residues 216-220 that confers vegetative incompatibility (*vic)* in *N. crassa* (Staben and Yanofsky, 1990; Glass *et al.*, 1990; Philley and Staben, 1994). In *Podospora anserine* a having two *het* loci, *het-s* and *het-e* are functional similar to the that of *het-c* and *het*-6 of *N. crassa*, and *vib-1*(vegetative incompatibility block-1) of *Aspergillus nidulans*; but all of these genes are not homologous to each other (Ferreira *et al.*, 1998; Saupe, 2000; Sarkar *et al.*, 2002). The distinguish mechanism of *het* incompatibility reconcile by allele of differences at the *het-c* loci of *N. crassa* that inhibits phenotypic aspects of *het-c* vegetative incompatibility (Xiang, 2002; Glass and Kaneko, 2003; Xiang, 2004). The molecular description of *het* loci and *het* genes participating in the incompatibility effect has been achieved for two ascomycete's *N. crassa* and *P. anserine* (Smith *et al.*, 2000; Dementhon *et al.*, 2003; Xiang, 2002; Xiang, 2004; Glass and Kaneko, 2003; Saupe, 2000).

Biological Significance of Heterokaryon Incompatibility

The biological significance of heterokaryon incompatibility, two different views was explained by subject. First, it has been proposed that heterokaryon incompatibility genes continue living limit nutrient situation, and heterokaryon development between different individuals (Debets, 1994; Glass *et al.*, 2004; Xiang, 2004). Second, the heterokaryotic cells strength boundary to the horizontal gene transfer of cytoplasmic infectious fundamentals elements such as senescence of plasmids, mycoviruses, transposes (Anagnostakis, 1977, 1983; Baidyaroy *et al.*, 2000; Biella *et al.*, 2002; Caten, 1972; Debets *et al.*, 1994; Van Diepeningen *et al.*, 1998; Hartl *et al.*, 1975; Hickey *et al.*, 2002; Glass *et al.*, 2004). On the other hand it has been proposed that mating type associated heterokaryon incompatibility might be maintaining out breeding by preventing the construction of different mating types of heterokaryons between siblings of the identical crosses (Debets and Griffiths, 1998; Worrall, 1997).

Present Understanding of Mating Type-Associated Het Incompatibility in N. Crassa

N. crassa has two mating (*mat*) types, *mat A* and *mat a*, both mating types are essential for sexual development, however, coexistence during the vegetative phase is lethal, and therefore display incompatibility. The importance of mating type associated heterokaryon incompatibility, as anticipated for other *het* genes, the appearance of mating type allied incompatibility might be chance (Sarkar *et al.*, 2002). The *tol* gene (*tol* gene, for tolerant) is a suppressor of mating-type-associated heterokaryon incompatibility in *N. crassa* (Saupe, 2000). The Ca^{2+}-signaling genes regulates numerous processes secretion, sporulation, cytoskeletal organization, circadian rhythm, hyphal tip growth and hyphal branching in *N. crassa* (Fig. 1). Does Calcium signaling genes impact on mating-type-associated heterokaryon incompatibility in *N. crassa*? On this foundation, we designed the experiment by using auxotrophic marker for testing for complementation answer on Ca^{2+}-signaling genes in *N. crassa;* and we crossed with Ca^{2+}-signaling knockout mutants with auxotrophic marker *leu-3*, and *his-3* strains (Garnjobst, 1953; Adams *et al.*, 1987; Coenen, 1994).

Materials and Methods

Strains and Growth Conditions

Growth and maintenance of *Neurospora crassa* strains on Vogel's medium supplement with glucose were essentially as described in Davis and De Serres (1970). The Ca^{2+}-signaling knockout mutants, genotypes indicate as a *Neurospora crassa* unit number (NCU) (Table1, and Table 2).

Table1: Ca^{2+}-signaling genes encodes proteins in *N. crassa*

S.N.	Ca^{2+}-signaling genes	No. of amino acid	Encodes Name of Proteins
1	NCU02283	467	calcium/calmodulin-dependent protein kinase type I
2	NCU05225	674	mitochondrial NADH dehydrogenase
3	NCU06366	505	Ca2+/H+ antiporter
4	NCU06650	186	Ca^{2+} and /or CaM binding protein, a secretory phosholipase A2
5	NCU07075	508	CAX , Ca^{2+} /H^{+} exchanger
6	NCU07966	1110	Calcium transporting ATPase 3, a cation -ATPase
7	NCU09655	598 625	plasma membrane zinc ion transporter, phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma 2

Note: Ca^{2+}-signaling genes encode proteins in *N. crassa*, available in site (http://www.broadinstitute.org/annotation/genome/neurospora/)

Table 2: list of strains used in this study.

S. No.	FGSC	Genotype	Source
1	12448 *a*	ΔNCU02283 *a*	FGSC
2	12449*A*	ΔNCU02283 *A*	FGSC
3	11405*a*	ΔNCU05225*a*	FGSC
4	11406*A*	ΔNCU05225*A*	FGSC
5	11407a	ΔNCU06366 *a*	FGSC
6	114084	ΔNCU06366 *A*	FGSC
7	11246 *a*	ΔNCU06650 *a*	FGSC
8	11247*A*	ΔNCU06650*A*	FGSC
9	11248 *a*	ΔNCU07075*a*	FGSC
10	11249 *A*	ΔNCU07075*A*	FGSC
11	11409 *A*	ΔNCU07966*A*	FGSC
12	11410 *A*	ΔNCU07966*A*	FGSC
13	11271*a*	ΔNCU09655*a*	FGSC
14	11272*A*	ΔNCU09655*A*	FGSC
15	1321 *A*	*leu-3A*	FGSC
16	3740 *a*	*leu-3a*	FGSC
17	6103 *A*	*his-3A*	FGSC
18	9716 *a*	*his-3a*	FGSC

Table 3: List of Ca^{2+}-signaling knockout mutants obtained intensity band by Southern hybridization

S. No	Gene	R. E	Probe	Wild type bands	Knockout bands
1	NCU02283*A*	*NcoI*	5F - 5R	10kb,9.5 kb	4kb
2	NCU02283*a*	*NcoI*	5F - 5R	10kb,9.5 kb	4kb
3	NCU05225*A*	*SalI*	5F - 5R	0.8kb	5.5kb
4	NCU05225*a*	*SalI*	5F - 5R	0.8kb	5.5kb
5	NCU06366*A*	*Sac I*	5F - hph R	5kb,1.5k b	6kb
6	NCU06366*a*	*Sac I*	5F - hph R	5kb,4kb	6kb
7	NCU06650*A*	*Sac I*	5F - hph R	6kb,0.8k b	6.5kb,3kb,a nd 0.8kb
8	NCU07075*A*	*Hind III*	3F - 3R	1kb	5kb,1kb
9	NCU07075a	*Hind III*	3F - 3R	1kb	5kb,1kb
10	NCU07966*A*	*Hind III*	5F - 5R	9.5kb,8k b,and 6kb	10kb,8kb,an d 0.7kb
11	NCU07966*a*	*Hind III*	5F - 5R	10kb,8kb ,and 5kb	-
12	NCU09655*A*	*Xma I*	5F - 5R	10kb,6kb ,and 5.5kb	5.5kb,4kb,2 kb,and 0.7kb
13	NCU09655*a*	*Xma I*	5F - 5R	10kb,6kb ,and 5.5kb	5.5kb,4kb,2 kb,and 0.7kb

The strains were obtained from the Fungal Genetic Stock Centre (FGSC), Kansas City, Missouri, USA. In this study we used two auxotrophic marker strains *leu-3* and *his*-3 (*leu-3*, which is deficient to isopropylmalate synthatase, isopropylmalate dehydrogenase and isopropylmalate isomerase; *his-3*, histidinol dehydrogenase, phosphoribosyl-ATP-pyrophosphohydrolase and phosphoribosyl-AMP-cyclohydrolase) respectively; two auxotrophic strains *leu-3* and *his*-3 were supplemented in the media with leucine (mg/ml) and histidine(mg/ml) nutrition for their proper growth. The crosses were performed by on Synthetic Crossing media 1X (SCM), and Sorbose-glucose-fructose media 1X (FGS) were used for germinating progeny/ascospore. The antibiotic hygromycin used at a working concentration 220 µg/ml for selecting Ca^{2+}-signaling knockout mutants strains form their colonies.

Test for Heterokaryon Incompatibility

Strains with auxotrophic markers were cultured in Vogel's medium with required supplements. Conidial suspension of two auxotrophic strains, shown in below, were mixed and placed on a Petridis containing Vogel's glucose media without any supplement.

HO [*leu-3*; ΔNCU02283A) + (*his-3*; ΔNCU02283a], C1 [*leu-3*; NCU02283A) + (*his-3*;ΔNCU02283a], C2 [*leu-3*; ΔNCU02283A) + (*his-3*; NCU02283a], and C3 [*leu-3*; NCU02283A) + (*his-3*; NCU02283a] i.e. Ho: Heterokaryon homokaryosis and Heterokaryon heterokaryosis knockout test controls and well wild type control, similarly we examined all genes. That is, HO [(ko1+;mat A) + (ko1+;mat a)] are viable, whereas test controls C1[(ko1+; mat A) + (ko1-;mat a)], C2 [(ko1-; mat A) + (ko1+; mat a)]; and C3 [(ko1-; mat A) + (ko1-; mat a)] as a wild type are inviable (Table4, Fig. 2, Fig. 4a).

Microscopic Analysis with Evan's Blue Staining

Sterile pieces of cellophane were spread on top of the surface of solid Vogel's glucose agar medium. Heterokaryons were enforced by co-inoculating conidia of two strains grown on the cellophane for 2 days. The cellophane contain hyphae was peeled off from the surface of the medium and stained with 1% Evan's Blue dye (GAFF, 1971; JACOBSON *et al.*, 1998). The hyphae were stained for 15 to 25 minutes, and after that the cellophane was placed in a Buchner funnel containing a piece of filter paper pre-wetted with sterile water. Sterile water was then gently pipette over the cellophane and very weak vacuum was applied in order to clean off overload dye. The cellophane was then sited on a glass slide, with 5% glycerol to avoid drying, and observed under the bright field phase contrast microscope (Jacobson *et al.*, 1998; Wu and Glass 2001; Xiang, 2002; Glass, 2002).

Table 4: Test for heterokaryon incompatibility activity

S. No.	Heterokaryon type	*het* incompatibility
1	[(*leu-3*;ΔNCU02283*A*(105))+(*his-3*;ΔNCU02283*a*(86))]	No
2	[(*leu-3*;NCU02283*A*(104)))+(*his-3*;ΔNCU02283*a*(86))]	Yes
3	[(*leu-3*;ΔNCU02283*A*(105))+(*his-3*;NCU02283 *a*(85))]	Yes
4	[(*leu-3*;NCU02283*A*(104))+(*his-3*;NCU02283*a*(85))]	Yes
5	[(*leu-3*;ΔNCU05225*a*(93))+(*his-3*;ΔNCU05225*A*(63))]	No
6	[(*leu-3*;NCU05225*a*(89))+(*his-3*;ΔNCU05225 *A*(63))]	Yes
7	[(*leu-3*;ΔNCU05225*a*(93))+(*his-3*;NCU05225 *A*(64))]	Yes
8	[(*leu-3*;NCU05225*a*(89))+(*his-3*;NCU05225 *A*(64))]	Yes
9	[(*leu-3*;ΔNCU06366*A*(13))+(*his-3*;ΔNCU06366 *a*(6))]	No
10	[(*leu-3*;ΔNCU06366*A*(13))+(*his-3*;NCU06366 *a*(38))]	Yes
11	[(*leu-3*;NCU06366*A*(14))+(*his-3*;ΔNCU06366 *a*(6))]	Yes
12	[(*leu-3*;NCU06366*A*(14))+(*his-3*;NCU06366)*a*(38))]	Yes
13	[(*leu-3*;ΔNCU06650*a*(37))+(*his-3*;ΔNCU06650*A*(17))]	No
14	[(*leu-3*;ΔNCU06650*a*(65))+(*his-3*;NCU06650 *A*(41))]	Yes
15	[(*leu-3*;NCU06650*a*(66))+(*his-3;* ΔNCU06650 *A*(44))]	Yes
16	[(*leu-3*;NCU06650*a*(66))+(*his-3*;NCU06650 *A*(41))]	Yes
17	[(*leu-3*;ΔNCU07075 *a*(22))+(*his3*;ΔNCU07075*A*(19))]	No
18	[(*leu-3* ;ΔNCU07075*a*(22))+(*his-3* ;NCU07075*A*(24))]	Yes
19	[(*leu-3* ;NCU07075*a*(25))+ (*his-3* ;ΔNCU07075*A*(19))]	Yes
20	[(*leu-3* ;NCU07075*a*(25))+(*his-3*;NCU07075*A*(24))]	Yes
21	[(*leu-3*;ΔNCU07966*a*(44))+(*his-3*;ΔNCU07966*A*(34))]	No
22	[(*leu-3* ;NCU07966*a*(31))+(*his-3*;ΔNCU07966*A*(34))]	Yes
23	[(*leu-3*; ΔNCU07966*a*(44))+(*his-3*;NCU07966*A*(8))]	Yes
24	[(*leu-3*;NCU07966*a*(31))+(*his-3*;NCU07966 *A*(8))]	Yes
25	[(*leu-3*;ΔNCU09655*a*(97))+(*his-3*;ΔNCU09655*A*(70))]	Yes
26	[(*leu-3*;NCU09655*a*(96))+(*his-3*; ΔNCU09655*A*(70))]	Yes
27	[(*leu-3*; ΔNCU09655*a*(97))+(*his-3*;NCU09655*A*(78))]	Yes
28	[(*leu-3*;NCU09655*a*(96))+(*his-3*;NCU09655*A*(78))]	Yes

Results and Discussion

In primarily, we screened for heterokaryon incompatibility, heterokaryons homokaryosis and heterokaryons heterokaryosis of 20 Ca^{2+}-signaling knockout mutants out of 48 merely in Yeast, Peptone and D-glucose (YPD) media, results showed *het* incompatibility. On this foundation, we crossed ΔNCU05225, ΔNCU06366, ΔNCU06650, ΔNCU07075, and ΔNCU07966 Ca^{2+}-signaling knockout mutants with the opposite mating types of *leu-3* and *his-3* auxotrophic marker strains (Table1 and Table 2). We found that the result heterokaryons homokaryosis for ΔNCU05225, ΔNCU06366, ΔNCU06650, ΔNCU07075, and ΔNCU07966 mutants overcomes *het* incompatibility, therefore display mating type associate *het* compatibility and form vigours conidia on Petridis (Fig.2, Test: HO, Table4). Whereas, heterokaryons heterokaryosis for ΔNCU05225, ΔNCU06366, ΔNCU06650, ΔNCU07075, and ΔNCU07966 mutants continue to showed mating type associated *het* incompatibility and showed killing of conidia on Petridis (Fig. 2, Test: HO, Test controls: C1 and C2; wild type control: C3; Table4). These results indicate that the knockout mutants of Ca^{2+}-signaling genes NCU05225, NCU06366, NCU06650, NCU07075, and NCU07966 suppressing the mating type associated *het* incompatibility in a recessive manner, and it showed genetically complementation in the heterokaryons of homokaryosis. The Ca^{2+}-signaling genes NCU05225, NCU06366, NCU06650, NCU07075, and NCU07966 encode proteins mitochondrial NADH dehydrogenase, Ca2+/H+ antiporter, Ca^{2+} and /or CaM binding protein a secretory phosholipase A2, CAX Ca^{2+} /H^{+} exchanger, and Calcium transporting ATPase 3 respectively(Table1). We have also tested two additional Ca^{2+}-signaling knockout mutants ΔNCU02283, ΔNCU09655. We found the results in both the cases heterokaryons of homokaryosis and heterokaryons heterokaryosis for ΔNCU02283, ΔNCU09655 mutants display mating type associated *het* incompatibility like the wild-type control (Fig.3.3a,Test:HO, test controls: C1 and C2; wild type control: C3). The Ca^{2+}-signaling gene deletion mutants of these acts as recessive phenotypic expression of mating type associated *het* incompatibility. Therefore showed cell death on Petridis (Fig.3.3a, test: Ho, test controls: C1 and C2, wild type control: C3). This programmed cell death was confirmed by staining with vital dye Evan's Blue (Fig.3 and Fig. 4b, test: HO, test controls: C1 and C2, wild type control: C3). These Ca^{2+}-signaling genes NCU02283 and NCU09655 encodes protein calcium/calmodulin-dependent protein kinase type I and plasma membrane zinc ion transporter, phosphatidylinositol-4, 5-bisphosphate phosphodiesterase gamma 2 respectively(Table1).

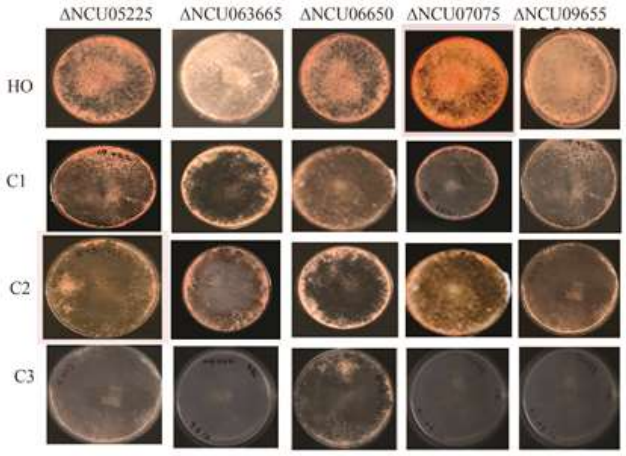


Fig. 2: Ca^{2+}-signaling knockout mutant strains display mating type associated *het* compatibility activity in *N. crassa*.Ho: [(*leu-3*; ΔNCU06650 a (37)) + (*his-3*; ΔNCU06650 *A* (17))];C1: [(*leu-3*; ΔNCU06650 *a* (65)) + (*his-3*; NCU06650 *A* (41))]; C2: [(*leu-3*; NCU06650 *a* (66)) + (*his-3;* ΔNCU06650 *A* (44))]; C3: [(*leu-3*; NCU06650 *a* (66)) + (*his-3*; NCU06650 *A* (41)0]. Here, heterokaryons homozygous for ΔNCU05225, ΔNCU06366, ΔNCU06650, ΔNCU07075, and ΔNCU07966 mutants display *het* compatibility, and C1, C2 and C3 heterokaryons heterozygous for ΔNCU05225, ΔNCU06366, ΔNCU06650, ΔNCU07075, and ΔNCU07966 mutants display *het* incompatibility.

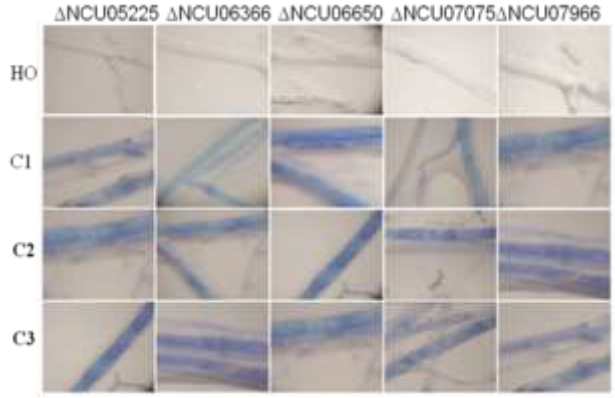
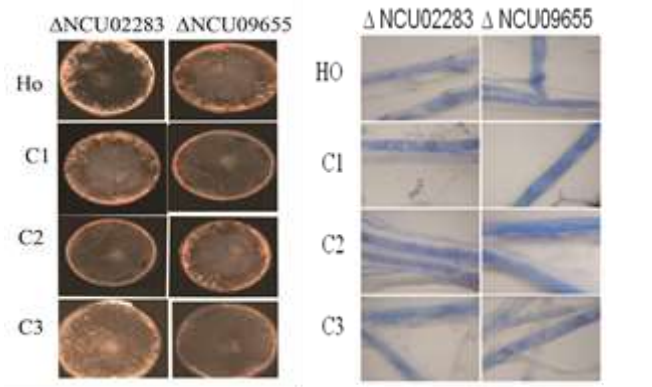


Fig. 3: Microscopic analysis of cell death mediated by heterokaryon incompatible, using Evan's blue staining. Ho: [(*leu-3*; ΔNCU06650 a (37)) + (*his-3*; ΔNCU06650 *A* (17))]; C1: [(*leu-3*; ΔNCU06650 *a* (65)) + (*his-3*; NCU06650 *A* (41))]; C2: [(*leu-3*; NCU06650 *a* (66)) + (*his-3;* ΔNCU06650 *A* (44))]; C3: [(*leu-3*; NCU06650 *a* (66)) + (*his-3*; NCU06650 *A* (41)0]. Here, blue staining indicates killing due to mating type associated *het* incompatibility; dye retains the portion and remaining portion exclude. Heterokaryons homozygous for ΔNCU05225, ΔNCU06366, ΔNCU06650, ΔNCU07075, and ΔNCU07966 mutants display *het* compatibility, therefore dye exclude within the portion.



A B

Fig. 4: Two Ca2+-signaling genes NCU02283, NCU09655 do not supress mating type associated het incompatibility

[a). Mating type associated heterokaryon incompatibility was observed for Ho: [(leu-3; DNCU02283 a (105)) + (his-3; DNCU02283 A (86))]; C1: [(leu-3;NCU02283 a (104))+(his-3; DNCU02283 A(86))]; C2: [(leu-3; DNCU02283 a (105)) + (his-3; NCU02283 A (85))]; C3: [(leu-3; NCU02283 a (104)) + (his-3; NCU02283 A (85))]; similarly for DNCU09655. Here, both the cases heterokaryons homozygous and heterozygous for □NCU02283, □NCU09655 mutants display het incompatibility. b). Microscopic analysis of heterokaryon incompatibility, using Evan's blue staining. Ho: [(leu-3; DNCU02283 a (105)) + (his-3; DNCU02283 A (86))]; C1: [(leu-3; NCU02283 a (104)) + (his-3; DNCU02283 A (86))]; C2: [(leu-3; DNCU02283 a (105)) + (his-3; NCU02283 A (85))]; C3: [(leu-3; NCU02283 a (104)) + (his-3; NCU02283 A (85))]; similarly for DNCU09655. Here, blue staining indicates killing due to mating type associated het incompatibility.]

The Ca^{2+}-signaling knockout mutants were isolated from progeny by *hph* screening method, and we used strategy to test involvement of Ca^{2+}-signaling genes in mating type of associate heterokaryons incompatibility. These Ca^{2+}-signaling knockout mutants were confirmed by PCR (Fig. 5), using PCR primers (Table 5) and Southern hybridization methods (Fig. 5, 6). The Ca^{2+}-signaling gene deletion mutants of these five acts as recessive suppressors of mating type associated *het* incompatibility; and result reveals their role in mutant's recessive phenotypes of mating-type-associated heterokaryon incompatibility in *N. crassa.*

Table 5: List of Primers used in this study

S.NO	Gene	Primers
1	NCU02283	5F' AGG AGA AGT CTG AGA AGA GG 5R' CTA GAG GCA CCG TAC TAT CG
2	NCU05225	5F' TGT GAT TCA GGA TGT GGA GG 5R' GTT AGT GCA GCC AGT AAA GG
3	NCU06366	5F' CGG TAC ACT TGG TAA AGA GG 5R' AGT TGT AGA CAG GTA GGT GG
4	NCU06650	5F' TAC CTT ACC CAC CAG TAA CG 5R' CCT TCT CTT CTA TGT GCC AG
5	NCU07075	3F' GAG GTT TTC TGG TAG GGA GC 3R' GTA CCT CTA CCT AGC CTT GC
6	NCU07966	5F' AAG TTG AGT GTT CGT CCT CC 5R' GGT TCT TCT GTT CCT GTT CC
7	NCU09655	5F' CTG ACA GAG ATC TTG GAA GC 5R' GAT GAC TGA TGA CTG TGA CC

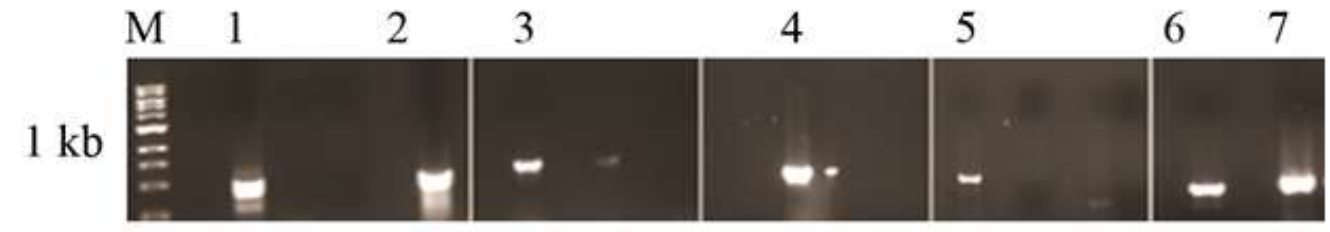


Fig. 5: PCR amplification of Ca^{2+}-signaling genes to confirm the knockout mutants;1) ΔNCU05225, 2)ΔNCU06366,3)ΔNCU06650, 4)ΔNCU07075, 5)ΔNCU07966, 6)ΔNCU02283; and 7)ΔNCU09655. Here, 'M' is1kb (NEB) marker.

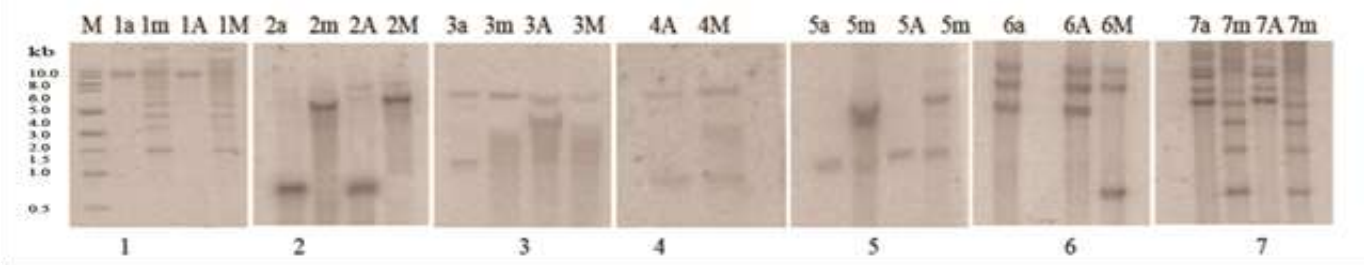


Fig. 6: Ca^{2+}-signaling knockout mutant's confirmation by Southern hybridization**.** 1)ΔNCU02283, 2) ΔNCU05225, 3) ΔNCU06366, 4) ΔNCU06650, 5) ΔNCU07075, 6) ΔNCU07966 and 7) ΔNCU09655 were digested with *NcoI, Sal I, Sac I, Sac I, Hind III, Hind III* and *Xma I* respectively, and resolved using of 1% agarose DNA was fragmented(Table3) . Here,'M' is 1kb (NEB) marker; 1(Wild types mating types 1*m* and 1*M*; mutant mating types 1*a* and 1*A*) 2, 3, 4, 5, 6 and 7 respectively. All probes obtained by gene specific PCR amplification with respective primers (except ΔNCU06366, ΔNCU06650 knockout probe, using primer *hph* R) 5F - 5R, 5F -5R, 5F - hphR, 5F - hphR, 3F - 3R, 5F - 5R, and 5F - 5R (Table4). Note that fragment resulting, respectively, from the digestion at the right and middle and the middle and left restriction sites of the intact gene is replaced in the disruption by the size of fragments duo to loss of the middle sites from the disrupted genes. Although the disruption bands is not seen as prominently in the DNA from wild type both mating types (1*m* and 1*M*), it is seen in its hygromycin-resistant mutant both mating types (1*a* and 1*A*).

Discussion

The found results showed heterokaryons for homokaryosis ΔNCU05225, ΔNCU06366, ΔNCU06650, ΔNCU07075, and ΔNCU07966 of suppression of mating type associated *het* compatibility(Fig.3.1, HO, Table4); and heterokaryons for heterokaryosis ΔNCU05225, ΔNCU06366, ΔNCU06650, ΔNCU07075, and ΔNCU07966 mutants display *het* incompatibility like the wild-type control (Fig.3.1, test: HO, test controls: C1 and C2, wild type control: C3;Table4). That is, HO [(ko1;mat A) +(ko1;mat a)] are viable, whereas controls [(ko1+; mat A) + (ko1;mat a)] and [(ko1+; mat A) + (ko1+; mat a)] are inviable, likewise, for controls C1,C2,and C3 (Fig. 2, test: HO, test controls: C1 and C2, wild type control: C3;Table4). Therefore NCU05225, NCU06366, NCU06650, NCU07075, and NCU07966 Ca^{2+}-signaling genes have some significant role in *het* compatibility. An additionally, same approach, we tested two more Ca^{2+}-signaling knockout mutants ΔNCU02283, ΔNCU09655 as control, for confirming of whether all calcium signaling genes showed heterokaryons compatibility (i.e. genetically complementation) or not. In this case two Ca^{2+}-signaling knockout mutants ΔNCU02283, ΔNCU09655 showed results heterokaryons for homokaryosis and heterokaryosis ΔNCU02283, ΔNCU09655 displayed *het* incompatibility (*vic*) as like the wild-type control (Fig. 4a, test: HO, test controls: C1 and C2, wild type control: C3). The microscopic analysis of the heterokaryons involving ΔNCU05225, ΔNCU06366, ΔNCU06650, ΔNCU07075, and ΔNCU07966 mutants were confirmed by using the vital dye Evan's blue, as well ΔNCU02283, ΔNCU09655 knockout mutants. Which revealed neither hyphal compartmentation nor cell death showed for the heterokaryon homokaryosis (Fig. 3, test: HO), whereas in heterokaryon heterokaryosis showed cell death. During the process of *het* incompatibility, some organelle modifications occurred like septal plugging, vacuolization, shrinkage of plasma membrane, organelle degradation, DNA fragmentation(Glass, 2006; Marek *et al.*, 2003) (Fig. 1), and accumulation of lipid bodies are common microscopic features associated with *het* incompatibility. Blue staining retaining was indication of killing due to mating type associated *het* incompatibility (Fig.3 and 4b, test: HO, test controls: C1 and C2, wild type control: C3), and live cells portion exclude the dye (Fig.3, test: HO). Both mating types are essential for sexual development, however, coexistence during the vegetative phase is lethal, and therefore display incompatibility (Fig. 1) (SAUPE, 2000; Garnjobst, *et al.*, 1956). The calcium signaling genes NCU05225 encode a 674aa residues of mitochondrial NADH dehydrogenase, NCU06366 encodes a 505aa residues of Ca2+/H+ antiporter, NCU06650 encode a of 186aa residues of novel Ca^{2+} and /or CaM binding protein, a secretory phosholipase A2, NCU07075 encode a of 508 aa residues of CAX that is a Ca^{2+}/H^{+} exchange, and NCU07966 encodes a 1110aa residues of calcium transporting ATPase 3 (Table 1) respectively. In additionally other two Ca^{2+}-signaling genes encodes proteins like NCU02283 encode a 467aa residues of calcium/calmodulin-dependent protein kinase type I, and NCU09655 encodes plasma membrane zinc ion transporter(598aa) and phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma 2(625aa)(Table 1) respectively. This calcium signaling genes encode protein might be involved in heterokaryons incompatibility process and further needs to be studied at the molecular level in *N. crassa*. Therefore, the result indicates suppression of mating type associated *het* incompatibility is not common phenomenon for all the Ca^{2+}-signaling knockout mutants in *N. crassa*.

Conclusion

Previous reports suggest that the nuclei from two different genotypic strains are incompatible within the same cytoplasm (Garnjobst *et al.*, 1956; Saupe, 2000). In *N. crassa* at least 11 het loci exist. Five of them, the mating type locus and het-c, -d, -e and –I were originally identified using forced heterokaryons between nearly isogenic strains. Here, we reported five Ca^{2+}-signaling knock mutant strains ΔNCU05225, ΔNCU06366, ΔNCU06650, ΔNCU07075, and ΔNCU07966 play important role in the mating-type-associated *het* incompatibility in *N. crassa*(Fig.2, test: HO, test controls: C1 and C2, wild type control: C3), additionally tested two more Ca^{2+}-signaling knockout mutants ΔNCU02283, ΔNCU09655; in both the condition like heterokaryons homokaryosis and heterokaryosis of ΔNCU02283, ΔNCU09655 mutants display *het* incompatibility like the wild-type control(Fig. 4a, test: HO, test controls: C1 and C2, wild type control: C3). In *N. crassa* heterokaryon compatibility is shown by strains with identical genotypic class of progeny, whereas different genotypes of progeny are *het* incompatible. But the five Ca^{2+}-signaling knockout mutant strains are *het* compatible suggesting that the knockout Ca^{2+}-signaling gene may interact with the genes in *het* domain that result in induced mating type-associated *het* incompatibility in *N. crassa*(Fig.1.1 sup.info). Therefore, the mutants of heterokaryons homokaryosis identical *het* loci specificity displayed development rate, normal conidiation, and aerial hyphae formation, suggesting that the mutation was compatibility (Fig.2, HO) and heterokaryotic homokaryosis auxotrophic mutant strains growth in limited nutrient condition. Whereas heterokaryons heterokaryosis different *het* loci specificity displayed decrease in the development rate like lack of growth, normal conidiation, and aerial hyphae formation, suggesting that the mutation was incompatibility (Fig. 2, and Fig. 4a, test: HO, test controls: C1 and C2, wild type control: C3).

During the time of experiment setup, we have taken more care in mixing of conidial cells from other conidial strains contamination, and we verified specific knockout mutants mating type as well. We reported here the involvement of Ca^{2+}-signaling gene in heterokaryon incompatibility as a phenotypic expression in *N. crassa* not yet reported and further needs to be studied at the molecular level.

Acknowledgement

I thank my senior colleagues Ravi Kumar & Rekha for performing of Southern hybridization at CCMB, Hyderabad, India. I thank Fungal Genetics Stock Center (FGSC), University of Missouri, Kansas City, USA, for providing strains for free of charges; as well DBT, DST,MHRD and IITG for financial support. We are not paying attention on conflict of interest.

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