Chapter 4

Results

4.1. Identification of *S. cerevisiae* FAR1 homolog in *M. oryzae*

A FAR1 homologue corresponding to MGG_00134 (GenBank accession number XP 369110.1) was identified in the *M. oryzae* genome database using NCBI BLASTP analysis. MGG_00134 showed 24% homology with *S. cerevisiae* FAR1 and no other obvious homologs were found in the available genome sequence thus making MGG_00134 the probable candidate for FAR1 homolog in *M. oryzae*. Also, the sequence similarity between the two proteins is comparatively very high between the conserved domains. On the basis of available genome sequence, this homolog of FAR1 in *M. oryzae* consists of 1161 amino acids with three introns (Fig. 5A). The length of the gene including the intragenic sequences is 3841 base pairs, whereas the total exonic region consists of 3486 base pairs. In order to confirm the length and sequence of this gene in B157 strain, genomic DNA was isolated from wild-type strain and this genomic DNA was used to amplify this FAR1 homolog and 3841 base pairs of amplicon was obtained (Fig. 5B). Similarly, total RNA was isolated from the wild-type strain B157and cDNA was prepared. Coding region of the gene was amplified from the cDNA and 3.5 Kb band was obtained. These results confirmed the presence and expression of the gene in the wild-type strain.

Although, there is only 24% similarity between FAR1 homolog in *M. oryzae* and *S. cerevisiae* FAR1, the BLASTP analysis showed that this protein is closest to similar proteins present in some closely related filamentous fungi. It showed almost 74%, 73% and 70% identity with *Gibberella zeae, Fusarium oxysporum* and *Neurospora crassa* respectively. These results indicate that *M. oryzae* MGG_00134 is more identical to similar proteins in other filamentous fungi than yeast.

A phylogenetic tree was prepared using ClustalX software (Thompson et al., 1997) by comparing the FAR1 homolog in *M. oryzae* with other similar proteins in other fungi (Fig. 6). The phylogram showed that FAR1 like protein in *M. oryzae* is a distant relative of *S. cerevisiae* FAR1 but is very closely related to similar but hypothetical proteins in other filamentous fungi like *N. crassa. F. oxysporum* and *G. zeae.*

NCBI and Smart Domain prediction tool shows that FAR1 homolog in *M. oryzae* contains the 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). These results indicate that even though there is not a high degree of identity between FAR1 and its corresponding homolog in *M. oryzae*, all the characteristic domains are conserved in a similar order (Fig. 7). Based on the sequence similarities and predicted characteristic domains, FAR1 homolog in *M. oryzae* (MGG_00134) was named as MFAR1. Predicted domain structure analysis of FAR1 and its homologs showed that zinc finger domain, PH domain and VWA domains are highly conserved throughout the fungal kingdom except *Schizosaccharomyces pombe* and few other closely related fungi.

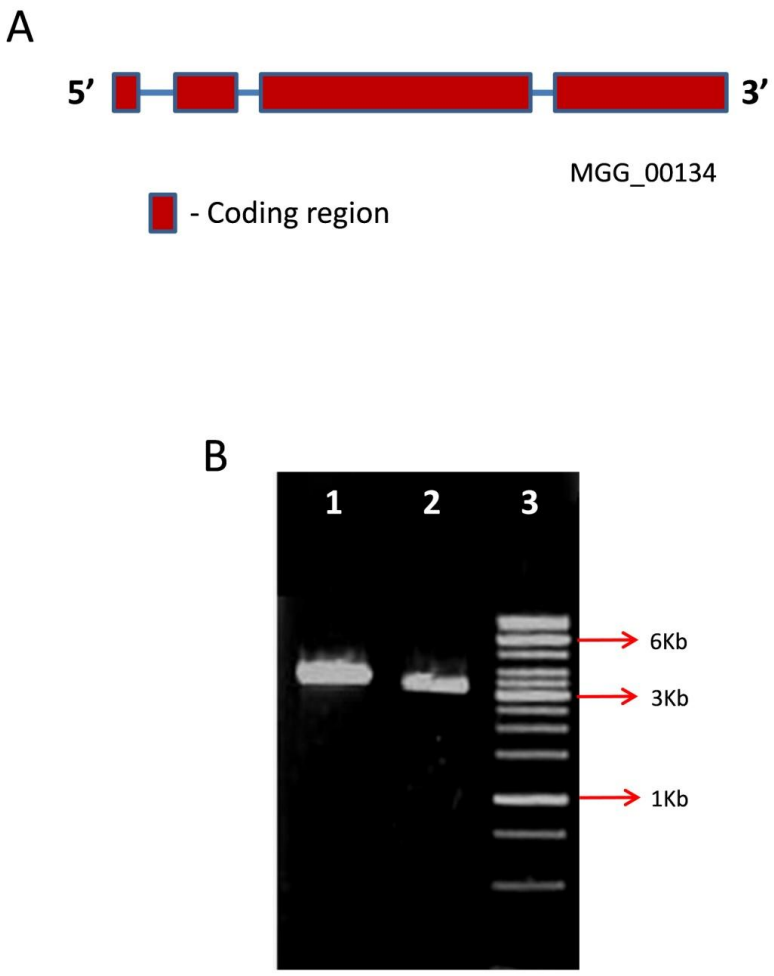

Figure. 5. Homolog for *S. cerevisiae* FAR1 in *M. oryzae*. (A) NCBI genome database indicates presence of three introns inside the predicted homolog. **(B)** The predicted gene with and without introns were amplified from genomic DNA and cDNA of wild B157 type strain. **Lane 1**: 3.8 Kb gene, lane **2**: 3.5 Kb coding sequence and **lane 3**: 1 Kb ladder.

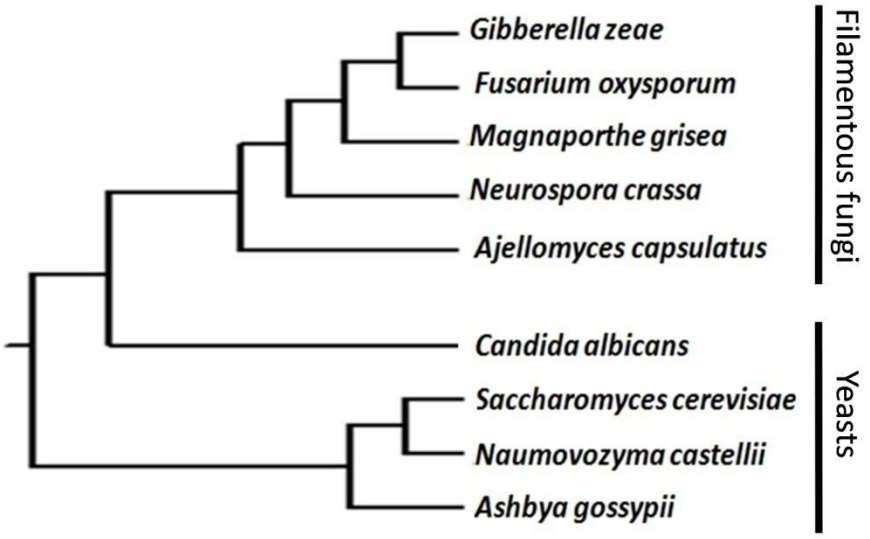

Figure. 6. Phylogenetic tree of fungal yeast FAR1 orthologs. ClustalX software dendrogram depicting the phylogenetic relatedness of *M. oryzae* FAR1 homolog with FAR1 like proteins from other fungi. Bootstrapping (500 trials) was utilized in generating the phylogenetic tree. Accession numbers for the sequences are: *Gibberella zeae* (XP_011326409.1), *Fusarium oxysporum* (EWZ44347.1), *Neurospora crassa* (XP_958610.1), *Ajellomyces capsulatus* (EEH03738.1), *Candida albicans* (XP_720396.1), *Saccharomyces cerevisiae* (NP_012378.1), *Ashbya gossypii* (AEY96883.1), *Naumovozyma castellii* (XP_003678289.1) and *Magnaporthe oryzae* (XP_369110).

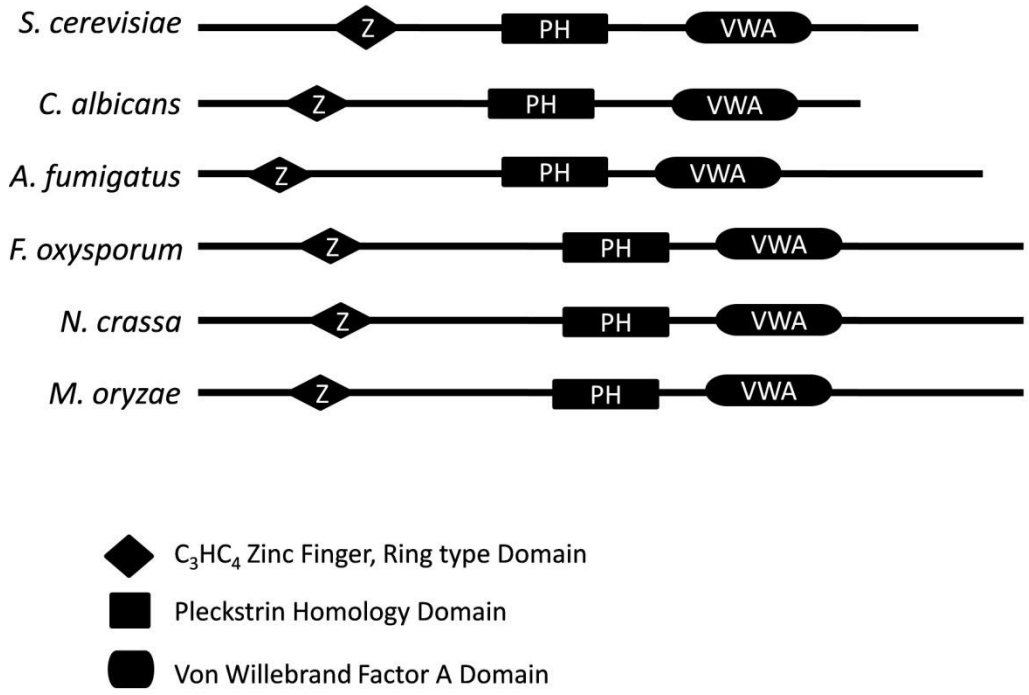

Figure. 7. Schematic representations of the structural organization. FAR1 homologs in different fungi contain the characteristic C3HC4 Zinc Finger domain, Pleckstrin Homology (PH) domain and Von Willebrand Factor A (vWA) domain, suggesting the evolutionary relatedness.

4.2. MFAR1 complements the mating defect in *far1Δ*mutant in *S. cerevisiae*

FAR1 in *S. cerevisiae* is involved in pheromone induced cell cycle arrest by inhibiting the activity of G1 cyclin–Cdc28p complex (Gustin et al., 1998). Yeast cells lacking FAR1 fail to show cell cycle arrest when treated with α-factor. On the basis of homology with yeast FAR1 and presence of characteristic domains, we wanted to assay whether MFAR1 can functionally complement Far1 function in *S. cerevisiae* deletion mutant. RNA was isolated from four day old B157 wild-type culture using Trizol method (Fig. 8A). cDNA was prepared from the RNA and used for amplification of MFAR1 using FAR1-XhoI-F and FAR1-*Xba*I-R primers (Appendix- 1). These primers were designed with incorporated terminal restriction sites, with *Xho*I in forward and *Xba*I in the reverse primers. Around 3.5 Kb PCR product was obtained after amplification, which is similar to the predicted size of coding region of gene i.e. 3481 bps (Fig. 8B). PYES2, which is a widely used expression vector in *S. cerevisiae*, was used for complementation studies (Fig. 8C). This vector contains GAL1 inducible promoter for inducible expression of proteins in the presence of galactose.

The amplified cDNA was digested with *Xho*I and *Xba*I restriction enzymes and cloned in PYES2 yeast expression vector under GAL1 inducible promoter (Fig. 9A). Plasmid was isolated from putative *E. coli* transformants using boiling lysis method and screening for clone was done using different restriction enzymes and gene specific PCR. The selected clones were further confirmed with *Xho*I and *Xba*I restriction enzymes. A release of 3.5 Kb, corresponding to the MFAR1, was obtained along with a 5.9 Kb vector backbone, confirming the PYES2-MFAR1 ligated product (Fig. 9B).

S. cerevisiae Far1 mutant strain, YMP18 (MATa farlΔ ura3Δ his2 adel trpl leu2 barl::LEU2), was used for the complementation studies. This strain was kindly provided by Ira Herskowitz lab, USA. The mutant strain was transformed with this PYES2-MFAR1 vector. Transformants were selected on SD medium deficient in uracil, and confirmed with gene specific PCR of MFAR1. After confirmation of yeast transformants, cells were assayed for cell cycle arrest assay, with untransformed far1Δ (YMP18) cells and far1Δ cells transformed with empty PYES2 vector as controls.

Farlp is required for two different events in *S. cerevisiae* during mating. It includes cell cycle arrest and polarization of yeast cells towards the mating partner (Valtz et al., 1995). Far1 deletion causes drastic decrease in the mating efficiency, due to involvement of the protein in cell cycle arrest and oriented polarization during mating. It has been previously reported that cell cycle arrest and cell cycle polarization are two independent functions performed by Far1 during yeast mating (Valtz et al., 1995). We investigated whether MFAR1 will be able to functionally complement mating defect in FAR1 deletion mutant. Mating assays were performed by streaking a-cells on lawns of wild-type α-strains on restrictive SD minimal medium on which only diploids are able to grow. Since, IH1793 (MATα lys1) is unable to grow on SD medium deficient in Lysine, similarly YMP18 expressing MFAR1 cells are unable to grow on SD medium deficient in Uracil, Histidine, Tryptophan and Leucine. But, if the two cell types are able to mate with each other, the diploid cell formed will be able to grow on SD medium deficient of all these amino acids due to complementation of marker genes.

10^5 MATα yeast cells from log phase cultures were plated on SD agar plates containing 2% galactose, with SD agar plates containing 1% glucose as control. far1Δ cells expressing MFAR1, along with far1Δ cells containing empty PYES2 vector and untransformed far1Δ cells were streaked on the lawns of MATα cells. Plates were incubated for 4 days at 28°C till colonies started appearing. Large number of colonies appeared in the sector containing MFAR1 expressing far1Δ cells, whereas very few colonies were found in sectors containing far1Δ cells containing empty PYES2 vector and untransformed far1Δ cells. Spot analysis of these cultures also showed that there are higher number of diploid cells formed in MFAR1 expressing far1Δ cells, whereas very few colonies were found in spots containing far1Δ cells containing empty PYES2 vector and untransformed far1Δ cells (Fig. 10A).

Quantitative mating assays were performed by mixing ~10^6 a-cells and α-cells from log phase cultures on nitrocellulose filters. The cells were allowed to mate for 4-6 h and plated on selective SD plates to determine total diploids. It was found that mating experiment between IH1793 MATα cells and MFAR1 expressing far1Δ cells produced almost 5 times ± 0.6 diploid cells in comparison to cross between MATα cells and far1Δ cells or MATα cells and far1Δ containing the empty vector (Fig. 10B).

Yeast α-cells produce a mating peptide pheromone known as α factor. a-cells respond to α-factor, by growing a projection known as a shmoo towards the source of α-factor. Far1 deletion mutants in yeast show drastic reduction in shmoo formation. Shmoo assay was performed to investigate whether MFAR1 can complement shmoo morphology defect in far1Δ cells. It was found after incubation of far1Δ cells and far1Δ cells expressing MFAR1

with α-factor for 6 hours only 8% ± 3 shmoo were formed in far1Δ cells whereas 22% ± 6 cells formed shmoo in far1Δ cells expressing MFAR1 (Fig.11A). It indicates that MFAR1 significantly enhances shmoo morphology in far1Δ mutants.

Cell cycle arrest (halo) assay was performed as previously described (Valtz et al., 1995). 10^4 cells from log phase cultures were plated on YEP-GAL plates containing 0.5% yeast extract; 1% peptone and 2% galactose. Yeast α-factor was spotted on a sterile filter disk already placed on the plates. Plates were incubated for 2 days at 28°C. Zone of clearance indicating cell cycle arrest was observed in FAR1 containing JC2-IB (MATa HMLa HMRa bar1-1 met1 ade2-101 ura3-52) control cells, however no such phenomenon was observed in far1Δ cells expressing MFAR1 (Fig. 11B). These results indicate that MFAR1 functionally complements mating defect but not cell cycle arrest defect in *S. cerevisiae* Far1 deletion mutants.

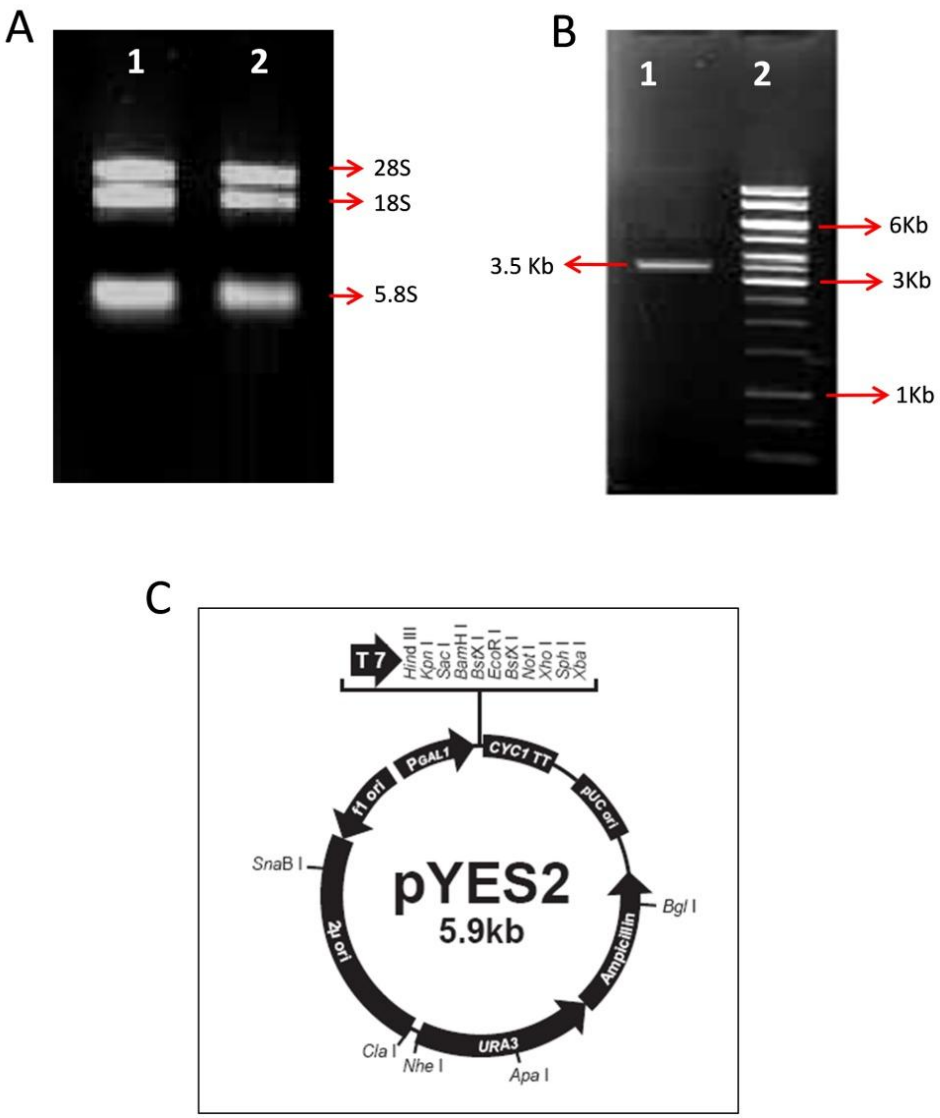

Figure. 8. PCR amplification of MFAR1 cDNA and PYES2 vector map. (A) RNA was isolated from a four day old culture of wild-type strain of *M. oryzae*. **(B)** Lane 1 and 2 contain RNA from the same sample. cDNA was prepared and used for the amplification of 3.5 Kb coding region. Lane 1 shows the amplified band and Lane 2 contains 1 Kb DNA marker. PYES2 yeast expression vector was used for the inducible expression of MFAR1. **(C)** pYES2 is a 5.9 kb vector designed for the expression of proteins in *S. cerevisiae.* GAL1 promoter present in the vector is responsible for inducible protein expression in yeast in the presence of galactose.

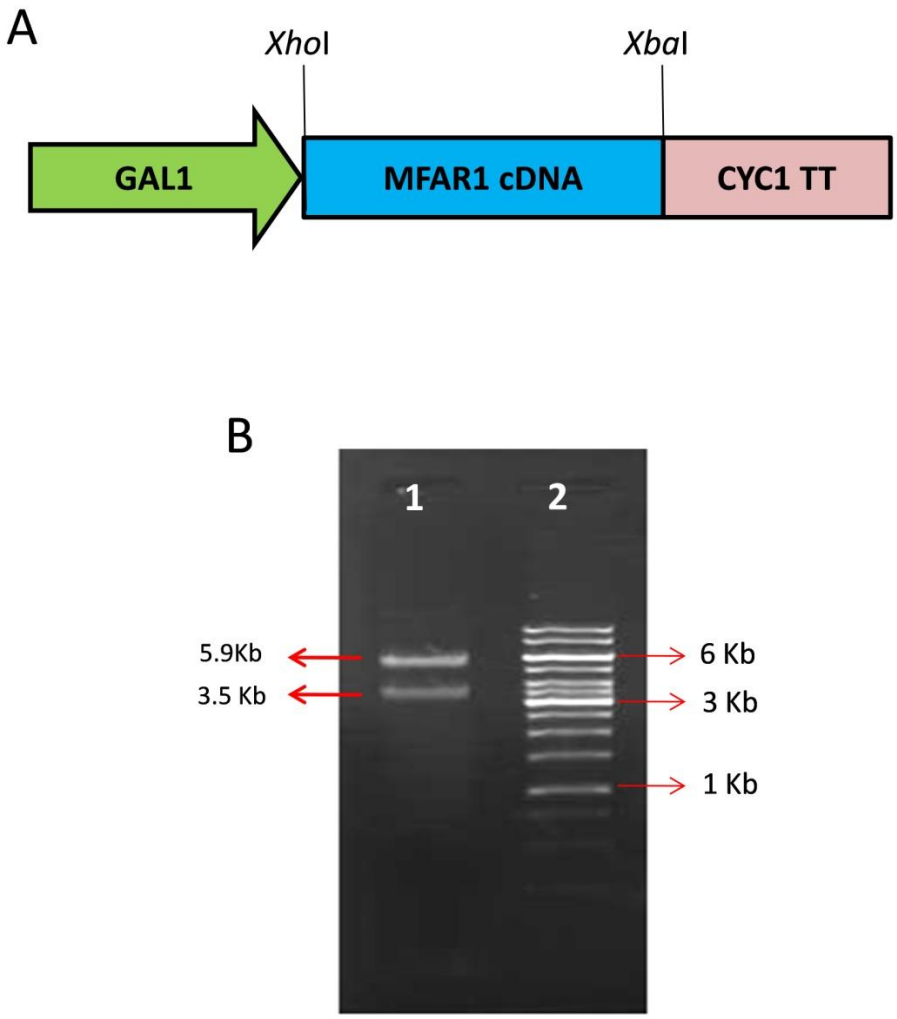

Figure. 9. Cloning of MFAR1 in PYES2 vector. (A) 3.5 Kb MFAR1 cDNA was cloned in PYES2 yeast expression vector at *Xho*I and *Xba*I sites under the GAL1 inducible promoter. **(B)** The clones were screened with gene specific PCR and confirmed with *Xho*I and *Xba*I restriction digestion. Lane 1 shows 5.9 Kb and 3.5 Kb bands corresponding to the vector backbone and gene respectively. Lane 2 shows 1Kb DNA ladder.

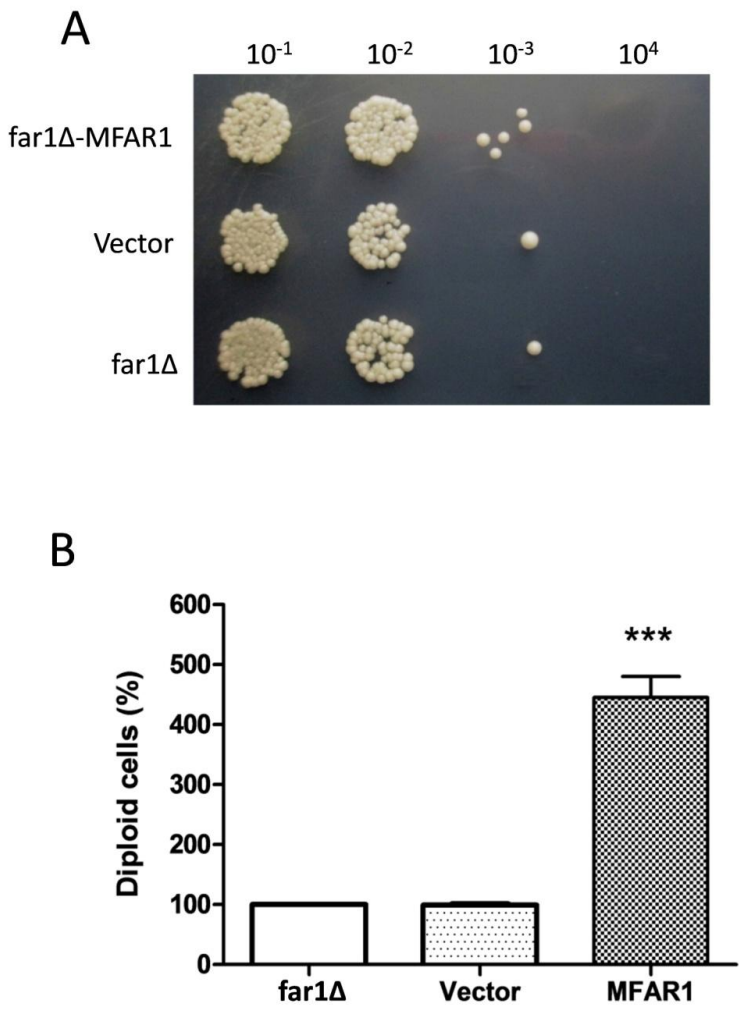

Figure. 10. Mating assay of far1Δ cells expressing MFAR1. (A) Spot test analysis was performed by spotting equal number of test and control cells on SD media containing α-cells (IH1793). **(B)** Quantitative mating assays were performed by mixing 10^6 a-cells and α-cells from log phase cultures on nitrocellulose filters. The cells were allowed to mate for 4-6 h and plated on selective SD plates to determine total diploids. Values are expressed as mean ± SD in triplicates where *** = $p < 0.001$ when MFAR1 expressing far1Δ cells were compared with untransformed far1Δ cells.

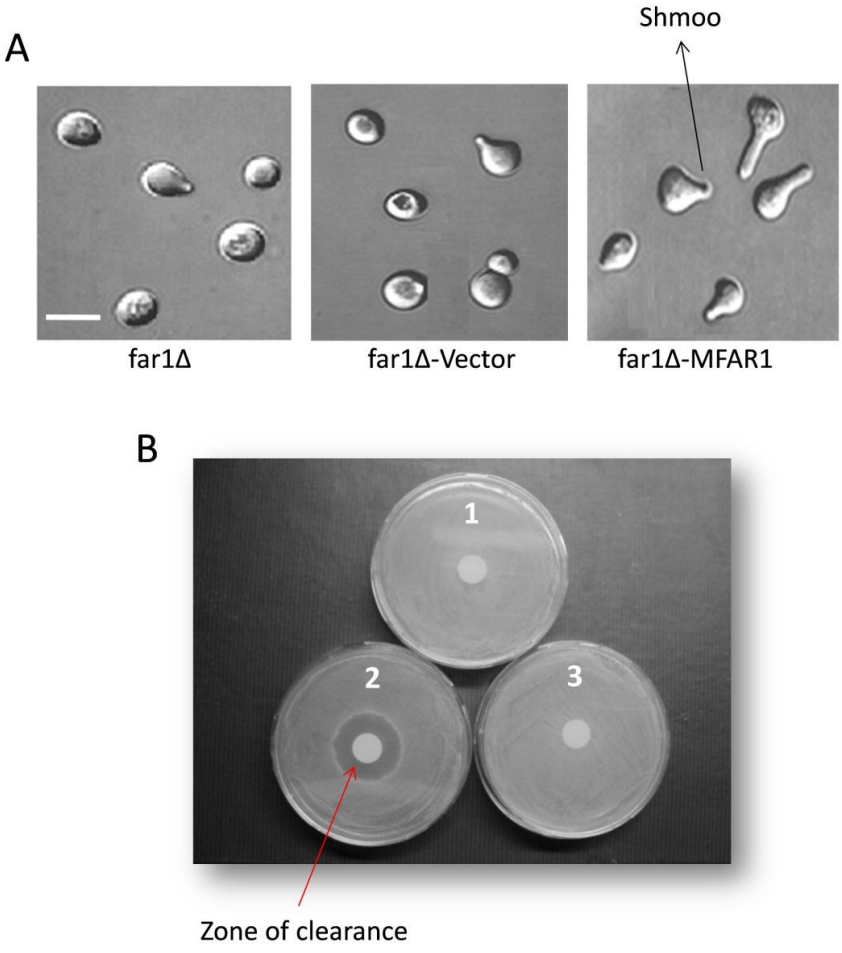

Figure. 11. Shmoo morphology and cell cycle arrest. (A) Shmoo morphology of yeast cells was determined by the addition of 10^{-6} M α-factor to the log phase cultures for 6 h at 28°C and viewed by differential interference contrast (DIC) microscopy. Bar = 10 µm. **(B)** 10^4 cells from log phase cultures were spread plated on YEPD plates and 1µg α-factor in 10 µl water was spotted on a sterile filter disk at the center of the plates. Plate 1: far1Δ cells transformed with PYES2-MFAR1; Plate 2: FAR1 containing JC2-IB cells (+ control) and Plate 3: untransformed far1Δ cells.

4.3. VWA domain of MFAR1 is essential for complementation of mating defect.

In addition to cell cycle arrest, Far1 plays an additional role in yeast mating as truncation of two-thirds of Far1 at COOH-terminal exhibits normal cell cycle arrest but are defective in mating (Chang, 1991; Chang and Herskowitz, 1990). The major cause for this mating defect has been reported to be due to the inability of cells to orient towards the mating partner (Chang, 1991).

Domain analysis shows that yeast Far1 and MFAR1 contain Von Willebrand factor type A (VWA) domain in two-thirds at COOH-terminal. We therefore tried to investigate whether MFAR1 without VWA domain can functionally complement mating defect in far1Δ cells. Strategy was designed to use almost two-thirds of MFAR1 excluding VWA domain for yeast complementation studies (Fig. 12A). A 2.7 Kb fragment of MFAR1 excluding the region that codes for VWA domain was amplified from B157 wild-type strain. The fragment was cloned in PYES2 vector at *Xho*I and *Xba*I sites and the resulting plasmid, PYES2-MFAR1 2.7, was transformed into far1Δ cells. Almost 10^5 MATα (IH1793) yeast cells from log phase cultures were plated on SD agar plates containing 2% galactose. far1Δ cells expressing MFAR1 2.7, far1Δ cells containing empty PYES2 vector, untransformed far1Δ cells and far1Δ cells expressing MFAR1 were streaked on the lawns of MATα cells. Plates were incubated for 4 days at 28°C till colonies started appearing. It was found that none of the cells except far1Δ cells expressing MFAR1 formed diploid colonies on SD restrictive medium (Fig. 12 B). Therefore, the results clearly show that far1Δ cells expressing MFAR1 2.7 gene fragment do not complement the mating defect in far1Δ cells. These results indicate that VWA domain of MFAR1 is essential for complementation of mating defect in far1Δ cells in yeast.

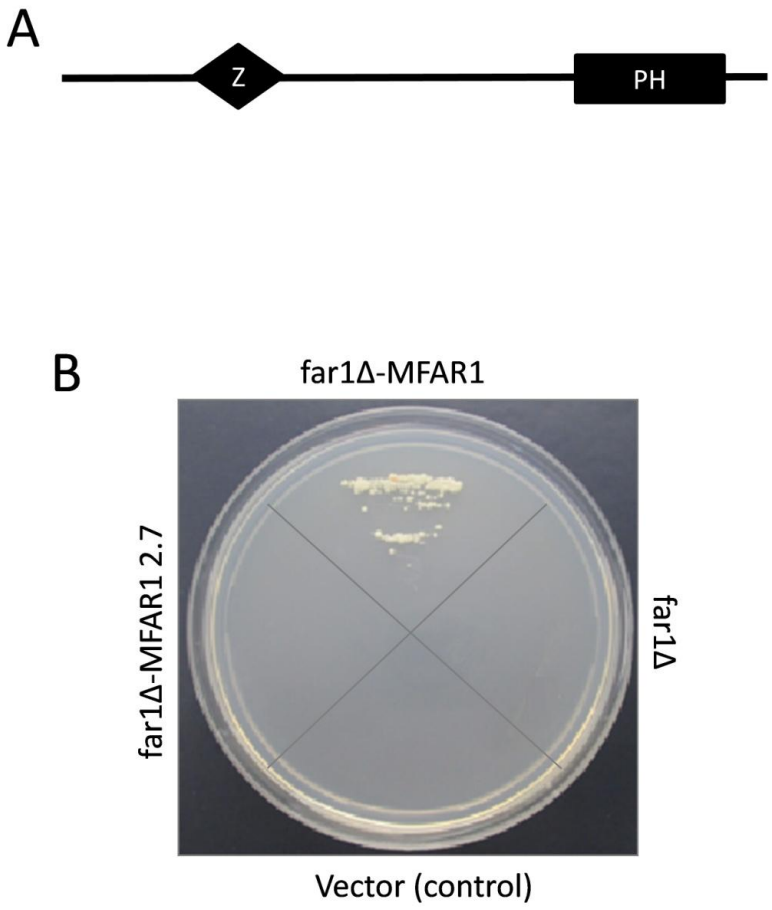

Figure. 12. Yeast complementation with MFAR1 without VWA domain. (A) 2.7 Kb N-terminal fragment of MFAR1 excluding VWA domain was amplified from B157 wild-type strain and cloned in PYES2 vector. **(B)** far1Δ, far1Δ cells containing empty PYES2 vector, far1Δ expressing 2.7 Kb MFAR1 and full MFAR1 gene (control) cells were streaked on restrictive SD minimal medium on lawns of wild-type IH1793 α-cells where only diploids can grow.

4.4. MFAR1 does not complement the mating defect in Ste5 deletion in *S. cerevisiae*

S. cerevisiae contains separate Far1 and Ste5 proteins and both the proteins are similar in structure but have different functions during yeast mating. However, filamentous fungi contain only a single protein identical to both Far1 and Ste5 in *S. cerevisiae*. There have been many speculations whether this protein in filamentous fungi can complement functions of both Far1 and Ste5 in *S. cerevisiae.* We wanted to investigate whether MFAR1 can functionally complement Ste5 deletion defect also in yeast. Blastp analysis showed that MFAR1 has 23% similarity to Ste5 in *S. cerevisiae*. Ste5 is a 917 amino acid protein in *S. cerevisiae* that contains several structural motifs. Like Far1, amino-terminal of Ste5 contains a RING-H2 Zn finger domain and a cryptic PH (Pleckstrin homology) domain, while as the carboxy-terminal half of the protein contains a vWA (von willebrand type A) domain (Fig. 13A). It indicates that FAR1 and Ste5 share similar domains in *S. cerevisiae.* This gives rise to a possibility that MFAR1 can be an ortholog of Ste5. We performed yeast complementation assay to study whether MFAR1 will be able to complement mating and cell cycle arrest assay defect in ste5Δ mutants. BYB69 (*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 lys2 hisG trp1-1 ura3-1 ste5 LYS2*) strain, provided by Prof. Jeremy Thorner, University of California, USA was used for the complementation studies. ste5Δ (BYB69) cells were transformed with PYES2-MFAR1 plasmid and selected on Dropout Medium (DM) minus uracil plates. The selected colonies were confirmed for the presence of MAFR1 gene with PCR using gene specific primers.

Mating assays were performed by streaking a-cells on lawns of wild-type α-strains on restrictive SD minimal medium on which only diploids are able to grow. 10^5 IH1793 (MA

Tα lys1) yeast cells from log phase cultures were plated on SD agar plates containing 2% galactose. ste5Δ cells expressing MFAR1, along with ste5Δ cells containing empty PYES2 vector, untransformed ste5Δ, and JC2-IB cells were streaked on the lawns of MATα cells. Plates were incubated for 4 days at 28°C till colonies started appearing. It was found that only control strain (JC2-IB) was able to form colonies on galactose containing SD medium (Fig. 13B). It indicates that MFAR1 is unable to complement mating defect of Ste5 deletion mutant in yeast.

In order to know whether MFAR1 can complement cell cycle arrest defect in Ste5 deletion mutant,10^4 cells from log phase cultures were spread plated on YEP-GAL plates containing 0.5% yeast extract; 1% peptone and 2% galactose. α-factor was spotted on a sterile filter disk already placed on the plates. Plates were incubated for 2 days at 28°C. Zone of clearance indicating cell cycle arrest was observed in JC2-IB (control) strains whereas no zone of clearance was observed in MFAR1 expressing Ste5 deletion cells or other control cells (Fig. 13C).

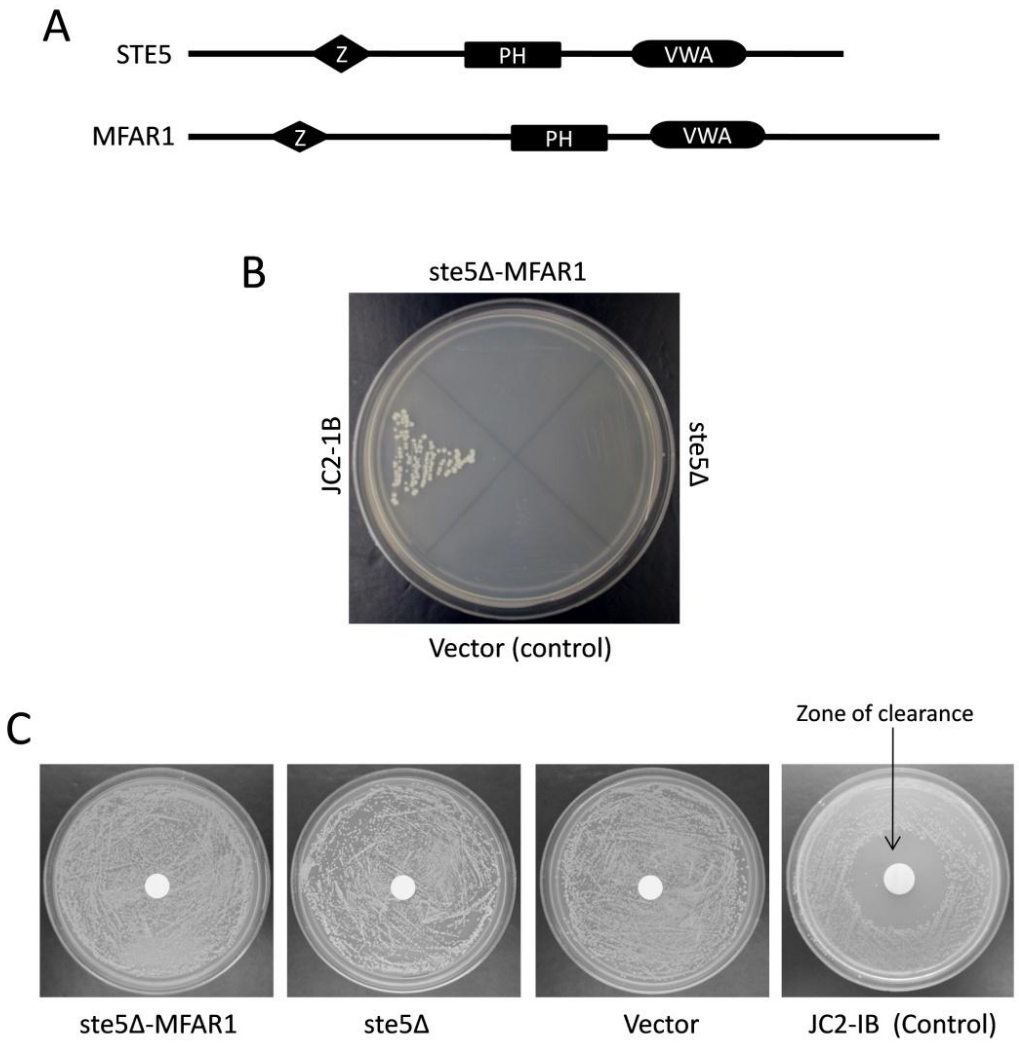

Figure. 13. Complementation of Ste5Δ mutant with MFAR1. (A) Domain arrangement of S. *cerevisiae* STE5 and *M. oryzae* MFAR1. **(B)** Mating assays were performed by streaking ste5Δ (BYB69) cells expressing MFAR1, ste5Δ cells containing empty PYES2 vector and untransformed ste5Δ cells on restrictive SD minimal medium on lawns of wild-type IH1793 α-cells . **(C)**. Cell cycle arrest assay was performed by spotting α-factor at center of the lawns of ste5Δ cells expressing MFAR1, untransformed ste5Δ, ste5Δ cells transformed with empty PYES2 vector and JC2-IB control strain.

4.5. Gene knockout studies

Initially, a classical method based on using an intact hygromycin-resistance gene for selection was used for gene disruption of MFAR1 in *M. oryzae*. Hygromycin phosphotransferase gene (hpt) was cloned between upstream and downstream flanking regions of pGKO2 vector and transformation was carried out by *Agrobacterium tumefaciens* mediated transformation (ATMT). However, in spite of screening 170 transformants, none of the transformants showed gene disruption and all of these transformants were found to be ectopically integrated by non homologous recombination.

In order to overcome the problem of non homologous ectopic integrations, a more efficient method for gene deletion based on split-marker recombination system was used to replace the MFAR1in the *M. oryzae*. The various steps involved in the split marker based gene knockout are shown in a flowchart diagram (Fig. 14). First, 1 Kb of 5' upstream region of MFAR1 was amplified from B157 wild-type using 5'FAR1-F and 5'FAR1-R primers (Appendix-1). Similarly 1 Kb of 3' downstream region was amplified using 3'FAR1-F and 3'FAR1-R primers (Appendix-1). Using pSilent1 vector as template, 800 base pairs of HY and 1.4Kb base pairs of YG were amplified (Fig.15 A). All the amplicons were digested with *Kpn*I restriction enzyme, as *Kpn*I restriction sites have already been incorporated in the primers during primer synthesis. After purification of digestion products 5' and HY fragments were ligated using T4 DNA ligase and similarly YG and 3' fragments were ligated. These 5' and HY ligation product was used for amplification of 1.8 Kb 5'-HY using 5'FAR1-F and HY800-R primers and the YG and 3' ligation product was used for amplification of 2.4 Kb YG-3' using YG-F and 3'-R primers (Fig. 15 B). PEG mediated protoplast transformation was carried out to transform B157 wild-type strain with 5'-HY

and YG-3'. 5µg each of 5'-HY and YG-3' were mixed and transformed into the wild-type strain. Transformants were selected on YEG medium containing 200 µg/ml of Hygromycin B (Fig. 16).

The number of transformants obtained from the split marker based approach was smaller in comparison to the classical methods where an intact hygromycin resistance gene is used. Using the traditional approach, 125 hygromycin resistant transformants were obtained from 10 mg of the disruption cassette DNA and in none of these transformants deletion of MFAR1 was obtained. In contrast, 12 hygromycin-resistant colonies were observed using 10 mg of each of the 5'-HY and YG-3' in split marker based gene replacement approach.

Genomic DNA was isolated from these transformants and gene specific PCR using FAR1-F and FAR1-R was carried out in order to identify transformants with MFAR1 deleted and replaced with hygromycin phosphotransferase gene (hpt). Using gene specific primers, 1.2 Kb fragment of gene was amplified for screening of transformants for presence and absence of MFAR1 gene. 1.2 Kb PCR product was obtained from the wild-type strain and ectopic transformants whereas no such amplified product was obtained from the BSM1, BSM2 and BSM11 transformants (Fig. 17A). RNA was isolated from wild-type strain as well as the transformants and cDNA was prepared. Reverse transcriptase PCR showed that only wild-type and ectopic transformants amplified the 900bp band corresponding to exon region of 1.2Kb fragment of MFAR1 gene. However, no such band was obtained in the BSM1, BSM2 and BSM11 transformants (Fig. 17 B). These results indicate that MFAR1 has been deleted from these transformants. Therefore, out of the 12 transformations selected on Hygromycin B, 3 transformants were identified with MFAR1 replaced with hygromycin phosphotransferase gene.

Transformants were further characterized for gene replacement and single copy integration using southern blot analysis. The strategy for southern blot analysis of wild-type and transformants has been depicted in a schematic diagram (Fig. 18). Genomic DNA from B157 wild-type strain and transformants i.e. BSM2 and BSM11 was digested with *Eco*RI for 12 hours. After complete digestion, blots were prepared and two different probes P1 and P2 were used to identify replacement of MFAR1 gene and number of integrations of hygromycin phosphotransferase gene in the genome respectively. Southern blot analysis showed 4.6 Kb bands in BSM2 and BSM11 transformants whereas 6 Kb band was observed in wild-type strain when P1 probe was used for detection (Fig. 18C). These results indicate replacement of MFAR1 with hygromycin phosphotransferase gene. Single band each of 4.6 Kb was identified in BSM2 and BSM11 transformants whereas no band was found in wild-type strain, when probe P2 was used for detection (Fig. 18D). It indicated that there is a single copy integration of hygromycin phosphotransferase gene in BSM2 and BSM11 transformants.

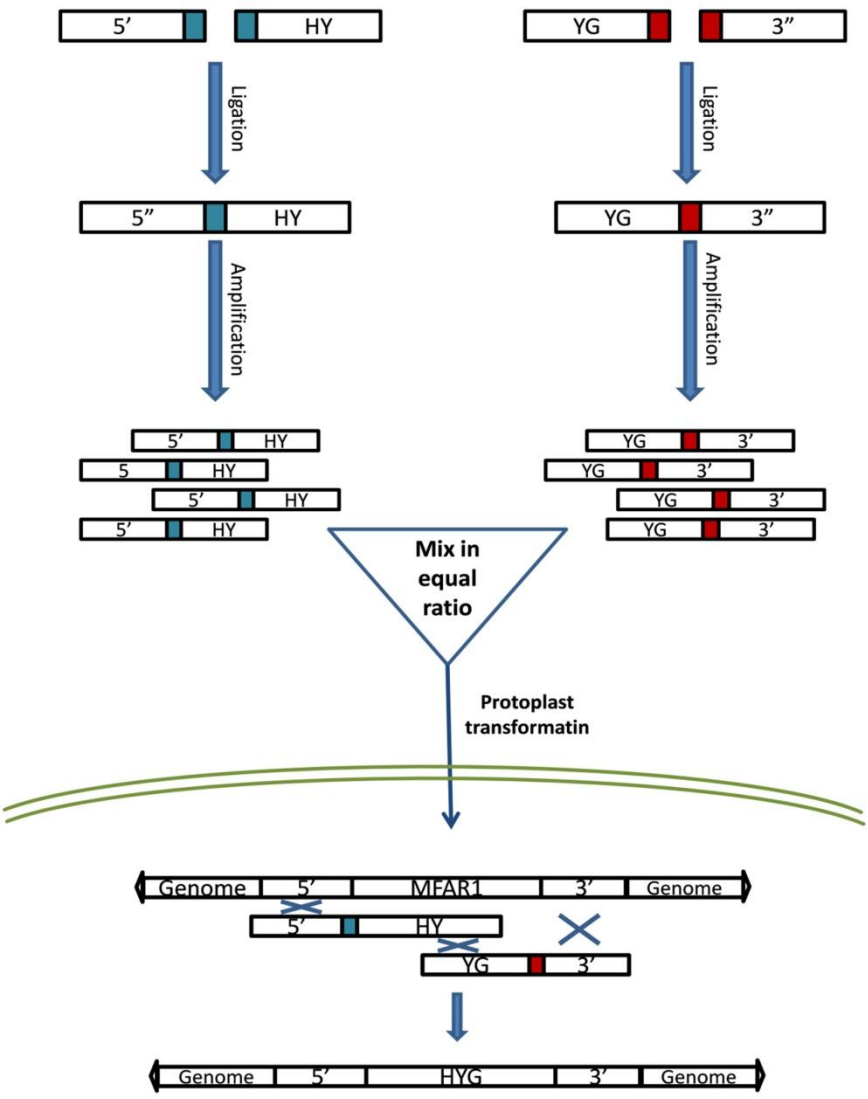

Figure. 14. Schematic representation of split marker based gene deletion. Almost 1Kb each from 5' upstream and 3' downstream of the gene of interest were amplified using specific primers. Then, two fragments with small overlapping region of the hygromycin phosphotransferase gene (HYG) from each end were amplified and designated as HY and YG. It is followed by restriction digestion and ligation of 5', HY, YG and 3' to form 5'-HY and YG-3' fragments. These fragments were mixed in equal ratio and used for transformation of wild-type strain. The three point homologous recombination takes place in the cells for gene replacement.

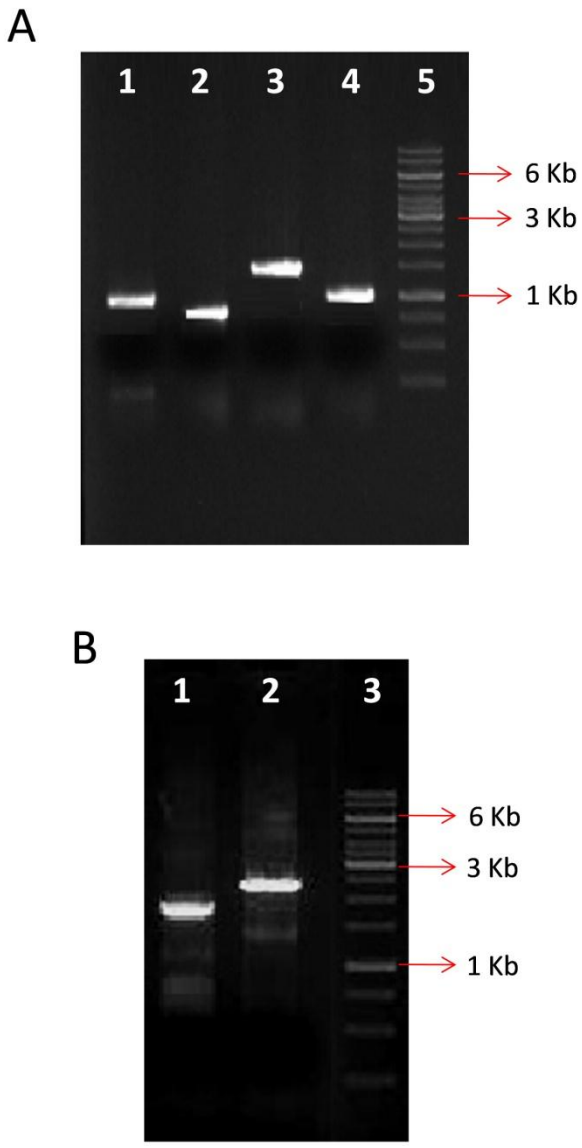

Figure. 15. Split marker gene knockout. (A). 1 Kb each of 5' and 3' were amplified from B157 wild-type strain. 800 base pairs of HY and 1450 base pairs of YG were amplified from pSilent 1 vector with an overlapping region of almost 250 base pairs between the two. Lane **(1)** 5'. **(2)** HY. **(3)** YG. **(4)** 3'. **(5)** 1Kb ladder. (**B)** After restriction digestion and ligation 5'-HY and 3'-YG were amplified using end primers. Lane **(1)** 5'-HY. **(2)** YG-3' and **(3)** 1Kb ladder.

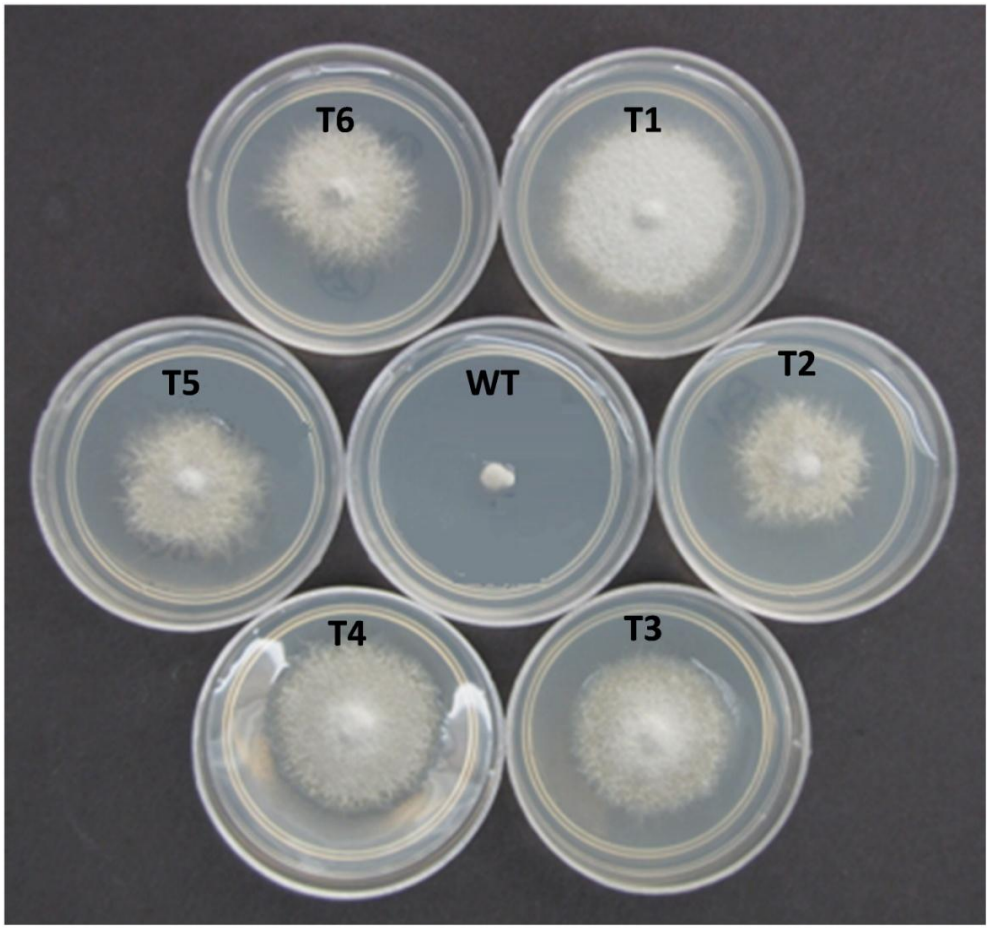

Figure. 16. Selection of hygromycin resistant transformants. After primary selection, transformants were subcultured on complete medium containing 200 mg/ml of hygromycin B. Plates T1 to T6 contain 5 day old cultures of transformants and WT B157 strain.

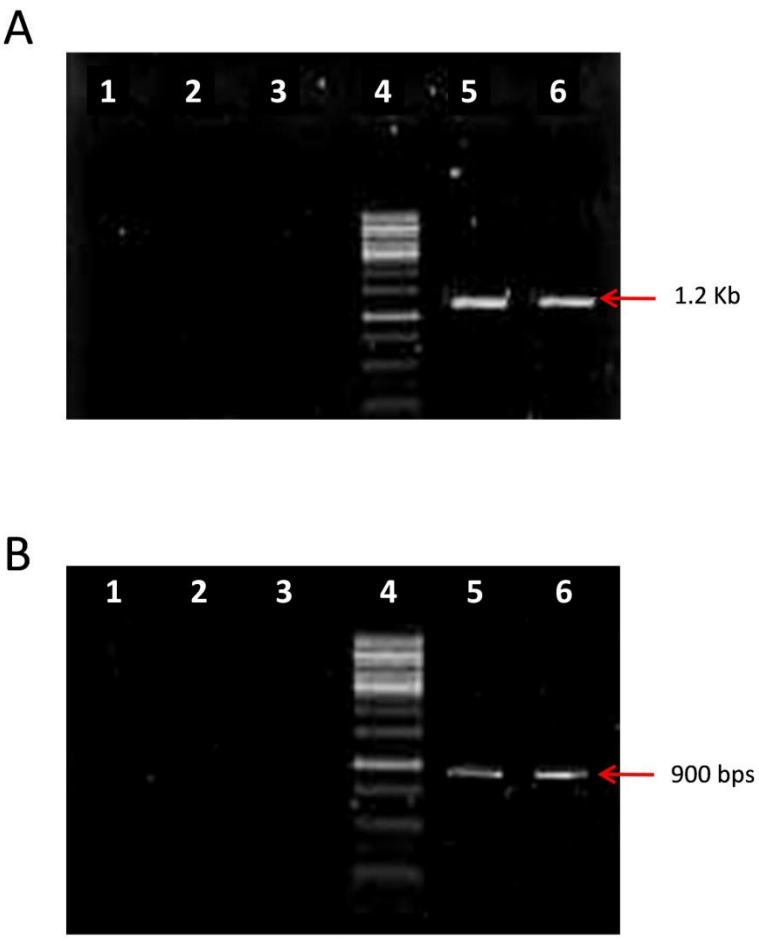

Figure. 17. Confirmation of transformants for MFAR1 deletion. (A) Transformants were screened by PCR for presence of MFAR1 gene using specific primer. **(1)** BSM1. **(2)** BSM2**. (3)** BSM11. **(4)** 1Kb DNA ladder**. (5)** BSM4. **(6)** B157 wild-type. **(B)** RNA was isolated and cDNA was prepared to investigate MFAR1 expression in transformants. 900 bps band was obtained due to presence of two intragenic introns in wild-type strain and ectopic transformants. **(1)** BSM1. **(2)** BSM2. **(3)** BSM11. **(4)** 1Kb DNA ladder. **(5)** BSM4. **(6)** B157 wild-type.

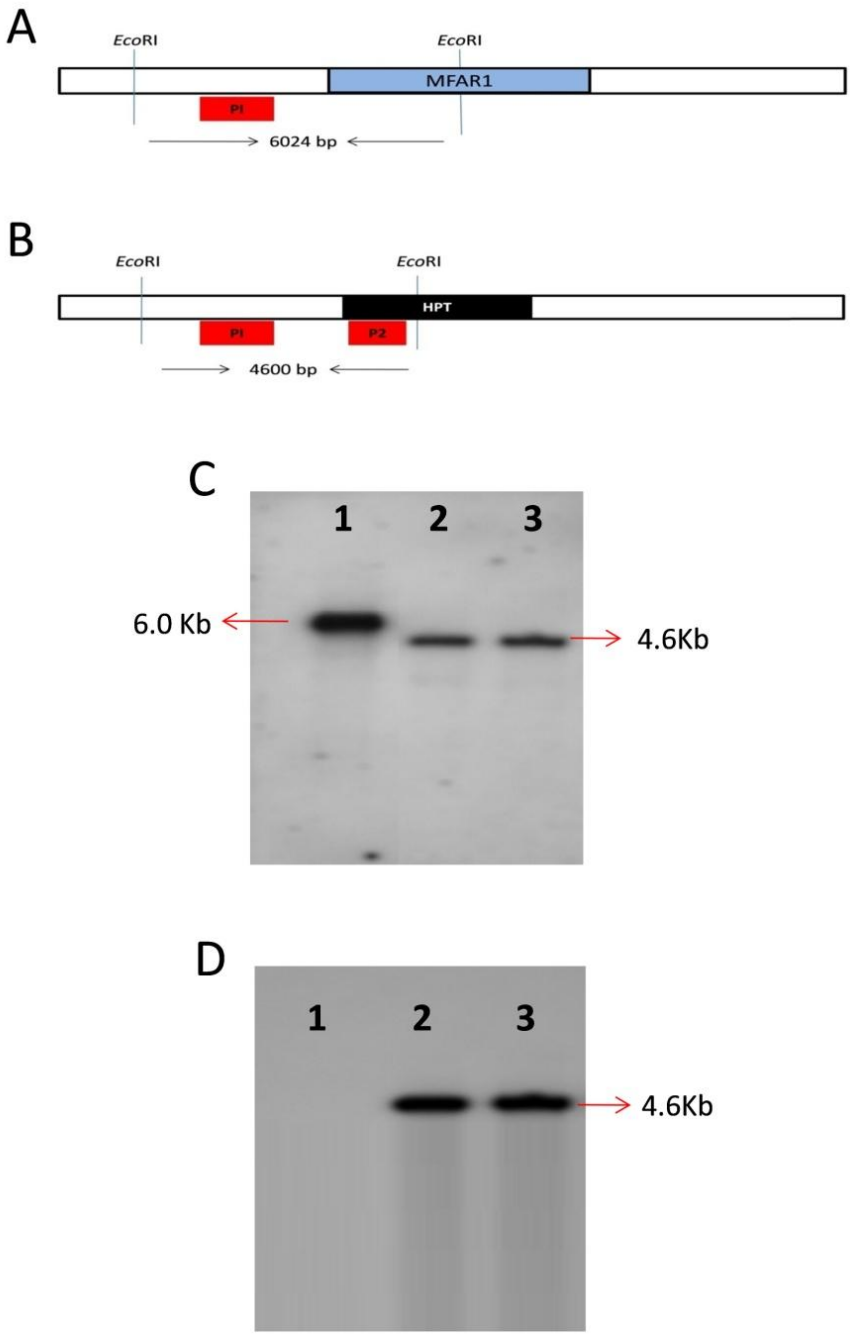

Figure. 18. Southern blot analysis. (A) Genome analysis indicates that wild-type strain contains one 5' upstream *Eco*RI restriction site and one internal *Eco*RI site in the MFAR1 gene. Probe P1 can detect this 6 Kb fragment between the two restriction sites. **(B**) Probe P2 can detect 4.6 Kb fragment between two *Eco*RI sites from upstream region and internal site present in the hygromycin phosphotransferase gene in knockout mutants of MFAR1 gene. No band will be obtained in the wild-

type strain with P2 probe. (**C)** Probe P1 was used for detection of gene replacement. Lane **1**: wild-type. **2**: BSM2. **3**: BSM11. (**D)** Same blot was subjected to detection with Probe P2 for investigation of the number of integration events of hygromycin phosphotransferase gene in genome of **(1)** Wild-type. **(2)** BSM2. **(3)** BSM11.

4.6. Phenotypic characterization of mutants

After confirmation of MFAR1 deletion mutants, BSM2 and BSM11 were renamed as *Δmfar1 #2* and *Δmfar1 #11*. Analysis of phenotypic characteristics of deletion mutants like growth rate, morphology and melanization was carried out. There was not a significant difference in growth rates between deletion mutants (*Δmfar1 #2* and *Δmfar1 #11*), ectopic transformant (eBSM4) and wild-type strain. One of the earlier observations evident from the deletion mutants was that they contained comparatively less melanin than wild-type strain or ectopic transformants (Fig.19). It was also found that deletion mutants produced comparatively more aerial hyphae than wild-type-strain.

Conidiation of the wild-type strain, MFAR1 deletion mutants (*Δmfar1 #2*, *Δmfar1 #11*), and ectopic transformants (eBSM4) on 10-day-old oatmeal agar cultures was determined. It was observed that deletion mutants produced 40-60% less conidia than wild-type strain as well as ectopic transformants where there was no gene deletion (Fig. 20A). It was also observed that MFAR1 deletion mutants exhibited an early autolysis of mycelia as compared to wild-type strain. Most of the deletion mutants showed autolytic mycelial zone after 12 days of inoculation on oat meal agar plates, whereas wild-type strain starts showing autolytic mycelial death only 20 days post inoculation (Fig. 20B). This kind of early autolytic cell

death has been observed in deletion mutants of many genes involved in cell cycle and MAPK pathway including MPS1 and MCK1 (Jeon et al., 2008).

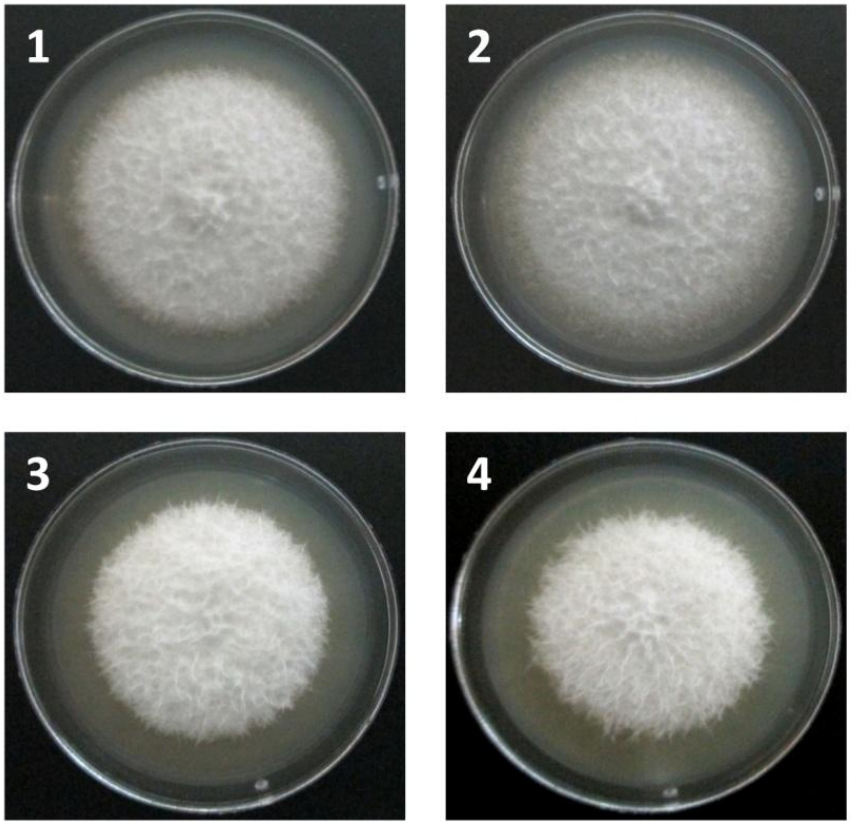

Figure. 19. Phenotypic characterization of mutants. After confirmation, transformants were grown on oat meal agar and phenotype of mutants was compared to B157 wild-type strain and ectopic transformant eBSM4. Plate: **(1)** B157 wild-type strain. **(2)** Ectopic transformant eBSM4. **(3)** Deletion mutant *Δmfar1 #2*. **(4)** Deletion mutant *Δmfar1 #11*.

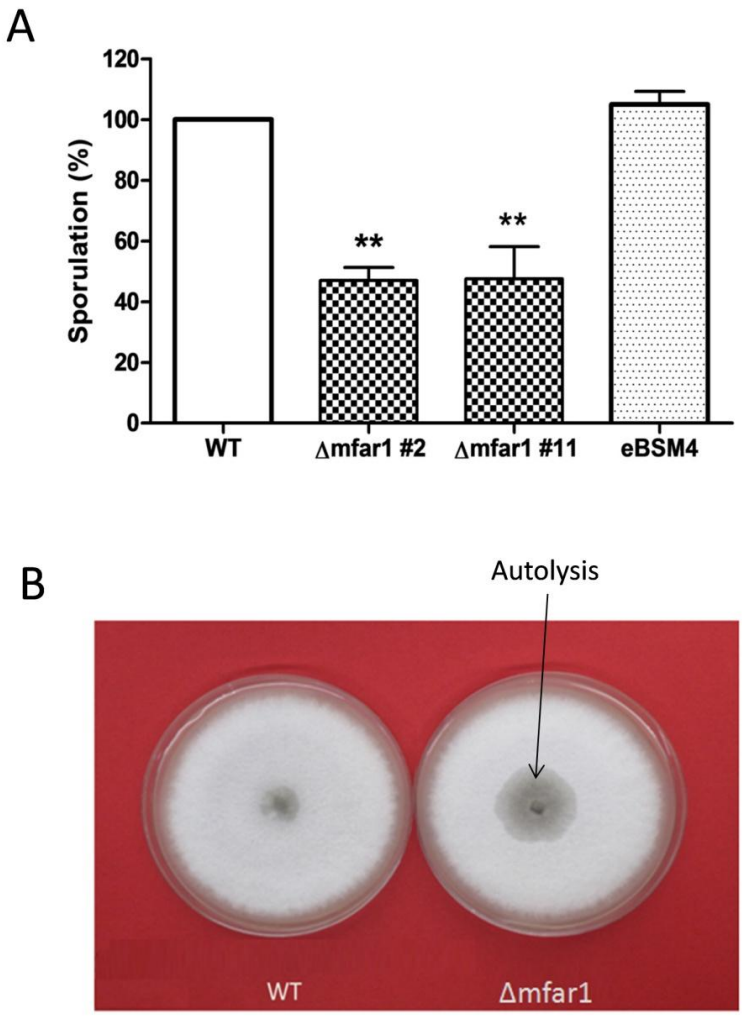

Figure. 20. *Δmfar1* shows reduction in conidia and early autolysis. (A) MFAR1 knockout mutants *Δmfar1 #2* and *Δmfar1 #11* were grown on oat meal agar for 8 days, conidia were isolated and counted using haemocytometer**. (B)** *Δmfar1*mutant strain and B157 wild-type were grown on oat meal agar for 12-15 days at 28°C and autolysis was monitored. Values are expressed as mean ± SD in triplicates where ** = $p < 0.01$ when number of conidia produced by deletion mutants was compared with B157 wild-type strain.

4.7. *Δmfar1* mutants are not affected in appressorium formation and virulence

PMK1, which is homolog for yeast FUS3 has been reported to be involved in appressorium development in *M. oryzae*. FAR1 acts downstream to FUS3 in yeast, therefore we presumed that MFAR1 which is homolog for yeast FAR1 in *M. oryzae* also must be involved in appressorial development and virulence. To investigate the role of MFAR1 in appressorial development, conidia were isolated from 8 day old culture of MFAR1 deletion mutants and wild-type strain and equal number of conidia were used for the appressoria assay. Appressorial development was observed under microscope after 8-10 hours and it was found that most appressorium development in mutants is indistinguishable from the wild-type strain (Fig. 21A). Almost 85% ±5 was observed in both mutants as well as wild-type strains. It indicates that unlike PMK1, MFAR1 is dispensable for appressoria formation under the conditions that were investigated.

To investigate the role of MFAR1 in virulence, young rice plants were spray inoculated with equal number of conidia from 8 day old culture of mutants and wild-type. Evaluation of plants 8-10 day post inoculation revealed that deletion mutants *Δmfar1 #2* and *Δmfar1 #11* did not show a significant difference in their ability to infect plants when compared to wild-type strain (Fig. 21B). These results indicate that MFAR1 may not be involved in pathogenesis related development of *M. oryzae.*

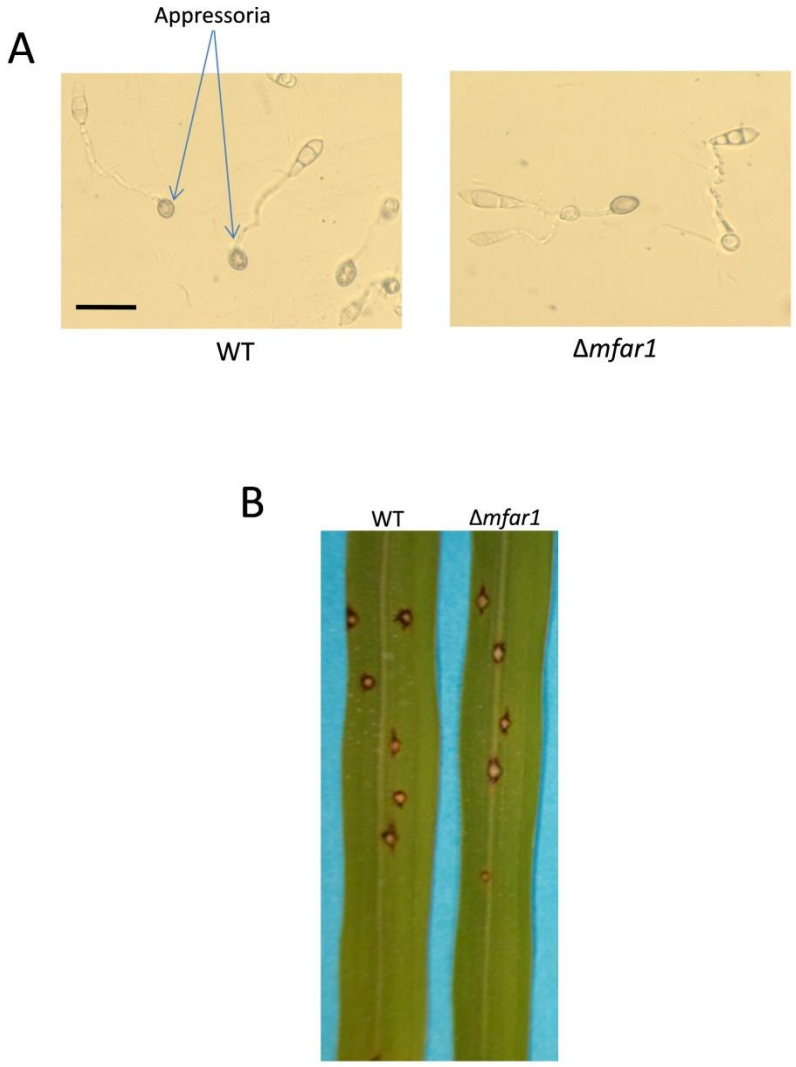

Figure.21. Appressoria and Infection assay of deletion mutants. (A) Conidia from *Δmfar1* mutant and B157 wild-type strain were harvested from oatmeal agar plates and appressoria assay was performed on synthetic gel bond film under moist conditions. **(B)** Rice plants were spray inoculated with conidial suspensions of B157 wild-type strain and Δ*mfar1*. Representative inoculated leaves were collected after 8 days of inoculation and photographed. Bar= 20 µM.

4.8. Complementation of *Δmfar1* mutants with MFAR1 in *M oryzae*

In order to investigate whether the *Δmfar1* phenotype in *M. oryzae* is specifically due to deletion of MFAR1 gene and not due to any other reason complementation studies were carried out. MFAR1 gene was ectopically expressed under native promoter in *Δmfar1*mutant *Δmfar1 #2*. MFAR1 native promoter (1.4 Kb) and MFAR1 gene (3.8 Kb) were amplified from B157 wild-type strain and cloned in PFC2 vector at *Spe*I and *Xba*I restriction sites (Fig. 22A) The resulting pFC2-Prom-MFAR1plasmid was used for transformation of Δ*mfar1* mutant strain *Δmfar1 #2*. The resulting transformants were selected on complete medium containing 50mg/ml Bialaphos. It was found that the Δ*mfar1/MFAR1* complemented strains were able to restore the wild-type like phenotype in Δ*mfar1* mutants (Fig. 22B). Melanin content of Δ*mfar1/MFAR1* transformants was also almost similar to the wild-type strain. Δ*mfar1/MFAR1* transformants also restored conidiation defect in *Δmfar1* mutants (Fig. 22C)

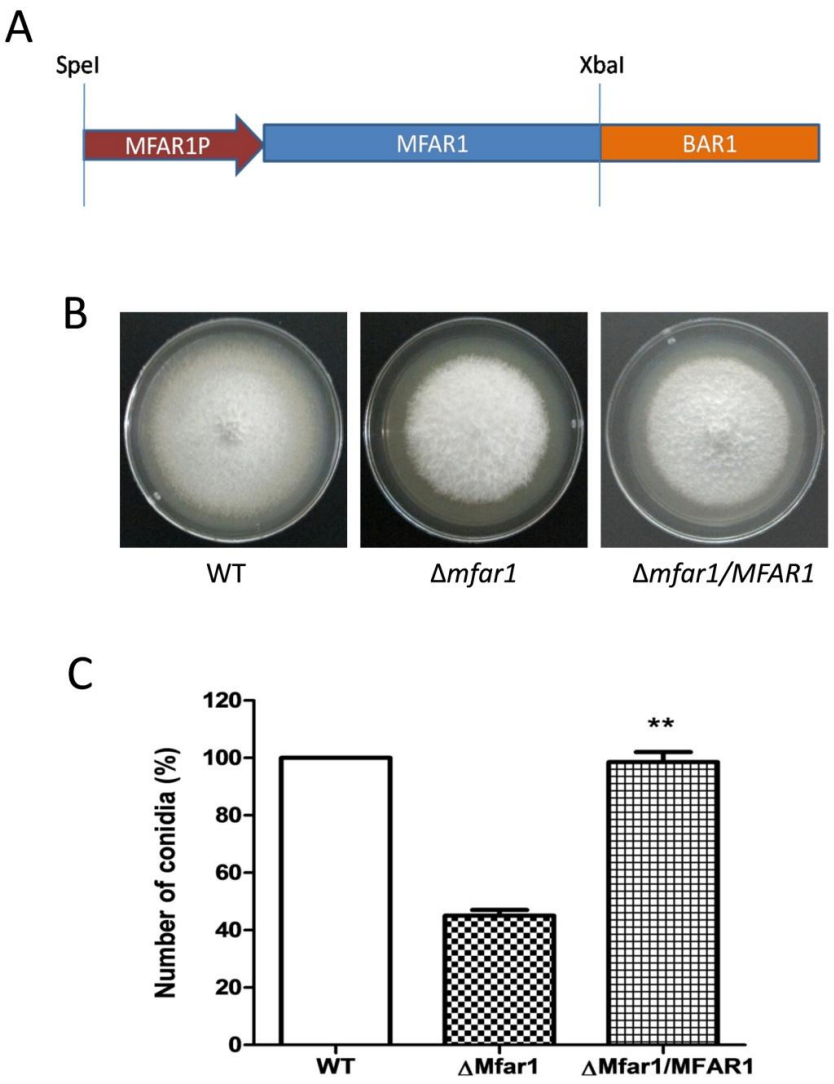

Figure. 22. Complementation of *Δmfar1*with MFAR1 gene. (A) MFAR1 gene along with 1.5 Kb MFAR1 promoter was cloned in PFC2 vector at *Spe*I and *Xba*I sites. pFC2 plasmid contains bialaphos resistance gene as selection marker**.. . (B)** Colony morphology and vegetative growth of **(1)** B157 wild-type strain **(2)** Δmfar1 and **(3**) complemented transformant Δ*Mfar1/MFAR1* on oat meal agar after 7 days of growth. **(C)** Conidia from B157 wild-type strain, Δ*Mfar1* and complemented strain Δ*Mfar1/MFAR1* were isolated and counted using haemocytometer. Values are expressed as mean ± SD in triplicates where ** = $p < 0.01$ when number of conidia produced by Δ*Mfar1/MFAR1* complemented strain were compared to that of Δ*Mfar1* deletion mutant.

4.9. Determination of mating type and fertility assessment of B157 strain

M. oryzae is a heterothallic ascomycetous fungus which exists in two opposite mating forms designated as MAT1-1 and MAT1-2. Sexual reproduction takes place *in vitro* between two strains of opposite mating forms. We tried to investigate mating type of *M. oryzae* B157 strain by amplification of mating type genes *mat1-1* and *mat1-2*, as described previously (Zheng et al., 2008). Genomic DNA was isolated from B157 wild-type strain and GUY11 control strain. Mating type determination was performed using primers described in materials and methods section. Approximately, 809 base pairs amplicon which corresponds to MAT1-1 locus in *M. oryzae* was obtained from B157, whereas an amplicon size of 940 base pairs corresponding to MAT1-2 locus was obtained in GUY11 strain, which was used as control (Fig.23). No band was obtained in B157 when MAT1-2 primers were used for amplification and similarly no band was obtained in GUY11 when MAT1-1 primers were used for amplification. These results indicate that B157 is a MAT1-1 mating type strain.

Crosses between *M. oryzae* strains were performed by placing small mycelial plugs of the test strain 4 cm from a mycelial plug of reference strain. A cross between two fertile strains of opposite mating type strains produces two lines of perithecia between them, as shown in the schematic representation (Fig. 24). Female fertility of strains was evaluated by their ability to produce perithecia in the presence of reference strains. Reference strains are the hermaphrodite strains of known mating type which produce abundant perithecia when crossed with a strain of the opposite mating type. Formation of two lines of perithecia indicates that both the strains are male and female fertile. However if one of the strains is able to induce perithecia formation in the other strain but does not form perithecia itself, it

means that the strain is male fertile and female sterile. A highly fertile MAT1-1 mating type reference strain TH12 was crossed with another fertile strain MAT1-2 reference strain GUY11. The plates were incubated at 20ºC in the continuous white and blue light. After 18 days of incubation, two lines of perithecia very clearly visible from each side of the reference strains (Fig. 25A). When GUY11 was crossed with B157 one dense line of perithecia was formed from GUY11, however almost 62% decrease in number of perithecia was found in B157 wild-type strain when compared to another MAT1-1 strain TH12 (Fig. 25B). It indicates that B157 possesses less female fertility compared to TH12, although male fertility is comparable to TH12 strain. When B157 was crossed with another MAT1-1 type strain TH12, no perithecia was observed on either of the two strains (Fig. 25C). It shows that mating did not take place between TH12 (MAT1-1) and B157 strain therefore indicating that B157 is a fertile MAT1-1 type strain.

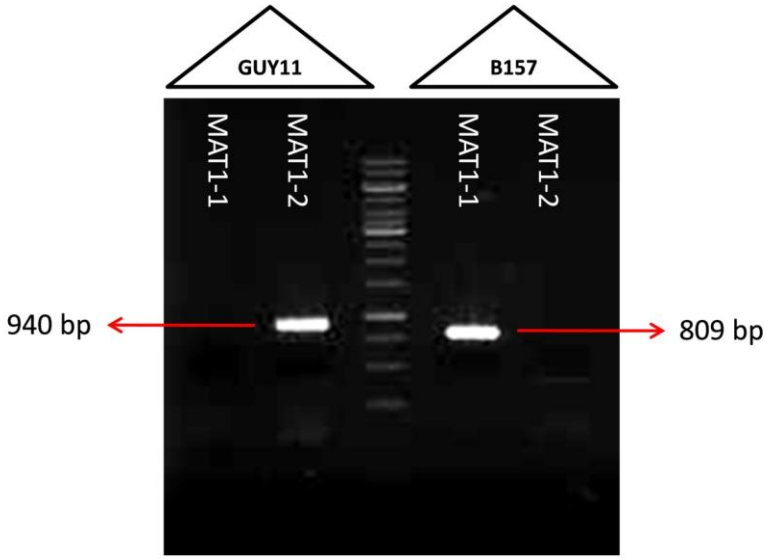

Figure. 23. Determination of mating type of B157. Mating type was determined by amplification of mating type genes using the MAT1-1F and MAT1-1R primers for MAT1-1 type strains; MAT1-2F and MAT1-2R primers for MAT1-2 type strains.

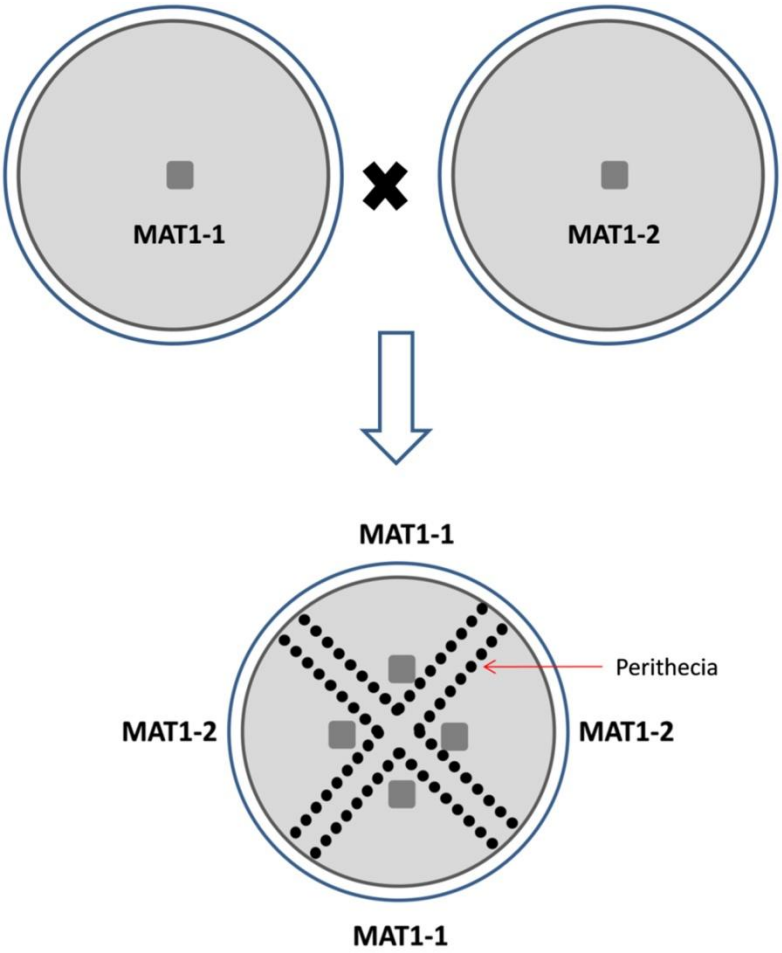

Figure. 24. Schematic representation of mating assay in *M. oryzae.* *M. oryzae* is normally a hermaphrodite which produces two lines of perithecia between MAT1-1 and MAT1-2 strains. It indicates that both the strains are male-fertile and female fertile. However if either of the strains induces formation of perithecia but is not able to produce perithecia, it indicates that the strain is a female sterile.

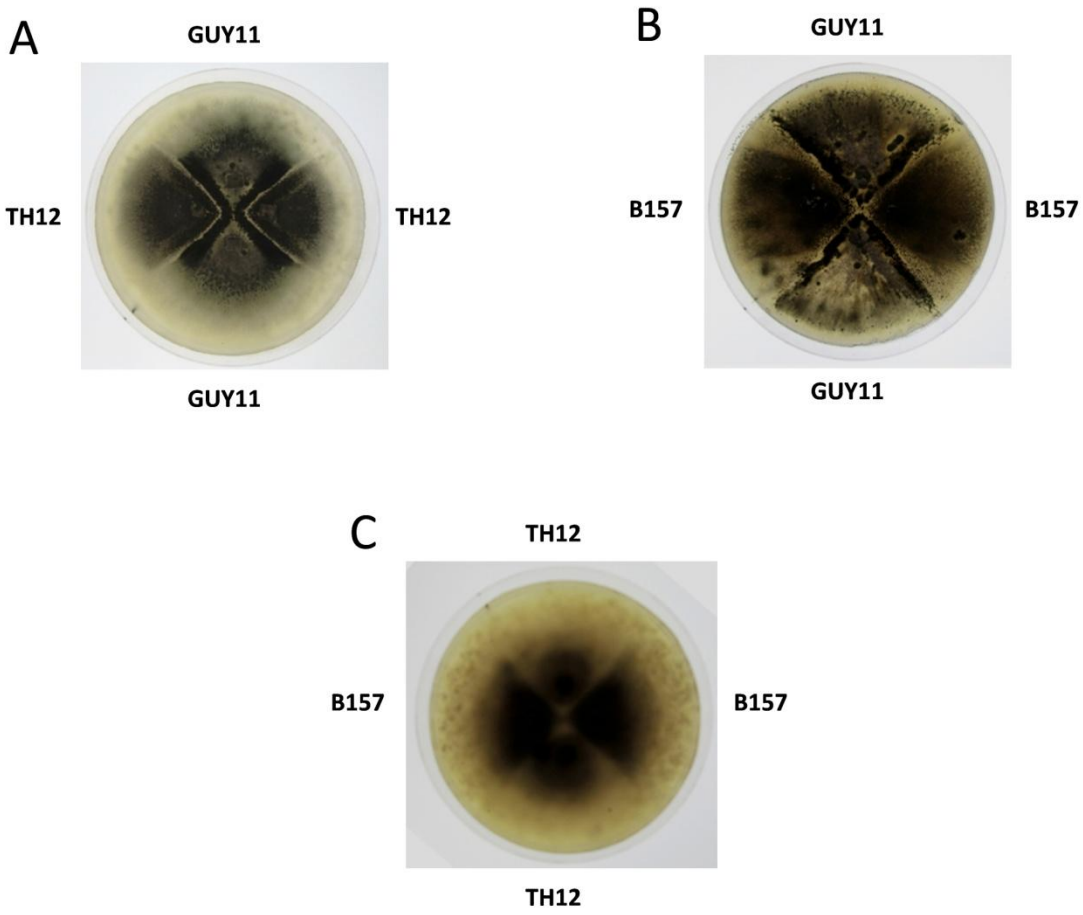

Figure. 25. Fertility assay of B157 strain. TH12 and GUY11were used as standard MAT1-1 and MAT1-2 reference strains for the assessment of fertility in B157 wild-type strain. Cross between **A.** GUY11 and TH12 reference strains. **B.** GUY11 and B157 strains. **C.** B157 and TH12 tester strains.

4.10. *Δmfar1* mutants show reduction in female fertility.

FAR1 is involved in cell cycle arrest and oriented polarization of cells during mating in *S. cerevisiae.* Far1 deletion mutants have been reported to have reduced mating ability and MFAR1 from rice blast fungus appears to complement this mating defect. Here, we tried to investigate whether deletion of MFAR1 leads to any effect on development of perithecia in *M. oryzae.* As demonstrated earlier, GUY11 forms large number of perithecia when crossed with B157 wild-type strain. However, B157 produces very small number of perithecia when crossed with GUY11 strain. The reason for this low female fertility is not clear however it has been reported that the continuous subculturing over a period of long time can lead to loss of female fertility (Saleh et al., 2012). Therefore, assessment of the fertility in deletion mutants generated in B157 strain would not be feasible. In order to overcome this limitation a MFAR1 deletion strain was generated in a highly fertile GUY11 strain using split marker gene knockout approach. After confirmation of one deletion strain (GSM3) mating assays were performed. Both GSM3 and GUY11 were crossed with TH12, a MAT1-1 type reference strain under similar conditions.

It was found that there is almost 62% ±12 decrease in formation of perithecia by GSM3 when compared to perithecia produced by GUY11 under the same conditions (Fig. 26) However, no significant effect was seen in the number of perithecia produced by TH12 strain. These results indicate that MFAR1 deletion leads to decrease in the female fertility of *M. oryzae*, however no significant effect is seen in male fertility of these mutants. Perithecia produced from the crosses of wild-type and GSM3 mutant with TH12 reference strain were examined under the microscope for the presence or absence of asci and ascospores. It was observed that almost 20-30% of the perithecia produced from cross between GSM3 and

TH12 do not produce asci and ascospores (Fig. 27). These results indicate that deletion of MFAR1leads to a decrease in fertility of *M. oryzae.*

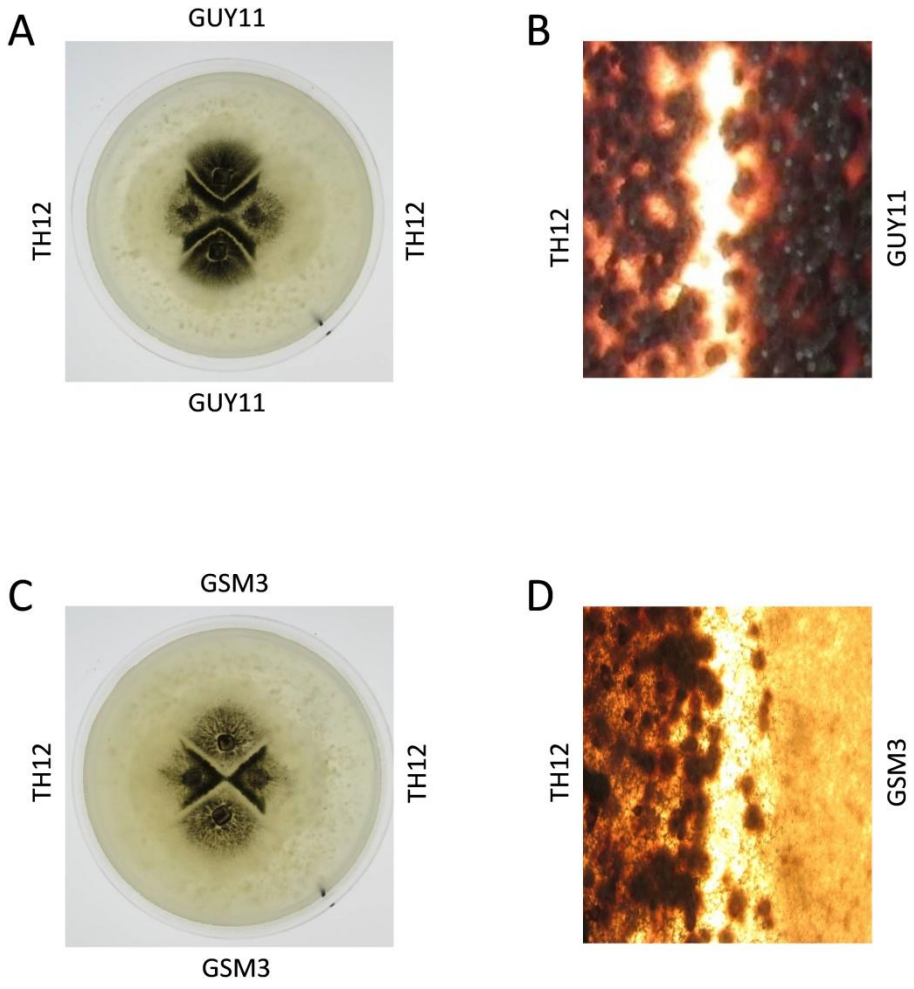

Figure. 26. Determination of effect of MFAR1 deletion on fertility. (A) GUY11 which is a MAT1-2 strain was crossed with another highly fertile MAT1-1 reference strain TH12. **(B**) Magnified view of perithecia from both the reference strains. **(C)** GSM3, the *Δmfar1*mutant in GUY11 background, was crossed with TH12 to assess the effect of MFAR1 deletion on fertility of GUY11. (**D)** Number of perithecia produced from reference strain and mutant strain were counted and compared to number of perithecia produced from cross between the reference strains.

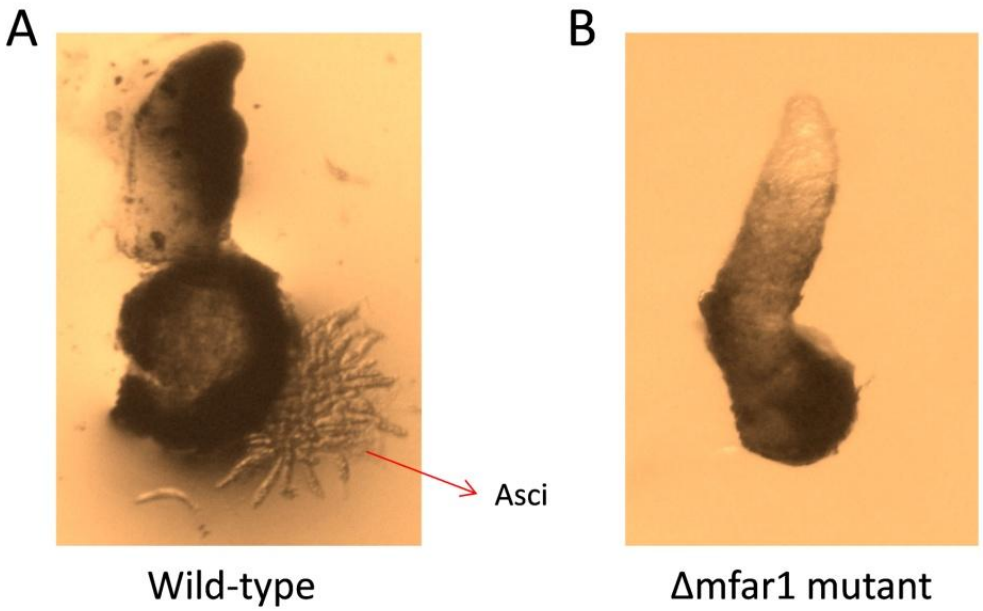

Figure. 27. Asci and ascospore development. Perithecia from the crosses of wild-type and mutant with TH12 reference strain were examined under the microscope for the presence or absence of asci and ascospores. Perithecia were mounted on a glass slide and gently squashed with a glass needle for the release of asci.

4.11. Accumulation of PMK1 mRNA in *Δmfar1* mutants

Fus3 acts upstream to Far1 in *S. cerevisiae,* and activates it by phosphorylation during mating. Pmk1 has been reported to functionally complement Fus3 deletion in budding yeast. We already that MFAR1 can functionally complement the mating defect of FAR1 mutant in *S. cerevisiae.* We investigated the effect of MFAR1 deletion on PMK1 mRNA expression. Quantitative real time PCR was performed for the expression analysis of PMK1 in *Δmfar1 #2* and *Δmfar1 #11* deletion mutants. Almost 4.5 ± 0.4 and 4.2 ± 0.3 fold accumulation of PMK1 mRNA accumulation was observed in *Δmfar1 #2* and *Δmfar1 #11* deletion mutants when compared to the wild-type strain (Fig. 28). The accumulation of PMK1 transcript level in *Δmfar1* mutants is not clearly understood. One of the possible reasons for this mRNA accumulation is that MFAR1 is expected to be substrate for PMK1 activity therefore in absence of MFAR1, PMK1 mRNA gets accumulated.

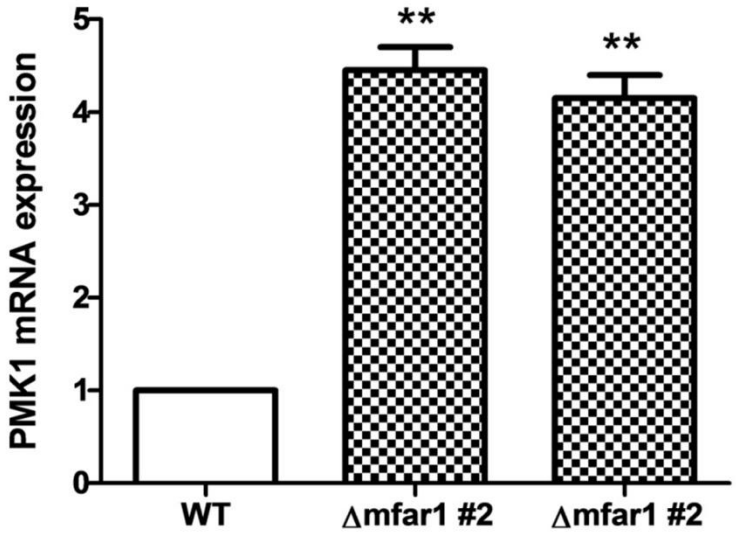

Figure. 28. PMK1 accumulation in *Δmfar1* mutants. Quantitative real time PCR was performed to investigate PMK1 mRNA expression in Mfar1 deletion mutants ***Δmfar1 #2*** and ***Δmfar1 #11***. Values are expressed as mean ± SD in triplicates where ** = $p < 0.01$ when PMK1 mRNA expression in MFAR1 deletion mutants was compared to the wild-type strain.

4.12. MFAR1 protein is localized in the nuclear region of *M. oryzae*

FAR1 in *S. cerevisiae* has been reported to be mainly localized in nucleus and cytoplasm depending on the stage of growth. To follow the expression of MFAR1 in *M. oryzae*, a MFAR1:GFP reporter plasmid was made. First of all, the 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 (Fig. 29A). The construct was confirmed using *Xba*I restriction enzyme (Fig. 29B). The reporter construct, pProMFAR1:SP:GFP, was introduced by protoplast transformation into B157 wild-type strain. Fluorescent microscopic analysis of transformants carrying the ProMFAR1:SP:GFP reporter construct confirmed that MFAR1 is expressed at detectable levels in mycelium of the fungus. It was observed that the protein is mainly localized in the nuclear region of the cells although some fluorescence was also detected in cytoplasm (Fig. 30A). Fluorescent microscopic analysis also showed that protein is more visibly expressed in conidia than mycelia (Fig. 30B). In conidia also most of the protein is localized to nuclear region only. Further analysis revealed that MFAR1 expression is not enhanced during appressorium development or plant infection indicting that its expression may not be required during plant infection.

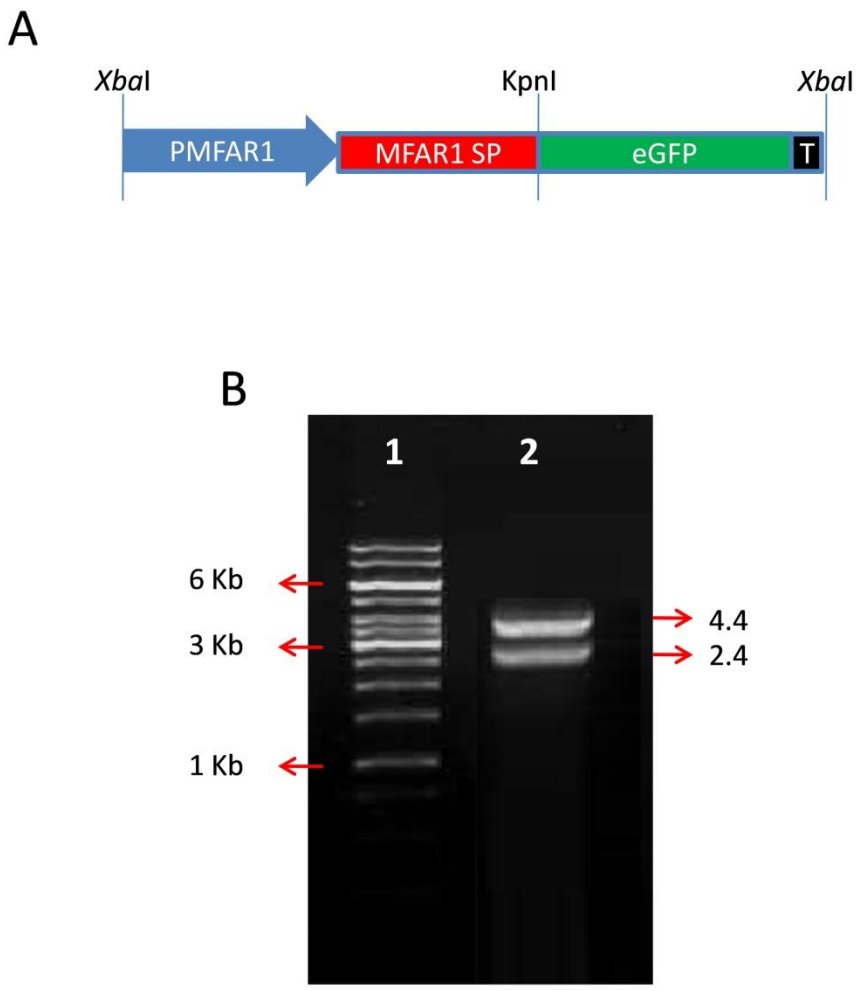

Figure. 29. Construction of MFAR1 localization plasmid. (A) MFAR1 promoter and signal peptide were ligated with enhanced GFP using *Kpn*I site. The fusion product was cloned in pSilent1 backbone at *Xba*I restriction site. **(B)** The construct was confirmed with *Xba*I restriction enzyme producing 2.4 Kb fragment release and the 4.4 Kb vector backbone fragment.

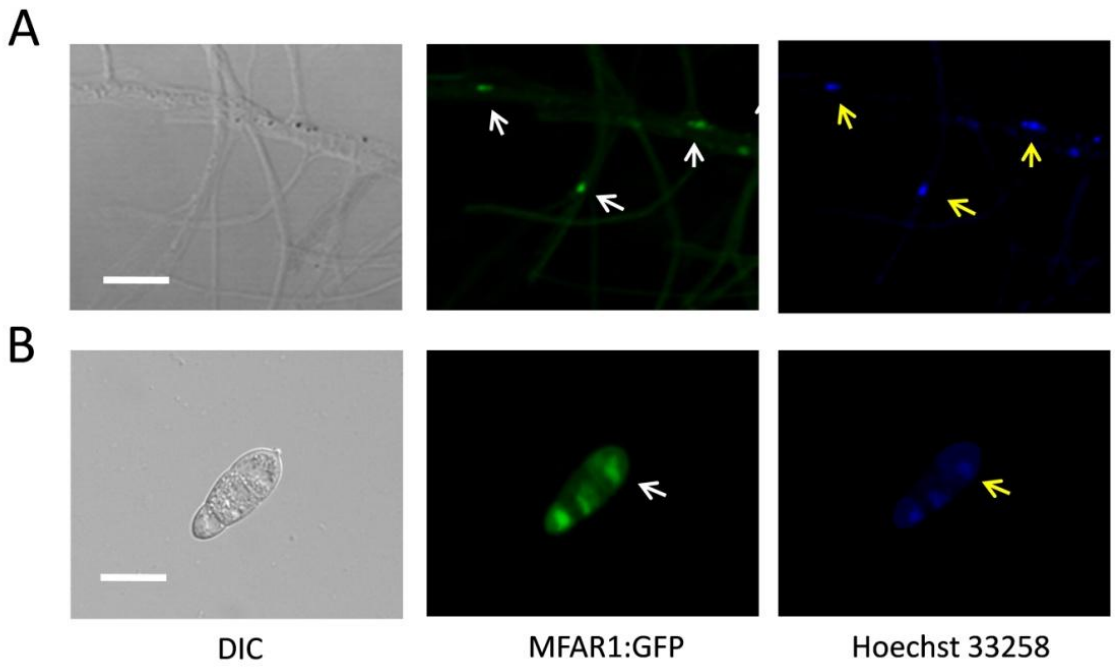

Figure. 30. Mfar1 is localized to nuclear region. (A) GFP fluorescence was detected at 488 nm in mycelia by confocal microscopy and its localization was compared with location of Hoechst 33258 nuclear stain. **(B**) Conidia showed comparatively more intense GFP fluorescence in and around the nuclear region of all the three celled conidia.

4.13. Yeast α-factor induces expression of PMK1 and MFAR1

It has also been reported that *S. cerevisiae* α-factor pheromone inhibits appressorium formation in a mating type–specific manner and inhibits plant infection by *MAT1-2* strains of *M. oryzae.* It was also demonstrated that supernatant of yeast a-cells inhibits appressorium development of MAT1-1 cells. (Beckerman et al., 1997a). In budding yeast, pheromones have been reported to induce expression of many MAPK genes including FUS3 and FAR1 through the STE12 mediated transcription activation (Roberts et al., 2000). Based on these previous studies, we tried to investigate the mRNA expression of some of the genes of MAPK pathway, including PMK1 (homolog of S. cerevisiae FUS3) and MFAR1 (homolog of yeast FAR1), in presence of *S. cerevisiae* a-cell and α- cell supernatant. Quantitative real time PCR showed that there is 4.5 ± 0.7 and 7.4 ± 0.6 fold induction in the mRNA expression of PMK1 and MFAR1 respectively when a MAT1-2 type strain GUY11 is co-cultured with Y187 α-cells for 6 hours, while no such effect was seen in fungus co-cultured with yeast a-cells (Fig. 31A).

Similar studies were carried out on MAT1-1 type strain of *M. oryzae* wild-type strain B157. It was found that there is a 3.9 ± 0.2 fold increase in the mRNA expression of PMK1 and almost 5.9 ± 0.6 fold induction in the expression of MFAR1 in MAT1-1 type strain in the presence of a-cell (Fig. 31 B). These results indicate mating type specific changes in the expression of PMK1 and MFAR1 in *M. oryzae* in the presence of yeast cells.

In order to investigate whether the changes in the expression of MAPK genes PMK1 and MFAR1 in rice blast fungus is due to pheromones released by the yeast cells, we treated GUY11 (MAT1-2) and B157 (MAT1-1) with different concentrations of synthetic α-factor.

Quantitative real time PCR analysis showed that there is 3.1 ± 0.4 and 4 ± 0.2 fold increase in the mRNA accumulation of PMK1 and MFAR1 in GUY11 strain treated with 25 μM of α-factor (Fig. 32). At 50 μM of α-factor, 4.4 ± 0.1 and 5.6 ± 0.3 fold expression of PMK1 and MFAR1was recorded. This result confirms that the change in the expression profile of these MAPK genes is due to interspecific pheromone response between *S. cerevisiae* and *M. oryzae*.

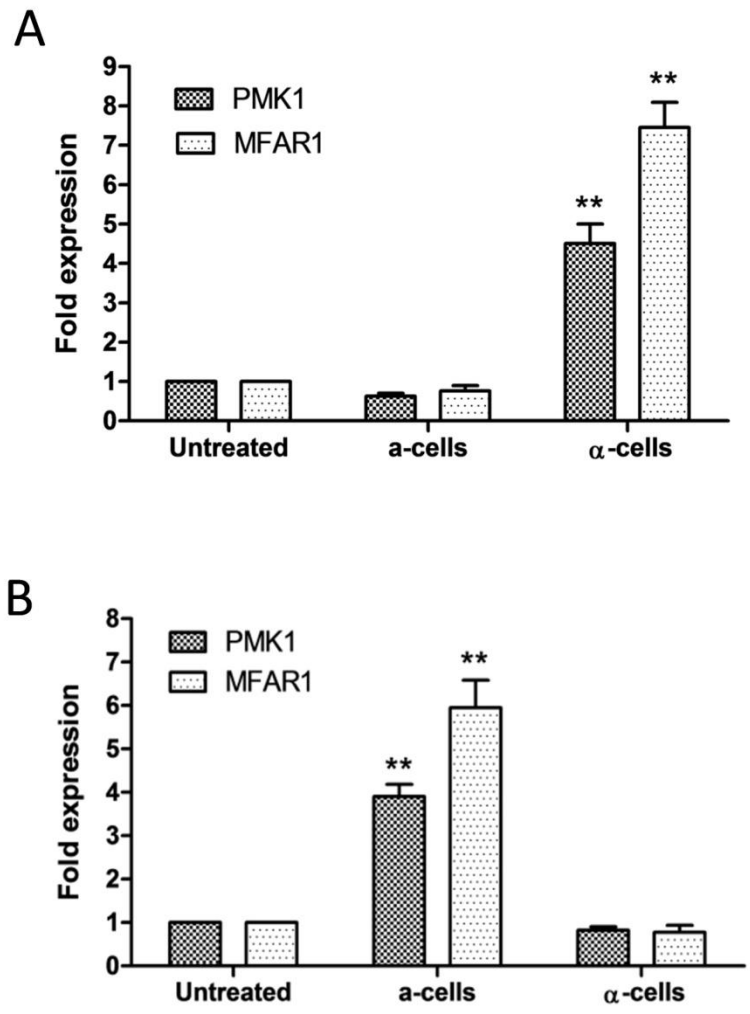

Figure. 31. PMK1 and MFAR1 mRNA expression in presence of yeast cells. (A) GUY11 (MAT1-2) was co-cultured with 10^7cells per ml of a and α cells of yeast for 6 hours and fold changes in mRNA expression of PMK1 and MFAR1 were determined by qRT PCR **(B)** Under similar set of conditions, B157 (MAT1-1) was co-cultured with a and α-cells and fold changes in mRNA expression of PMK1 and MFAR1 were determined. Values are expressed as mean ± SD in triplicates where ** = $p < 0.01$, when a and α-cell co-cultured groups were compared with untreated group.

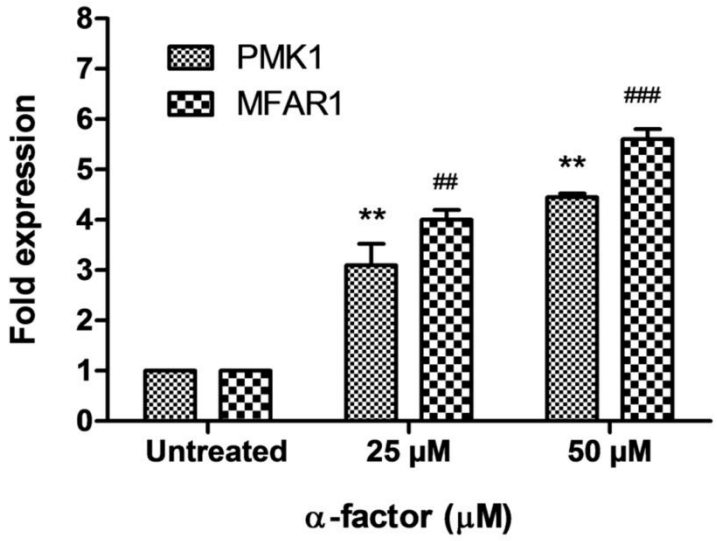

Figure. 32. Yeast α-factor induces expression of PMK1 and MFAR1 in *M. oryzae*. GUY11 wild-type strain was treated with 25 and 50 µM of synthetic α-factor and changes in the mRNA expression of PMK1 and MFAR1 were determined using real time PCR. Values are expressed as mean ± SD in triplicates where ** = $p < 0.01$, when α-factor treated groups were compared with untreated group for expression analysis of PMK1 and $^{\#\#}$ = $p < 0.01$, ### = $p < 0.001$ when α-factor treated groups were compared with untreated group for expression analysis of MFAR1.

4.14. Overexpression of MFAR1 causes reduction in appressorium formation

As reported previously. *S. cerevisiae* pheromone inhibits appressorium formation and plant infection in a mating type–specific manner in *M. oryzae* (Beckerman et al., 1997a). We wanted to investigate whether upregulation of MFAR1 due to yeast pheromones is somehow related to inhibition of appressorium formation in rice blast fungus. One of the strategies to study if expression levels of MFAR1 are related to appressorium formation in M. oryzae is overexpression of the gene using a strong constitutive promoter. 3.8Kb gene was cloned under constitutively expressing TrpC promoter in pSilent1 vector backbone at *Xho*I and *Apa*I restriction sites (Fig. 33A). The clone was initially screened by gene specific PCR and then confirmed using restriction digestion at *Xho*I and *Apa*I sites (Fig. 33B).

The clone was transformed in wild-type B157 strain using protoplast transformation and transformants were selected on complete medium containing hygromycin. After hygromycin selection monoconodial isolation was carried out to obtain pure cultures. On the basis of hygromycin selection and presence of gene cassette, a total of 7 transformants were carried forward for further characterization. Quantitative real time PCR was performed to investigate expression of MFAR1 in overexpression transformants. It was found that there is 5.1 ± 0.4 and 4.1 ± 0.5 fold increase in the mRNA expression of OF3 and OF13 (Fig. 34A). Based on the expression levels of MFAR1 in OF3 and OF13, they were carried forward for further characterization. There was not a significant difference in morphology of overexpression transformants and B157 wild-type strain (Fig. 34B).

According to our results, yeast α-factor induces mating type specific expression of MFAR1 in *M. oryzae.* Also, it has been previously demonstrated that yeast α-factor causes inhibition

of appressorium development and plant infection in rice blast fungus (Beckerman et al., 1997b). We tried to investigate whether overexpression of MFAR1 is related to appressorium development and plant infection. Two overexpression transformants with maximum expression of MFAR1 gene were selected for the study. It was found that there is 49% ± 8% and 34% ± 6% decrease in formation of mature appressorium in OF3 and OF13 overexpression transformants, when number of appressorium formed in B157 wild-type strain was considered as 100% (Fig. 35A). It was also observed that almost 25% of the conidia in the overexpression transformants form very long germ tubes before hooking and formation of appressoria. In case of B157 wild-type strain, 20- 40 µm long germ tubes were formed before appressorium formation, whereas 15-20% of the conidia in OF3 overexpression transformant formed more than 100 µm long germ tubes before forming appressorium (Fig 35B). It was also observed that size of appressorium in the overexpression transformants was smaller when compared to wild-type strain. These results indicate that overexpression leads to defect in development of appressorium in *M. oryzae.*

In order to investigate effect of MFAR1 overexpression of rice blast fungus on plant infection, young rice plants of a susceptible variety CO39 were spray inoculated with conidia from 8 day old culture of overexpression transformants and B157 wild-type strain. Evaluation of plants 8-10 day post inoculation revealed that overexpression transformants (OF3 and OF13) showed a significant decrease in the plant infection when compared to wild-type strain (Fig. 36). Almost 50-60 % decrease in the number of infection lesions was found in overexpression transformants compared to wild-type strain.

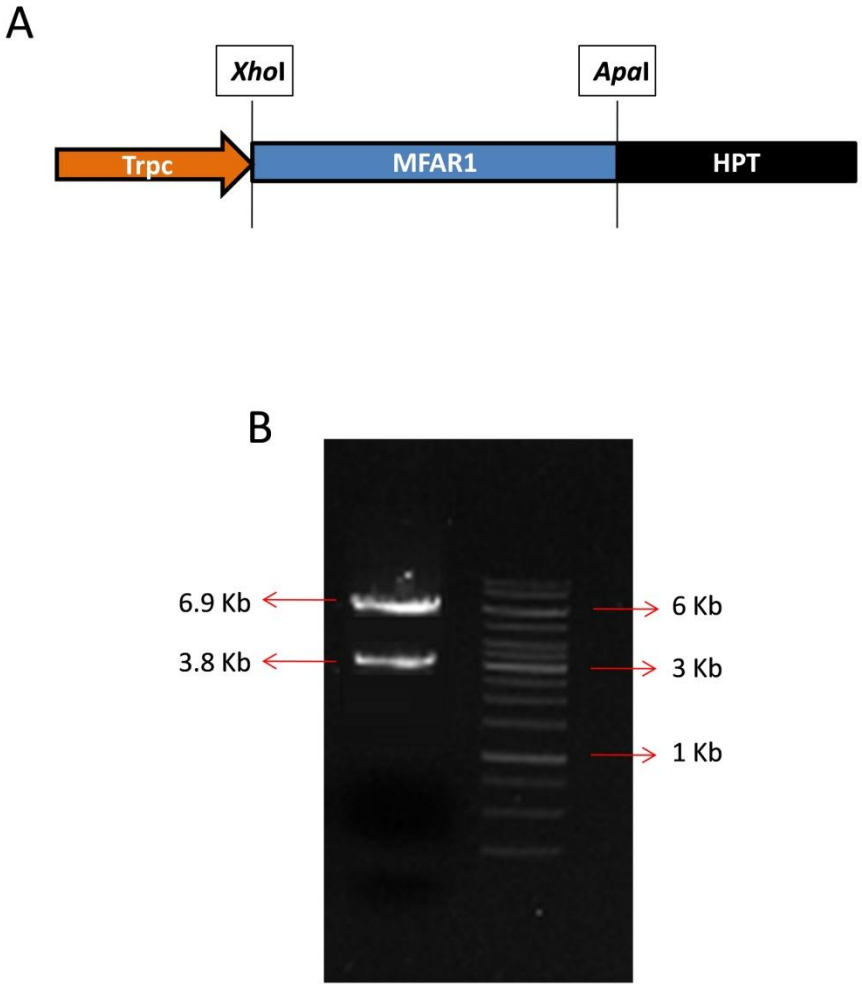

Figure. 33. Overexpression of MFAR1 in *M. oryzae*. (A) MFAR1 gene was cloned under TrpC constitutive promoter in the pSilent1 vector at *Xho*I and *Apa*I sites**. (B**) The construct was confirmed using *Xho*I and *Apa*I restriction enzymes. Two bands of 6.9 Kb and 3.8 Kb corresponding to vector backbone and MFAR1 gene were detected**.**

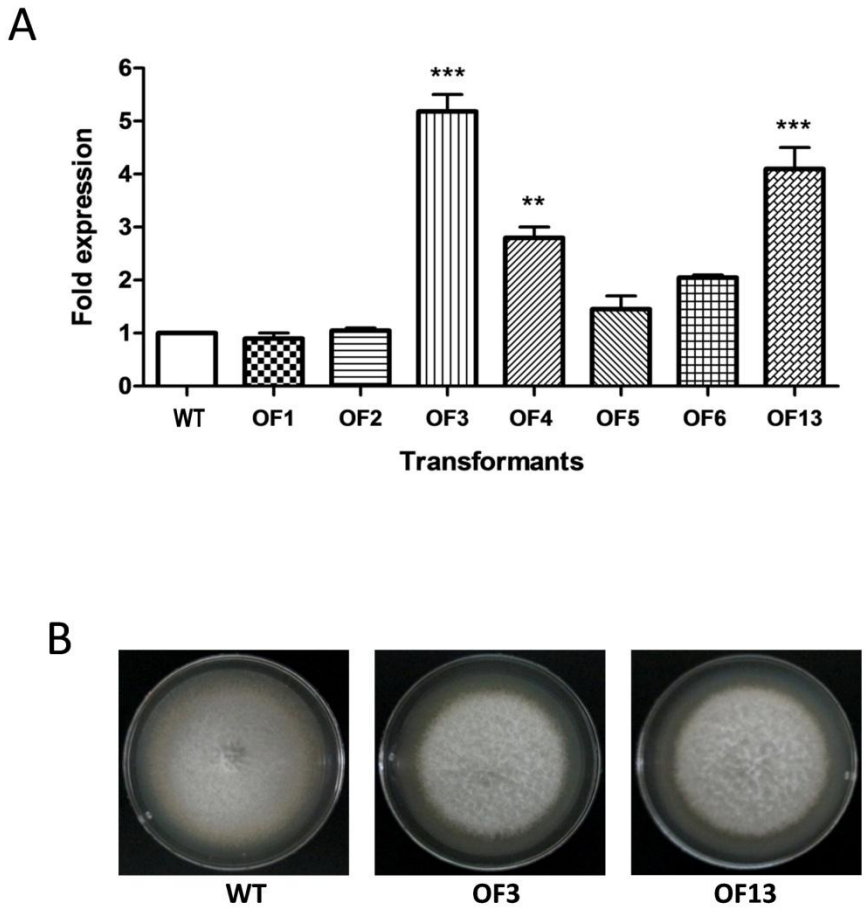

Figure. 34. Characterization of MFAR1 overexpression transformants. (A) Real time PCR was carried out to investigate fold increase in the mRNA expression of MFAR1 in overexpression transformants in comparison to wild-type strain. **(B)** Overexpression transformants were grown on OMA for 6 days for phenotypic characterization. . Values are expressed as mean ± SD in triplicates where ** = $p < 0.01$, *** = $p < 0.001$ when mRNA level of transformants was compared with wild-type.

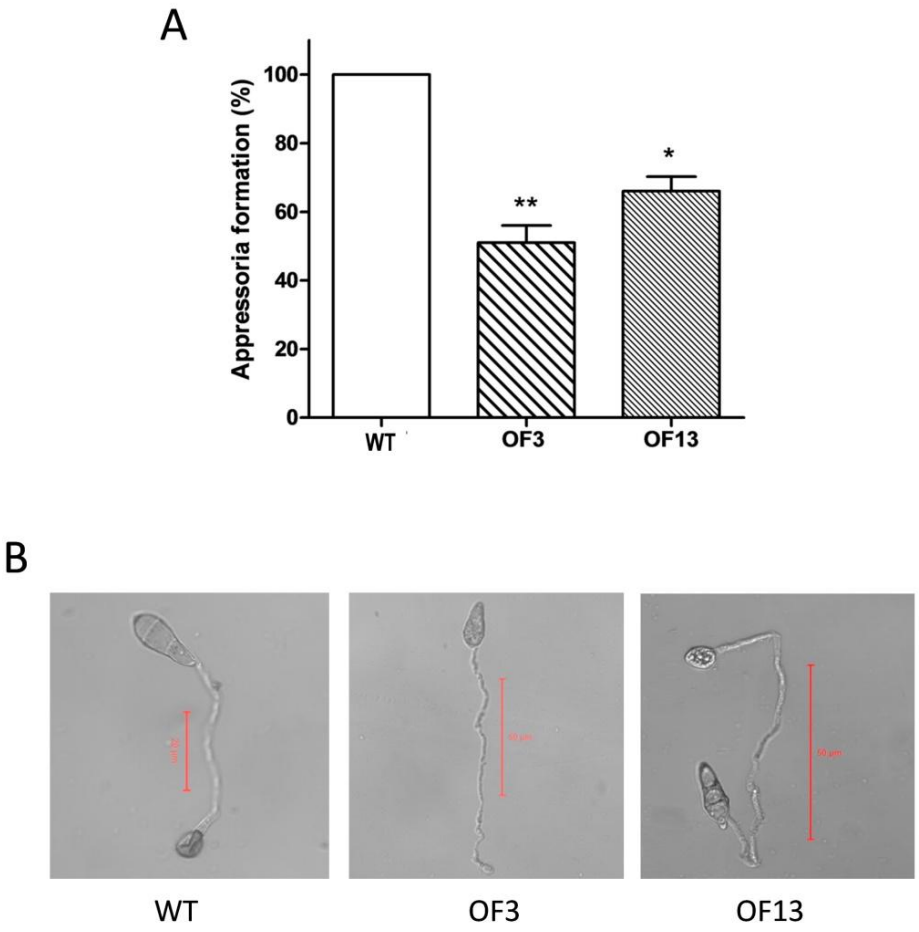

Figure. 35. Appressoria formation in MFAR1 overexpression transformants. (A) Appressorial assay was performed and number of appressoria formed by OF3 and OF13 overexpression transformants was represented as percentage of appressoria produced in B157 wild-type strain (control). The data was represented as the total number of appressorium forming conidia per 100 conidia observed under microscope. **(B)** Overexpression transformants produce comparatively longer germ tube than wild-type strain. Values are expressed as mean ± SD in triplicates where * = $p < 0.05$, ** = $p < 0.01$ when percentage of appressorium formed in overexpression transformants OF3 and OF13 was compared to B157 wild-type control. Bar = 10 µm.

Figure. 36. Overexpression transformants show reduced virulence. Rice plants were spray inoculated with conidial suspensions of B157 wild-type strain and overexpression transformants. Representative inoculated leaves were collected after 8 days of inoculation and photographed. White arrows indicate lesions due to rice blast infection.

4.15. Cyclic AMP restores appressorium development in overexpression transformants

It has also been demonstrated that the exogenous addition of cAMP to germinating conidia induces appressorium development on non-inductive hydrophilic surfaces, indicating the significance of cAMP mediated signaling during plant infection (Lee and Dean, 1993). cAMP is believed to exert its effect via activation of cAMP-dependent protein kinase A (PKA) and binding of cAMP to inactive enzyme causes the release of the catalytic subunit of protein kinase A (CPKA). cAMP has been demonstrated to restore the appressorium defect in Δpmk1 and some other deletion mutants of MAPK pathway. Here, we tried to investigate whether cAMP can restore the appressorium defect in MFAR1 overexpression transformants. 10mM cAMP was supplemented to OF3 and OF13 overexpression transformants during appressoria assay and it was found that 82% ± 4 of the OF3 conidia formed appressorium compared to 45% ± 2 when no cAMP was supplemented. Similarly, 85% ± 6 of that conidia formed appressorium in presence of 10mM cAMP compared to 57% ± 2 without cAMP supplementation (Fig. 37A). It was also found that the average size of the germ tubes formed in overexpression transformants was reduced to that of wild-type strain after application of cAMP (Fig. 37B). These results indicate that cAMP can override the MFAR1 induced inhibition of appressoria development in overexpression transformants.

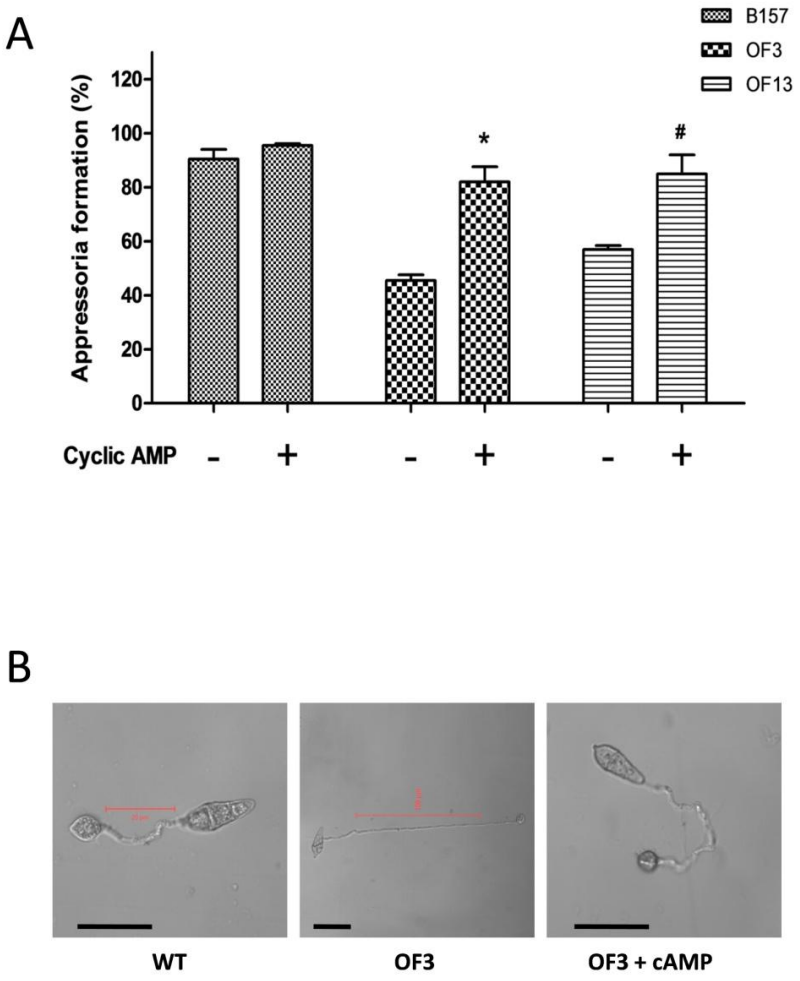

Figure. 37. Cyclic AMP restores appressoria defect in overexpression transformants. (A) Appressorial assay was performed from cyclic AMP treated and untreated overexpression transformants OF3, OF13 and B157 wild-type strain. **(B)** Overexpression transformant OF3 was supplemented with 10mM cAMP to evaluate its effect on length of germ tube formed during appressorium assay. Values are expressed as mean ± SD in triplicates where * = $p < 0.05$ when cAMP treated OF3 transfromant was compared to untreated OF3 and # = $p < 0.05$ when cAMP treated OF13 transfromant was compared to untreated OF13. Bar = 10 µm

4.16. *Δmfar1* mutant does not show pheromone induced inhibition of appressorium formation

In *S. cerevisiae*, the cell cycle arrest at G1 phase during mating is caused due to inactivation of Cdc28-Cln2 complex by FAR1 (Mendenhall, 1998). Initiation of the cell cycle at G1 has been demonstrated to be a key step towards development of appressorium in rice blast fungus (Saunders et al., 2010). Since, MFAR1 can functionally complement mating defect of Far1 mutants in yeast; we tried to investigate whether MFAR1 is directly involved in inhibition of pheromone mediated appressorium development of *M. oryzae*. Conidia from GSM3, *Δmfar1* mutant in GUY11 (MAT1-2) background and GUY11 wild-type strain were supplemented with 300 μM synthetic yeast α-factor during appressorium development. It was found that there is almost 85% decrease in the appressorium formation in GUY11 wild-type strain. However, interestingly only 15% decrease in the appressorium formation was observed in *Δmfar1* mutant (Fig. 38). Also, *Δmfar1* mutant didn't show elongated germ tube phenotype when conidia were treated with 100 μM concentrations of yeast α-factor. These results suggest that MFAR1 directly mediates inhibition of appressorium formation by yeast α-factor.

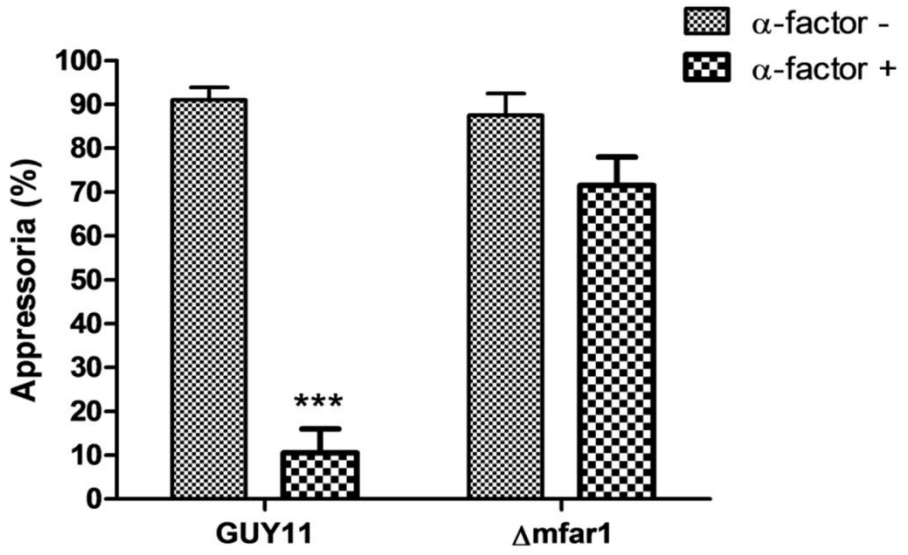

Figure 38. *Δmfar1* mutant does not show appressorium inhibition by α-factor . Conidia from GUY11 wild-type strain and *Δmfar1* strain were supplemented with 300 µM α-factor during appressorium assay and numbers of appressorium produced per 100 conidia were counted. Values are expressed as mean ± SD in triplicates where *** = $p < 0.001$ when cAMP treated group was compared to untreated group.

4.17. Expression analysis of cyclins in MFAR1 overexpression transformants

In *S. cerevisiae,* the ultimate targets of the pheromone mediated signal transduction pathway are the Clnl and Cln2 G1 cyclins. It has been demonstrated that α-factor-induced arrest is restored in far1deletion 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). Far1 in yeast inhibits cln1 and cln2 cyclins leading to cell cycle arrest (Tyers and Futcher, 1993). These results indicate that Far1 is involved in down regulating the expression of cln1 and cln2, an important step for cell cycle arrest. Cyclins Cln1 and Cln2 have also been reported to be involved in pseudohyphal and invasive growth in *S. cerevisiae* (Loeb et al., 1999). We tried to find out if overexpression of Mfar1 will affect mRNA expression of cyclins in *M. oryzae.* NCBI blast analysis was carried out to find homologs for yeast Cln2 in *M. oryzae* and three cyclin like homologs were selected for further analysis on the basis of sequence similarity with Cln2. The selected homologs include Cyclin-B (Gene ID: MGG_07065), G2/mitotic-specific cyclin-B1 (Gene ID: MGG_03595) and G2/mitotic-specific cyclin-B2 (Gene ID: MGG_05646). All these homologs are uncharacterized hypothetical proteins in *M. oryzae*. NCBI BLASTP predicts that Cyclin-B, G2/mitotic-specific cyclin-B1 and G2/mitotic-specific cyclin-B2 show around 28%, 31% and 27% identity with yeast Cln2. These putative cyclins were designated a short form of MoCyb, MoCyb1 and MoCyb2 respectively.

Real time PCR was carried out for expression analysis of these cyclins with respect to their expression in Wild-type strain. It was found that OF3 overexpression transformant showed 17% ± 3.5 MoCyb1 mRNA expression in comparison to B157 wild-type (Fig. 39A).

However, no significant changes in the expression of MoCyb and MoCyb2 were found in OF3 overexpression transformant. These results indicate that overexpression of MFAR1 represses the expression of yeast Cln2 homolog MoCyb1in a similar way that yeast Far1 causes inhibition of expression and activity of Cln2. However, Cln2 repression in yeast is related to cell cycle and growth and MoCyb1 downregulation is linked to inhibition of appressorium development in rice blast fungus.

Yeast Cln2 is a 545 amino acid protein and contains a characteristic cyclin domain at its N-terminal half and its homolog in *M. oryzae* MoCyb1, which is predicted to be a 406 amino acid protein also contains a cyclin domain at its N-terminal half (Fig. 39B). Even though the two proteins share only 31% homology but expression pattern and domain arrangement of MoCyb1 suggests that MoCyb1 can be an important target protein for functional characterization to study its role in cell cycle and infection related development in rice blast fungus.

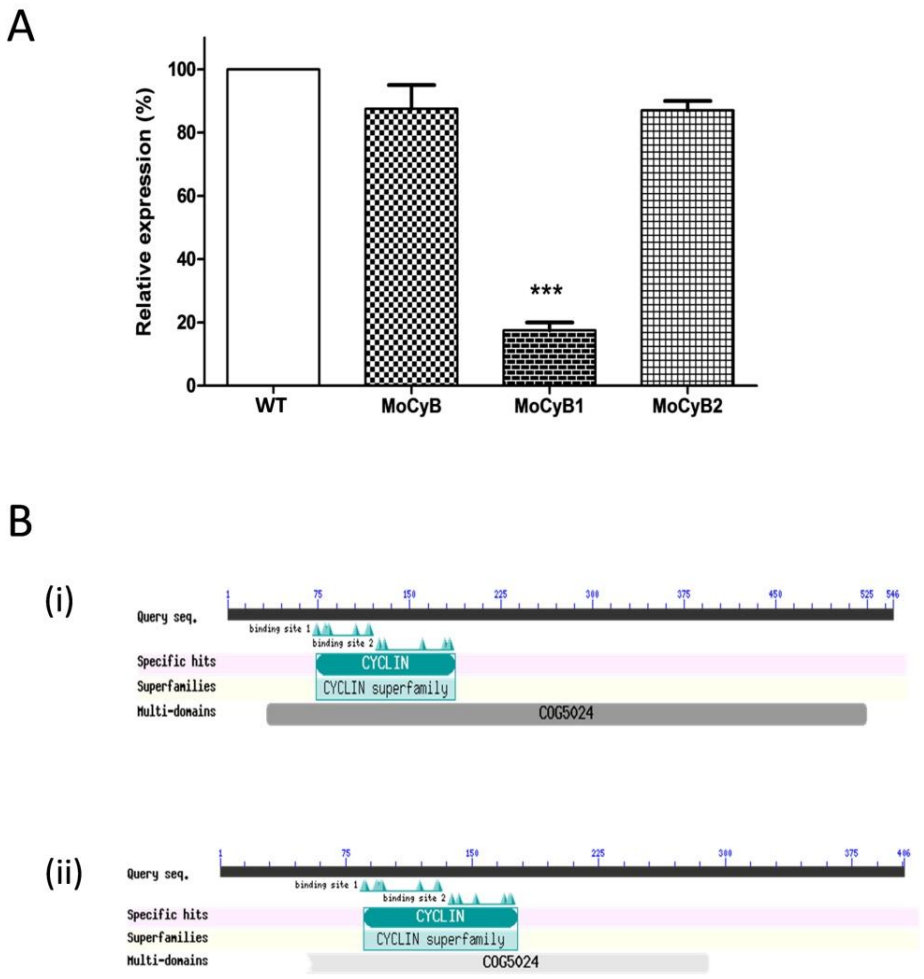

Figure. 39. Effect of MFAR1 overexpression on expression of putative cyclins. (A) Quantitative real time PCR was performed for relative expression analysis of putative cyclin genes, MoCyb, MoCyb1 and MoCyb2. **(B)** NCBI conserved domain prediction tool was used to predict the arrangement of characteristic domains of yeast cyclin Cln2 and its homolog in *M. oryzae.* **(i):** *S. cerevisiae* Cln2 and **(ii):** Cln2 homolog MoCyb1 in *M. oryzae.* Values are expressed as mean ± SD in triplicates where *** = $p < 0.001$ when expression of MoCYB1 in OF3 overexpression transformant was compared with the B157 wild-type strain.