4. DISCUSSION

4.1. Ubiquitin mediated proteasomal degradation

Targeted protein degradation by the ubiquitin-proteasome pathway is extremely specific with respect to target, time and space and plays a pivotal role in monitoring the levels of most of the short lived proteins in the cell. Thus, the proper functioning of this pathway is important for many different physiological and developmental processes. Once the signal for degradation of a particular protein is 'on', a cascade of three enzymes (E1, E2, E3) work on it and finally tag the substrate protein with ubiquitin, a small 76 amino acid heat stable protein, at an internal lysine residue. This tagging is an important step and is carried out by a specialised protein, E3 ubiquitin ligase, which is a multiprotein complex (Skp1-Cul1-F-box) also known as SCF. Skp1 is the core protein of this complex and stabilises the E3 ubiquitin ligase enzyme. Apart from the SCF complex, Skp1 may have novel functions in mitotic exit and cytokinesis, regulating assembly of vacuolar ATPase, connecting cell cycle regulation to the ubiquitin proteolysis machinery. Cell cycle regulator involved in DNA damage checkpoint, regulation of kinetochore, cell separation and mitotic exit have been reported to be subjected to control by the ubiquitin proteolysis machinery (Bai *et al*. 1996, Kaplan *et al*. 1997, Hermand *et al*. 2003, Lehmann *et al*. 2004, Brace *et al*. 2006, Kim *et al*. 2006).

In *Saccharomyces cerevisiae,* in which the Skp1 homologue has been studied extensively, it was named Cbf3d, due to its role as an important component of CBF3-*CEN* complex (Stemmann 1996). In *S. cerevisiae*, this protein is essential for growth and executing kinetochore function by interacting with cyclin A/CDK2 (Zhang *et al*. 1995). It

has been found that, CLB1-4/CDC28 complexes or unidentified cyclin/CDK–like protein might interact with Cbf3d directly or indirectly (Nasmyth 1993).

In *Arabidopsis thaliana*, regulated protein degradation by the ubiquitin/26S proteasome contributes significantly to the development by affecting a wide range of processes, including embryogenesis, hormone signalling and senescence. More than 1400 genes (5% of the proteome) encode ubiquitin/26S proteasome components (Smalle and Vierstra, 2004). Approximately 90% of these genes encode subunits of the E3 ubiquitin ligases, which confer substrate specificity to the pathway. Identification of the substrates and characterisation of the E3 ubiquitin ligases of the Ub/26S proteasome pathway are among the primary objectives in the proteosomal degradation pathway (Moon *et al*. 2004). Various stress-related E3 ligases identified to date facilitate responses to environmental stimuli by modulating the abundance of key downstream stress-responsive transcription factors. The regulatory role of ubiquitin during plant responses to abiotic stress have been highlighted (Lyzenga and Stone, 2011). Being a well-established major modifier of signalling in eukaryotes, ubiquitination is also involved in sensing of environmental nutrient levels in plants (Yates and Sadanandom, 2013). These observations provide insight into a new role of ubiquitination in nutrient homeostasis. Among the various protein modifications, such as phosphorylation, methylation, acetylation, myristoylation and glycosylation, the functional analysis of ubiquitination associated proteins from plants and pathogens shows that ubiquitination plays an important role in plant-microbe interactions (Zeng *et al*. 2006). In some cases, plant pathogens have the ability to hijack host SCF ubiquitin ligase machinery (Magori and Citovsky, 2011).

The Ubiquitin-proteasome protein degradation system plays an important role in response to nitrogen starvation in the white rot fungus *Trametes versicolor*. The fungus is an efficient lignin degrader with ecological significance and industrial applications (Staszczak 2008). The SCF E3 ligase and its F-box proteins have been extensively studied in *Saccharomyces cerevisiae*, but the role of F-box proteins in other fungi is not very well understood and so the involvement of proteosomal degradation remains to be investigated. Recently, a number of studies revealed that F-box proteins are required for fungal pathogenicity in *Fusarium graminearum*, *Fusarium oxysporum*, *Botrytis cinerea* and *Magnaporthe oryzae* (Liu and Xue, 2011). In *M. oryzae* PTH1 is a homolog of Grr1 (a F-box protein in *S. cerevisiae* and is involved in protein ubiquitination) and is required for regulating maturation of the appressorium. The pth1Δ mutant fails to penetrate the host leaf surface and to establish a successful host-pathogen interaction (Sweigard *et al*. 1998, Silva 2004). *M. oryzae* being a model organism to study plant-pathogen interaction can be utilised to investigate the role of SCF E3 ubiquitin ligase and its components in the development and pathogenicity of the fungus.

4.2. Silencing of *MoSKP1* gene in *Magnaporthe oryzae* B157

RNA interference (RNAi) is a naturally occurring post-transcriptional gene-silencing (PTGS) phenomenon. The double stranded RNA (dsRNA) triggers the degradation of a specific mRNAs with complementary sequence, thus reducing gene expression. RNAi is becoming a potential tool in biotechnological applications in fungi (Salame *et al*. 2011). More than 40 species have been proven to have RNAi machinery. In fungi, the use of RNAi as a tool for reverse genetics is gaining more popularity. This technology has the ability to simultaneously down-regulate and silence the expression of several genes in

fungi (Nguyen *et al*. 2008). Several vectors constructs have been developed to study RNAi mechanisms and for functional analysis of several genes involved in growth and development of the fungus. A series of pSilent vectors have been used in *Magnaporthe oryzae* as an effective way to study the functionality of a specific gene (Nguyen et al. 2011). Silencing technology has the advantage over total gene knock-out because it provides an opportunity to investigate housekeeping genes and genes important for viability. The pSilent-Dual1 (pSD1) vector have been generated by cloning two PtrpC promoter and Pgpd promoter in inverted repeat orientation containing a multiple cloning site in between which ease the construct generation time for functional genomics research in filamentous fungi (Nguyen *et al*. 2011).

In this study with *M. oryzae* B157, the pSilent-Dual2 RNAi vector was used, where the TrpC promoters (constitutive strong promoter), is expected to give same amount of sense and antisense transcripts. The TrpC promoter has been shown to work well in *M. oryzae* (Nakayashiki *et al*. 2005a&b, Kadotani *et al*. 2004, Nguyen *et al*. 2008, Nguyen *et al*. 2011). In the present study, the target selected was *MoSKP1*, a member of E3 ubiquitin ligase gene. The *MoSKP1* is an essential gene for the normal physiology and development of the fungus, so RNAi and antisense technology is the most suitable strategy to study the functions of this gene. To compare the silencing efficiency and their phenotypes, both RNAi and antisense technology have been used in this study. However the silencing efficiency in *MoSKP*1 RNAi transformants was found to be higher when compared with silencing in antisense transformants. The transcript levels of RNAi transformants vary from 15% to 40% of those in the parent strain, whereas in antisense transformants it goes up to 60%. Decrease in *MoSKP1* transcript levels in the RNAi

transformants leads to abnormal phenotypes consistent with the decrease in the level of transcripts, where transformants with the lowest transcript levels have minimum growth and sporulation and transformants with intermediate transcript levels were able to grow and sporulate but unable to form appressoria. These results were consistent with the reduced transcript levels in the RNAi transformants when checked by quantitative real time PCR using SYBR green chemistry. Transcript level evaluation of *MoSKP*1 in RNAi and antisense transformants confirmed that RNAi transformants had lower transcript level (minimum transcript level ~15%) whereas antisense transformants had comparatively higher *MoSkp*1 transcript level (minimum transcript level ~40%). Five RNAi transformants that showed *MoSkp*1 transcript levels in the range of 15% to 40% were analysed in this study. The silencing efficiency was comparable to previously reported research (Nakayashiki *et al*. 2005b, Nguyen *et al*. 2008). Small interfering RNAs (siRNA) are key molecules in post transcriptional gene silencing mechanism. Although, involvement of siRNA in PTGS has been previously shown (Kadotani *et al*. 2003), we examined the presence of *MoSKP1* siRNA in the silenced transformants. The *MoSKP1* small RNA below 26 bp was detected in silencing transformants with pSD2-MoSkp1 by northern blot analysis, thus indicating that gene silencing induced by this vector involved PTGS.

4.3. Phenotypic and molecular characterisation of *MoSKP1* transformants

The objective of the present investigation was to identify and characterise a gene involved in the growth, development and pathogenicity in the rice blast fungus *M. oryzae*. Although the genome sequence of *M. oryzae* is available, functions of nearly 80% of the

genes remains unknown. Very few reports on the role of ubiquitination in growth and development of *M. oryzae* have been published (Oh *et al*. 2012). *SKP1* is an important component of E3 ubiquitin ligase and our analysis shows that *M. oryzae* has only one orthologue of *SKP1* (MGG_04978). Several reports indicate that E3 ubiquitin ligase mediated protein degradation is involved in plant-pathogen interactions (Rosebrock *et al*., 2007, Singer *et al*., 2013). We characterised the function of this gene using a gene silencing approach, since the gene appears to be essential for viability. Our study shows that *MoSKP*1 silencing leads to pleiotropic effects. Cell wall integrity of the fungus was compromised and there was defective spore formation. MoSkp1 is localised from spore to germ tube and abundantly expressed in appressoria. Various RNAi and antisense transformants of *MoSKP1* in a B157 background showed reduced sporulation, defective spore morphology, less septation and diffused nuclei. Further the silenced transformants showed elongated germ tube and were unable to form appressoria on inductive surface. Transformants arrested in G1/S stage of the cell cycle during initial spore germination had similar phenotype as wild type spores treated with Hydroxyurea (HU). Reduced *MoSKP1* transcript and protein levels in knockdown transformants result in atypical germ tube development. The MoSkp1 protein interacts with a putative F-box protein (MGG_06351), revealing the protein complex forming ability by protein-protein interaction. Thus, these results show that reduced levels of *SKP1* lead to a variety of developmental defects in the fungus.

Phenotypic characterisation of the *MoSKP1* transformants was followed by molecular analysis. A quick and easy technique for the large scale screening of putative transformants was developed in our lab (Tendulkar *et al*. 2003). 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 good quality genomic DNA from biomass as little 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. In this study, the genomic DNA obtained by this method was used for the analysis of a large number of putative transformants by PCR, which facilitated the screening of *M. oryzae* transformants in a short time.

Southern blot analysis and western blot analysis was performed to confirm the integration and expression of the *MoSKP1* constructs in various silencing transformants. RNAi and antisense transformants were also checked for the presence of siRNA by enriching total small RNA, running on 16% denaturing PAGE and northern blot analysis. The detection of siRNA was found to be comparable to previously published data (Fire *et al*. 1998, Goto *et al*. 2003). Total protein ubiquitination pattern was detected by ubiquitination enrichment assay of MoSkp1 RNAi transformants as described earlier in various model systems (Ciechanover 1998). The number of bands found was less, probably because the enrichment was done for higher molecular weight ubiquitinated protein (80 kDa to 220 kDa). However, this indicates that the silencing of *MoSKP1* leads to reduction in ubiquitination pattern which in turns leads to various phenotypic defects including loss of pathogenicity. The quantitative PCR was performed on RNAi, antisense and over expression transformants of *MoSKP1* in high throughput PCR system by monitoring the increase in fluorescence of the SYBR Green dye in real time. Fold change of target gene transcript was calculated by using the formula as described by Van der velden *et al*. 2003, Gabert *et al*. 2003.

4.4. Role of *MoSKP1* in growth, sporulation, development and pathogenesis

Given the fact that ubiquitination controls cell cycle progression and development of cells, we believe that our characterisation of the *SKP1* homologue in *M. oryzae* will pave the way to study its potential role in morphogenetic programming of appressorial development. The S-phase kinase-associated protein 1A (Skp1) is predicted to be 19.363 kDa protein with tetramerisation and dimerisation domain that is a characteristic of the family. Various temperature sensitive mutants have been generated for *SKP1* in *S. pombe* and *S. cerevisiae*. In *S. pombe*, there are several F-box proteins out of which three specific F-box proteins, two essential (Pof1, Pof3 involved in maintaining genome integrity) and one non-essential (Pof10) are found to be involved in cell cycle. The binding of Skp1 with these three specific F-box proteins was reduced in skp1 temperature sensitive mutants, as a result of which cells exhibit G2 cell cycle delay (Lehmann 2004, Hermand 2003). We have used *skp1 A7* mutant of *S. pombe* for the interspecies gene complementation study. The wild type phenotype was largely restored in this mutant when it was complemented with *MoSKP1,* although the growth was slow and sizes of cells were slightly smaller than wild type, possibly due to overexpression of the protein because of high copy number of the vector. Phylogenetic analysis revealed that *MoSKP*1 is closest to *NcSkp*1 of *N. crassa. MoSKP*1 is able to complement the function of *S. pombe* even though this is not a very near relative. This shows that functionally *SKP1* is well conserved but we cannot rule out the possibility of other novel functions of *MoSKP1*.

Sequence analysis of *MoSKP*1 suggests a strong possibility of phosphorylation sites, and along with dimerisation domain the protein has opportunity to perform various functions.

Analysis of the phosphorylation sites in *M. oryzae*, gave us several hits, most of which were serine residues. Three Serine residues at position 77, 81 and 83 are most likely to be phosphorylated along with two threonine residues at 76 and 86 positions just in the vicinity of serine residues. All the phosphorylation sites fall in the stretch of amino acids which are more strongly conserved and participate in protein-protein interactions. Silencing of the *MoSKP*1 gene in the RNAi transformants and decrease in transcript level lead to an abnormal phenotype of transformants. Observation indicate that increased severity of defects in RNAi transformants is consistent with the decrease in the level of transcripts, with transformant with the least transcript levels have minimum growth and sporulation and transformants with intermediate transcript levels were able to grow and sporulate but were unable to form appressoria but instead formed elongated germ tubes. This suggests that the level of *MoSKP1* is critical to its diverse functions. While 40% of transcript is sufficient for sporulation, much higher levels are required for appressoria development and further reduction to 20% leads to complete abolition of function. Mitosis is an important step in the development of appressoria and it is likely that Skp1 stabilises the SCF E3 ubiquitin ligase complex which plays an important role in the progression of the cell cycle. Presence of MoSkp1 protein in spore, germ tube and in appressoria when compared with mycelia provides strong evidence that MoSkp1 is necessary for the initial development from spore to appressorium thus controlling the development of the infection structure. Further, phenotype of the silenced transformants similar to the cell cycle defective mutant of *S. pombe* (*skp1 A7*) and to hydroxyurea treated *M. oryzae*, provides an additional insight into the nature of defect in absence of MoSkp1 protein.

Skp1 is extensively involved in protein-protein interaction and has affinity to bind with a subset of F-box proteins which is another characteristic feature of the family. MoSkp1 interacts with a hypothetical protein which is supposed to be an F-Box protein in *M. oryzae* on the basis of bioinformatics and interaction study in another phytopathogenic fungus *F. oxysporum*. In our study interaction of MoSkp1 and MoFrp1 is unable to give β-galactosidase assay but was able to grow on quadruple dropout media indicating that two reporter genes, *His3* and *Ade*2 (a tightly regulated gene) were active whereas the third reporter *lacZ/MEL1* is not active possibly because of weak interaction. Further the co-immunoprecipitation of MoSkp1-myc protein with MoFrp1-HA protein and pull down assay using anti-MoSkp1 antibody provides evidence for the association of two proteins. Our finding suggests that the interaction between MoSkp1 and MoFrp1 is either weak or transient as most of the biological interactions are dynamic in nature, although further experiments are required to address this issue.

Current investigation on the role of MoSkp1 suggests that reduction of MoSkp1 manifests in reduced total protein ubiquitination pattern and subsequently defective cell cycle and appressorial development. Thus, MoSkp1 plays important roles in growth, sporulation, appressorial development and pathogenicity of *M. oryzae* B157.

4.5. Molecular basis of appressorium development and pathogenicity

Appressorium formation in *M. oryzae* is regulated by cell cycle progression. Emergence of the fungal germ tube from the three-celled conidium is followed by migration of one nucleus into the developing germ tube, where it undergoes mitosis 4–6 hours after germination (Saunders *et al*. 2010, a&b). Following mitosis, a daughter nucleus enters the

developing appressorium, while the other returns to the conidium. The three nuclei in the conidium are then degraded together with the rest of the spore contents, leaving a single nucleus in the mature appressorium. The appressorium nucleus later migrates into the penetration peg, where it undergoes further rounds of mitosis as invasive hyphae develop. The nucleus in the appressorium is therefore the source of all genetic material for subsequent *in planta* colonisation.

MoSKP1 RNAi transformants showed defective cell cycle and aberrant appressorial development. *MoSKP1*-RNAi transformants and antisense transformants of *M. oryzae* showed similar phenotypes as the wild type B157 strain of *M. oryzae* when treated with the Hydroxyurea (HU, 200mM), a known inhibitor of cell cycle progression which inhibit DNA replication and activate the G1/S phase checkpoint (Singer and Johnston, 1981, Koc *et al*. 2004),. This observation clearly suggests the involvement of MoSkp1 in the cell cycle progression and involvement in the appressorial development. The elongated germ tube phenotype of the knockdown transformants of *MoSKP1* RNAi is supported by *S. pombe MoSKP1* complementation data (Bai *et al*. 1996, Connelly and Hieter 1996, Kaplan *et al*. 1997, Skowyra *et al*. 1997). Defect in cell cycle also explains the reduction of sporulation in the several MoSkp1 transformants. The arrangement of conidia on the conidiophore was observed and *MoSKP1* RNAi transformants showed single defective unhealthy conidium on each conidiophore. It seems that less number of conidia on each conidiophore results in reduced sporulation (Oh *et al*. 2012).

The signalling pathways responsible for regulating appressorium formation have been investigated in *M. oryzae* and it is known that a large number of signalling processes are involved in the development and maturation of appressoria including the cAMP response

pathway are triggered soon after the attachment of the fungus on the leaf surface (Saunders *et al*. 2010); these includes a regulatory subunit of PKA (cAMP-dependent protein kinase A) (Xu *et al*. 1996, Choi *et al*. 1997, Adachi *et al*. 1998), and a MAP kinase (mitogen-activated protein kinase) cascade involving the Pmk1 MAP kinase (Thines *et al*. 2000). Appressorium development can be broadly divided, into two different stages: the initiation of appressorium development, which requires cAMP signalling, and appressorium morphogenesis, which requires the presence of the Pmk1 MAP kinase pathway (Skamnioti *et al*. 2009, Wang *et al*. 2005). Following mitosis, autophagic cell death of conidia occurs with a single nucleus remaining in the appressorium with one daughter nucleus migrates to the swollen germ tube tip while the other returns to the original conidial cell. This phenomenon has been shown to be necessary for appressorial development (Veneault-Fourrey *et al.* 2006). Live-cell imaging of *M. oryzae* expressing a histone H1-enhanced red fluorescent protein (RFP; tdTomato) fusion (Shaner *et al*. 2004) and β-tubulin-synthetic green fluorescent protein (sGFP) fusion showed nuclear division and its migration during appressorium formation. Targeted deletion of the *M. oryzae ATG8* gene, which is functionally related to the *ATG8* (also known as *AUT7*) gene in budding yeast (*Saccharomyces cerevisiae*) and is required for the generation of autophagosomes, or the ATG1 kinase-encoding genes, resulted in mutants that were unable to undergo autophagy and could not infect plants. A temperature-sensitive mutation in the *MgNIM-A* gene, which encodes a protein kinase that is necessary for mitosis, prevents appressoria formation (Saunders *et al*. 2010). In the filamentous fungus *Aspergillus nidulans*, the temperature-sensitive *nimO18* mutant (for never in mitosis) is unable to replicate DNA, at the restrictive temperature (James *et al.*

1999). NimO is functionally related to *Saccharomyces cerevisiae* Dbf4p, the regulatory subunit of the Cdc7pDbf4p kinase complex, which is required for Cdc7p kinase activity and initiation of DNA replication (Jackson *et al*. 1993). Analysis of the *NIM1* sequence revealed a BRCT domain that is characteristic of cell cycle regulators involved in the DNA damage checkpoint (Bork *et al*. 1997), the Dbf4 M motif, and a zinc finger domain, all of which are conserved in Dbf4-related proteins (Ogino *et al.* 2001). Initiation of appressorium morphogenesis is regulated at S-Phase. In the investigation of cell cycle progression of appressorium development, DNA synthesis inhibitor hydroxyurea (HU) arrests conidia predominantly with undifferentiated germ tubes. This is in contrast with perturbation of the cell cycle in *M. oryzae* with either benomyl or *nimA* mutation, both of which cause a late G2 arrest but allow the formation of swollen germ tube tips (Veneault-Fourrey *et al*. 2006). Thus, DNA replication (S-phase) is necessary for formation of the terminal swelling that later develops into an appressorium. The process of appressorium morphogenesis is tightly coupled to cell division, as mitosis is always observed within germ tubes of the fungus before the appressorium development (Ebbole *et al*. 2007).

The present studies clearly established the role of *MoSKP1* in *M. oryzae* B157 in ubiquitination as well as in various processes including the progression of cell cycle, sporulation and appressorial development. E3 ubiquitin ligase serves as a fundamental element for protein ubiquitination leading to protein degradation, maintaining proteostasis. Thus, Skp1 might be essential for all the processes where an optimal protein turnover is to be maintained. In *M. oryzae* during the initial stages of infection the organism undergoes a number of essential processes so as to produce a mature infection structure and further establish itself in the host tissue. Proteostasis is therefore important

for fundamental processes like cell separation, mitotic exit, DNA damage check and repair, V-ATPase assembly, cell cycle progression (Connelly *et al*., 1996., Seol *et al*., 2001, Harmend *et al*., 2002, Lehmann *et al*., 2004., Kim *et al*., 2006). These processes might be important not only during vegetative growth, but also the biotrophic phase of the pathogen. Since the knock-out would be inviable and, therefore, would not give any information about other processes that *MoSKP1* might be involved in, we intended to study the phenotype by silencing the gene.