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ADAPTATIONS OF NITROGEN METABOLITE REGULATOR GENE UNDER AZOLE STRESS IN NEUROSPORA CRASSA

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ABSTRACT: *:Fungal development critically depends on nitrogen source utilization for the production of secondary metabolites in fungi. Nitrogen metabolite regulating gene may reduce stress adaptations in the fungal growth. In Neurospora crassa deletion in the nitrogen metabolite regulating gene retarded the cell growth in reduced nitrogen contents in the culture media. Which proposed the indication of loss of stress adaptations in deletion mutant as compare to the wild type. Nmr loss of function mutant in Neurospora crassa also represses secondary nitrogen regulating genes in fungi. Nitrogen starvation in fungi may lead to the pathogenesis. In Neurospora crassa NCU04158 Nmr mutant induce growth arrest as compare to wild type in ketoconazole stress. Azole sensitive nitrogen repressor mutant in Neurospora crassa accumulated sterol derivative eburicol in fungal membrane under antifungal stress. It can be suggested that Nitrogen metabolite repressor mutant induce eburicol accumulation that reduces the fungal growth in the mutant as compare to Neurospora crassa wild type.*

Keywords: Nitrogen metabolite repressor, pathogenesis, nitrogen starvation, secondary metabolites

1. INTRODUCTION

Fungi exhibit varied adaptations when they are persistently confronted by environmental stressors for example temperature, Ultra Violet rays exposure, extreme pH and nutrient deficiency. Nitrogen is a vital necessity for growth and metabolism. Fungi can inhabit in broad range of nitrogen sources that facilitates in various environmental niches and keep the fungal survival in nutrient scarcity. Ammonia and glutamine are used preferentially because they are the favored nitrogen sources, while gene expression essential to utilize different secondary nitrogen sources exposed in the mechanism called nitrogen metabolite repression (NMR) (Bettina Tudzynski, 2014). Studies suggested that nitrogen regulating proteins has been observed as the virulent factors in basidiomycetes and ascomycetes. The nitrogen metabolite repression (NMR) controller protein shows a significant activity in regulation of the GATA transcription factor AreA by nitrogen metabolism.(Jiang N, Xiao D, Zhang D, Sun N, Yan B, et al. 2009; Caddick, M. X., Peters, D., and Platt, A. 1994)). NmrA interrelates with AreA to inhibit nitrogen catabolic expression of the gene in the existence of ammonium and glutamine; but, NmrA dissociate from AreA, with of less favored type of nitrogen for example nitrate, allowing AreA to trigger the genes expression related to the substituted nitrogen source usage(Lamb HK, Ren J, Park A, Johnson C, Leslie K, et al. 2004;Fernandez et al., 2012; B Tudzynski, 2014). In NmrA the characteristics ther are two motifs a glycine-rich NAD(P)- and another binding motif (GxxGxxG) as well as transformed active site motif (HxxxK) that to be found, alongside the DNA-binding residues (T11, R39, D40 and A45). While, bioinformatics computing specified that the gene was stable in vitro, hydrophilic long with very low disorder ability (Lee, I. R., Lim, J., Morrow, C. A., & Fraser, J. A. 2012; Stammers DK, Ren J, Leslie K, Nichols CE, Lamb HK, et al. 2001).

In the model *Ascomycetes group of fungi*, two positively regulating GATA factors proteins *GLN3* and *GAT1* are present in the genome of the yeast *Saccharomyces cerevisiae* whereas single positively regulating factors *nit-2* and *areA* are present in the genomes of filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* (Dabas N, Morschhauser J 2007;Fu & Marzluf, 1990; Kudla et al., 1990; Minehart & Magasanik, 1991; Magasanik B, Kaiser CA (2002). AreA transcription factor is important nitrogen metabolic gene in *Aspergillus nidulans.* AreA consisted on a C-terminal GATA zinc finger DNA-binding domain which stimulates gene expression in nitrogen acquisition. (Platt A, Langdon T, Arst HN Jr, Kirk D, Tollervey D, et al. (996)AreB is a negatively controlling gene of nitrogen catabolism. (Coffman JA, Rai R, Cunningham T, Svetlov V, Cooper TG 1996) It has similarity with Penicillium chrysogenum NreB and *Neurospora crassa* ASD4 (Wong, Hynes, Todd, & Davis, 2009; Young JL, Jarai G, Fu YH, Marzluf GA 1990). In zoophilic infectious fungi microsporum canis there is another well studied Dnr1 gene have homolog of nitrogen regulatory genes areA from *Aspergillus nidulans* and nit-2 from *Neurospora crassa*. This gene has similar amino acid sequence to the zinc-finger domains of AREA and NIT-2, which codes for 761 amino acid residues containing a single zinc-finger DNA-binding protein (Limjindaporn T, et al 2003; Yamada, Makimura, & Abe, 2006 and Stewart V, Vollmer SJ 1986). This study has been focused on Negative transcriptional regulator protein mutant, which is a GATA type transcription factor modulate the post transcriptional modification. It have a fairly conserved domain in filamentous fungi (Wong KH, Hynes MJ, Todd RB, Davis MA2007;Xiao X, Fu YH, Marzluf GA 1995). Protein functions as repressor of nitrogen metabolite. Nmr gene works with Nit gene as co repressor (Pan H, Feng B, 1997; Marzluf GA 1997). Nmr gene mutation may result in depression of various secondary metabolites regulatory mechanism in the presence of alternative Nitrogen conditions (Lee IR, Chow EW, Morrow CA, et al 2011; Kmetzsch L, Staats CC, Simon E, Fonseca FL, Oliveira DL, et al.2011). But how Nmr respond to the Nitrogen containing conditions under azole stress has not been investigated yet. In order to investigate the adaptations of *Nmr* mutant in azole response. Global azole responsive sterol biosynthesis has been under taken. Mechanism of sterol biosynthesis initiated by the up regulation of the genes based on efflux pumps CDR (family in *Candida albicans*), associated with ABC transporters and

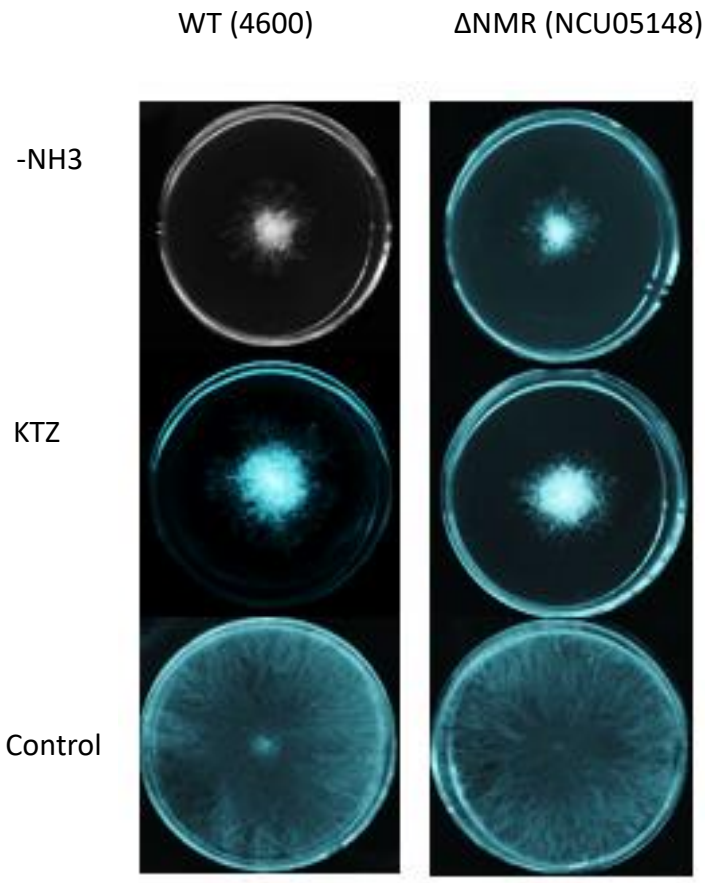

Figure 1. NMR deletion mutant under ketoconazole stress and in (-NH3) reduced Nitrogen contents in vogels media.NMR deletion mutant phenotype hasbeen compared with wild type after the incubation of 5 days at 28°C.

MDR1, that probably formulate the alterations of transcriptional factors. The paper is arranged as following. Section I describes Nmr architecture, section II presents the methods and results, the III section presents the discussion and section V concludes the paper.

1. MATERIAL AND METHODS

2.1 Fungal strains and media conditions

Neurospora crassa Knockout mutant and the wild type strain used in the study was acquired from FGSC (Fungal Genetic Stock Centre) USA. For strain inoculation Vogel's medium have been used with 2% supplemented w/v sucrose for solid medium while 2% glucose for liquid medium for culturing Neurospora crassa and incubated at 28°C (Sun, X., et.al 2014).

2.2 Azole Sensitivity Tests

For the drug sensitivity test ketoconazole has been dissolved in DMSO (dimethyl sulfoxide) and added in autoclaved medium aseptically in the plates. For drug sensitivity test 2µg/ml ketoconazole, was used. Drug sensitivity test was done on the vogel medium after addition of the drugs. 2 µl of the (2×10-6) Neurospora crassa conidial suspension was inoculated on to the middle of the plates and incubated at 28 degree Centigrade. Drug is tested for the drug phenotype test with triplicates (Sun, X., et.al 2014). However, for the role of nitrogen metabolite regulatory. Nitrogen contents were reduced to half in the Vogel's media.

2.3 Sterol Analysis by HPLC-MS

Sterol derivatives have been analyzed with dried crushed mycelia. Mycelial powder was weighed for 0.5g and added into the 1.5ml of chloroform and incubated for 12 h. Dried mycelial mass was subsequently been added with 150ul of methanol and kept on concentrator for 1h again following ultrasonification. Methanol containing extract was filtered through milipore filter and subjected to analysis on HPLC-MS as (Sun, X., Wang, W, et al. 2013)

2.4 RESULTS

We find out a mutant FGSC#14030 confer sensitivity with ketoconazole. NCU04158 is annotated as nitrogen metabolite regulation protein having 488 amino acids. RNA sequencing of the gene in wild type shown 24.57 fold change of transcriptional levels after 12h Ketoconazole treatment as compare to the ketoconazole untreated condition. Nitrogen metabolite regulatory protein is a conserved domain among filamentous fungi and yeast as mentioned in figure 3. It is emerged in the five groups including Eurotiomycetes, Sordariomycetes, Saccromycetes, Eucascomycetes and Eurotiomycetes. (fungi.db.org).

Table 1. Study has been undertaken on two fungal strains

No. of Strain	Wild type	NMR deletion mutant
Srain Number	*Neurospora crassa* (4200)	NCU04158

2.5 Deletion of NCU04158 does accumulate sterol derivatives in Ketoconazole stress in *Neurospora Crassa*

To analyze the availability of abnormal sterol derivatives. The azole target mechanism of sterols biosynthesis was determined by HPLC–MS in wild type and deletion mutant using Ketoconazole. Our findings revealed, eburicol a product of erg6 and substrate of erg11 in the NCU04158 mutant after KTC stress as compare to WT. Increase of eburicol in the membrane with 423 molecular mass a substrate of erg11 (14 α demethylase enzyme in sterol biosynthesis pathway) can disturb the cellular fluidity that leads to the minor growth arrest under ketoconazole stress as mentioned in (Fig.2). Hence it may intended that NCU04158 mutant induce azole adaptibility by accumulating eburicol in the membrane and resulted azole responsive phenotype as indicated in (Fig.1).

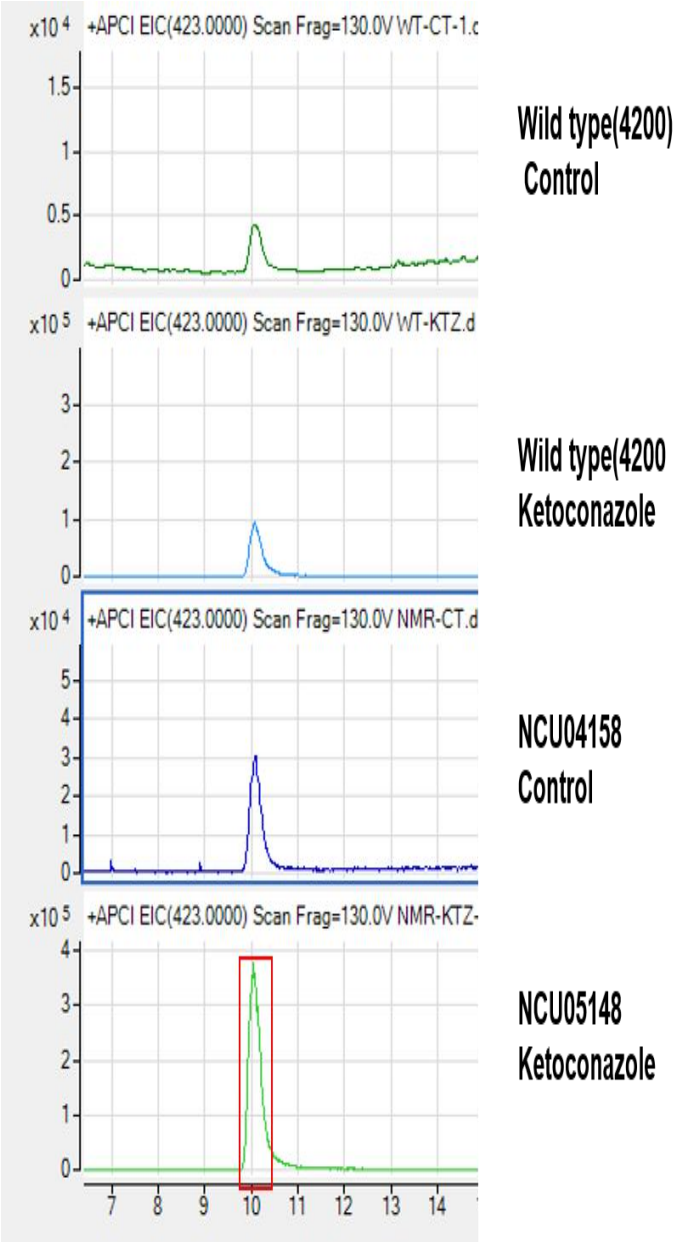

Figure. 2. Deletion of NCU04158 accumulated eburicol in significant levels as compare to *Neurospora crassa* wild type in control and under KTC stress.

3. DISCUSSION

Negative transcriptional regulator protein NCUO4158 is a GATA type transcription factor modulate the post transcriptional modification. It have a fairly conserved domain in filamentous fungi. Gene contain 488 amino acid. It has two binding sites on 165 and 215 position. Protein functions as repressor of nitrogen metabolite. In order to investigate the response of *Nmr* mutant in azole response. Global azole responsive sterol biosynthesis was analyzed. Mechanism of sterol biosynthesis initiated by the up regulation of the genes based on efflux pumps CDR (family in *Candida albicans*), associated with ABC transporters and MDR1, that probably depends on the alteration of transcriptional activators. In azole stress Erg11 and other sterol regulating erg genes may triger the accumulation or depletion of sterol derivatives in the membrane that induces azole resistance or sensitivity in fungi (Sun, X., et.al 2014).

In *N. crassa* NCU04158 shown growth adaptation in the presence of ketoconazole as compare to the wild type. Under ketoconazole (KTZ) in wild type NCU04158 shown 24.57 fold altered transcriptional levels as compare to wild type control. However, in order to investigate azole responsive mechanism. We investigated the azole responsive mechanism azole targeted sterol biosynthesis pathway. In current study NCU04158 *Nmr* protein revealed azole sensitive phenotype as the eburicol was accumulated after 24h treatment of the Ketoconazole by late step genes of ergosterol biosynthesis *erg6* (C-24 methyl transferase).

This indicated that *Nmr* deletion increases eburicol accumulation as compare to the wild type under KTC stress by late step sterol biosynthesis gene. In *N.crassa Nmr* has shown a little higher peak for eburicol (Mw.423) after KTZ stress encoded by erg 6 as compare to wild type.

Hence sterol derivative accumulations in the membrane in azole exposure add to the abnormal sterol supply in the membrane and induce growth retardation. Thus it is suggested that azole sensitivity in NCU04158 mutant is due to the accumulation of sterol derivative in *N. crassa*.

4. CONCLUSION

Nitrogen metabolite regulating gene deletion mutant shown adaptations in the fungal growth in KTZ stress. It is suggested that deletion in the nitrogen metabolite regulating gene retarded the cell growth in reduced nitrogen contents in the culture media. Which proposed the indication of loss of stress adaptations in deletion mutant as compare to the wild type under azole exposure. However in order to know the azole targeted role in the mutant of nitrogen metabolite regulatory protein. Sterol biosynthesis was analyzed and found eburicol accumulation in the mutant after the ketoconazole stress. Eburicol is the substrate of ERG6 C5 sterol desaturase a sterol regulating gene in the downstream of azole responsive mechanism.Which proposed that in the fungal nitrogen metabolite deletion mutant the accumulation of eburicol is the key phenomena of reduced adaptations in ketoconazole stress. Ketoconazole exposure increases the transcriptional response of NMR mutant due to the accumulation of eburicol in the fungal cell. Study may also propose that Nit a co regulatory gene of Nitrogen metabolism do not constantly regulate the fungal growth in Nitrogen deficit condition. However in order to investigate the role of Nit gene transcriptional levels in NMR mutant should be investigated.

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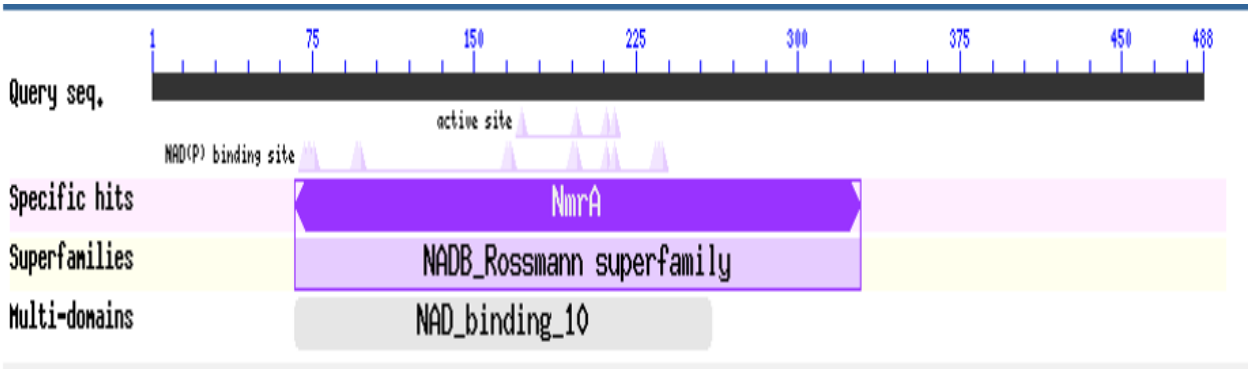

Figure 3. NmrA (Negative transcriptional regulator involved in the post-translational modification of the transcription factor AreA). NmrA has a multi protein domain consisting on NAD binding and significant matches to Rossmann fold super familiy.