



A ROLE OF ENVIRONMENTAL FACTORS ON CALCIUM SIGNALING GENES IN *NEUROSPORA CRASSA*

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ABSTRACT

Neurospora crassa possesses a complex of Ca^{2+} -signaling system consisting of 48 Ca^{2+} -signaling proteins. The Ca^{2+} -signalling protein plays an important role in a range of processes such as a Ca^{2+} stress tolerance, hyphal tip branching growth, cytoskeletal organization, cell cycle progression, circadian clocks, sporulation, sexual development, and ultraviolet (uv) survival. The environmental factors, broadly defined to include chemical, physical, nutritional, and behavioral factors...etc. In this article, we are reporting here a role of physico-chemical environmental factors pH, glucose and ultraviolet (UV) affect on $\Delta\text{NCU06366}$, and $\Delta\text{NCU05225}$ Ca^{2+} -signaling knockout mutants in *N. crassa*. The verified result showed that, $\Delta\text{NCU06366}$ and $\Delta\text{NCU05225}$ Ca^{2+} -signaling knockout mutants slower growth rate at pH (7.6), and glucose starvation against to the control wild type respectively. In addition to that the found results showed, ultraviolet (UV) survival, there is no UV radiation affects on $\Delta\text{NCU06366}$ and $\Delta\text{NCU05225}$ Ca^{2+} -signaling knockout mutants as evaluate to the positive and the negative controls in *N. crassa*. Along with that, In-silico analysis Multiple sequence analysis and Phylogenetics tree for conserve domain of NCU05225 (NADH dehydrogenase) and NCU06366 ($\text{Ca}^{2+}/\text{H}^{+}$ anti-porter) Ca^{2+} -signaling genes encodes proteins in *N. crassa*, showed high sequence similarity and 68-100% and 89% homology to the other class of fungi respectively. It indicates that, NCU05225 (Mitochondrial NADH dehydrogenase) and NCU06366 ($\text{Ca}^{2+}/\text{H}^{+}$ exchangers) Ca^{2+} -signaling gene encoding conserve domain widespread in other class of fungi as well.

Key words: *Neurospora crassa*; Ca^{2+} -signaling genes; *Neurospora crassa* unit number (NCU); ultraviolet (UV); Glucose; knockout mutants

Academic Discipline And Sub-Disciplines

Environmental Biology

SUBJECT CLASSIFICATION

Calcium signaling genes

TYPE (METHOD/APPROACH)

Regular Article

Neurospora crassa possesses a complex of calcium (Ca^{2+})-signaling system, that appears to be significantly different from plant and animal cells; especially in relation to the second messenger systems responsible for Ca^{2+} -release from internal stores (Galagan et al., 2003). The genome analysis of *Neurospora* has been revealed three Ca^{2+} -channel proteins, nine Ca^{2+} /cation-ATPases, six recognizable $\text{Ca}^{2+}/\text{H}^{+}$ exchangers, two novel putative $\text{Ca}^{2+}/\text{Na}^{+}$ exchangers, four novel phospholipase C-d subtype (PLC-d) proteins, 23 Ca^{2+} and/or calmodulin (CaM) binding proteins, and one CaM (Galagan et al., 2003; Borkovich et al., 2004). The Cytosolic Ca^{2+} plays a central role as an intracellular signal, however, high concentrations of Ca^{2+} are toxic to the cell, and therefore, cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) is effectively regulated in *Neurospora*, *Arabidopsis*, and human (Cornelius and Nakashima, 1987; Sanders et al., 2002; Berridge et al., 1998).

The yeast *Saccharomyces cerevisiae* has emerged as a major model system for eukaryotic V-ATPases, the importance of V-ATPases for acidification of the vacuole/lysosomes, Golgi apparatus, and endosomes of eukaryotic cells is well established (Forgac, 2007; Kane, 2006). The multiple cellular processes, including the secondary transport of ions and the metabolites, maturation of iron transporters, endocytic and biosynthetic protein sorting, and zymogen activation depend on compartment acidification and have been linked to V-ATPase activity (Forgac, 2007; Klionsky et al., 1990). The V-ATPases function with Pma1p, an essential P-type of proton pump localized to the plasma membrane, to help control pH (Serrano et al., 1986; Serrano, 1991). The microbial growth is determined by as a function of temperature, pH, and water activity (aw) (Mc Meekin et al., 1993). In fact, the optimal pH range was found at pH 3.5 to 5, and at pH 2.5 is showing 30% reduction in the growth rate (Jeanne et al., 1999). In Yeast *vma* mutants exhibit a set of distinctive phenotypes, it showed inability to grow at pH values (<3 or >7) and sensitivity to high extracellular calcium concentrations (Kane, 2006).

Glucose, the preferred carbon source for *S. cerevisiae*, is metabolized through fermentation and respiration. The yeast cells produce huge amounts of carbonic and organic acids by using energy metabolism as a glucose, and these constitute utilized protons as a main source (Serrano, 1991; Conway and Brady, 1950). The *N. crassa* grows in the medium containing low level of glucose, and which is catalyzed the accumulation of L-Sorbose against to the concentration gradient (Scarborough, 1970), it could be an employ unique strategy for carbon assimilation and glucose regulation of gene expression (Monod, 1950; Novick and Szilard, 1950; Beck and Meyenburg, 1968; Springer, 1993). The glucose addition to glucose-starved *S. cerevisiae* cells, it triggers a quick and transient influx of calcium from the extracellular environment (Silvia Groppi et al., 2011). The microbial balanced growth requires the coordination of nutrient assimilation, energy generation, biosynthesis, and the cell division cycle (Monod, 1950; Novick and Szilard, 1950; Beck and



Meyenburg, 1968; Rhee 1973; Senn et al., 1994). Glucose stimulates Pma1 H₂O-ATPase activity by inducing phosphorylation (Lecchi et al., 2007), the result is a decreasing Km for ATP and an increased Vmax, and V-ATPases are also activated by glucose (Kane, 1995; Parra and Kane, 1998; Eraso et al., 2006; Portillo et al., 1991). In *N. crassa*, glucose starvation induces asexual sporulation (conidiation), and mutation of a putative glucose sensor (RCO-3), leads to conidiation in glucose-rich liquid cultures (Madi et al., 1997).

The process of DNA repair has been extensively studied in *N. crassa*; where the three genes such as *upr-1* (*ncrev3*), *mus-42* (*ncrev1*), and *mus-26* (*ncrev7*) proved to be induced by DNA damage and function in the mutagenic translesion DNA synthesis (TLS) pathway (Sakai et al., 2002; Sakai et al., 2003). The versatility of Ca²⁺-signaling proteins, Ca²⁺-Calmodulin binding protein (CaM) playing a crucial role in modulating of DNA repair, DNA synthesis, and cell proliferation in the human cell lines (Chafouleas et al., 1984; Pavelic, 1987; Chard 1987; Mirzayans et al., 1995), along with that mediating the effects of calcium concentration [Ca²⁺]_c in the cell (Chafouleas et al., 1984; Pavelic, 1987; Chard 1987; Mirzayans et al., 1995). The sensitivity of mutagens is observed many of the repair-defective rad mutants, when the exposure of ultraviolet radiation (uv) (Brychcy, 1974; Brychcy and Borstel, 1977; Hasting et al., 1976; Borstel et al., 1971; Inoue and Ishii, 1984; Kato and Inoue, 2006). When the strains of yeast having enhanced mutation rates were found that a minimum of 10 genetic loci were responsible for the effect of ultraviolet radiation (Hasting et al., 1976; Ord, 1980), most of these mutant strains are sensitive to mutagenic agents such as ultraviolet radiation (uv), gamma radiation and methyl methanesulfonate (mms) (Brychcy, 1974; Brychcy and Borstel, 1977; Hasting et al., 1976; Borstel et al., 1971). However, it is still not known whether and how CaM or any other Ca²⁺-signaling protein plays a role in DNA damage repair process in *N. crassa*.

On this basis, we setup an experiment for verification of environmental factors such as pH, Glucose and ultraviolet radiation (uv) affect on Δ NCU05225, Δ NCU06366, Δ NCU06650, Δ NCU07075, and Δ NCU07966 Ca²⁺-signaling knockout mutants. The verified initially screened results showed environmental factors plays role on Δ NCU05225 and Δ NCU06366 Ca²⁺-signaling knockout mutants in *N. crassa*. In addition to that, we did computational analysis on Multiple sequence analysis and Phylogenetics tree analysis for NCU05225 (Mitochondrial NADH dehydrogenase) and NCU06366 (Ca²⁺/H⁺ exchangers) Ca²⁺-signaling genes encoding proteins, for authentication, how much these conserve domains showed to sequence similarity and homology with other class of fungi.

Materials and methods

Strains, growth, and crosses

The wild-type and Ca²⁺-signaling mutant strains were obtained from the Fungal Genetics Stock Center (FGSC), University of Missouri, Kansas City, MO 64110 (McCluskey, 2003). The Ca²⁺-signaling mutants were generated using a high-throughput gene knockout procedure, developed by the Neurosporagenome project (http://www.dartmouth.edu/neurosporagenome/proj_overview.html; Colot et al., 2006). The growth, crossing, and maintenance of Neurospora strains were essentially as described by Davis and De Serres (1970). The growth was initially measured by placing either conidia or a plug of agar containing mycelium in the center of a Petri dish and colony diameter was measured every 2–3 hrs to obtain linear rates of diameter increase over a period of 28 hrs. The strains that show lower growth rate on Petri dish were further analyzed by using standard race tube assay (Ryan et al., 1943; Ryan, 1950). The growth rates were calculated as cm/h in both cases.

Verification of UV sensitivity Test

UV sensitivity was essentially as described by Kato and Inoue (1984, 2006). In briefly, the conidia were grown in flasks containing Vogel's glucose medium at 30 °C for 5 days, harvested and assayed for UV sensitivity. For UV dose dependency of the survival of *N. crassa*, conidia were irradiated at various doses of UV and aliquots were sampled and plated after appropriate dilution. UV sensitivity was investigated by spotting a conidial suspension onto an agar plate and irradiating at 50, 100, 150, 200 and 300 J/m². The plates were grown at 30 °C for 3 days in dark place, and number of colonies on each plate was counted.

Sequence analysis

BLAST (Altschul et al., 1990) analysis was performed using software tools available from NCBI (<http://www.blast.ncbi.nlm.nih.gov/Blast.cgi>), the Conserved Domain Database (CCD; Marchler-Bauer and Bryant, 2004; Marchler-Bauer et al. 2009) was used to identify conserved domains in the protein. The protein sequences were aligned with ClustalX 1.83 (Thompson et al., 1997) and transferred to GeneDoc for visualization (Nicholas et al., 1997). The phylogenetic trees were constructed from these alignments by using the minimum-evolution method (Rzhetsky and Nei, 1992), with 500 bootstrap value replications as a test of phylogeny (Felsenstein, 1985) and the software MEGA5.05 (Tamura et al., 2007).

RESULTS

We have verified the role of environmental factors affect on Δ NCU05225, Δ NCU06366, Δ NCU06650, Δ NCU07075, and Δ NCU07966 Ca²⁺-signaling knockout mutants in *N. crassa*. These Ca²⁺-signaling knockout mutants has been showed results interestingly on heterokaryon incompatibility in *N. crassa* (R. Gedela and R. Tamuli (2015)). On this basis we have checked for what could be the environmental factors affect on Δ NCU05225, Δ NCU06366, Δ NCU06650, Δ NCU07075, and Δ NCU07966 Ca²⁺-signaling knockout mutants. The evaluated results showed at pH-7.6 for NCU05225, and



NCU06366 Ca^{2+} -signaling genes slower growth rate against to the control wild type (Fig.3.2), and we verified for 72hrs in Race Tube experiment (Fig.3.3) as well. While, at pH-5.8 for $\Delta\text{NCU06366}$, and $\Delta\text{NCU05225}$ Ca^{2+} -signaling knockout mutants, showed as a normal growth as like control wild type (Fig.3.1), there is no growth rate affect on NCU05225, and NCU06366 Ca^{2+} -signaling genes in *N.crassa*; that's why at this pH-5.8, all the conidia cells grows as like wild type. The verification of chemical factor such as Glucose affect on $\Delta\text{NCU06366}$, and $\Delta\text{NCU05225}$ Ca^{2+} -signaling knockout mutants in *N.crassa*, the evaluated results showed slower growth rate in the absence of glucose (Vogel's glucose medium: Glucose -1.5% w/v) (Fig.4.1, Fig.4.2). Where as in the presence of glucose in media (VG+Glu, Vogel's glucose medium: Glucose -1.5% w/v), all the conidial cells grows linearly on Petri dish (Fig.4.1, Fig.4.2), the conidial cells, showed a vigorous growth rate as like control wild type (Fig.4.2) in *N.crassa*. In addition to that testing of another environmental factor such as ultraviolet (UV), the exposure of UV radiation energy doses for 10 minutes (0Jm^{-2} , 100Jm^{-2} , 200Jm^{-2} and 300Jm^{-2}) on NCU05225, NCU06366 Ca^{2+} -signaling genes in *N.crassa*, the results showed UV survival at 100Jm^{-2} to 200Jm^{-2} (Fig.5), against to the Wild type as a positive control (which is resistance to the exposure of UV doses at 150 or 300Jm^{-2}) and Rev-3, as a negative control (which is sensitive to the exposure of UV doses at 150 or 300Jm^{-2}) (Fig.5) (Inoue and Ishii, 1984; Chard 1987; Mirzayans et al., 1995; Kato and Inoue, 2006). In this study, we exposed UV radiation energy doses up to 100Jm^{-2} , 200Jm^{-2} and 300Jm^{-2} , till conidia grow on the Petri dish (Fig.5). When we exposed to UV radiation at 150Jm^{-2} to 300Jm^{-2} energy for 10 minutes, all the conidial has to die, if they are sensitive to UV radiation (Inoue and Ishii, 1984; Kato and Inoue, 2006; Chard 1987; Mirzayans et al., 1995). Here we found the results showed UV survival (Fig.5). Thus, these results suggested to that NCU05225 and NCU06366 Ca^{2+} -signaling genes showed UV resistance (Fig.5). In *N.crassa*, NCU05225 (Mitochondrial NADH dehydrogenase, 674 aa) and NCU06366 ($\text{Ca}^{2+}/\text{H}^{+}$ exchangers, 505 aa) Ca^{2+} -signaling genes encodes a conserve domains respectively (Table1). A computational analysis for conserve domains studies for Multiple sequences alignment and Phylogenetics tree, the evaluated results showed sequence with high similarity (Fig.6b, Fig.7b) and 68-100% and 89% homology (Fig.6a, Fig.7a) with other class of fungi respectively. These results suggested to that NCU05225 and NCU06366 Ca^{2+} -signaling genes encoding functional unit wide spread in other class of fungi as well (Fig. 6a, 6b, Fig.7a, 7b).

These Ca^{2+} -signaling knockout mutants were confirmed by PCR (Fig.1) and Southern hybridization methods (Fig.2) by using PCR primers (Table2), Restriction enzymes Sal I and Sac I (Table3) respectively. The NCU05225, NCU06366 Ca^{2+} -signaling genes, deletion mutants of these two acts as their role in recessive manner of phenotypic expression on availability of environmental factors.

Discussion

The Ca^{2+} -signaling genes of NCU05225, and NCU06366 in *N.crassa*, the evaluated verified results showed slower growth rate at pH-7.6 (Fig.3.1, 3.2 and 3.3), whereas, there was a normal growth takes place at pH-5.8. The reviving result tells about, this Ca^{2+} -signaling knockout mutant's requires an acidic condition, while basic or alkaline condition, that showed adversely affect to their growth. The optimal pH range was found to be pH 3.5 to 5, and at pH 2.5 showed 30% reduced in the growth rate (Jeanne et al., 1999; Mc Meekin et al., 1993), as well includes the inability to grow at pH values lower than 3 or higher than 7 and sensitivity to high extracellular calcium concentrations (Kane, 2006). In this connection, two Ca^{2+} -signaling genes NCU05225, and NCU06366 in *N.crassa*, showed slower growth rate at pH-7.6. Therefore, these NCU05225, and NCU06366 Ca^{2+} -signaling knockout mutant strains are acidophilic nature. The Ca^{2+} -signaling genes for NCU05225, and NCU06366, for verification of Glucose, in the absence of sugar in the media, showed slower growth rate (Fig.4.2 and 4.1), against to the presence of sugar in the media, in this circumstance increased linearly growth rate was observed on the Petri dish (Fig.4.1 and 4.2). When the nutrient availability falls to the microorganisms, the point of impair biosynthetic activity takes place and then decreasing their growth rate (Monod, 1950; Novick and Szilard, 1950; Beck and Meyenburg, 1968; Rhee, 1973; Senn et al., 1994). The glucose concentration at which half-maximum glucose uptake occurs markedly different depending upon whether cells have been grown in the presence of high or low levels of glucose (Monod, 1950; Novick and Szilard, 1950; Jeanne et al., 1999; Mc Meekin et al., 1993; Scarborough, 1970). In addition to that, for verification of ultraviolet (UV), the exposure of UV energy doses for 10 minutes (0Jm^{-2} , 100Jm^{-2} , 200Jm^{-2} and 300Jm^{-2}) on NCU05225, NCU06366 Ca^{2+} -signaling genes in *N.crassa*, the evaluated results showed UV survival at 100Jm^{-2} to 200Jm^{-2} radiation energy, against to the positive control and the negative control (Fig.5). The Ca^{2+} -modulated protein, CaM might be playing an important role in modulating DNA repair (Chafouleas et al., 1984; Pavelic 1987; Chard 1987; Mirzayans et al., 1995). Thus, the Ca^{2+} -signaling genes of NCU05225, and NCU06366, deletion of conserved domain region of these two mutants showed a reduced growth rate at pH 7.6 (Fig.3.1, 3.2 and 3.3), and in the absence of Glucose (Fig.4.1 and 4.2) respectively. Whereas in the presence of glucose and exposure of ultraviolet (UV) radiation, showed illustrate growth rate as like wild type (Fig.4.1 and 4.2; Fig.5). These two Ca^{2+} -signaling genes NCU05225, and NCU06366 deleted mutation of conserved domain region acts as a recessive manner of phenotypic expression (Fig.3.1, 3.2 and 3.3; Fig.4.1 and 4.2; Fig.5) in *N.crassa*.

Conclusion

The evaluated results showed slower growth rate at pH 7.6 (Fig.3.2 and 3.3), and in the absence of Glucose conidial cells showed slower growth rate, against to the presence of Glucose on Petri dish (Fig.4.1 and 4.2). The exposure of ultraviolet (UV), showed there is no effect on conidial cells, that why in all conidia grow on Petri dish (Fig.5). The NCU05225 (Mitochondrial NADH dehydrogenase), and NCU06366 ($\text{Ca}^{2+}/\text{H}^{+}$ exchangers) Ca^{2+} -signaling genes in *N.crassa*, encoded conserve domains, showed high sequence with similarity (Fig.6b and 7b) and 68-100% and 89% homology (Fig.6a and 7a) with other class of fungi. These results suggested that an environmental factors pH, and glucose playing a role for their growth of NCU05225, and NCU06366 Ca^{2+} -signaling genes in *N.crassa*. These results showed initially screening, further need to study at molecular level.



Acknowledgement

I thank my sincere to Dr. Rajan Tamuli dept of Bioscience and Bioengineering , IIT Guwahati and 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, IITG for financial and RGUKT IIT Nuzvid for partial financial support.

Conflict of interest:

The authors declare that, we have no conflict of interest.

Tables

Table1: Ca²⁺-signaling genes used in this study and encode proteins in *N.crassa*

S.No	Ca ²⁺ -signaling genes	No.of amino acid	Encodes Name of Proteins
1	NCU05225	674	Mitochondrial NADH dehydrogenase
2	NCU06366	505	Ca ²⁺ /H ⁺ exchangers (anti-porter)

Note: Ca²⁺-signaling genes encode proteins in *N.crassa*, available in the site (<http://www.broadinstitute.org/annotation/genome/neurospora/>).

Table2: List of Primers used in this study

S.NO	Gene	Primers
1	NCU05225	5F' TGT GAT TCA GGA TGT GGA GG 5R' GTT AGT GCA GCC AGT AAA GG
2	NCU06366	5F' CGG TAC ACT TGG TAA AGA GG 5R' AGT TGT AGA CAG GTA GGT GG

Table3: List of Ca²⁺-signaling knockout mutants obtained intensity band by Southern hybridization

S.No	Gene	R.E	Probe	Wild type bands	Knockout bands
1	NCU05225	<i>Sal I</i>	5F -5R	0.8kb	5.5kb
2	NCU06366	<i>Sac I</i>	5F - hphR	5kb,1.5kb	6kb

Figures:

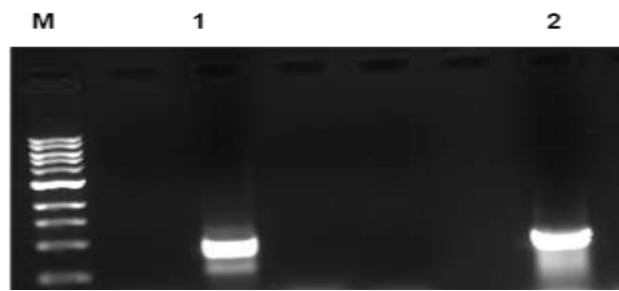


Fig: 1 1) Δ NCU05225, 2) Δ NCU06366; Here, 'M' is 1kb (NEB) marker.

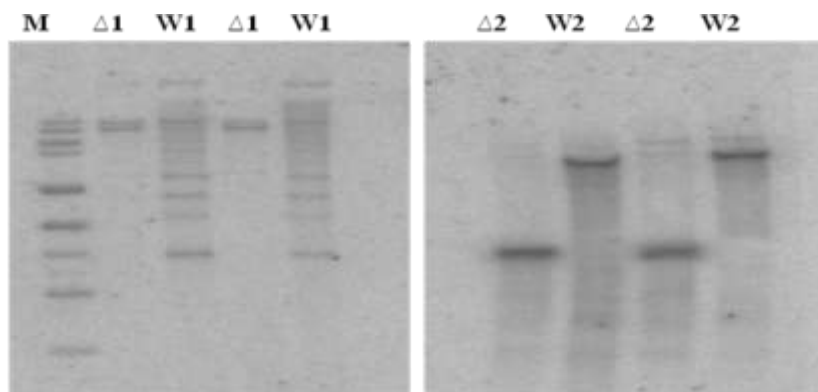


Fig: 2 $\Delta 1$ NCU05225, $\Delta 2$ NCU06366 were digested with Sal I, Sac I; M is 1kb (NEB) marker; W1 & W2, wild type.

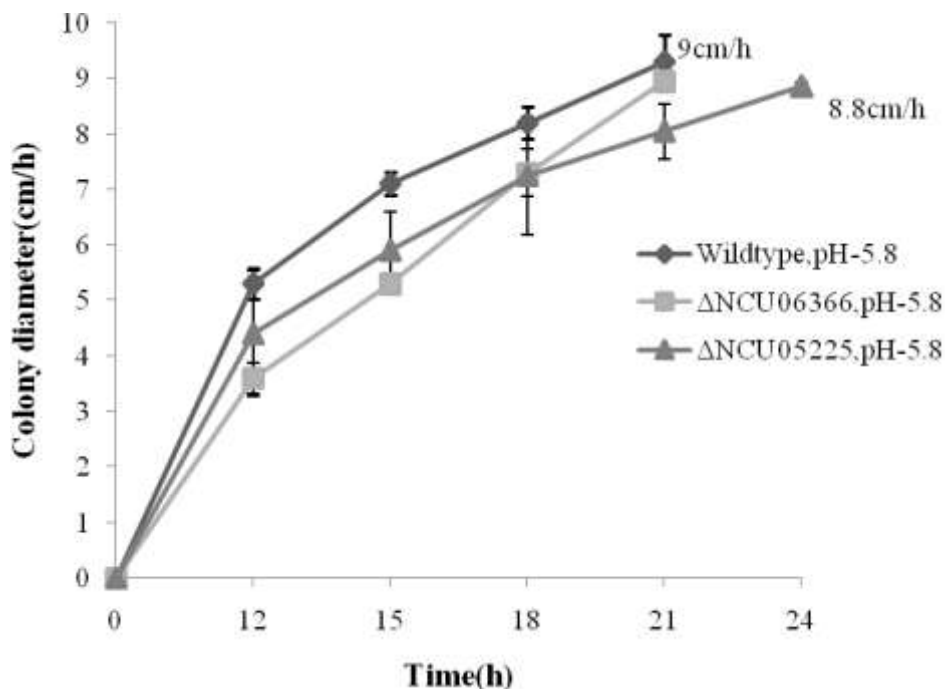


Fig: 3.1 Verification of pH-5.8 effect on Ca^{2+} -signaling knockout mutant's in *N. crassa*, Δ NCU05225, and Δ NCU06366 Ca^{2+} signaling genes showed almost similar growth as compared to that of the wild type.

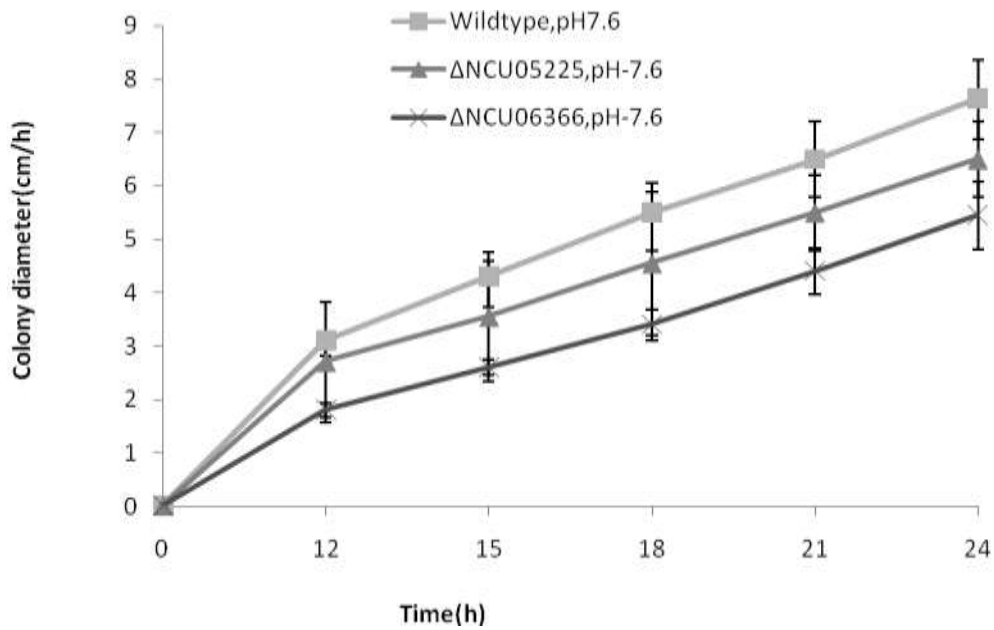


Fig: 3.2 Verification of pH-7.6 effect on Ca^{2+} -signaling knockout mutant's in *N.crassa*, ΔNCU05225, and ΔNCU06366 Ca^{2+} -signaling genes showed slower growth as compared to that of the wild type.

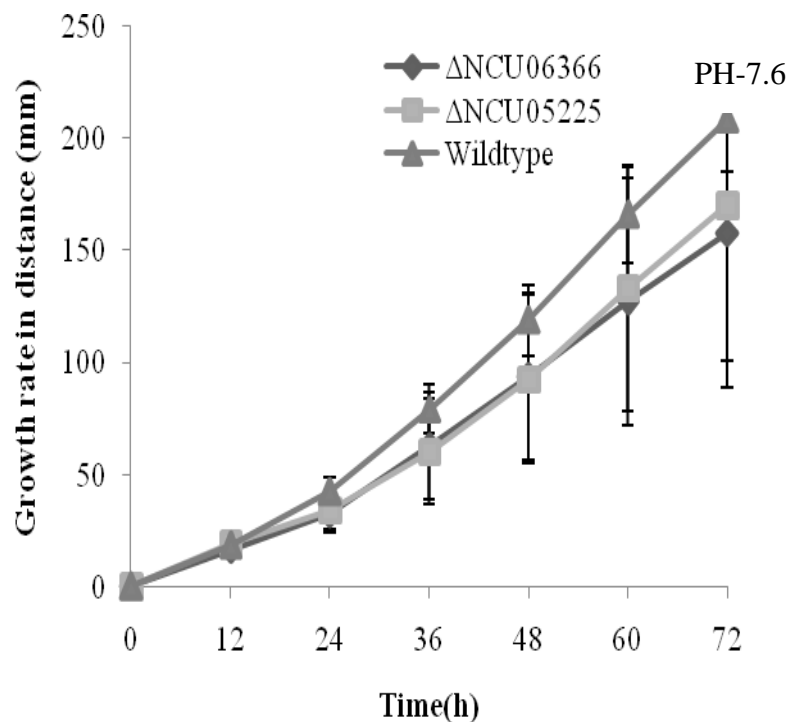


Fig: 3.3 Verification of pH-7.6 effect on Ca^{2+} -signaling knockout mutant's in *N.crassa*, ΔNCU05225, and ΔNCU06366 Ca^{2+} -signaling genes showed slower growth as compared to that of the wild type in Race Tube experiment for 72h.

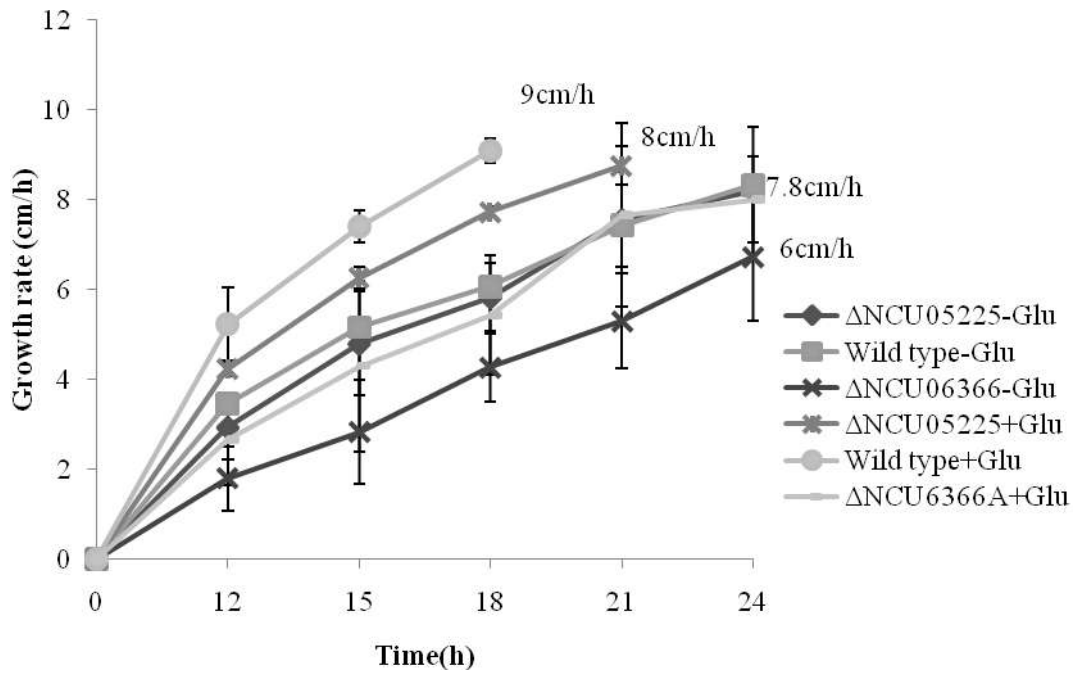


Fig: 4.1 Verification of glucose on Δ NCU05225, and Δ NCU06366 Ca^{2+} -signaling genes, showed slower growth rate in a absence of glucose.

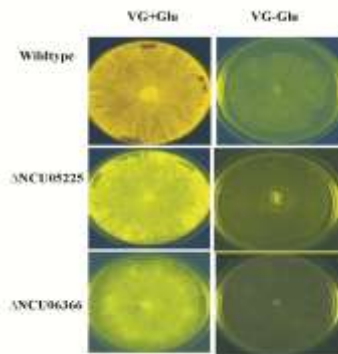


Fig: 4.2 Verification of glucose on Δ NCU05225, and Δ NCU06366 Ca^{2+} -signaling genes, showed slower growth rate in the absence of glucose.

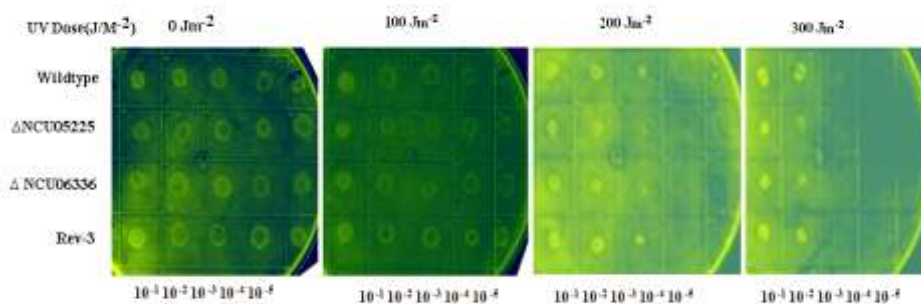


Fig: 5 Verification of UV (Jm^{-2}) radiations affect on NCU05225, and NCU06366 Ca^{2+} -signaling genes in *N. crassa*

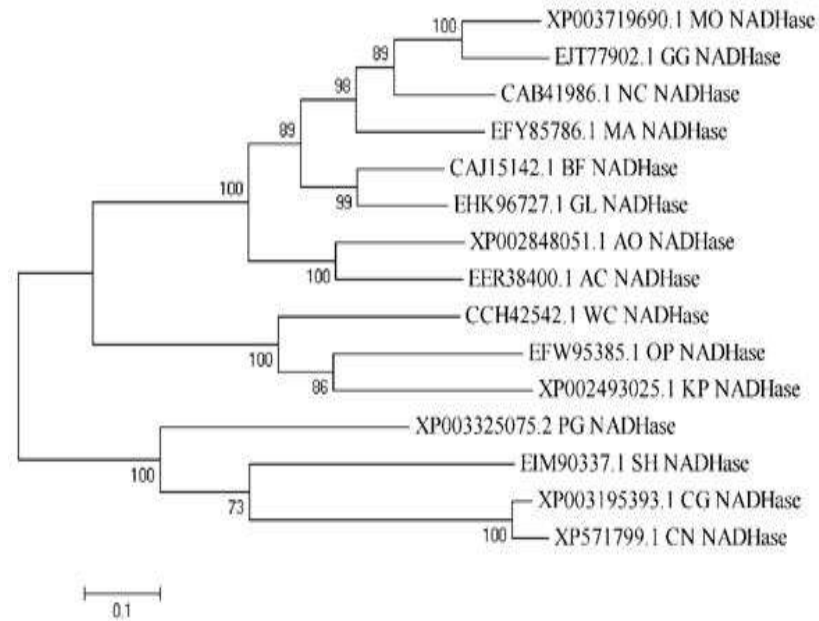


Fig: 6a Phylogenetics analysis of NCU05225 Ca^{2+} -signaling genes encode protein NADHase in *N.crassa*; using the minimum-evolution method, 500 Bootstrap replications (bootstrap values are indicated in the point at nodes) as test of phylogeny, and the software MEGA5.02

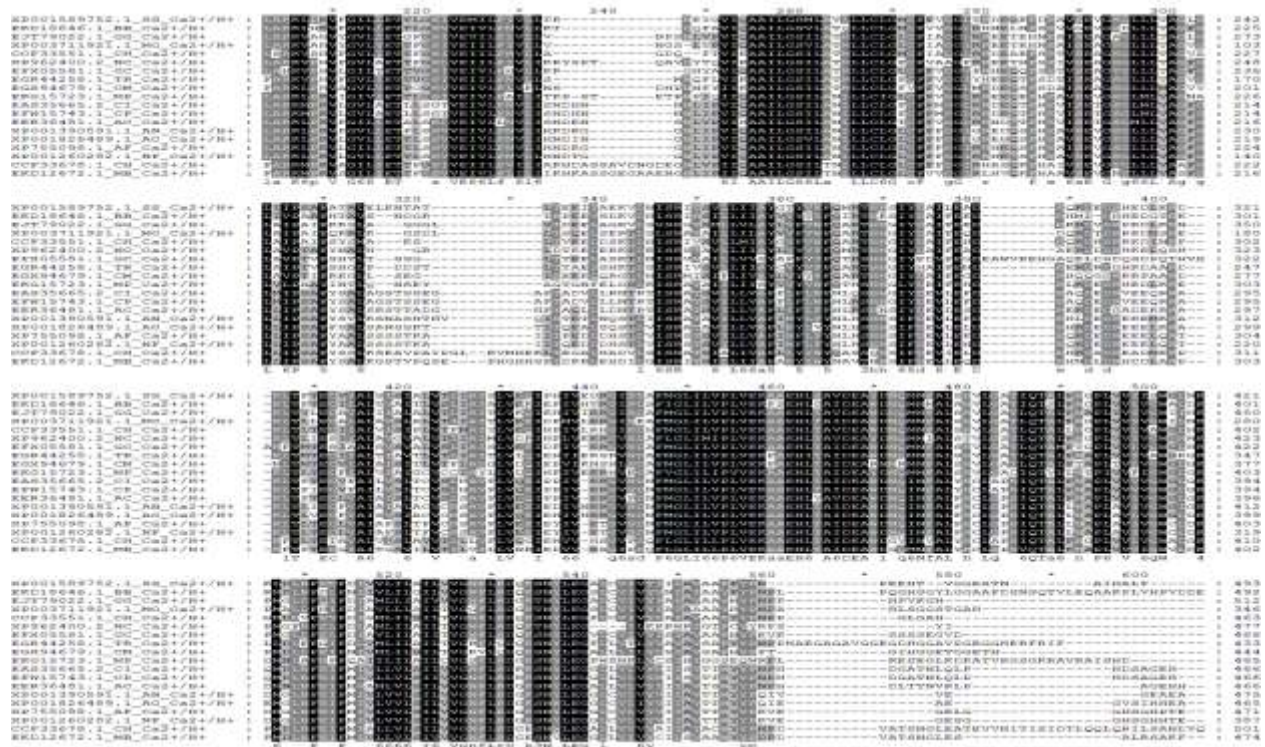


Fig: 6b Multiple sequence of NCU05225 Ca^{2+} -signaling genes encode protein NADHase in *N.crassa* (block color indicates 100% sequence similarity, brown color indicates 80% sequence similarity).

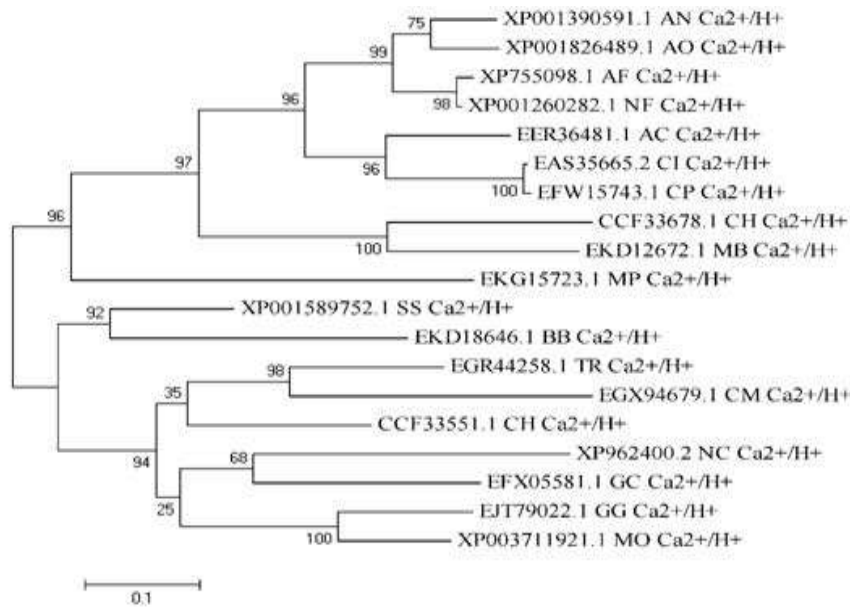


Fig: 7a Phylogenetics analysis of NCU06366 Ca^{2+} -signaling genes encodes protein Ca^{2+}/H^{+} in *N.crasa*; using the minimum-evolution method, 500 Bootstrap replications (bootstrap values are indicated in the point at nodes) as test of phylogeny, and the software MEGA5.02.

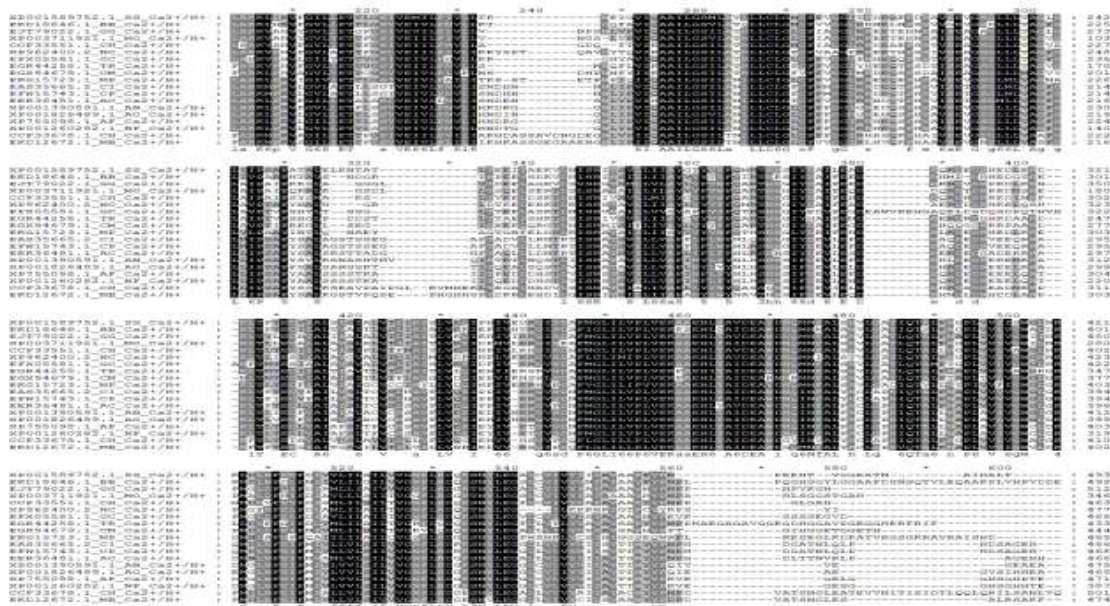


Fig: 7b Multiple sequence analysis of NCU06366 Ca^{2+} -signaling genes encode protein Ca^{2+}/H^{+} in *N.crasa* (block color indicates 100% sequence similarity, brown color indicates 80% sequence similarity).

Reference

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