Chapter 5

Discussion

Magnaporthe oryzae is responsible for blast disease in rice and other cereal crops. The infection process in rice blast disease has been associated with MAPK signalling pathway mediated by MAPK kinase kinase (MAPKKK, encoded by MST11), MAPK kinase (MAPKK, encoded by MST7), and MAPK (encoded by PMK1) (Jin et al., 2013) . PMK1 (Pathogenicity MAP kinase1) MAP kinase (MAPK) gene is essential for appressorium formation and plant infection (Xu and Hamer, 1996). Pmk1 is a homologue of yeast Fus3 and can rescue the mating defect in a fus3 kss1 double mutant. Fus3 regulates the mating process by phosphorylating downstream proteins like Ste12 and Far1 (Kusari et al., 2004). Ste12 is a transcription factor which is involved in regulation of expression of genes related to mating process in *S. cerevisiae* (Errede and Ammerer, 1989). MST12 (a homolog for *S. cerevisiae* STE12) has been functionally characterized in *M. oryzae.* Gene deletion mutants of MST12 have been found to be non-pathogenic even though they could form appressoria (Park et al., 2002). This defect in virulence is due to inability of Δ*mst12* mutants to form penetration peg, an essential stage for plant infection.

Another downstream protein of FUS3, FAR1 (factor arrest resistant) is responsible for the cell cycle arrest at G1 phase due to inactivation of Cdc28-Cln2 complex in yeast (Mendenhall, 1998). Most of the homologous proteins of yeast MAPK pathway have been functionally characterized and reported to be involved in early stages of infection. We therefore predicted that FAR1 homolog in *M. oryzae* may also be involved in pathogenicity related development in *M. oryzae*. NCBI BLASTP analysis suggested a hypothetical protein (MGG_00134) as the probable candidate for FAR1 homolog in *M. oryzae.* Although, MGG_00134 showed only 24% homology with *S. cerevisiae* FAR1 but due to absence of any other obvious homologs in the available genome database, makes it the obvious candidate for FAR1 homolog in *M. oryzae*. On the basis of the homology with yeast FAR1

this hypothetical protein (MGG_00134) was designated as MFAR1. Although, there is less than 30% similarity between MFAR1 and yeast FAR1, the BLASTP analysis showed that this protein is closest to the similar proteins present in some closely related filamentous fungi. It showed more than 70% identity with *Gibberella zeae, Fusarium oxysporum* and *Neurospora crassa*. A phylogenetic tree was prepared using ClustalX software by comparing the MFAR1 with similar proteins in other fungi. It also indicated that MFAR1 is closer to similar proteins in filamentous fungi like *N. Crassa, F. oxysporum* and *G. zeae* than *S. cerevisiae* or *C. albicans.*

Yeast FAR1 consists of 830 amino acids, whereas FAR1 homolog in *M. oryzae* (MFAR1) consists of 1161 amino acids with three introns. Due to presence of these introns, coding region of the MFAR1 gene consists of 3486 base pairs in comparison to the 3841 base pairs of total gene. Expression analysis and confirmation of size of coding region of the gene were carried out by reverse transcription PCR (RT PCR). The results suggested that the size of MFAR1 coding region is indeed around 3.5 Kb in comparison to 3.8 Kb band obtained from amplification of gene from genomic DNA.

NCBI and Smart Domain prediction tool showed that MFAR1 contains a characteristic ring zinc finger domain, a pleckstrin homology (PH) domain and a von Willebrand factor type A (VWA) domain. Based on the structural and bioinformatics analysis it has been previously demonstrated that FAR1 in yeast contains ring zinc finger domain, a pleckstrin homology (PH) domain and a von willebrand factor type A (VWA) domain (Côte and Whiteway, 2008). Therefore, even though homology between FAR1 and MFAR1 is not very high but all the characteristic domains are conserved between them. Bioinformatics analysis of similar proteins also revealed that these proteins are highly conserved throughout the fungal kingdom except *Schizosaccharomyces pombe* and few other closely related fungi. Lately,

the investigation of the FAR1 like proteins containing RING/PH domain has been extended to different fungal species. The *Candida albicans* Far1 ortholog has been analyzed for its roles in mating and pheromone mediated arrest (Côte and Whiteway, 2008). FAR1 deletion mutants of the *C. albicans* show defect in mating and pheromone mediated cell cycle arrest, suggesting that FAR1 like proteins may be conserved in other fungal species.

In *S. cerevisiae*, Far1p plays two independent functions in the pheromone response process during mating (Elion, 2000). It acts as a cyclin-dependent kinase inhibitor that triggers cell cycle arrest in response to pheromones and it also regulates pheromone-induced cell projection during mating (Chang and Herskowitz, 1990; Shimada et al., 2000). Yeast cells lacking FAR1 fail to show cell cycle arrest assay in presence of pheromones like α-factor and also show a drastic decrease in the mating efficiency with opposite mating type cells. We wanted to investigate whether MFAR1 can functionally complement mating and cell cycle arrest defect in Δfar1 yeast mutants. Coding region of MFAR1 was cloned in a galactose inducible expression vector and transformed into YMP18 far1 deletion mutants. After confirming the yeast transformants for expression of MFAR1, cell cycle arrest assays were carried out using synthetic α-factor. It was found that none of the transformants showed any zone of clearance except for positive control containing intact yeast FAR1 gene. These results indicate that MFAR1 is not able to functionally complement cell cycle arrest defect in FAR1 deletion mutants in yeast.

In addition to cell cycle arrest, Farlp is required for polarization of yeast cells towards the mating partner during mating (Valtz et al., 1995). Therefore, yeast cells deficient of FAR1 gene show drastic decrease in mating efficiency. We investigated whether MFAR1 will be able to functionally complement mating defect in FAR1 deletion mutant in yeast. IH1793

(MA Ta lys1) yeast cells from log phase cultures were spread on SD agar plates containing galactose, with SD agar plates containing glucose as control. *S. cerevisiae* Far1 mutant strain, YMP18 (MATa farlΔ ura3Δ his2 adel trpl leu2 barl::LEU2) expressing MFAR1, along with far1Δ cells containing empty PYES2 vector and untransformed far1Δ cells were streaked on the lawns of IH1793 cells. Intriguingly, it was found that colonies developed from MFAR1 expressing far1Δ cells whereas no colonies were found in far1Δ streaked sector containing empty PYES2 vector and untransformed far1Δ streaked sector. Quantitative mating tests indicated that MFAR1 expressing far1Δ cells have almost five time higher mating efficiency than far1Δ cells containing empty PYES2 vector and untransformed far1Δ cells. These results indicate that MFAR1 can complement mating defect of far1 deletion mutants in yeast. These results further confirm that two functions of FAR1 i.e. cell cycle arrest and oriented polarizations of yeast cells during mating are distinct and independent functions of the same gene.

In *S. cerevisiae*, Far1 is structurally similar to the Ste5 scaffold protein , even though the overall sequence identity between the proteins is low but both the proteins have similar RING and PH domains. However, the proteins interacting with Far1 are different from those interacting with Ste5 (Côte et al., 2011). MFAR1 also shows 23% identity to the STE5 scaffold protein in *S. cerevisiae* which is a 917 amino acids protein and has all the characteristic domains like RING-H2 Zn finger domain , PH (Pleckstrin homology) domain and vWA (von Willebrand type A) domain. Based on these similarities between Yeast Ste5 and MFAR1, we wanted to investigate whether MFAR1 can complement mating, shmoo formation and pheromone mediated cell cycle arrest defects in yeast STE5 deletion mutant. Ste5 deletion mutant, BYB69 (*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 lys2 hisG trp1-1 ura3-1 ste5 LYS2*) was transformed with PYES2-MFAR1 plasmid and transformants

were confirmed for presence and expression of MFAR1 gene. The transformants were analysed for their ability to complement mating defect in ste5 deletion mutant, BYB69. It was found that unlike FAR1, MFAR1 is not able to functionally complement STE5 mating defect. After mating defect, we wanted to investigate whether MFAR1 can rescue ste5 deletion mutants from defect in pheromone mediated cell cycle arrest and shmoo formation but none of these functions were complemented by MFAR1. From FAR1 and STE5 complementation assays it can be concluded that MFAR1 is functionally related to yeast FAR1 rather than STE5.

On the basis of bioinformatics analysis, it is evident that unlike *S. cerevisiae*, a second RING/PH/VWA containing Ste5 like protein is absent in the genomes of all filamentous fungi including *M. oryzae*. It may be hypothesized that MFAR1 functions as both STE5 and FAR1 in *M. oryzae* and after the whole genome duplication (WGD) led to the duplication of MFAR1 allowing its subfunctionalization into the Far1 and Ste5 proteins. Our results based on complementation studies do not support this hypothesis as MFAR1 complements the mating defect in far1Δ mutant but not in case of ste5Δ mutant.

Generation of knockout strains can be a major bottleneck in most of the filamentous fungi including rice blast fungus *M. oryzae*, thus posing a limitation in functional analysis of the genes under study. Split marker recombination has been recently reported as an efficient method for targeted gene deletion in fungus (Catlett et al., 2003). In the present study, a split-marker based recombination technology was employed to replace the MFAR1 from the *M. oryzae* genome with a hygromycin phosphotransferase gene. The method of gene deletion in split marker gene replacement technology is comparatively easier and more efficient than the vector based classical method. The transforming amplicons can be rather

easily generated either using a recombination fusion PCR strategy or ligation-based fusion PCR, without going through tedious process of gene cloning steps. The number of hygromycin resistant transformants generated from the split marker based technique was smaller when compared to the classical vector based methods where an intact hygromycin phosphotransferase gene is used for selection. Using the traditional approach, 125 hygromycin resistant transformants were produced from 10 mg of the deletion vector based on pGKO2 vector. After molecular characterization of these 125 transformants, none of them was the deletion strain of MFAR1. On the contrary, only 12 hygromycin resistant transformants were generated using 5 mg of each of the transforming amplicons, 5-HY and YG-3 and 3 of them were identified as replacing MFAR1. These results demonstrate that split marker based technology for gene replacement is highly efficient and less cumbersome than pGKO2 based dual selection method of gene disruption.

In *S. cerevisiae*, FAR1 was identified in a mutant screening for growth of cells in presence of mating pheromone. During mating, the cell cycle arrest at G1 phase is caused due to inactivation of Cdc28-Cln2 complex by FAR1 (Mendenhall, 1998). Yeast *Δfar1* are defective in cell cycle arrest in presence of pheromones and also show a drastic decrease in the mating efficiency with opposite mating type cells but does not affect the normal growth or morphology.

In *M. oryzae a*nalysis of growth characteristics, like the growth rate and melanin content of BSM2 and BSM11 mfar1 deletion strains indicated that *Δmfar1* mutants grow at a similar rate as that of the wild type strain but produce comparatively less melanized mycelia. It was also observed that after many generations of subculturing, *Δmfar1* mutants show unstable and abnormal growth patterns like melanin deficient aerial hyphae whereas wild type strain

showed a stable growth pattern after similar number of subculturing. Also, our results indicated that *Δmfar1* mutants produce comparatively less number of conidia than wild type strains and this phenotype was relatively clearer when the mutants were subcultured for more than 15 generations. These phenotypic traits of *Δmfar1* mutants are different from *Δfar1* mutants in yeast; therefore the reason behind such phenotype is not very clear.

Sexual reproduction in fungi starts with the fusion of two morphologically identical haploid cells. However, mating takes place only when the two participating cells belong to different mating types (Bölker and Kahmann, 1993). The mating type of an organism is determined by the specific genetic information present at the mating type locus called as MAT locus. In budding yeast, two different mating types, a and α, have been identified and cells belonging to the mating types are termed as a-cells and α-cells. The mating type in yeast is determined by a single MAT locus, which codes for the regulatory proteins that control the expression of many genes in a mating type specific manner. Nature has always devised a mechanism to ensure the maintenance of either two or more mating types (Bölker and Kahmann, 1993). In fission yeast *S. pombe*, sexual differentiation and mating-type are determined by four different genes located on the mating-type locus, mat1 (Kelly et al., 1988). The cells belong to either plus (P) or minus (M) mating types, and mating takes place only under the conditions of nitrogen starvation. In contrast to budding yeast, the diploid cells resulting from mating are short lived and undergo meiosis and sporulation immediately after mating.

Rice blast fungus, *M. oryzae* is a hermaphrodite, heterothallic ascomycetous fungus which reproduces mostly asexually in nature. There are normally two mating type strains identified in *M. oryzae*, MAT1-1 and MAT1-2. Out of the two strains, at least one should be female-fertile strain , which means it should have ability to form perithecia (Saleh et al.,

2012) . One gene with two different alleles namely MAT1-1 and MAT1-2 controls its mating type during mating (Notteghem and Silue, 1992). Mating between the two strains of opposite mating types has been demonstrated exclusively under laboratory conditions and has never been reported in field conditions.

In *S. cerevisiae,* Fus3 is a key member of the mitogen activated protein kinase (MAPK) pathway which mediates phosphorylation reactions of downstream elements Ste12 and Far1 (Kusari et al., 2004). PMK1 (Pathogenicity MAP kinase1) is a homolog of *S. cerevisiae* FUS3 and it has been reported to be involved in regulation of appressorium development and plant infection (Xu and Hamer, 1996). Also, Pmk1 has been shown to be related to production of perithecia in rice blast fungus as *Δpmk1* mutants are female sterile but are not affected in male fertility. Therefore, we hypothesized that the downstream protein MFar1 may also have some role in sexual reproduction of rice blast fungus. Our results suggest that *Δmfar1* mutants are defective in female fertility as there is a significant decrease in the number of perithecia production when Δmfar1 mutants are crossed with opposite mating type strain. However, no evident effect was seen in male fertility of *Δmfar1* when crossed with opposite mating type strain. It indicates that like yeast FAR1, MFAR1 is an essential element in sexual reproduction in rice blast fungus.

Specific cell recognition during mating in many fungal species is mediated by diffusible peptide mating factors, termed as pheromones (Bölker and Kahmann, 1993). The best characterized system in terms of the molecular mechanisms of pheromone production and response is the budding yeast *S. cerevisiae*. Existence of peptide pheromones in yeast came first from the observation that a diffusible substance can act from a distance in a cell type specific manner (Levi, 1956). It was observed that supernatant from a-cell and α-cell

cultures contain some factors that can induce certain morphological and physiological changes including cycle arrest in the opposite mating type cells. These secreted factors from both a-cell and α-cell cultures were purified and characterized. α- factor was identified as a 13-amino acid peptide, secreted by the classical secretory pathway (STÖTZLER et al., 1976). It has been reported that MFa1 and MFa2 genes code for 165 and 120 amino acids precursors which contain the tandem copies of the α-factor coding sequence (Kurjan and Herskowitz, 1982).

Recent studies suggest that peptide pheromones are ubiquitous in the fungi and have been found in fungal species in which even the physiological studies didn't support the view of their presence (Bölker and Kahmann, 1993). There are also indications that in addition to cell-cell recognition, pheromones are essential for other aspects of development, like pheromones induce meiosis in *S. pombe* and are involved in maintenance of the filamentous growth in *Ustilago maydis*. As morphogenetic events are at times associated with pathogenesis related growth, it would be interesting to investigate whether pheromones are involved in such transitions directly or indirectly.

In *M. oryzae,* no pheromone like peptides have been purified till date but two putative pheromone precursor genes have been identified (Shen et al., 1999). Although, these genes are present in the genome of both mating types but their expression is regulated in a mating type specific manner. MF1-1 gene is expressed in MAT1-1 strains and it is predicted to encode a 26-amino-acid peptide that is processed to form a lipopeptide pheromone. The MF2-1 gene on the other hand is expressed in MAT1-2 strains only. This gene is predicted to code for a polypeptide that is processed to yield a pheromone. One the basis of sequence analysis it has been assessed that the MF2-1 coded pheromone has a striking similarity to a

predicted pheromone sequence of another fungus, *Cryphonectria parasitica* (Shen et al., 1999).

An exciting observation has been reported in rice blast fungus that yeast α-factor pheromone can inhibit formation of appressorium in a mating type specific manner (Beckerman et al., 1997). The basis for this inhibition is largely unknown; however, some results indicate that the trait that determines α-factor sensitivity segregates as a single locus independent from the mating type locus (Shen et al., 1999). It indicates that yeast pheromone α -factor is not recognized as a sex pheromone by *M. oryzae*. However, these observations clearly contradict the previous data that indicated a mating type-specific response of rice blast fungus to yeast α-factor. The reason for the different results from the two independent studies has not been resolved, therefore needs a broader study at the molecular level. Our results however support the previous findings which state that yeast pheromone response in *M. oryzae* is not general but specific in nature. Quantitative Real Time analysis data suggested that α-factor induces expression of PMK1 and MFAR1 specifically in MAT1-2 cell type and expression of same genes in B157 MAT1-1 type strain was not upregulated at all. On the other hand there was a minute decrease in the expression of PMK1 and MFAR1 when MAT1-1 was treated with yeast α-factor or α-cell supernatant. The reason for this slight downregulation however is not clear as such kind of response has never been reported in *S. cerevisiae* or any other fungi. Our results demonstrating that yeast pheromone induce expression of genes in another species of fungus are novel and have not been reported previously in any system.

Pheromones in fungi are recognized by specific G protein-coupled receptors (GPCRs), which are present on the surface of recipient cells (Xue et al., 2008). GPCRs are the largest

family of cell receptors and are generally efficient targets for antifungal compounds. GPCRs interact with small molecules with the transmembrane domains whereas they interact with large ligands through their extracellular domains (Eglen and Reisine, 2009). The best characterized GPCR in fungal system is Ste2, the receptor for α factor in *S. cerevisiae* (Eilers et al., 2005). Yeast pheromone α-factor is recognized by the Ste2 receptor on the surface of a-cells, initiating the mating signalling pathway. Even though α-factor is a short peptide, different regions of this pheromone have defined roles in Ste2 activation. The C-terminus of α-factor is involved in the physical interaction with Ste2 receptor, while the N-terminus is more related to some of the downstream signalling events of the pathway (Naider and Becker, 2004). Ste2 like receptor has not been identified in rice blast fungus till date and one of the main reasons being low degree of sequence similarity of Ste2 with the proteins present in *M. oryzae.* One of the probable candidates for putative Ste2 in rice blast fungus is a hypothetical protein (Gene ID: MGG_04711). Functional characterization of this protein can provide us with new insights towards understanding of pheromone response in *M. oryzae* and its relation with appressorium development.

In yeast, FARl transcription is induced by four to five folds in presence of the mating pheromone α-factor by Stel2 regulated gene expression (Chang and Herskowitz, 1990). When FAR1 was overexpressed using pGAL1 promoter, it didn't have any drastic effect on the growth, mating frequency and morphology of wild-type cells. However, the cells expressing pGAL-FAR1 were slightly larger and more elongated in comparison to control cells carrying the empty vector (Chang and Herskowitz, 1992). In *M. oryzae,* we found that yeast α-factor induces expression of PMK1 and MFAR1 by multifold. Also, it is known that α-factor causes inhibition of appressorium formation in a mating type specific manner (Beckerman et al., 1997). We wanted to find out whether MFAR1 overexpression alone can

lead to appressorium defect in rice blast fungus. When MFAR1 was expressed under strong constitutive promoter TrpC, it was found that overexpression of MFAR1 leads to defect in appressorium formation and caused reduction in ability to infect plants. It was also noted that many of the conidia from some of the overexpression transformants form a very long germ tube before forming the appressorium. These results indicate that overexpression of MFAR1 is sufficient to cause cell cycle arrest in rice blast fungus.

Cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger produced by most cell types in response to external signals including nutrients (Daniel et al., 1998). In rice blast fungus, it has been demonstrated that contact of germ tube with a hydrophobic surface is adequate for appressoria formation, however this recognition step is efficiently bypassed by the addition of exogenous cAMP. It was also demonstrated that 3-isobutyl-1-methylxanthine (IBMX), which is a specific phosphodiesterase inhibitor and causes an accumulation of intracellular cAMP in cells induces high levels of appressorium formation (Lee and Dean, 1993). Spore germination and appressorium formation in rice blast fungus are closely linked with each other through several signal pathways including the cAMP pathway and of the Pmk1 and Mps1 MAP kinase cascades (Dean, 1997; Thines et al., 2000; Xu, 2000) The cyclic AMP signalling pathway is important for surface recognition and appressorium formation and turgor generation in rice blast fungus (Mitchell and Dean, 1995; Thines et al., 2000; Xu et al., 1997).The PMK1 MAP kinase plays an essential role in appressorium formation and the pmk1 deletion mutant is unable to form appressorium and infect plants. However, the mutants recognize hydrophobic surfaces and form appressorium in presence of exogenous cAMP (Xu and Hamer, 1996). As reported previously, yeast α-factors inhibits appressorium formation in *M. oryzae*, however supplementation of exogenous cAMP can override this inhibitory activity of α-factor (Beckerman et al., 1997).

Our results show that cAMP can significantly induce appressorium formation in MFAR1 overexpression transformants, therefore it indicates that cyclic AMP signalling works in coordination with MFAR1 and other members of MAPK pathway during appressorium formation and plant infection.

Pheromone activation of the GPCRs initiate a signal transduction cascade involving the mitogen-activated protein kinase (MAPK) pathway (Bardwell, 2004). This pathway involves kinases which activate Ste12 and Far1. Far1 is a cell cycle arrest protein which exerts its effect by inhibiting cyclin-dependent kinase activity of CDC28 and its association with G1 cyclin Cln2. It has been demonstrated that α-factor-induced arrest is restored in far1Δ mutants when CLN2 is deleted (Chang and Herskowitz, 1990). Application of α-factor in yeast cells nearly eliminates CLNI and CLN2 mRNA expression and their associated kinase activities (Tyers et al., 1993; Wittenberg et al., 1990). These results indicate that Far1 is involved in the downregulation of Cln1 and Cln2, an important step for cell cycle arrest. Our results show that overexpression of MFAR1 leads to downregulation of MoCyb1, a Cln2 homolog in *M. oryzae.* This result confirms that MFAR1 has functional similarity with yeast FAR1, as far as regulation of cyclin expression is concerned. Cyclin Cln2 has also been reported to be involved in pseudohyphal and invasive growth in *S. cerevisiae* (Loeb et al., 1999). Our results demonstrate that overexpression of MFAR1 leads to reduction in the appressorium development and plant infection in the rice blast fungus. Therefore, functional characterization of MoCyb1 can provide us the understanding of how cell cycle arrest, mating and cyclins are linked to the infection related development in rice blast fungus.