Chapter 3

Materials and Methods

3. MATERIALS AND METHODS

3.1. Microbial cultures and growth media

3.1.1. *Escherichia coli*

Luria Bertani (LB medium)

25 g of LB (HiMedia, India) were dissolved in 900 ml distilled water and the volume was brought up to 1 litre. It was autoclaved at 121 °C for 15 minutes at 15 psi. Solution was allowed to cool down to 55 °C and appropriate antibiotics were added before pouring into sterile petri dishes (tarsons, India). LB agar was prepared by adding 20 g/l Agar powder (HiMedia, India) to LB. Melted agar medium was allowed to cool down to 55 °C prior to addition of antibiotic and then poured in the sterile petri dishes.

3.1.2. *Saccharomyces cerevisiae*

YEPD (yeast extract peptone dextrose) Medium

5 g of YEPD (HiMedia, India) was dissolved in 900ml distilled water and volume was brought up to 1 litre. The medium was autoclaved at 121 °C for 15 minutes at 15 psi. The broth was allowed to cool to room temperature and appropriate antibiotics were added to prevent bacterial contamination. Yeast was grown overnight at 28°C in a shaking incubator at 200 rpm prior to transformation. YEPD agar was prepared by adding 2% agar to YEPD broth before autoclaving.

Synthetic defined (SD) medium for yeast

6.7g of YNB (Yeast nutrient base) without amino acids (Hi-Media, India), 10g of glucose, and 20 g of agar were added to 900 ml of distilled water and final volume was brought up to 1 litre. The medium was autoclaved at 121°C for 15 minutes at 15 psi. After autoclaving, appropriate amino acids from stocks (100X) were added to the media in aseptic conditions. The media was poured in sterile petri dishes and allowed to solidify.

3.1.3. *Magnaporthe oryae*

Oatmeal agar

35 g oatmeal agar (Hi-Media, India) was added to 800 ml distilled H_2O. The volume was made up to 1 litre and autoclaved at 121°C for 15 minutes at 15 psi. Appropriate antibiotics were added to avoid the bacterial contamination and the media was poured in sterile petri dishes and allowed to solidify.

Complete medium (CM)

The CM broth was prepared by dissolving 10 g glucose, 2 g peptone, 1 g yeast extract, 1 g casamino acids, 6 g NaNO3, 0.5 g KCl, 0.5 g MgSO4, and 1.5 g KH2PO4 in 1 litre distilled water. The pH was adjusted to 6.5. It was followed by autoclaving at 121 °C for 15 minutes at 15 psi.

3.2. Bacterial strains

***Escherichia coli* DH5α**

E.coli DH5α (F^-, *end*A1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *thi*-1, λ^-, *rec*A1, *gyr*A96, relA1 Δ(*lacZYA*-*arg*F) U169 *deo*R (ϕ80d*lac*Δ(lacz) M15; Bethesda Research Laboratories) was used for bacterial transformation, cloning and plasmid isolation. Cells were grown at 37^{o}C in LB broth and *E. coli* transformants carrying plasmid vectors were grown on LB agar containing either ampicillin or kanamycin at a concentration of 100 μg/ml or 50 μg/ml, respectively. Bacterial cultures were maintained at 4^{o}C as slants or stab cultures on LB agar medium. Cells were stored in 25% glycerol solution at -80^{o}C for long term preservation.

3.3. Yeast strains

S. cerevisiae strains mentioned in Table.1 were used in our study. The yeast strains were grown at 28^{o}C and maintained on YEPD medium. Streptomycin (50 µg/ml) was added to the autoclaved medium to avoid bacterial contamination.

Strain	**Genotype**	**Source**
S288C	*MAT α, SUC2, gal2, mal, mel, flo1, flo8-1, hap1, ura 3-52*	Lab stock
IH 1793	*MA Tα lys 1*	Ira Herskowitz lab, USA
YMP18	*MATa farl A ura3A his2 adel trpl leu2 barl::LEU2*	Ira Herskowitz lab, USA
JC2-IB	*MATa HMLa HMRa bar1-1 met1 ade2-101 ura3-52*	Ira Herskowitz lab, USA
BYB69	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 lys2 hisG trp1-1 ura3-1 ste5 LYS2*	Jeremy Thorner lab, USA
AH109	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1UASGAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3 : : MEL1UAS-MEL1TATA-lacZ*	Clonetech, Inc.
Y187	*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met–, gal80Δ, URA3 : : GAL1UAS-GAL1TATA-lacZ*	Clonetech, Inc.

Table. 1. Yeast strains used in the study

3.4. Fungal cultures

Magnaporthe oryzae B157 strain (MTCC accession number 12236), corresponding to the international race IC9 was used for this study. The fungus was grown on either YEG agar or Oat meal agar, at 28-30^{o}C. Mycelial plugs were inoculated on OMA plates for 8-10 days and conidia were harvested by scraping the biomass with a sterile surgical blade. The biomass was resuspended in sterile water, and passed through autoclaved Mira cloth to obtain mycelia free conidia. The different transformants were maintained as monoconidial isolates and stored on paper strips in vacuum.

3.5. Competent cell preparation and transformation of *E. coli*

A single colony of *E. coli* DH5α was inoculated in 3 ml of LB broth and grown overnight at 37^{o}C. An overnight grown culture (1 ml) was inoculated in 100 ml of fresh LB broth and incubated at 37^{o}C in a shaking incubator till $O.D_{550}$ = 0.4. The culture was incubated on ice for 10 min and the cells were pelleted down by centrifugation at 2012 x g for 5 min at 4^{o}C. The cells were resuspended in ice-cold 0.1 M $CaCl_2$ and incubated on ice for 30 min. The cells were harvested by centrifugation and resuspended in ice-cold 0.1 M $CaCl_2$ with 20% glycerol. The competent cells were stored at -80^{o}C in 100µl aliquots. Transformation was carried out by adding plasmid DNA or ligation mixture to the 0.1 ml aliquot of competent cells and incubated at 4^{o}C for 30 min. Cells were subjected to heat shock at 42^{o}C for 90 sec, followed by 2 min. incubation on ice. 1 ml of LB broth was added to the above suspension and incubated at 37^{o}C for 45 min. About 0.1 ml aliquot of transformation mixture was plated on LB agar medium containing either ampicillin (100 µg/ml) or kanamycin (50 µg/ml).

3.6. Transformation of *S. cerevisiae* with plasmid DNA

Yeast transformation was carried out using 'One-step transformation' method with some modifications (Chen et al., 1992). A single yeast colony was inoculated in YPD broth and incubated in a shaking incubator at 28°C till stationary phase (10^8 cells/ml). Cells were centrifuged, washed twice with sterile water and mixed with one-step buffer containing 0.2 M lithium acetate, 40% polyethylene glycol (PEG 3350), 1-2 µg of plasmid DNA and 50 µg of single-stranded carrier DNA (ssDNA). The cell suspension was mixed by vortexing and incubated at 42°C for one hour. After incubation the cell suspension was directly plated onto the selective medium and incubated at 28°C for 3-5 days.

3.7. Isolation of plasmid DNA

A single bacterial colony was grown overnight in LB broth at 37°C in a shaking incubator. The culture was centrifuged in a 1.5 ml microfuge tube at 13414 x g for 20 sec and the bacterial pellet was resuspended in 300 µl of STET (8% Sucrose, 0.1 % TritonX-100, 50 mM Tris pH 8.0, 50 mM EDTA pH 8.0). Lysozyme (10 µl of 50 mg/ml) was added to cell mixture, vortexed and boiled in a water bath for 45 sec, followed by centrifugation at 13414 x g for 10 min. The resulting snot was removed with a tooth pick and 20 µl of 5% CTAB was added followed by centrifugation for 10 min. 300 µl of 1.2 M NaCl and 750 µl of 100% ethanol was added to the pellet followed by centrifugation for 10 min. The DNA pellet was washed with 70% ethanol, air dried, resuspended in 50 µl of TE (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) and allowed to dissolve at 68°C for 5-10 mins.

3.8. Nucleic acid manipulations

Restriction enzyme digestion of DNA was carried out in reaction buffers, supplied with the corresponding enzymes, as recommended by the suppliers. Sticky and blunt ended ligations were carried out at 16^{o}C and 20^{o}C respectively for 3-4 h.

3.9. Gene deletion by split marker technology

The split-marker recombination method was used for targeted deletion of MFAR1. The hygromycin phosphotransferase gene, which confers resistance to Hygromycin B (HYG) was used in our study. Two split templates (HY and YG) were amplified from HYG gene from pSilent-1 vector using HY-F, HY-R and YG-F, YG-R primers respectively. 1kb upstrean (5') and 1kb downstream (3') of gene were amplified from genomic DNA of *M. oryzae* wild type strain using 5'F, 5'R and 3'F, 3'R primers. 5' and HY fragments were digested with *Kpn*I using restriction sites present in the primers and ligated using T_4 DNA ligase (Fermentas, Germany). 5'::HY was amplified from the ligation mixture using 5'-F and HY-R primers. Similarly 3' and YG were digested with *Kpn*I and ligated, using this ligation mixture as template YG::3' was amplified using YG-F and 3'-R primers.

PCR was performed in a 20 µl reaction volume containing 100 ng of template DNA, 1X PCR buffer, 10 pico moles each of forward and reverse primers, 0.2 mM dNTPs, and 1U of Taq DNA polymerase. PCR was performed using Veriti PCR thermal cycler (Applied Biosystems, , California, USA) . After a single step of initial denaturation at 95 °C for 5 min, twenty six cycles of amplification were performed in a PCR program. Each cycle consisted of a denaturation at 95°C for 30 s, annealing at 56 °C for 30 s and an extension at 72 °C for 2 min.

Final extension step was performed at 72 °C for 5 min. The PCR products were fractionated by electrophoresis in 1% agarose gel, stained with ethidium bromide and the gels were scanned using a Gel Doc XR documentation system (Bio-Rad, CA, USA).

After preparation of split marker amplicons (5'::HY and YG::3'), 5 µg of DNA containing equal proportions of both 5'::HY and YG::3' was used to transform *M. oryzae* protoplasts (Leung et al., 1990). Transformants were selected in regeneration agar medium with 200 µg/ml of hygromycin B. Integration of the hygromycin cassette was confirmed by PCR using hygromycin-specific primers. Targeted gene deletion was confirmed by gene specific PCR of MFAR1 and its transcript.

3.10. Fungal transformation

Fungus was grown on OMA plates for 8-10 days and conidia were harvested. 10^5 conidia were inoculated in 30 ml of CM in 100 ml conical flask. The flask was incubated at 28°C for 3-4 days with shaking at 200 rpm. The resulting mycelia was harvested by filtration, washed with distilled water and resuspended in 30 ml of 1 M sorbitol. Lysing enzyme (20 mg/ml in 1M Sorbitol) was added at the rate of 1.5 ml of lysing enzyme per 3 g of mycelium. The mixture was incubated at 28^{o}C in a shaking incubator at 100 rpm for 3-4 hours. The protoplasts were harvested by filtering through Mira cloth (Calbiochem, USA). The protoplast suspension was centrifuged at 3000 x g for 10 minutes at 4^{o}C. The pellet was resuspended in 10 ml of 1 M sorbitol and centrifuged at 3000 x g for 10 minutes at 4 ^{o}C again. This step was repeated and pellet was resuspended in 10 ml of STC (1M sorbitol, 50mM Tris HCl pH 8.0, 50mM$CaCl_2$). Protoplasts were diluted to $3x10^7$/ml in STC buffer and approximately 5 µg (20 µl) of

appropriate plasmid was added to 200 ml protoplast solution. The mixture was incubated on ice for 30 min, 1 ml of filter sterilized PTC buffer (40 % PEG 3350, 1 M sorbitol, 50 mM Tris-HCl, 50 mM $CaCl_2$, pH 7.5) was added and the mixture was incubated at room temperature for a further 30 min. 3 ml of CMS (Complete media with 1M Sorbitol) was added and the protoplast mix was incubated at 28^{o}C in shaking incubator at 100 rpm for 12-14 h. The protoplast suspension was then centrifuged as before and the pellet was resuspended in 0.1 ml of STC. Molten regeneration medium was added and the protoplasts were poured on CM agar plates containing 200µg/ml of hygromycin B (MP Biomedicals, USA). Transformants were picked after 4-6 days and again subcultured on CM agar plates containing 200 µg/ml of hygromycin B for secondary selection. Single conidia were isolated from each transformant to ensure that each mutant was derived from single nucleus.

3.11. Phenotypic characterization of the transformants

Plant infection and appressorium assay

Harvesting of spores was carried out by scrapping mycelia from 7-8 day-old plates and mixing it with 1ml of sterile water and counting the spores using haemocytometer. Infection assays were performed as previously described (Xu and Hamer, 1996), using barley (*Hordeum vulgare*) and rice (cultivar CO39). Briefly, conidia were harvested from 8-10 day old cultures and resuspended in 0.2% gelatin to a concentration of $5x10^5$. 21 days old rice plants were spray inoculated with the conidial suspension using an artist's airbrush. Virulence was assessed as dark brown lesions appearing on leaves after 7-10 days.

In vitro appressorial assays were performed using hydrophobic gel bond film as described previously (Hamer et al., 1988). 50µl of 10^3 spores/ml were carefully placed on the hydrophobic side of gel bond film (Amersham Biosciences, England) and incubated at 28^{o}C under moist conditions for 8-14 h. The conidia were observed under light microscope (Optiphot-2*;* Nikon, Japan) and the frequency of appressorial formation was determined by counting the number of mature appressoria emerged from 100 conidia after 14 hours.

3.12. DNA isolation from *M. oryzae*

Extraction of genomic DNA from *M. oryzae* was carried out as described previously, with some modifications (Dellaporta et al., 1983). Fungal spores were inoculated in CM broth for 4-5 days and harvested. The biomass was frozen in liquid nitrogen and ground into a fine powder in a pre chilled mortar and pestle. The powder was transferred to a 1.5 ml microfuge tube containing 1 ml of extraction buffer (0.1 M Tris, pH 8.0; 0.05 M EDTA, pH 8.0; 0.5 M NaCl; 0.01 M β-mercaptoethanol). 100 µl of 20 % SDS was added, mixed and the tubes were incubated at 65^{o}C for 10 min. 200 µl of 5 M potassium acetate was added to the mixture and incubated on ice for 20 min, followed by centrifugation at 10,000 rpm for 10 min. The supernatant was collected in a clean 1.5 ml tube containing 500 µl of isopropanol, mixed and incubated at -20^{o}C for 30 min. It was followed by centrifugation at 13,000 rpm and the supernatant was discarded and tubes were air dried. The DNA was resuspended in 200 µl of TE buffer and incubated at 65^{o}C for 10 min. All the insoluble debris was removed by a tooth pick and 10 µl of RNase (10 mg/ml) was added to the solution and was incubated at 68^{o}C for 10 min. 200 µl of chloroform: isoamylalcohol (24: 1) was added to these tubes, mixed well and

centrifuged at 13,000 rpm for 5 min. Upper aqueous phase was transferred to a fresh 1.5 ml tube and 50 µl of 3 M sodium acetate, pH 5.2, was added. After that, 500 µl of isopropanol was added, mixed gently, centrifuged at 13,000 rpm for 10 min to pellet down the DNA. The pellet was washed with 70% ethanol, air dried and dissolved in 100 µl of TE buffer.

3.13. Southern blot analysis

Genomic DNA of wild type strain and transformants was isolated and Southern blot analysis was carried out as described previously (Sambrook et al., 1989). Briefly, 20 µg of the genomic DNA from wild type strain and transformants was digested with restriction enzymes as recommended by the supplier. The digested DNA was electrophoresed on 0.8% agarose gels, stained with ethidium bromide, visualized in Gel Doc XR systems (Bio-Rad, USA) and blotted onto nylon membrane (Hybond N^+, Amersham Biosciences, England). DNA was fixed to the membranes by UV crosslinking (12 x 10^4 µJ/cm^2) using UV cross-linker (Spetrolinker, Spectronics Corporation, USA). Hybridisation probe was labeled nonradioactively using Gene Images AlkPhos Direct Labelling and Detection System as per manufacturer's instructions (Amersham Biosciences, England). The hybridization and detection was followed according to the manufacturer's instructions.

3.14. Complementation of Δ*mfar1* mutant with native gene

MFAR1 native promoter (1.4 Kb) and MFAR1 gene (3.8 Kb) was amplified from B157 wild type strain using FP1.5-SpeI-F and FAR1-XbaI-R primers (Appendix-I) using XT-5 Taq DNA polymerase (Bangalore Genei, India). The gene cassette was cloned in pFC2 vector at *Spe*I and

*Xba*I sites. Fungal transformation was carried out and transformants were selected on complete medium containing 50 mg/ml of Bialophos.

3.15. RNA isolation and Real time-PCR analysis

Mycelia, harvested from 3-4 days old culture was crushed in liquid nitrogen for RNA isolation using TRIzol (Invitrogen, USA). The first-strand cDNA was synthesized with M-MLV reverse transcriptase (Bangalore Genei, India). 10 µl reaction mixture in each well contained 5 µl of Power 2× SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 2 µl of cDNA (12.5 ng/µl), and 15 pmol of each primer. Real-time PCR was performed using ABI-Prism 7900HT system (Applied Biosystems, CA, USA) according to the protocols recommended by the manufacturer. The thermal cycling program was set as 10 min at 94°C followed by 41 cycles of 15 s at 94°C and 1 min at 56°C. All amplification curves were analyzed with a normalized reporter threshold of 0.1 to obtain the threshold cycle (Ct/CP) values. Ct values were normalized using ß-tubulin as internal reference, and fold change in the expression level was calculated (Livak and Schmittgen, 2001). qRT-PCR was performed three times with two replicates in each experiment, and graphs were prepared as average value for all the replicates. The primers used for different qRT PCR reactions are mentioned in appendix-I.

3.16. Overexpression of MFAR1

MFAR1 gene was amplified from B157 wild type strain using FAR1-XhoI-F and FAR1-XbaI-R primers (Appendix-I). Amplified product was cloned at *Xho*I and *Apa*I restriction sites of pSilent1 vector under strong constitutive TrpC promoter. The resulting construct was

transformed into B157 wild type strain (Leung et al., 1990). Transformants were selected on complete medium containing 200µg Hygromycin B and screened for integration of gene cassette using TrpC-F and FAR1-R primers. Expression analysis of resulting transformants was carried out using quantitative real time PCR.

3.17. Pheromone Response Assays

Cell cycle arrest (halo) assays was performed as previously described (Valtz et al., 1995). 10^3-10^4 cells from log phase cultures were plated on YEPD plates. 1µg α-factor (Sigma Chem. Co., St. Louis, MO) in 10 µl water was spotted on a sterile filter disk already placed on the plates. Plates were incubated for 2 days at 30°C and zone of clearance around filter disc was considered as cell cycle arrest. Shmoo morphology was determined by the addition of 10^{-6} M α-factor to 100µl log phase cultures for 6 h at 28°C and viewed by differential interference contrast (DIC) microscopy.

3.18. Mating assays in *S. cerevisiae*

Mating assays were performed by streaking a-strains on lawns of wild-type α-strains on restrictive SD minimal medium on which only diploids are able to grow. Plates were incubated for 2 days at 30°C. Quantitative mating assays were performed as described previously (Valtz et al., 1995). ~3 $\times 10^6$cells from log phase cultures of a and α strains were mixed and filtered onto nitrocellulose filters (Millipore Corp., Bedford, MA). The cells on filters were allowed to mate on YEPD plates for 4-6 h at 28°C. Cells were resuspended in 5 ml sterile water by vigorous vortexing for 30 seconds, and plated on selective SD plates to determine total

diploids. Plates were incubated for 2 days at 28°C.and diploid cells were counted to determine mating efficiency.

3.19. Mating type determination and fertility assessment of *M. oryzae*

Mating type determination of *M. oryzae* strains was carried out by the amplification of mating type genes *mat1-1* and *mat1-2*, as described previously (Zheng et al., 2008). The primer pairs used for the determination of mating type are:

- (MAT1-1-F 5'TCAGCTCGCCCAAATCAACAAT3')
- (MAT1-1-R 5'ACTCAAGACCCGGCACGAACAT3')
- (MAT1-2-F 5'GAGTTGCCTGCCCGCTTCTG3')
- (MAT1-2-R 5'GGCTTGGTCGTTGGGGATTGT3')

PCR was performed in a 20 µl reaction volume containing 100 ng of template DNA, 1X PCR buffer with 1.5 mM MgCl2, KCL, 10 pico moles each of forward and reverse primers, 0.2 mM dNTPs, and 1U of Taq DNA polymerase. PCR was performed using Veriti PCR thermal cycler (Applied Biosystems, , California, USA) . After a single step of initial denaturation at 95 °C for 5 min, twenty six cycles of amplification were performed in a PCR program. Each cycle consisted of a denaturation at 95°C for 30 s, annealing at 56 °C for 30 s and an extension at 72 °C for 1 min. Final extension step was performed at 72 °C for 5 min. The PCR products were fractionated by electrophoresis in 1% agarose gel, stained with ethidium bromide and the gels were scanned using a Gel Doc XR documentation system (Bio-Rad, CA, USA).

Sexual crosses were performed on rice flour agar (RA) medium (20 g rice flour, 2 g yeast extract, 15 g agar and 1 l water). Crosses of transformants were performed by placing small mycelial plugs of the test strain 4 cm from a mycelial plugs of reference strain on rice agar in a

90 mm petri dish as described previously (Saleh et al., 2012) . Initially, cultures were incubated for two days at 25°C and were then placed under continuous white and blue light at 20°C. After 18-21 days of incubation at 20°C, perithecia were observed. If there are two rows of perithecia formed between the tested strain and the reference strain, it indicates that both tested and tester strains are male-fertile and female-fertile. However, if a single row of perithecia is produced by reference strain, it indicates that the tested strain is female sterile but male-fertile. Total absence of perithecia from either side will indicate that the tested strain is both female sterile and male-sterile. The numbers of perithecia formed in a uniform area are estimated by counting the perithecia in three 3 × 3 sq. mm area of each line.

3.20. Subcellular localization of MFAR1

Signal peptide of MFAR1 gene was predicted by using bioinformatics tool SignalP. 1.4 Kb MFAR1 promoter along with 180bp signal peptide was amplified from wild type B157 strain. eGFP gene was amplified from pEGFP plasmid using gene specific primers. Green fluorescent protein (GFP) reporter gene was ligated with the 1.4 Kb MFAR1 promoter and 180 bps MFAR1 signal peptide using restriction enzyme *Kpn*I. The resulting fusion construct was introduced into pSilent1 vector at *Xba*I sites, replacing the TrpC promoter with MFAR1 promoter:Signal peptide:GFP. The reporter construct, pProMFAR1:SP:GFP, was introduced by protoplast transformation into B157 wild type strain. For fluorescent microscopy, GFP expressing mycelia were observed under LSM 700 confocal microscope (Carl Zeiss, Germany) at Excitation/Emission 488/530 nm. Nuclear staining with Hoechst 33258 was observed at 405 nm.

3.22. Statistics

Statistical analysis of the data was carried out by one way ANOVA followed by Tukey's HSD (Honest Significant Differences) test and two way ANOVA followed by Bonferroni's post-hoc test. The results were represented as mean ± S.D using Graph Pad Prism version 5.0 Graph Pad Software, San Diego, USA.