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Role of Sterol Derivatives Depletion Under Azole Stress in Fungi

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Abstract: Azole antifungals target the sterol biosynthesis in fungal cell membrane and disrupt sterol biosynthesis pathway. In response to antifungal azoles fungal cell upregulate the transcriptions of drug efflux pumps in the membrane or the sterol regulatory gene in the fungal cell membrane. These transcriptional upregulationsare generated due to accumulation or the reductions of the sterol derivatives in azole stress. Study demonstrated the azole target ERG 11 similar protein mutant response in Neurosporacrassa under azole stress. Which revealed the transcriptional upregulation of all three cytochrome P450 homolog genes mutants in ketoconazole stress as compare to the controls using qRT-PCR. That were further justified by HPLC-MS. That suggested that ketoconazole responsive gene transcriptions pattern were similar in cyp450 homolog genes mutants for ERG11, ERG6, ERG25, ERG5 and ERG 3 gene in sterol biosynthesis. Whereas the accumulations of the sterol derivatives lanosterol, eburicol was increased in the CYP 450 mutants after ketoconazole stress. On the other hand the toxic derivative fecosterol and the ergosterol contents were reduced after ketoconazole stress and didnot altered the fungal morphology. Study indicated that in azole stress ERG11 similar protein mutants deplete sterol derivatives and do not induce antifungal drug resistance in Neurosporacrassa.

Keywords. Cytochrome 450, Ketoconazole, Drug Resistance, Sterol Derivatives, Cell Membrane.

1. INTRODUCTION

The Cyp450 family proteins are the most active group of drug target proteins mainly involved in sterol biosynthesis pathways taking place in eukaryotes. Cytochrome p450 family proteins have been studied in 82 species among all biological kingdoms. The number of identified sequences has been increased more than 100. However, the functional existence of the homolog genes in one species is variable, and the precise role of existence is still unknown (Martel, *et al.,* 2010). (Kim, *et al.,* 2005),. Study on CYP51 in A. Thaliana had two homolog genes, only one of which was potentially functional (Rozman, and Strömstedt, 2005),. The average sequence similarity in Cyp51 family proteins is approximately 30%, which is different in closely related species to 95% and decreased to 23% across the biological kingdom (Galina 2007).The major metabolic function of P450 family proteins is oxygenation, which takes place in three steps one with oxygen and two NADPH based reducing substances (Waterman, and Lepesheva, 2005). Cytochrome p450 family (Cyp450) proteins are the major target of antifungal azoles that inhibit Sterol biosynthesis required in various metabolic pathways. Sterol is a major component of plasma membrane that controls membrane permeability (Pena Diaz 2005), as well as regulates the derivatives of the steroid hormones in mammalian and plant cells and controls various metabolic processes (Nes, *et al.,* 1977). The azoles are the highly studied Cyp450 inhibitors that direct the heme iron from nitrogen and block the substrate binding thus disrupting sterol metabolism (Jackson, *et al.,* 2002). The azoles are the most widely useable antifungals with low toxicity while, long term treatment would cause inhibition of other Cyp450 enzymes and induce drug tolerance in pathogens due to the increased gene transcription and mutations of Cyp450 (Aoyama, *et al.,* 1987) (Zhang, *et al.,* 2002) (Wy, and Brown, 2005). The azoles including imidazoles and triazoles are active against lanosterol 14 α demethylase encoded by erg11 belonging to the Cyp450 enzymes involved in sterol biosynthesis while allylamines constrain erg1, mopholines inhibits erg2 and erg24 that are downstream to erg11 (Matsumoto, 2002). Various molecular mechanisms of antifungal resistance are identified in fungi as C.albicans resistant isolates showed higher expression level of ATP binding cassette efflux pumps CDR1 and CDR2 gene expression (White, *et al.,* 1998).. A reported GAL1 promoter which mediates the increased expression level in erg11 gene in S.cerevisiae (Albertson, *et al.,* 1996). (Franz, *et al.,* 1998). Whereby depletion of ergosterol encoding genes transcriptions are the main functions that leads to ergosterol accumulation in the cell. The ergosterol biosynthesis considered specific to the fungal texaA.fumigatus. The existence of various enzymes showed duplicated function due to mutations. Azole sensitive metabolic pathway revealed 14 similar sterol derivatives of ergosterol as major sterols in the membrane. The sensitivity of strains differentiates the involvements of multiple genes in different Cyp51A

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mutations (Kontoyiannis, *et al.,* 1999).. Mutants having 14-α sterol demethylase deletion usually reveal reduced ergosterol concentrations as compared to the normal strain and accumulate mainly C-4 and C-14 methyl sterol essentially eburicol similar to the erg11 downstream ergosterol synthesis that involves in the alterations of the isomers in C-8 to C-7 followed through the desaturation at C-5 and C-22, while reduction at C-24(16). The last three steps of ergosterol biosynthesis pathway differentiate between fungal taxa. Deletion of the erg3 (C-5 sterol desaturasesparalogs exhibit altered sterol profile with varied phenotype in various species (Sarmiento, *et al.,* 2011),. Genome based studies explore more association between cellular biosynthesis of sterol and other cellular metabolic pathways and bring into more elaborative perspective strategies for studying invasive antifungal resistance pathways (Livak and Schmittgen 2001). . Current study has been focused on azole targeted responses in cytochrome p450 homolog mutant genes to uncover the azole targeted role in Neurosporacrassa.

2. MATERIALS AND METHODS

Strain Inoculation

Neurosporacrassa wild type and the knockout mutants were inoculated on the Vogel's slants at 28℃ for a week. Spores were further collected from the slants and inoculated on the plates containing 20 ml Vogel's liquid media and incubated at 28℃ for 24 hour at dark to grow the mycelial mate (Sun *et al.,* 2013).

Drug Sensitivity

Vogel's media is prepared using 2% sucrose for w/v 2% Vogel's reagent ,1.5 % agar as (23)and autoclaved at 115℃ for 30minutes. Sterilized media was used to prepare Vogel's plates and 2µl conidial suspension containing 2×10^6/ml. 2µl conidia was poured onto the middle of the plate with 2.5ug/ml Ketoconazole added media. While the control groups were not treated with ketoconazole and incubated at 28℃. In order to observe the drug sensitivity test fungal growth Plates were scanned after 60h for azoles and 24h for control. Each sensitivity test has been done three times independently.

RNA Extraction

Mycellial mate was cut into the diameter of 1cm hole and tear the disc into pieces. Torn mycelial discs was further transferred into the100ml liquid medium containing flask and incubated at 28℃ with shaking 200 rpm for 12hrs. To see the transcriptional response 2.5ug/ml Ketoconazole was added into the mutants and the wild type flasks containing mycelial growth after 12 hours while the control groups were not treated with ketoconazole.After 24hour further incubation mycelia were extracted by vacuum pump filtration and mycelia were transferred into the 1.5 ml eppendorff tubes and frozen by liquid nitrogen.

Mycelia were gently grinded with the liquid nitrogen in the tubes and powdered with round bottom glass rod. 1ml TRIzol reagent was mixed while oscillating for 30s at the room temperature for 5 minutes and centrifuged for 15 minutes at 4℃. The supernatant was further added with 250µl chloroform and mixed with slight oscillations for 30s at room temperature. The centrifugation was repeated and the supernatant was added with 400µlof isopropyl alcohol, following Centrifuge at 12000rpm for 15 minutes at 4℃. The supernatant was discarded as washed with 70% alcohol twice and the tubes were dried at room temperature for 40 minutes. The RNA pellet was diluted with 40µl RNA free water. The concentration of RNA was measured at OD 260/280 in range of 1.9-2.1absorbance and cDNA was prepared using reverse transcriptase enzyme and q-RT PCR was done at BioRad system. Results output was calculated on the base of CT values using ∆2-CT method to analyze the relative expression level as (Sun *et al.,* 2013). The primer used in the expression analyses are mentioned in the table 1. Beta tubulin gene was used to normalize the transcriptional expression of ERG genes from the CYP450 mutants.

Mycelial Collection and sample preparation for HPLC analysis

Neurosporacrassa mutants and the wild type spores were inoculated on the plates containing 20ml liquid media and incubated for 24 hours at 28℃ in dark. 1cm diameter discs of the mycellial mate were cut and shredded into pieces and 12 pieces were suspended into the 100ml Vogel's liquid media containing flasks and incubated at 28℃ 200rpm for 12 hours.2.5µg Ketoconazole was added into the flasks after 13.5 hours while DMSO were added as control and further incubated at 28hours 200rpm for next 24 hour.

HPLC Analysis

Mycelia were collected from liquid media by vacuum pump filtration. Heat-dried mycelium was ground into fine powder and about 7-10 mg was weighed for sterol extraction in 2 mL Agilent vials with 1.5 ml of chloroform and incubated at room temperature overnight. mycelial extract was vacuum filtered and volatilized following sonication. The concentrate was further Diluted with 300µl of HPLC grade methanol and non-dissolved residues were removed. The filtration subjected for the sterol determination on HPLC MS as descripted before mate (Sun *et al.,* 2013). (Vogel 1956). (Cañabate *et al.,* 2007). The results has been computed after acquiring the specific peak of each molecular weight of sterol derivative lanosterol (MW.409), euburicol (MW.423), fecosterol (MW.395)

and ergosterol (MW.397) Graphical expressions were presented using peak area of individual derivatives.

3. RESULTS.

The ERG 11 protein homolog Three Cytochrome 450 (CYP450)protein mutants NCU08128, NCU09115 and NCU5376 were selected to test the sterol regulatory mechanism under ketoconazole stress as compare to wild type strain and the control (with out ketoconazole). The drug sensitivity test have shown the similar phenotypic morphology in three CYP 450 mutant and wild type except the control plates as mention in **(Fig. 1).** That depicted the efficiency of ketoconazole sensitivity as compare to control.

Sterol Biosynthesis

The sterol biosynthesis in the Neurosporacrassa wild type and the ERG 11 homolog gene mutants under ketoconazole stress revealed 2 fold higher lanosterol levels in NCU08128 than Neurosporacrassa wild type and other cytochrome 450 gene mutants. Which means in lanosterol biosynthesis CYP450 gene deletion did not altered any significant responses in cytochrome 450 gene regard less of the ERG 11 upregulated gene transcriptional levels as in **(Fig. 3).** The eburicol accumulation was 5 fold higher in NCU08128 as compare to the other genes of CYP450 protein mutants and the Neurosporacrassa wild type under ketoconazole stress. Which revealed that ERG 25 gene transcriptional upregulations but did not accumulate the eburicol in CYP450 mutant as in figure 2. As well as the increased eburicol levels in NCU08128 fungal mutant did not effected the growth morphology in fungi. The fecosterol levels after ketoconazole treatments were reduced in all three CYP 450 mutants except NCU08128 after ketoconazole stress. However the ergosterol levels was reduced in the fungal strains including deletion mutants and the wild type in the fungi. Thus study can suggest that upregulation of ergosterol regulating genes reduced the ergosterol in Neurosporacrassa that lead to no morphological change in the fungal growth three cyp450 proteins as mentioned in **(Fig. 1).** Ketoconazole regulating ERG 11 transcriptional responses in cytochrome conserved gene mutants was upregulated in as in the Neurosporacrassa wild type as compare to the control.

Gene transcriptional responses

Ketoconazole regulating ERG 11 transcriptional responses in cytochrome conserved gene mutants was upregulated in as in the Neurosporacrassa wild type as compare to the control. The ketoconazole up regulating ERG 6 gene transcriptional responses were also upregulatedin all three cytochrome conserved gene mutants in Neurosporacrassa wild type as compare to the control **(Fig. 4).** The ERG 25 relative gene expression was also upregulated in three CYP450 genes of Neurosporacrassa wild type as compare to the control figure 6. Similarly the ketoconazole ERG 5 and ERG 25 relative gene expression have shown the similar up regulated pattern of the cytochrome similar genes figure 5,6. Whereas, ERG 3 relative gene expression was down regulated in NCU08128 in contrast to other NCU05376 and NCU09115 the CYP 450 similar gene mutants under Ketoconazole responses in Neurosporacrassa **(Fig.7).**

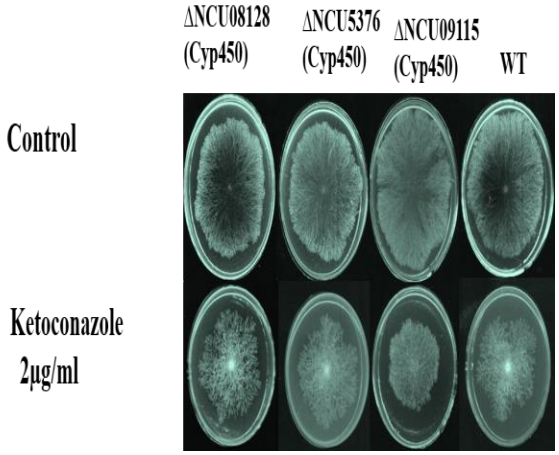

Fig. 1. Cytochrome homolog gene mutants in Neurosporacrassa

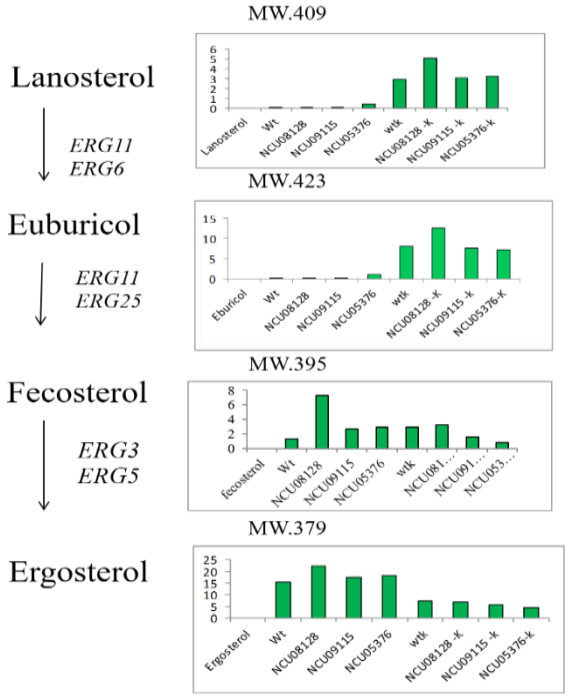

Fig. 2. Sterol responding mechanism of the cytochrome p450 mutants NCU08128, NCU09115, NCU05376 and Neurosporacrasssa (WT) wild type under (-K) Ketoconazole stress.

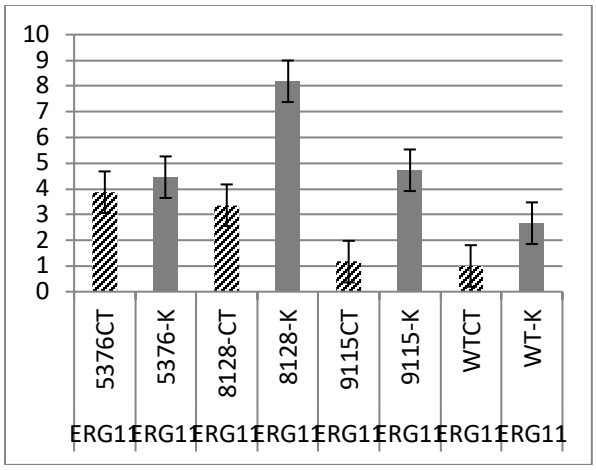

Fig. 3. Relative gene expression of ERG11 in the cytochrome 450 homologgene mutants NCU05376(5376)NCU08128(8128) NCU09115(9115) and WT (wild type) with -K (Ketoconazole) and CT (control).

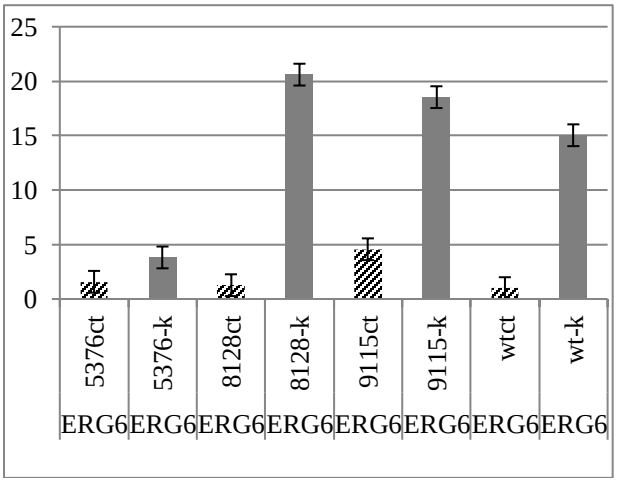

Fig. 4. Relative gene expression of ERG6 in the cytochrome 450 homologgene mutants NCU05376(5376)NCU08128(8128) NCU09115(9115) and WT (wild type) with -K (Ketoconazole) and CT (control).

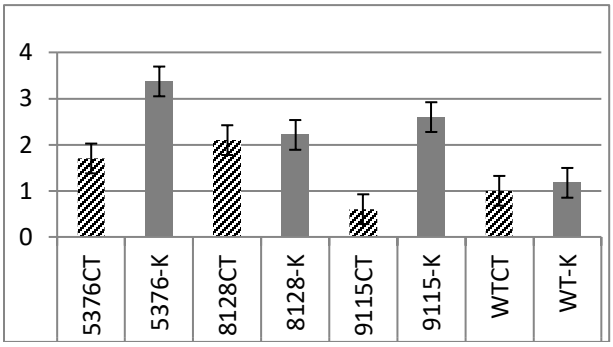

Fig. 5. Relative gene expression of ERG25 in the cytochrome 450 homologgene mutants NCU05376(5376)NCU08128(8128) NCU09115(9115) and WT (wild type) with -K (Ketoconazole) and CT (control).

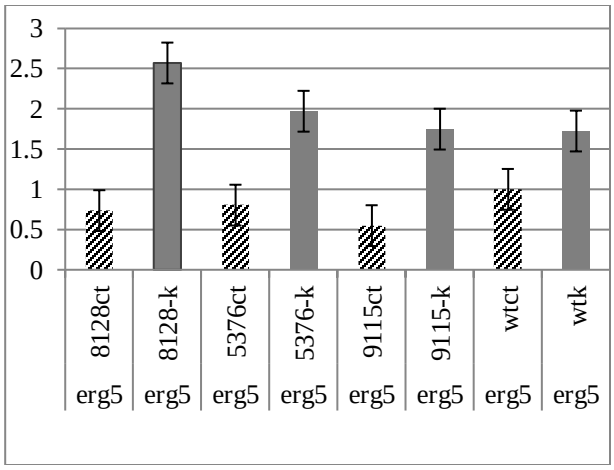

Fig. 6. Relative gene expression of ERG5 in the cytochrome 450 homologgene mutants NCU05376(5376)NCU08128(8128) NCU09115(9115) with -K (Ketoconazole) and CT (control).

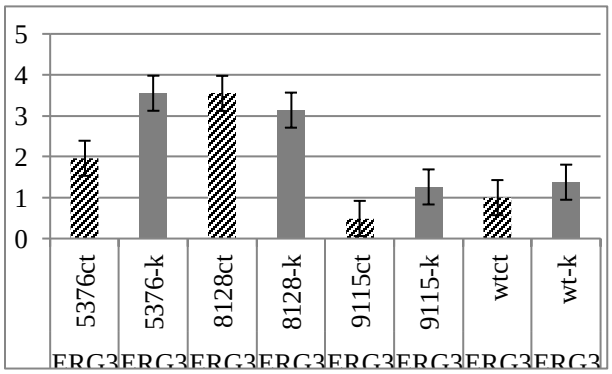

Fig. 7. Relative gene expression of ERG3 in the cytochrome 450 homologgene mutants NCU05376(5376)NCU08128(8128) NCU09115(9115) with -K (Ketoconazole) and CT (control).

4. DISCUSSIONS

Ergosterol, biosynthesis is critical for the fungal membrane fluidity, stability and survival under stress conditions. As well as, a target pathway for antifungals drugs (Liu *et al.*, 2010). The existing trends of resistance among current antifungals demand study more related genetic aspects of antifungal resistance. Mutants in particular derivatives of the ergosterol biosynthesis pathway are more potent to uncover the under lying phenomena of various genes. The antifungal azole, commonly interact with sterol demethylase enzyme ERG11 and catalyze the oxidative demethylation of C-14 of lanosterol derivatives of sterol. This study was focused on the erg11 similar protein domain CYP450 mutants in order to investigate the sterol regulating gene transcriptional levels in antifungal stress by ketoconazole regulating genes and sterol derivatives accumulation in Neurosporacrassa, a model fungi. Under azole treatment N.crassa wild type

culture led 3 fold reduction of ergosterol while, accumulation of lanosterol and eburicol respectively. However the accumulation of these derivative not detected in without drug treatment cultures and toxic sterols were only detected under Ketoconazole treatment (Vollmer, and Yanofsky,1986) (Larkin, *et al.*, 2007). (Liu Jiang and Ma 2010). (Francois *et al.*, 2009). The sterol biosynthesis in the Neurosporacrassa wild type and the ERG 11 homolog gene mutants under ketoconazole stress revealed 2 fold higher lanosterol levels in NCU08128 than Neurosporacrassa wild type and other cytochrome 450 gene mutants. Which means in lanosterol biosynthesis CYP450 gene deletion did not altered any significant responses in cytochrome 450 gene regard less of the ERG 11 upregulated gene transcriptional levels as mention in **(Fig.2).**

The eburicol accumulation was 5 fold higher in NCU08128 as compare to the other genes of CYP450 protein mutants and the Neurosporacrassa wild type under ketoconazole stress. Which revealed that ERG 25 gene transcriptional upregulations but did not accumulate the eburicol in CYP450 mutant. As well as the increased eburicol levels in NCU08128 fungal mutant did not effected the growth morphology in fungi. The fecosterol levels after ketoconazole treatments were reduced in all three CYP 450 mutants except NCU08128 after ketoconazole stress. However the ergosterol levels was reduced in the fungal strains including deletion mutants and the wild type in the fungi. Thus study can suggest that upregulation of ergosterol regulating genes reduced the ergosterol in Neurosporacrassa. Whereas, Ketoconazole regulating ERG 11 transcriptional responses in cytochrome conserved gene mutants were upregulated as in the Neurosporacrassawild type as compare to the control (Hu *et al.*, 2018).

On the other hand interestingly a transcriptional factor ccg-8 deletion mutant accumulated more lanosterol, eburicol and toxic sterol 14α-methyl-3,6-diol under ketoconazole treatment than wild type. Since, ergosterol level reduced under KTC in CCG-8 mutant and evident more KTC penetrations in ccg8 mutant as compare to the wild type that was (6 fold to 1fold) (Hu, *et al.*, 2018). However, The comparison of the sterol derivatives accumulation under normal conditions in ccg8 mutant and over expression strain showed reduced accumulations of lanosterol and eburicol from (4 to 2 fold) in ccg-8 mutant (Xue *et al.*, 2019).

5. CONCLUSION

In fungi ketoconazole stress respond to the disruption of sterol biosynthesis pathway in the membrane which has the connections of two patterns, the first is the azole targeted gene transcriptional levels and their ultimate influence on the sterol derivatives depletion or accumulation. The second one is the upregulation of the drug efflux pumps in the fungal cell membrane. However, this study has focused on the ERG 11 similar gene transcriptions under ketoconazole stress in Neurosporacassa. Which revealed the transcriptional upregulation of all three cyp450 genes mutants in ketoconazole stress as compare to the controls. That suggested that ketoconazole responsive gene transcriptions pattern was similar in cyp450 homolog genes mutant for ERG11, ERG6, ERG25, ERG5 and ERG3. Whereas the accumulations of the sterol derivatives lanosterol, eburicol was increased in the CYP 450 mutants after ketoconazole stress. While the toxic derivative fecosterol and the ergosterol contents were reduced after ketoconazole stress and did not altered the morphology due to the possible reduced penetrations of antifungal drug. This conclude the idea that depletion in the sterol derivatives fecosterol and ergosterol do not induce antifungal drug resistance in Neurosporacrassaa model fungi.

Table. 1 Gene Primers

Encoding gene primers	Primer name	Primers
Q-PCR-PRIMERS		
NCU03006 (erg6)	**F-**	TCAGCTCAAGTTCGTCAAGGGTGA
	R-	TTCATAGACACCAAAGGTACCGCC
NCU05278(erg5)	**F-**	TTTCACCTTCCTCTTCGCTTCCCA
	R-	TCATCGACTCAAGCTGCTCCATGT
	F-	AAATCGATTACGGCTACGGTCTCG
NCU02624(erg11)	**R-**	TATCGCTACCATCCACGTTCCTGA
NCU06207(erg3)	**F-**	CGGTTCACCACTTCGCCTTCA
	R-	CATCCTCACCCTCCACCTCCTT
NCU06402 (erg25)	**F-**	ACCTCCATCTCGCCACCATGTA
	R-	CTCGGTGTCGAGGCAGTAATCC
β tubulin	**QbtubF**	CCCAAGAACATGATGGCTGCTT
	QbtubR	TTGTTCTGAACGTTGCGCATCTG

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