4. DISCUSSION

4.1. Agrobacterium tumefaciens mediated transformation of M. grisea

Fungal plant pathogenesis is a complex biological process requiring diverse pathogenicity and virulence factors. Identification of such pathogenicity genes using functional genomics approach involves the creation of large single gene mutant collection. Recently, Agrobacterium has been used as a method of choice for creating random disruptions in various eukaryotes. In this study, ATMT was successfully carried out in the fungus M. grisea in order to create mutant affected in pathogenicity. Using this method, the use of protoplast for transformation was avoided as it is quite time consuming and laborious. ATMT was used to directly transform the fungal conidia. The transformation efficiency varied from 200-300 transformants for every $10^6$ conidia taken for transformation, which was comparable to that of Fusarium oxysporum (Mullins et al. 2001) where 200-400 transformants per $10^6$ conidia were obtained. For M. grisea transformation, although the presence of AS (acetosyringone) during co-cultivation was essential for successful transformation, the inclusion of AS during the growth of A. tumefaciens cells in induction medium (IM) for 6 h prior to co-cultivation was not essential (Rho et al. 2001). AS, which is required for the activation of virulence genes, was added in the co-cultivation medium but excluded from IM medium in order to increase the efficiency of single copy integration of T-DNA. Southern analysis of the transformants showed that ~62 % of the
transformants (17 out of 27 transformants) had single copy of T-DNA per genome. This was also observed in *F. oxysporum* (Mullins et al. 2001) where 19 out of 28 transformants (67.9%) and *M. grisea* (Rho et al. 2001) where 9 out of 15 transformants (60.0%) had single copy of T-DNA.

Vectors harbouring *hpt* gene under different promoters were tried for transformation. The vector pCAMBIA 1200 was used having hygromycin resistance gene (*hpt*) under CaMV 35S promoter. In vector pABC, *hpt* was cloned under *Aspergillus nidulans gpd* promoter. Earlier, *A. nidulans gpd* promoter has been demonstrated to work well in *M. grisea* (Kachroo et al. 1997). The transformation efficiency of *M. grisea* achieved with pCAMBIA1200 was comparable to that with pABC, demonstrating the application of CaMV35S promoter in *M. grisea*. The CaMV35S promoter is the most popular promoter for driving a selectable marker gene in plant transformation, and binary vectors carrying the hygromycin B resistance gene under the control of the CaMV35S promoter are readily available and can be used for fungal transformation. Transformants harbouring *GFP* (from jellyfish *Aequorea victoria*) under 35S promoter showed a very weak fluorescence when compared to the transformants harbouring *SGFP* under Tox A promoter. In addition, expression of *GFP* in filamentous fungi requires a *GFP* variant that is efficiently translated in fungi. *SGFP* contains the S65T mutation as well as plant-optimised codon usage that also deletes a cryptic intron splice site reported to reduce *GFP* expression in *Arabidopsis* (Chiu et al. 1996) and has been the *GFP* gene most often used for transformation of filamentous fungi.
4.2. Phenotypic and molecular characterisation of transformants

The aim in the present investigation was to define genetically controlled factors, critical in pathogenesis of *M. grisea*, by using ATMT as a random mutagenesis method. A number of pathogenicity mutants were created via ATMT, which were grouped on the basis of various factors critical for pathogenicity. These included phenotypic characters like growth, appressorium formation, melanin production and sporulation. Some of the transformants deficient in melanin production also lacked laccase activity as the gene coding for *M. grisea* laccase *MGL1* lies in the melanin biosynthesis pathway (Iyer and Chattoo 2003). When the various appressorial mutants were compared, it was found that the appressorial mutants also showed variation in their other phenotypes indicating that they were affected in the development of appressorium due to mutations in different genes. The transformants were also checked for auxotrophy but no auxotrophic mutant could be obtained.

Phenotypic characterisation of the mutants was followed by molecular analysis. A quick and easy technique for the large scale screening of putative transformants was developed (Tendulkar et al. 2003). Microwave radiation has been shown to be effective in killing *Escherichia coli* and lysing several other bacterial strains (Fujikawa et al. 1992, Bollet et al. 1991) as well as total DNA from *E. coli*, and other Gram negative bacteria (Fujikawa et al. 1992). The technique has also been applied for genomic DNA extraction from
plants, animal cells and fungi such as *Xyleria hypoxylon*, *Heterotextus alpinus*, *Crucibulum leave*, *Lycoperdon* spp (Goodwin and Lee 1993). However, the purification using phenol/chloroform extraction and isopropanol precipitation was performed prior to PCR amplification. Though it reduced time required for genomic DNA extraction, hazardous chemicals such as phenol were recommended. Here, it was demonstrated that microwave treatment alone, gives high yields of good quality DNA suitable for RAPD, or PCR amplification of a single copy gene such as hydrophobin. The method was used to isolate genomic DNA from spores and cultures grown in liquid media as well as biomass scraped from solid media. This approach yielded a good quality of genomic DNA from biomass as less as 2 mg. Since the technique is very fast and efficient, it can be used for various studies such as DNA fingerprinting analysis of a large number of isolates. The method yielded sufficient amount of genomic DNA for RAPD as well as Pot 2 fingerprinting analysis to study the population structure of the blast fungus isolates obtained from different geographical regions (Tendulkar et al. 2003). In this study, the genomic DNA obtained by this method was used for the analysis of a large number of putative transformants in a dot blot assay, which facilitated the screening of *M. grisea* transformants in a short time.

The sequences flanking the inserted T-DNA were identified using different PCR approaches. Initially, inverse PCR was carried out where sequences flanking the right border in 2 of the mutants were amplified. More sequences could not be identified using inverse PCR because of non-specific amplification or no amplification. No amplification
could be a result of large fragments generated after ligation which were difficult to amplify. Using vectorette the non-specific amplifications were much reduced and there was more choice of enzymes to be used so that the size of the fragments to be amplified is not very large. PCR products from the first round of amplification were re-amplified with nested primers to ensure specificity of amplified fragments.

4.3. Mutant mgal and role of MGA1 in pathogenesis

Using the forward genetics approach, a mutant strain mgal was identified due to its failure to form appressorium and cause infection. Vectorette PCR revealed that the T-DNA insertion in the mutant disrupted the genomic region corresponding to the locus MG09245.4 coding for an unknown protein (MGA1). The gene was unique with not any known homologues. A comparative analysis of fungal genome sequences and ESTs with known genes from other organisms has shown that a large number of the fungal sequences exhibit no similarity to DNA or protein sequences present in databases. Such genes have often been called orphans (Oliver 1996) and may represent genes that rapidly diverge between closely related species (Schmid and Aquadro 2001). Approximately 30% of the predicted genes of M. grisea have no significant homologs in other organisms and most of these genes encode proteins of unknown function (Xu et al. 2006). Presence of consensus kinase dependent phosphorylation motifs in MGA1 and the importance of kinase signaling in M. grisea disease pathway signify a possible role of this protein in plant infection process. MGA1 apparently codes for a putative phosphoprotein, which
Discussion showed very less identity to the known proteins in Swiss-Prot database. Nevertheless, analysis of the role of this protein in appressorium formation was carried out.

The role of MGA1 in invasive growth in plants was tested by inoculating mutant and control strain into wounded barley leaves. Injection with B157 resulted in lesions in and around the wound sites. Inoculation with the mutant resulted in slight necrosis but lesions did not spread around the wound site. Thus, MGA1 is required for both penetration and invasive growth in plants. It has been shown that *M. grisea* not only causes foliar infection, but can also infect the roots (Dufresne and Osbourn 2001; Sesma and Osbourn 2004). The germ tube arising from the conidia gives rise to appressoria during foliar infection, whereas it leads to hyphopodia formation during root infection. The fact that the mutant *mgal* does not cause either foliar or root infection suggests that *MGA1* is not only essential for appressorium mediated infection, but also required for infection via roots. When the sections from roots challenged with mutant were seen under microscope, unlike the wild type B157, no hyphopodia were observed. Thus, MGA1 plays an important role during early infection when the germ tube is not differentiated to form either appressoria or hyphopodia. This also explains the presence of MGA1 in the germ tube and appressoria as observed in the localisation experiment. It has been shown that the MAP kinase gene *PMK1* is also required for infection of both leaves and roots (Dufresne and Osbourn 2001). Thus, MGA1 appears to be a key molecule required for the formation of infection structures leading to pathogenicity.
Both targeted disruption and complementation analysis confirmed the function of this gene in formation of appressorium. To determine the degree of enrichment of gene replacement mutants, Hygromycin B resistant transformants in the absence of F2dU were first isolated and subsequently analysed for their sensitivity to F2dU and the presence of target mutation. During targeted disruption, of 25 transformants growing on hygromycin, 11 were found to be insensitive to F2DU (44%). One representative transformant was analysed further and confirmed were confirmed by Southern analysis, appressorial assay and infection assay. During complementation, after transformation with GFP as a marker gene, all viable spores grew up (transformed as well as untransformed) due to absence of any other selectable marker. Transformation was carried out using the optimised protocol and 10^6 spores/ml were taken for transformation, however, since there was no selectable marker, around 100 spores were plated. Out of these, 18 were randomly selected and 4 showed GFP fluorescence when observed under microscope. One transformant was selected further for characterization.

The mutant *mgal* was not only impaired in appressorium formation, but also in pigmentation, as compared to untransformed fungus. The adherence of conidia to the hydrophobic surface was not affected. Both, cAMP and 1, 16-hexadecanediol, induce appressorium formation on noninductive surfaces in *Magnaporthe grisea* (Lee and Dean 1993; Gilbert et al. 1996). However, appressorium formation in the mutant was not restored by either of the compounds; although in presence of cAMP, the germ tube underwent hooking and swelling. These results show that MGA1 is not involved in
surface sensing, but plays a role during early stages of appressorium formation and might be acting downstream of cAMP.

Osmoregulation and signal transduction is relayed through MAP kinase network. When cells are exposed to hyperosmotic stress, the pathway phosphorylates a signal that leads to the activation of genes whose expression results in the synthesis of compatible intracellular solutes. The compatible solutes generated are used by the cells to prevent water loss and regulate cellular turgor (Brewester et al. 1993). *M. grisea* accumulates a number of polyols including glycerol during hyperosmotic stress (Dixon et al. 1999). The effect of hyperosmotic stress on the mutant *mgal* was monitored and the growth of the mutant was greatly reduced in presence of sorbitol. A few appressorial mutants of *M. grisea* have also been shown to be sensitive to hyperosmotic stress. These include *pmkl*, *mst7*, *mst11* and *mmtl* mutants involved in the MAP kinase (*PMK1*) pathway (Tucker et al. 2004; Zhao et al. 2005), suggesting that *MGA1* might be involved in an osmoregulation response pathway controlling cellular turgor, an important factor in appressorium development in *M. grisea*. The glycerol content in the mutant was approximately half than that of the wild type under sorbitol stress. The expression of *MGA1* in *M. grisea* mycelia grown under sorbitol stress was also seen to be more as compared to complete media.

The enzymes involved in glycerol production from glycogen during hyperosmotic stress are present in mycelia as well as appressoria (Thines et al. 2000). Glycogen and lipid
mobilisation from the spores to developing appressoria is a part of appressorium differentiation process. cAMP and MAP kinase pathways are involved in appressorium development and a complex relationship exists between these pathways (Dean 1997; Kronstad 1997; Xu 2000). The mutant \textit{mga1} was unable to mobilise glycogen and lipids required for appressorium differentiation; as such it was unable to form appressoria. cAMP and \textit{PMK1}-dependent mobilisation of carbohydrate and lipid reserves has been demonstrated in \textit{M. grisea} (Thines et al. 2000), suggesting \textit{MGA1} to be a downstream effector of a kinase involved in appressorium differentiation.

Therefore, in absence of \textit{MGA1}, the fungus is not synthesising the appropriate amount of glycerol required to overcome hyperosmotic stress conditions or metabolising glycogen required for appressorium differentiation. The results here suggest that \textit{MGA1} plays a role in signal transduction during appressorium differentiation. Fig. 39 shows the probable location of \textit{MGA1} in the pathway for appressorium morphogenesis. Its activity is essential for the metabolism of glycogen and lipids, which is a part of appressorium differentiation process. The inability of the mutant to cause foliar as well as root infection signifies its role in both the pathways of infection.
Figure 39: Probable location of MGA1 in appressorium morphogenesis pathway.
cAMP-adenosine 3'-5' cyclic monophosphate, CPKA-catalytic subunit of cAMP
dependent protein kinase A, MAC1-adenylate cyclase, MagB-G protein α subunit,
MAPK, MAPKK, MAPKKK, MPS1, MST12-mitogen activated protein kinases,
PTH11-transmembrane protein, SUM1-regulatory subunit gene of PKA.
Signalling Model for Appressorium Development in *M. grisea*

Surface Hardness

MAPKKK → MAPKK → MAPK → PMK1

MPS1 → CPKX → Appressorium morphogenesis

MST12 → Penetration Peg Emergence

Turgor

Nutritional signal

Pth1

cAMP

SUM1

CPKX

CPKA

Talbot 2003
4.4. Role of ABC4 in pathogenesis

Sequence obtained from another appressorial mutant (abc4) showed homology to gene coding an ABC family of proteins. The ATP-Binding Cassette (ABC) superfamily, also known as ‘traffic ATPases’, comprises an extremely diverse class of membrane-transport proteins. ABC transporters are transmembrane proteins that couple the energy of ATP hydrolysis to the selective transfer of substrates across biological membranes (Higgins, 1992). ABC superfamily, also known as ‘traffic ATPases’, comprises an extremely diverse class of membrane-transport proteins. These transporters are responsible for the controlled efflux and influx of substances across cellular membranes. ABC transporters have been identified in a wide variety of organisms, including mammals, yeast, fungi, bacteria and insects (van Veen & Konings, 1998) and play a major role in multidrug resistance (MDR). These proteins act as virulence factors and provide protection against plant defense compounds during pathogenesis (Stergiopoulos et al. 2002).

ABC4 showed highest similarity to *N. crassa* (59%) ABC transporter but not to the transporters reported earlier from *M. grisea* (Urban et al. 1999; Lee et al. 2005; Sun et al. 2006). ABC4 is related to Ste6 protein from *S. cerevisiae* (22%) which is an ATP-binding cassette transporter required for the export of a-factor during mating in MAT a cells (McGrath and Varshavsky, 1989). The locus (MG0937.5) which harbors the ABC4 gene in *M. grisea* has been reported to be one of the ESTs from *M. grisea* mated culture library (Unisequence ID: Mag30405205; Cogeme database).
Blast search suggests that apart from rendering MDR function, *M. grisea abc4* may be involved in lipid as well as Na$^+$ transport. Domain spanning from 460 to 684 and 1289 to 1503 residues show homology to ABC sub-family A, which is known to mediate transport of a variety of lipid compounds. The ABCA protein in humans is suggested to be involved in the removal of cholesterol and phospholipids from cells onto high-density lipoprotein particles (Young and Fielding 1999). Cdr1p and Cdr2p confer azole resistance and act as phospholipid translocases in *C. albicans* (Smriti et al. 2002). Regions from 460 to 761 and 1048 to 1507 residues are predicted to be similar to CcmA and Mdl B, respectively, which are ATPase component of ABC-type multidrug transport system. The region over 1289 to 1507 aa shows homology to ‘NatA’ that is a ABC-type Na$^+$ transport system. Similar predictions are made for ABC homologues in *Neurospora crassa* (CAD 79694), *Aspergillus fumigatus* (XP_753691), *Ustilago maydis* (XP_759601), and *Yarrowia lipolytica* (XP_504037).

The mutant was affected in appressorial development as well as pathogenicity implying the role of this transporter, ABC4 in pathogenic development of *M. grisea*. As a consequence of the defective appressorium formation, the mutant was also not capable of infection. Nevertheless, lesser intensity disease lesions were observed when infection was done on wounded leaves indicating that ABC4 is indispensable for appressorium mediated penetration but may cause infection once inside the host cells. The mRNA expression of the *ABC4* gene was increased in presence of various drugs while it was absent in the mutant, confirming the expression and role of ABC4 in multidrug transport system.
resistance. In real time PCR, the expression of ABC4 was induced in presence of various drugs confirming the multidrug resistance phenomena of ABC4. The maximum induction of ABC4 was found in presence of resveratrol, a phytoalexin from grapevine, where ABC4 was 10.50 fold induced as compared to untreated sample. The ABC transporter from Botrytis cinerea (accession no. AJ006217), showing 29% identity to ABC4, has been reported to affect the sensitivity towards resveratrol and the mutant was reduced in virulence on grapevine (Schoonbeek et al. 2001). Phytopathogenic fungi encounter toxic environments during plant invasion as a result of plant defense response. The interaction between rice and M. grisea is accompanied by production of an array of antifungal proteins and phytoalexins in host (Kodama et al. 1992). Increased sensitivity of the mutant to grapevine phytoalexin resveratrol may be the cause of loss of virulence of the mutant.

In conclusion, ABC4 is essential for the pathogenic development of M. grisea inside the host. It imparts multidrug resistance and is required to cope with cytotoxic environment by providing protection against plant defense mechanisms.