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The Regulatory Mechanism of Cytochrome P450 Family Proteins under Azole Resistance in Neurospora Crassa

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Abstract: The azoles are the most widely used antifungal drugs. Their target ERG-11 and its downstream protein ERG-5 play significant roles in resistance to the azoles. Both of them belong to the cytochrome P450 family proteins. Except them, there are many other P450 family proteins, but their contribution to fungal drug-resistance is not clear. In this research, we analyzed the effects of cytochrome P450 family proteins on fungal drug-resistance to azoles using the model filamentous fungus *N. crassa* as research material. Through the azole susceptibility test to the knockout mutants of 18 P450 family proteins including ERG-5, we found that the other 17 P450 family proteins except ERG-5, have the similar susceptibility to azole drugs to the wild-type. Furthermore, we determined the sensitivity of these knockout mutants to benzoic acid, and screened a sensitive mutant to that drug. The corresponding gene (NCU08128) encodes a phenyl-4-monooxygenase.We further analyzed the possible mechanism of the sensitivity of this gene to benzoic acid. We confirmed the roles of the other cytochrome P450 family proteins except ERG-11 and ERG-5in fungal resistance to azole drugs, using the model filamentous fungus *N. crassa*as research material. The above researches enriched the drug resistance mechanism of filamentous fungi and provided new targets for the development of novel antifungal drugs.

Keywords: *Neurosporacrassa*, Drug resistance, Stress adaptation, Transcription factor, Cytochrome P450

1. INTRODUCTION

The increased exposure of antifungal agents has diversified the antifungal resistance and heightened the interest to study and analyze other multiple focusing angles (Lepesheva, and Waterman, 2007) (Morikawa, *et al.*,2009) (Melo, *et al.*, 2009) Generally there are multiple mechanisms which contribute in antifungal resistance e.g. change in drug target ,alteration in the level of sterol biosynthesis, reduced concentration of the enzymes or the increased in the expression level of target enzyme. Cytochrome P450 homolog *erg11*gene is the first direct target of antifungal azole. Cytochrome P450 homolog CYP51 subfamily proteinis highly conserved proteins majorly involved in the sterol biosynthesis. That enables fungi to protect the cell wall stability in the spore formation and crucially required in the fungal morphology Homolog members of the Cytochrome P450CYP56 from the pathogenic *Candida albicans*and the saccharomyces also revealed sensitivity in the homolog homozygous mutant as compared to the wild type under antifungals stress of echinocand in, (cell wall glucan synthase inhibitor). Filamentous fungi are the diverse group of lower eukaryotic organisms has extraordinary adaptability (Melo, *et al.*, 2009) (Sanglard,and Loper, 1989) (VanBogaert, *et al.*, 2009) Among that *Neurosporacrassa*, a well-known fungus has the similar azole responsive mechanism as pathogenic fungi and have availability of number of deletion mutants to study the molecular mechanisms (Sanglard, and Loper,1989) (VanBogaert, *et al.*, 2009) (Payne, *et al.*,1998) Fungal P450 family genes involved in the secondary metabolites are also required in the several oxygenation steps metabolized by several enzymes that release toxins (Bhatnagar, *et al.*, 2003) (Wen, *et al.*,2005) Filamentous fungi is also involved in detoxifying several aromatic compounds as member of the CYP53A sub division in ascomycetes and basidomycete fungi (Jawallapers *et al.*, 2014) (Faber *et al.*, 2001) CYP450 class of proteins are localized in mitochondria and also involved in the metabolism of various drugs. Antifungal azole are mainly regulated by CYP450 proteins that interact with the nitrogen atom of the azole drugs and get oxygen from the Fe catalytic region of the P450 protein. This leads to the inhibition of the sterol biosynthesis and interrupt the membrane fluidity (White *et al.*, 1998 (Mo *et al.*, 2002 Laude, *et al.*, 2004 Hand *et al.*,2003 Song, *et al.* ,2016). Fungal sterol biosynthesis enzymes belong to cytochrome P450 super family has membrane bound proteins among major the raptic target group as mainly erg5 and erg11. Ergosterol biosynthesis pathway is well studied in fungi but the potential regulatory mechanism needs some additional aspects for better management. Among CYP450 family there are several homolog proteins but in azole ergosterol regulatory mechanism only *erg5* and *erg11*are reported in *Neurosporacrassa*. In order to find the azole regulatory mechanism among homolog proteins we focused on *erg5* and *erg11* homolog proteins and find out 25 CYP450 homolog proteins deletion mutants on the base of the amino acid blast and

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differential expressions in ketoconazole exposure. in *Neurosporacrassa.* Furthermore in this study we have also find outCYP450 homolog protein deletion mutant of Benzoate 4 monoxygenase protein on the base of the amino acid sequence similarity among homolog proteins in *N.crassa*and fungi. Benzoate revealed toxicity by disrupting the membrane by PH variations that inhibit the required cellular activities in fungi (Lapadatescu, *et al.,* 2000) Lepesheva, *et al.,* 2007). Benzoat 4 moxygenase have (78%) conserved amino acids in active site that indicated that any inhibitor designed from this class can serve against range of animals and plant pathogenic fungi (Wright 1993) . A comparison study on the gene structure (exons size and the location of the introns) revealed high similarity among Ascomycota and basidomycota (Lepesheva, *et al.,* 2007). (Fraser *et al.,* 2002) where by the deletion of the CYP53 further proved the lethal phenotype of deletion mutant and predicted the CYP53 family a new antifungal drug target among fungal pathogens as CYP53 family (Ortiz de Montellano, *et al.,* 2005). Benzoate 4 monoxygenase belong to CYP53 are the most active group of enzymes involved in cellular metabolism and mycotoxin production in plants pathogenic fungi (Gillam, and Hunter 2007, Hannemann *et al.,* 2007, Fukuda, *et al.,* 1996, Faber, *et al.,* 2001 Matsuzaki, and Wariishi, 2005 Podobnik, *et al.,* 2008 Brankova, *et al.,*2007). Benzoic acid and its derivatives exert antifungal activity with reduced dissociation of lipophilic acidity and leads to the oxidative stress, protein aggregation and acidification of lipid in membrane that influence the membrane permeability (Kim, *et al.,*2010, Lmdelserone 1999)

2. MATERIALS AND METHODS

*Neurosporacrassa*strains used in this study were obtained from the Fungal Genetics Stock Center (University of Kansas Medical Center). Vogel's minimum medium was supplemented with 2% (w/v) sucrose for slants or 2% glucose for plates and the liquid medium, was used for culturing strains. All cultures were grown at 28°C in light.

Phylogeneticanalysis

To analyze the sequence similarity among various fungal species, protein sequences were aligned with the software Clustal X (2.1) as (36). Neighbor-joining (NJ) tree was constructed with MEGA 5 as previously mentioned (Cañabate-Díaz, *et al.,* 2007). For more assurance of the phylogenetic association 1000 resampling were directed for bootstrap tests.

Azolesensitivity tests

For the drug sensitivity test of corresponding cytochrome P450 homolog proteins of *Neurosporacrassa* antifungal drugsketoconazole have been dissolved in DMSO (dimethyl sulfoxide) and poured into the sterilized medium aseptically. Drug sensitivity test have been conducted on the Vogel's medium after addition of the drugs. 2 µl of the (2×10^{-6}) conidial suspension have been inoculated on the middle of the plates and compared with wild type. Every drug is tested for the drug sensitivity test with triplicates.

Benzoic acid sensitivity tests

In order to investigate the benzoic acid deletion mutant phenotype response, benzoic acid was dissolved in DMSO and aseptically added to the Vogel's media for 1 µ l/ml for sensitivity test. However, in order to analyze the azole response with addition of benzoic acid 1 µg/ml Ketoconazole was added in the media and benzoic acid concentrations was reduced to half. 2µl conidial suspensions were inoculated on the middle of the Vogel's media plates and incubated at 28°C in dark. Sensitivity test was done in triplicates for each individual strain.

Complementation of benzoate 4 monoxygenase

To complement the benzoate 4 monoxygenase deletion mutant whole length 2kb upstream and 2KB downstream coding sequences of benzoate 4 monoxygenase were under taken and a complemented fragment was amplified using forward AACGACGGCGGTCATAAACAT and CGAGTTCGG-AGACACGCACAG reverse Primer pair The PCR purified fragment have been inserted to PCB1532 plasmid at the *EcoR-5* site to get the complementation plasmid which then have been transferred to the deletion mutant benzoate 4 monoxygenase by using previously reported method (Lmdelserone 1999) Trans formants were screened by using chlorimuron ethyl resistant marker in Vogel's medium and further verified after using complementation F-(Forward) and R-(Reverse) primers

3. RESULTS

Cytochrome P450 proteins are essential for the steroids biosynthesis in plants and fungi required for the cellular stability as well as essential in detoxifying the drugs. CYP50 proteins are bound in the membrane containing a pigment that absorbs light at 450 nm so named the protein CYP450 proteins. In eukaryotes there are more than 50 CYP450 proteins have been studied as drug metabolizing proteins. However in filamentous fungi *Neurosporacrassa* have only two well-studied CYP450p *erg11* and *erg5*genesunder azole stress. Although *Neurosporacrassa* have several CYP450 homolog proteins in the genome that further required to be identified.

CYP450 conserved Protein

To identify other azole target homolog proteins among CYP450 proteins as azole target

*erg11*NCU02624 and *erg5*NCU05278 mentioned in previous study (Wang *et al.*,2015). We collected all the available homolog proteins from *Neurosporacrassa* by (NCBI BLAST) and aligned the amino acid sequence along with *erg11* and *erg5*genes as mentioned in **(Fig.1).** Furthermore 17 proteins were picked with highest similarity of amino acid alignment in CYP450 proteins. Among these NCU08112, NCU05848 and NCU05376were annotated as P450 monoxygenase. NCU06526, NCU09103, NCU02031, NCU9665 were hypothetical proteins and two proteins NCU05006 and NCU02852 CYP450 annotated as CYP P450p. Furthermore, there is one CYP52 NCU09115 and a CYP450 2C30 NCU07092 as well as NCU08128 benzoate 4 monoxygenase. There are four other annotated proteins NCU04089 Pistatindemethylase which is responsible for the catalysis of the 3-O- methyl group from the phytoalexins that induce tolerance mechanism in *Fusarium* spp. (plants pathogen fungi). Fungal species poses this gene generally reported resistant to pisatin (Sun *et al.*, 2014). While in *Neurosporacrassa*pistatindemethylase deletion mutant was not sensitive under azole stress and another CYP450 homolog protein NCU05001 encodes ent-Kaurene oxidase in GA biosynthesis in plants pathogens fungi. However in *Neurosporacrassa* deletion mutant of ent-kaurene oxidase deletion was resistant relative to wild type under KTC stress. Moreover, NCU05185 a fully equipped Bi functional P-450:NADPH-P450 reductase enzyme revealed wild type phenotype under azole stress Thus *in Neurosporacrassa* only NCU02624 *erg11* andNCU05278 *erg5* azole target revealed KTC sensitive phenotype as compared to wild type as mentioned in **(Fig. 2).** Other CYP450 homolog proteins wild type similar phenotype in KTC stress, while deletion of erg11 in *Neurosporacrassa* do not grow even in normal conditions. However, it is suggested that CYP450 homolog proteins sharing same CYP450 conserved domain are functionally not conserved.

Drug sensitivity test among CYP450 conserved domainproteins

Azole drug sensitivity test was conducted to identify the azole responsive phenotype of CYP450 homolog proteins in *Neurosporacrassa*for 17 homolog proteins. Our drug sensitivity suggested that in *Neurosporacrassa* only *erg5* was sensitive to KTC while azole target *erg11* do not grow even in normal conditions that suggest*erg11* deletion suppress the conidiation in *Neurosporacrassa* (unpublished data). Although NCU05001ent-kaurene oxidase was resistant to KTC as compared to the wild type. While, other CYP450 homolog proteins in *Neurosporacrassa* shown wild type similar phenotype under azole stress as shown in **(Fig.2).** This means CYP450 homolog proteins are not conserved functionally.

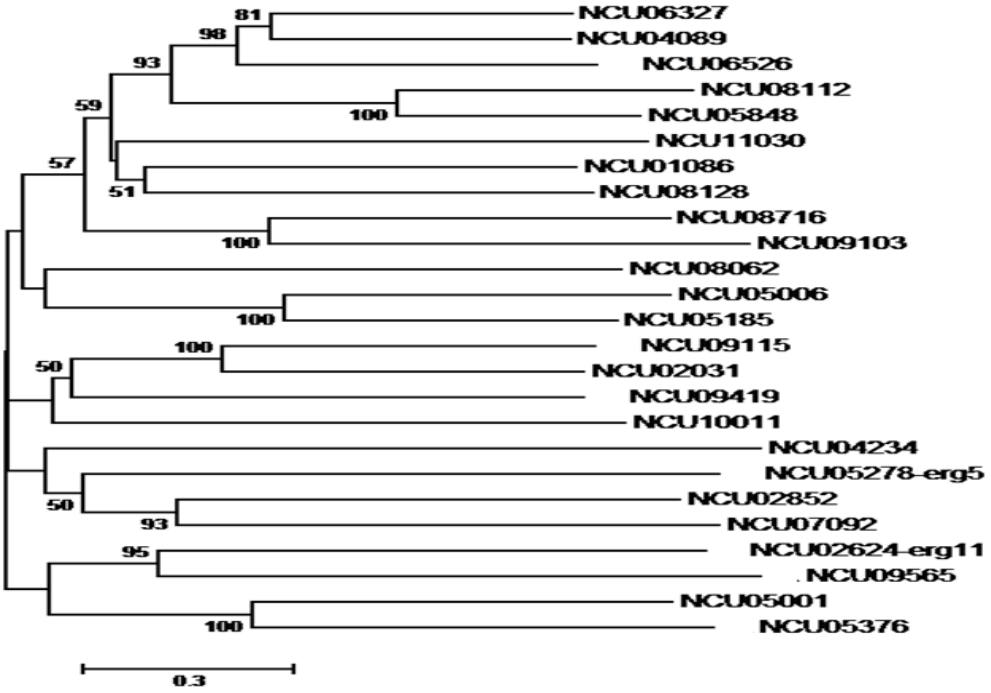

Fig. 1 Phylogenetic analysis of CYP450 proteins in *Neurosporacrassa*. On the base of azole target ERG-11(NCU02624) and its sown stream protein ERG-5(NCU05278), 25CYP450 family proteins were selected and their amino acid sequences were aligned in neighbor-joining (NJ) tree with 50% values from 1000 replicates by MEGA 5 software as (Tamura et al 2011).

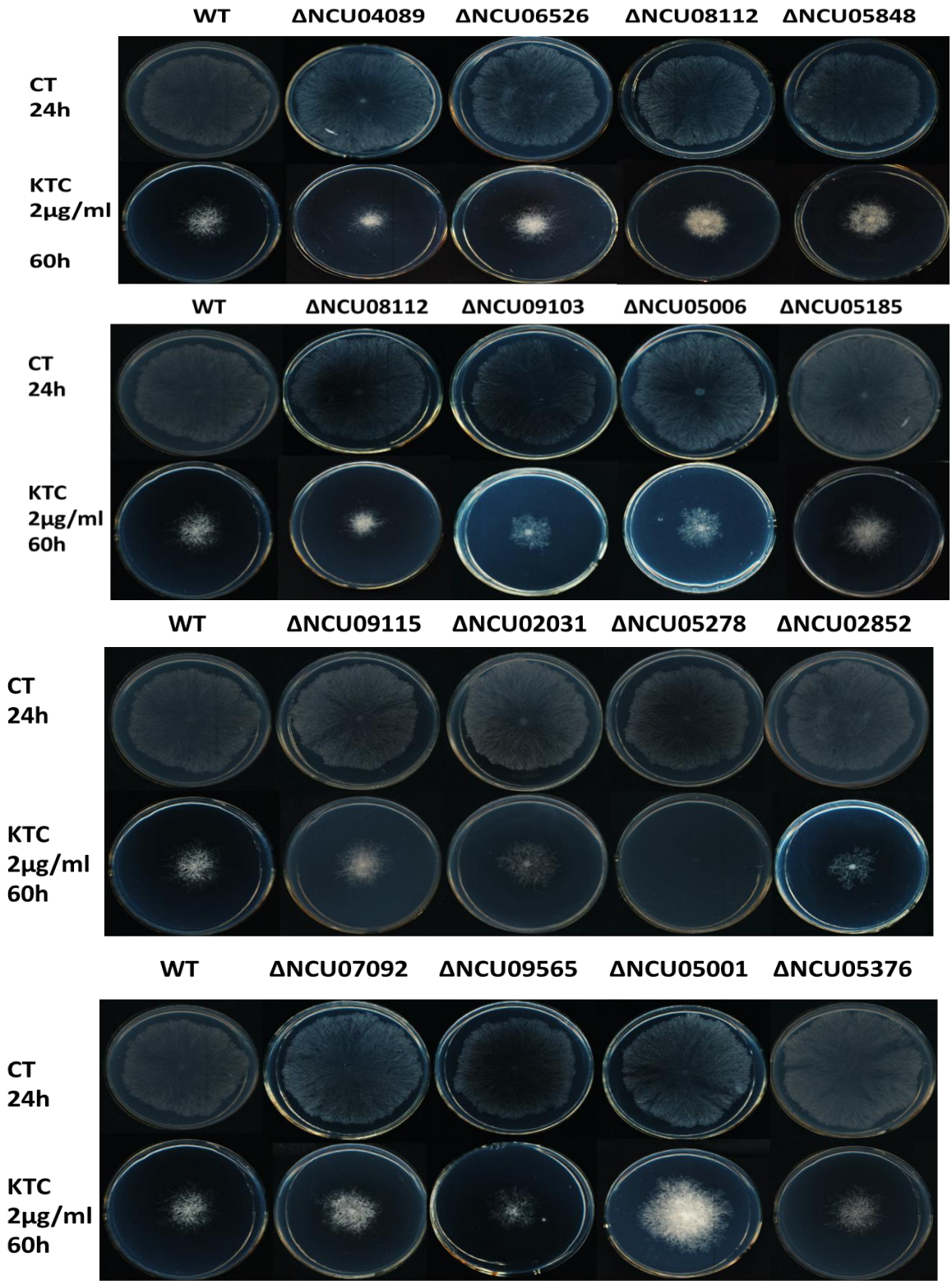

Fig.2. KTC sensitivity test of deletion mutants ofCYP450family proteins in *Neurosporacrassa*. 17 available deletion mutants of CYP450 family proteins were selected. Ketoconazole drugs sensitivity test was conducted using 2µg/ml in Vogel's media and incubated at 28°C. Plates were scanned after 24 hours for control and 60 hours for KTC treated plates. Each test was repeated independently three times

Benzoate 4 monoxygenase belong to antifungal-theraptic group

Among the screening of CYP450 similar domain proteins, we further found out a deletion mutant FGSC #21115 belong to the antifungal theraptic important group of monoxygenase enzymes among CYP450 homolog proteins. That does not show azole sensitive phenotype but confers sensitivity to benzoic acid. ΔNCU08128 is annotated as benzoate 4 monoxygenase cytochrome P450 protein having 557 amino acids. Under normal conditions NCU08128 gene in wild type shows 9.28 fold changes of transcriptional levels after 12h However, wild type transcriptional levels with time have consistently increased after the ketoconazole treatment for 2 h, 4 h, 6 h and 12 h with 4 fold, 2 fold and 10.68 fold change till 12 h respectively as compared to untreated control as mentioned in **(Fig.4)** which shows the significant RNA transcriptional up regulation of the CYP450 domain protein in azole adaptive mechanism in N.crassa but does not show azole sensitive phenotype of the deletion mutant as mentioned in **(Fig.3)**

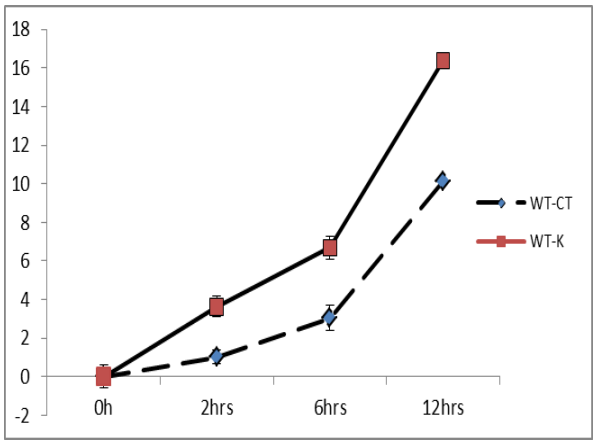

Fig.3: Time-course of expression of benzoate 4 monoxygenase-encoding gene in wild type treated with ketoconazole or not. *Neurosporacrassa* wild type strain was cultured at 28°C for 12 hours at 200 rpm. Ketoconazole was added with 2.5µg/ml final concentration and incubated for another 24 h while control with no drug. Gene transcriptional levels were determined after 2, 6and 12 h to analyze the relative gene expression with time. While control with no ketoconazole addition. However, the gene transcript level at 2 hour in control condition was set as 1. Experiment was repeated three times, statistical values were calculated and standard error is mentioned with error bars.

Benzoate 4 monoxygenaseis highly conserved in filamentous fungi

Benzoate 4 monoxygenase cytochrome P450 protein is a conserved domain among filamentous fungi and yeast. The phylogenetic analysis is comprised of four major groups including *Eurotiomycetes*, *Sordariomycetes*, *Saccromycetes*and *Eucascomycetes* **(Fig.4),** among which *Eurotiomycetes* contains *A.Clavatus*, 214/561 (38%) identity 311/561 (55%) positive sequences, *A.Terrous*, 214/535 and (40%) identity 291/535 (54%) positive *A.Oryzae*, 235/523 (45%) identity321/523 (61%) senquence positivity. However, A.*fumigatus*, 52/183(28%) identity 85/184 (46%) positive sequence *A.flavus*, 52/221 (24%) identity and 98/221 (44%) positive sequence, *A.niger* 51/185 (28%) identity, 88/185 (47%) positive sequence while, *A.nidulans* have 197/520 (38%) identity 288/520 (55%) positive sequence and *Neosartoryafischeri*. 217/479 45% identity 298/479 (62%) positive sequences. *Sordariomycetes* have *F.verticillioidies, F.oxysporum, F.graminearium, T.ressei, Neurosporacrassa* which shares 40%, 41% and 39% identity while 54%, 55% and 56% positive sequences respectively. However, *Saccromycetes* have only one ortholog in *C.Albicans* having 132/513 26% identity 207/513 40%positive sequences. *Eucascomycetes* have CYP450ortholog protein in *T.rubrum*with 200/536 (37%) identity 294/536(54%) positive sequence while in *Fusarium* spp. *Fusariumverticillioides* have 219/547 40% identity, 301/538 55% positive sequence.

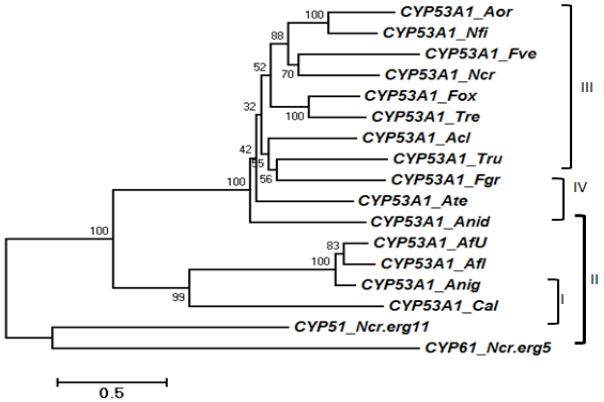

Fig. 4. Phylogenetic analysis of benzoate 4 monoxygenaseorthologs from filamentous fungi and yeast.CYP53A1 family Benzoate 4 monoxygenaseorthologs from 15 species were aligned and the condensed neighbor- joining (NJ) Tree was built up with values of 50% obtained from 1000 replicates by MEGA 5 software as (Tamura et al 2011) with (*Ncr, N. crassa; Afu, A. fumigatus; Afl, A. flavus; Anid,A. nidulans; Cal, C. albicans; Anig, A. niger; Acl, A. clavatus; Ate, A. terreus; Aor, A.oryzae; Nfi, N. fischeri; Fve, F. verticillioides; Fox, F*)

Bezoate 4 monoxygenasedeletion mutant in *N.crassa* shows sensitivity to benzoic acid stress.

Benzoic acid an oxidative agent builds up a concentration gradient in cells that effects the glycolysis by accumulation of hexose monophosphate and reduces the ATP falling into the pathway which partially influence intracellular pH. Subsequent reduced PH suppresses the glycolytic enzymatic potentials and inhibit the glycolysis that leads to growth retardation (38,39). Benzoate 4monoxygenase deletion mutant in *N.crassa*showed benzoic acid sensitive growth arrestas compared to the wild type. When grown on the plate with the media in addition with 0.5mM benzoic acid as

mentioned in **(Fig.5).** Mutant growth was further reduced when benzoic acid was added with ketoconazole in the media as compared to the wild type while the mutant growth on the Vogel's media without any addition revealed the same growth rate as compared to the wild type. Benzoic acid stress in *N. crassa* benzoate 4 monoxygenase deletion mutant and NCU08128::NCU08128 revealed the same phenomena as were seen in the previously mentioned study in fungi (40). However benzoic acid deletion and complemented strain also did not show azole sensitivity (as mentioned in Fig..2).

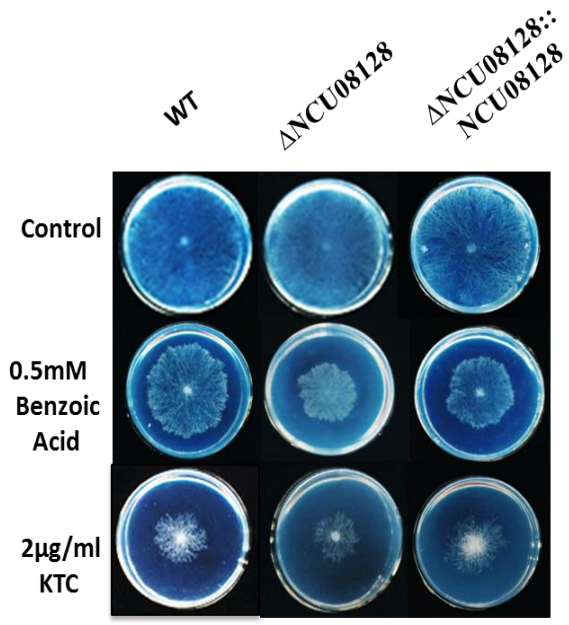

Fig. 5: Benzoate4 monoxygenase response to Benzoic acid stress. Benzoic acid sensitivity test for NCU08128 deletion mutant and the complemented strains of *Neurosporacrassa* was carried out at the Vogel's media with 0.5mM of benzoic acid on the Vogel's media and $2x10^6$ conidial suspension was inoculated on the center of the plates and incubated at the 28°C for 28 h for benzoic acid and controls and 60h for KTC addition. Each test was repeated three times independently

4. DISCUSSION

CYP450 domain proteins are the cellular target of azole antifungals. In urge to find more CYP450 homolog azole target proteins in *Neurosporacrassa.* We have selected 25 genes on the base of amino acid similarity as mentioned in **(Table .1),** and among these 17proteins were further selected on the base of more than 90% similarity with CYP450 proteins in *Neurosporacrassa* as shown in (Figure.1). According to reported studies CYP450 domain proteins are the cellular target of azoleantifungals also being cross resistant to various azoles on increase use in *C.glabrata* and *S.cerevisae* by altered transcription of *erg11* and other erg genes in azoles susceptibility and cause accumulation and depletion of different sterol contents in the membrane that induces azoles resistance or sensitivity. CYP450 homolog protein in *Neurosporacrassa* revealed higher transcription level as compared to the wild type under KTC but do not show azole sensitive phenotype. That further requires explaining the KTC non responsive mechanism in CYP450 homolog proteins among the under taken CYP450 homolog protein most of the member belong to the monoxygenase enzymes which are capable to hydroxylate the substrates. In order to investigate this domain we selected a benzoate 4 monoxygenase protein belong to the Cytochrome 53A1 domain have a fairly conserved domain in filamentous fungi. In *N.crassa* Benzoate 4 monoxygenase shown growth sensitivity in the presence of benzoic acid in the media but do not shows azoles sensitivity. However, in current study benzoate monoxygenase belong to CYP53A1 mutant of *N.crassa* shown growth sensitivity to benzoic acid response but does not confer azole sensitivity. Thus we can suggest that the less transcriptional levels of the azole responsive mechanism of the mutant are the main cause of wild type similar responses

Table 1. CYP450family proteins in *Neurosporacrassa*

NCU Number	Functional annotations
	Benzoate 4-monooxygenase
NCU06327	pisatindemethylase
NCU04089	Hypothetical Protein
NCU06526	Cytochrome P450 monoxygenase
NCU08112	Cytochrome P450 monoxygenase
NCU05848	Hypothetical Protein
NCU11030	Benzoate 4-monooxygenase
NCU01086	Benzoate 4-monooxygenase
NCU08128	CND11P
NCU08716	Hypothetical Protein
NCU09103	Cytochrome P450 monoxygenase
NCU08062	Cytochrome P450
NCU05006	Bi functional P450:NADPH-P450
NCU05185	reductase
NCU09115	Cytochrome P450 52A11
NCU02031	Hypothetical Protein
NCU09419	Cytochrome P450 3A4
NCU10011	Hypothetical Protein
NCU04234	Hypothetical Protein
NCU05278	Cytochrome P450 61
NCU02852	Cytochrome P450
NCU07092	Cytochrome P450 2C30
NCU02624	Cytochrome P450 51
NCU09565	Hypothetical Protein
NCU05001	ent-kaurene oxidase
NCU05376	P450 monooxygenase

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