Summary

Candida species are important agents of hospital-acquired infections and also, there is an increasing problem of antifungal drug resistance among the species. Therefore, there is a need to study these species at genetic level which involves the use of gene deletion or gene overexpression system. Different types of selectable markers are available but to study clinical isolates, dominant selection markers will be of use (El-Kirat-Chatel et al., 2011). This study deals with the development and testing of selection markers with special emphasis on dominant selection markers and tools for gene deletion or regulated gene expression for three major fungal pathogens i.e. *C. lusitaniae*, *C. albicans*, and *C. tropicalis*.

C. lusitaniae is known to cause severe fungemia in immunocompromised hosts and is also known to acquire resistance to antifungal drugs (Francois et al., 2001). Being haploid, it is convenient to study this species at genetic level. Therefore, to start with, we tested the utility of already available markers *NAT1* and *HygB* (Basso et al., 2010; Shen et al., 2005) in *C. lusitaniae* using *ADE2* gene. We could successfully achieve deletion of *ADE2* with these markers; however, the efficiency of homologous recombination (HR) was very low. Therefore, in an attempt to increase the efficiency of HR, we deleted yeast orthologs of C-terminus residues of *KU80* and entire ORF of *DNL4* separately in *C. lusitaniae* and further compared the efficiency of HR in the parent strain and two mutated strains. We observed that in both the strains carrying a deletion of either C-terminus of *KU80* or that of *DNL4*, there was a decrease in the NHEJ events. Further, we also checked the efficiency of HR in the presence of certain NHEJ inhibitors which are known to block the activity of protein kinases involved in the process in mammalian cell lines. However, we could not achieve either a decrease in NHEJ or increase in HR efficiency in *C. lusitaniae*.

C. albicans is the major fungal pathogen which is responsible for nearly 80% of all candidemias. Apart from this, there is also an increase in the number of infections caused by non-*C. albicans Candida* species (NCAC) (Calderone, 2002). Among NCAC species, infections caused by *C. tropicalis* are more prevalent due to the resistance exhibited by this species to fluconazole (Negri et al., 2012). There is a need to study these species at molecular and genetic level due to an increasing number of infections caused by them and

also due to increasing problem of antifungal drug resistance. However, being diploid, it is difficult to manipulate these species since there is no known sexual cycle and it is cumbersome to delete both the alleles of a gene. Therefore, in our studies with diploid *Candida* species, i.e. *C. albicans* and *C. tropicalis*, we developed a cassette for single-transformation gene deletion. One such cassette called as HAH2 was developed in our lab for studies with *C. albicans* (Sharma et al., 2014). This cassette utilizes arginine and histidine auxotrophies and is flanked at its ends by *loxP* sites which aid in eviction of markers after expressing Cre recombinase. We checked the utility of HAH2 cassette in *arg4*Δ *his1*Δ *C. tropicalis* strain to check if this system of one-step gene deletion works in this species or not. With the use of this cassette, the first allele was deleted by transformation of split markers of the cassette followed by deletion of second allele by mitotic recombination/ gene conversion. Further, markers could be evicted successfully after deletion of both the alleles of the gene by integration and expression of Cre recombinase.

Since HAH2 cassette cannot be used to study clinical isolates, we developed another cassette SHS based on similar strategy but by making use of dominant selection markers *SAT1* flipper and *HygB*. The first allele of the gene was similarly deleted using split markers of the cassette followed by deletion of second allele by mitotic recombination/ gene conversion. The homozygous mutants were selected in the presence of hygromycin and nourseothricin. This was followed by eviction of markers by inducing the expression of Flp recombinase (in the presence of maltose since it is under the control of *CaMAL2* promoter) which will lead to recombination between *FRT* sites flanking the markers. This cassette was successfully used for *C. albicans* but with *C. tropicalis*, markers could not be evicted efficiently. To utilize it for *C. tropicalis*, we replaced *CaMAL2* promoter upstream of *FLP* gene with the *CtMAL2* promoter. The modified cassette CtSHS was then tested in *C. tropicalis* and we could obtain homozygous *ADE2* mutant followed by marker eviction. Still, there was an issue of the instability of this cassette when maintained in *E. coli* due to leaky expression of *MAL2* promoter. To overcome this problem, we placed an intron (*CaTUB2* intron) in between the *FLP* gene in both SHS and CtSHS similar to that reported by Shahana et al. (Shahana et al., 2014). Then we tested the two cassettes in *C. albicans* and *C. tropicalis* respectively and obtained homozygous *ADE2* mutants in both the species. However, the markers could only be evicted from *C.*

albicans but not in *C. tropicalis* after induction of expression of Flp recombinase in the presence of maltose which could be due to inefficient splicing of the intron from *C. tropicalis*.

It is deleterious for the host if an essential gene is completely deleted; therefore, we further modified SHS-TI and CtSHS cassettes for single-transformation conditional gene expression. This was done by cloning *CaMET3* and *CaPCK1* promoter downstream of *FRT* site of SHS-TI and *CaMET3* promoter downstream of *FRT* site of CtSHS for conditional gene expression in *C. albicans* and *C. tropicalis* respectively. With these cassettes, we replaced the promoter of *ADE2* gene with *MET3* or *PCK1* promoter by a single-transformation step. This was followed by eviction of markers in the presence of maltose leaving behind the regulatable promoter along with a single *FRT* site. With *MET3* and *PCK1* promoters, gene expression was repressed by adding methionine and cysteine or by adding dextrose as a carbon source to the media respectively. We observed that the *MET3* promoter was tightly regulated compared to *PCK1* promoter since leaky expression could be observed with *PCK1* promoter even under repressing conditions.

It is not always feasible to add or remove metabolites from the media; therefore, a system is needed where gene expression is regulated by a substance which does not affect the host metabolism. The tetracycline-regulatable system is one such system where gene expression can be induced or repressed by addition or removal of doxycycline or vice-versa (Park and Morschhauser, 2005). Tet-On system is widely used for *C. albicans*, however, it is not advantageous for studying essential genes since a very high concentration of doxycycline, i.e. 50-100 µg/ml needs to be present continuously for induction of gene expression which can reduce cell growth. It is preferable to use Tet-Off system in such cases where gene remains active in the absence of doxycycline and its expression can be shut down by adding doxycycline when required. The Tet-Off system which is already available has certain limitations; the transactivator is under the control of *ENO1* promoter which is repressed by gluconeogenic carbon sources and also it requires 20 µg/ml doxycycline to repress gene expression (Nakayama et al., 2000). Tet-Off system which is available for S. cerevisiae requires as low as 1 µg/ml doxycycline to repress gene expression (Gari et al., 1997). Therefore, we developed another Tet-Off system by creating certain mutations: G12S, G19E, P56A, E148D and R179H in TetR of the both the Tet-On systems (pNIM1 and pNIMX) based on the study by Urlinger et al. (Urlinger

et al., 2000). This system can be used to study essential genes since the gene expression is induced in the absence of doxycycline and can be shut down by adding nearly 1-2 µg/ml doxycycline.

Tet-On systems which are available contain the transactivator *rtTA* under the control of *ADH1* or *TDH3* promoters. It was observed that the gene expression levels were not uniform when *rtTA* was under the control of *ADH1* promoter (Park and Morschhauser, 2005). Chauvel et al. replaced the *ADH1* promoter with *TDH3* promoter and observed a 5-fold increase in gene expression (Chauvel et al., 2012), however, it is known that *TDH3* is enriched in the stationary phase (Kusch et al., 2008). Therefore, we modified both Tet-On and Tet-Off cassettes containing P_{tet}-GFP such that the cassette was integrated at *RP10* locus and transactivator placed under the control of *RP10* promoter since this locus is well established for reliable and high level of gene expression (Backen et al., 2000; Brand et al., 2004; Care et al., 1999; Swoboda et al., 1995). Using GFP as a reporter, we checked the utility of the three Tet-On and three Tet-Off systems with the difference in the promoter regulating the transactivator. We observed that in Tet-On systems when transactivator is under the control of *ADH1* or *TDH3* promoter, significant GFP expression was observed only after 6-8 hrs. We reasoned that this might be due to the expression level of transactivators which is causing a delay in increase in GFP expression levels. The lower levels of expression with *rtTA* under *ADH1* promoter might be due to limiting levels of the transactivator since it is regulated by the carbon source and also during batch growth (Bertram et al., 1996). A decline in GFP expression was also observed at 6 hr with the Tet-On system where the transactivator is under the control of *TDH3* promoter. Since this cassette is also integrated at *ADH1* locus, we speculate that this locus might influence the expression from *TDH3* promoter to some extent. With *CaRP10* promoter controlling the transactivator expression, GFP expression increased steadly after transfer to inducing conditions and also the expression levels were higher and uniform in all the cells compared to *TDH3* and *ADH1* promoter. Similarly, with the Tet-Off system, there was a lag of 6-8 hrs before a significant gene expression was seen with *ADH1* and *TDH3* regulating the transactivator. With *RP10* promoter regulating the transactivator, GFP expression was observed within 2 hrs of transfer to inducing conditions. Overall, expression levels with Tet-Off systems were higher under inducing conditions as compared to their respective Tet-On versions. With Tet-Off system, GFP

levels with *RP10* promoter at 24 hrs was nearly 3-fold and 30% higher than that with *ADH1* and *TDH3* promoters regulating the transactivator respectively. With all the above constructs, gene expression was repressed completely within 8-12 hrs. Thus, induction of gene expression was rapid with *CaRP10* promoter and Tet-Off system with transactivator under the control of *RP10* promoter constructed in our lab is highly efficient to study essential genes.

The effect of different minimal promoters was also studied on gene expression and it was concluded that *OP4* was much more efficient in inducing gene expression compared to other minimal promoters tested. We further tested Tet-On and Tet-Off system in *C. tropicalis* after integrating the cassette at *CtRPS10* locus such that the transactivator is regulated by *RPS10* promoter. Tet-Off system was found to work efficiently in the species where induction of GFP expression was observed within 2 hrs of transfer to inducing conditions.

The functionality of tetracycline-regulatable system was also confirmed in *C. albicans* using *CaADE2* and *CaTUP1* where transactivator under the control of *TDH3* promoter was integrated at *ADH1* locus and the genes were placed under the control of P_{tet} using HAH2- P_{tet} cassette. The conditional mutants obtained worked in a doxycycline-dependent manner. In the case of Tet-On system, gene expression was induced at 100 µg/ml doxycycline and in the case of Tet-Off system, gene expression was repressed by the concentration of doxycycline as low as 2 µg/ml as evident by the loss of viability in *ade2* mutant and filamentation in *tup1* mutant. Finally, the utility of Tet-Off system was also tested for amphotericin B resistance gene *PDR16* in *C. albicans* and *C. tropicalis* since it requires very low concentration of doxycycline which will not interfere with the susceptibility patterns of the antifungal drugs. Therefore, this system can be successfully used to study essential genes, genes involved in virulence and antifungal drug resistance since such low concentration of doxycycline will not alter any of its properties.