Characterisation of genes involved in development and virulence of pathogenic fungi

Introduction

Fungi form a diverse group of eukaryotic organisms, including unicellular yeasts, yeasts that can undergo hyphal transition and multi-cellular filamentous fungi. Fungi are suited to lifestyles in a wide range of habitats/niches that call for efficient adaptation in the acquisition of nutrients, response to environmental triggers and stresses and maintenance of basal homeostasis and growth. Excellent examples of such adaptability are found in the class of fungal pathogens that have managed to infect plant, animal and human hosts, where successful colonisation is often achieved through the development of several distinct morphological stages with specialised function. Development of resistant conidia, elongation of germ tube, specialised infection structures that bring about chemical and physical disruption of host tissue, hyphal extension and branching, and production of aerial hyphae are key to fungal dispersal and virulence, especially in filamentous fungi. One such filamentous fungus with multiple morphological transitions is the rice blast fungus, *Magnaporthe oryzae* that has topped the list of scientifically and economically important plant fungal pathogens (Dean, Van Kan et al. 2012).

In *M. oryzae* the infection process starts when the three-celled conidium lands on the rice leaf surface and germinates by polarised extension of a narrow germ tube from the apical or basal cell. After sufficient elongation, the germ tube ceases to grow and switches from polarised to isotropic growth and the tip swells to form a dome-shaped infection structure called the appressorium. Maturation of the appressorium involves transport of large quantities of solute from the conidium to the appressorium, autophagic cell death of the conidium and melanisation of the appressorial cell wall that allows the establishment of a high turgor pressure. Once again the appressorium switches to polarised growth to form the penetration peg that breaches the host epidermis, and elaborates into primary infection hyphae. Secondary hyphae subsequently develop and spread to the adjacent cells. Typical eye shaped disease lesions with a brown border and grey centre appear. At this stage conidiation takes place allowing spread of the fungus to neighbouring plants and a new infection cycle is initiated. *Magnaporthe oryzae* infects rice and several other cereal crops in India and across the world causing negative impact on global food security. The millions of hectares of crops lost every year to rice blast disease caused by *M. oryzae* call for a better understanding of the disease. Though several aspects of the life cycle like conidia formation and germination, formation of appressoria and pathogenesis of this devastating ascomycete have been studied before, the function of a large proportion of the 38Mb genome still remains unknown. Even with the availability of the genome sequence, the centromeres of *M. oryzae* have still not been identified. A detailed investigation of the factors affecting fungal development and virulence will provide the basis for overcoming these devastating organisms and in general provide a better understanding of fungal development.

Mitosis plays a crucial role in regulating these morphological transitions in the *M. oryzae* infection cycle. There is some information available about nuclear and microtubule organisation during mitosis during morphological switches in *M. oryzae* which can be used as a starting point for the study. Mitosis has been studied using histone H1-mCherry and β-tubulin-GFP tagged strain. Tagging of nuclear and cytoskeletal proteins with fluorescent protein labels has proved to be an integral tool in studying nuclear dynamics. The first round of mitosis is consistently

observed in the germ tube during appressorium formation. Interestingly, this nuclear division precedes and is spatially uncoupled from cell division which takes place at the base of the developing appressorium after a long-distance nuclear migration of the daughter nucleus into the appressorium (Saunders, Dagdas et al. 2010). Initiation of appressorium formation takes place when the germ tube ceases to elongate further and begins to swell at the tip, in a switch from polarised growth to isotropic development. This stage is often referred to as hooking and is characterised by the formation of the incipient appressorium. As later demonstrated using temperature sensitive alleles of Nim1, a regulatory subunit of Dbf4 kinase required for replication, entry of the germinating cell nucleus into S-phase is required for swelling of the germ tube. Further, it was shown using *nimA* mutants that entry into mitosis was necessary for development of appressoria. The diploid nucleus of the germinating cell migrates to the germ tube within 4-6 hrs and then undergoes mitosis. One daughter nucleus migrates into the newly developed incipient appressorium while the other returns back to the conidium. The role of cell cycle regulation in infection-related development was also observed using stage-specific cell cycle blockers, hydroxyurea (HU) and benomyl, which inhibit the G1/S and G2/M phases respectively. Exit from mitosis, as shown using mutants of *bim1*, analogous to the large subunit of the Anaphase Promoting Complex (APC), is required for appressorium maturation and function (Saunders, Aves et al. 2010). Nuclear return to the respective cells and simultaneous autophagic nuclear degeneration in the conidium cells is required to form a functional appressorium and for host invasion (Veneault-Fourrey, Barooah et al. 2006). Nuclear degeneration occurs via non-selective macro-autophagy and is characterised by the presence of a diffused nuclear signal in the cytoplasm and vacuoles. Thus, appressorium development is tightly coupled to the cell cycle at multiple stages.

The next round of mitosis is observed in the mature appressorium which contributes a nucleus to the invasive hyphae (IH) during penetration of leaf tissue. The invasive hyphae undergo periodic nuclear division with each round of mitosis lasting roughly 3 minutes (Jones, Jenkinson *et al.*, 2016). During host invasion, *M. oryzae* undergoes semi-closed type of mitosis where the nuclear membrane opens up partially with a transient closing of the septal pores. The appressorium retains mitotic potential even after several rounds of division in the hyphae and can contribute a second nucleus if the first IH fails to elaborate. During host invasion, the nucleus displays great plasticity and is capable of extreme constriction in crossing cell-to cell barriers (Jenkinson, Jones *et al.*, 2017).

Mitosis involves the equal distribution of the duplicated eukaryotic genome to the two daughter cells. In mitosis, anaphase is characterised by the precise segregation of chromosomes to the respective spindle poles. Anomalies in chromosome division have been previously associated with malfunction and disease even in multi-cellular organisms. In unicellular yeasts, they can often prove critical and lead to loss of viability. To avoid mis-segregation, mitosis is tightly regulated by the spindle assembly checkpoint, through a series of kinases and phosphatases that prevent chromosome segregation in case of improper kinetochore-spindle attachments. The kinetochore is a multi-protein complex that drives high fidelity chromosome segregation during mitosis. The multi-storey kinetochore structure is organised into the inner chromatin-associated zone, followed by the middle scaffold and the outer microtubule (MT) or spindle associated layer. The inner kinetochore is fairly conserved across eukaryotes and is marked by the presence of a specialised centromeric nucleosome composed of a histone H3 variant referred to as Cse4/CenpA. CenpC is in direct association with CenpA. Both these proteins are associated with the centromere throughout the cell cycle and across all systems studied so far, including yeast,

worms, flies, plants and mammals and hence have become markers for centromeric regions in systems that lack any centromere-specific DNA sequences. The middle kinetochore is made up of multi-subunit complexes, each made up of several proteins. Central among these is the MIND complex, made up of Mis12/Mtw1, Mis13/Dsn1, Nnf1 and Nsl1. The outer kinetochore NDC80 complex of 4 proteins - Ndc80, Nuf2, Spc24 and Spc25 - directly interacts with the microtubules. Both these complexes interact with the microtubule-interacting DASH/DAM/DDD Complex that has so far only been identified in fungi. The DASH complex is made up of 10 subunits, namely, Ask1, Dad1-4, Dam1, Duo1, Hsk3, Spc19 and Spc34. Though a good deal is known about mitosis in *M. oryzae*, as far as kinetochore proteins are concerned, little work has been done. One kinetochore component that has been studied is Duo1. Though not essential for viability, it is required for conidiophore and conidial morphology and full virulence in rice (Peng, Feng *et al.*, 2011). However, the focus of this study was towards pathogenicity and not its role in kinetochore function.

The DASH complex has so far been studied in the yeasts *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans* and *Cryptococcus neoformans* with regard to its composition, role, localisation and structure. It is a complex specific to fungi, though it may have functional similarities to the Ska Complex found in humans. Studies on the DASH complex have largely centred on Dam1, and the complex is often also referred to as the DAM complex. In *S. cerevisiae* Dam1 (*Duo1 and Mps1 interacting*) is the largest member. It is involved in association with α- & β-tubulin of MTs, other DASH complex members and also between complex monomers to form DASH complex oligomers (Legal, Zou et al. 2016). In *S. cerevisiae*, Dam1 is required for proper spindle shape and orientation (Hofmann, Cheeseman et al. 1998). In *S. cerevisiae* dam1 temperature sensitive mutants, Dam1 mislocalises from the kinetochore spindle at non-permissive temperature and the mutants show spindle defects with aberrant shapes, size and orientation, suggesting a role for Dam1 in both spindle assembly as well as elongation during anaphase (Cheeseman, Brew et al. 2001). Ask1 seems to be required for proper bipolar attachment as in *ask1* mutants, chromatids separate but remain in the same cell due to failure to segregate (Janke, Ortiz et al. 2002).

In *S. pombe DAM* mutants display chromosome segregation delay and ectopic septation (Sanchez-Perez, Renwick et al. 2005). In addition Dam1 is required for resistance to the microtubule poison thiabendazole and environmental stresses like low temperature and osmolarity, phenotypes that are also associated with other DASH complex members. These phenotypes are also shared by genes affecting MT stability and dynamics. *DAM1* was also discovered as a multicopy suppressor of mutations in genes like *CDC13*, which regulates spindles, and *MAL3*/*EB1*, which encodes a protein that binds to plus ends of MT. *ASK1* also, plays a role in cell shape and polarity: *ask1*Δ mutants cells are often Y/T shaped compared to the normal oval shape of *S. pombe*. The DASH complex also plays a role in kinetochore clustering and biorientation. In addition to maintenance of intact spindle, they also regulate MT dynamics by altering the rate of polymerisation and depolymerisation. In *S. pombe*, this is not restricted to spindle MTs, but is also seen with cytoplasmic MTs during interphase (Gao, Courtheoux et al. 2010). Loss of DASH members affects the localisation of other members of the complex. The similarity in phenotypes of different members of the DASH complex and interdependent localisation further strengthens their role in a common function as a complex. Dam1 also acts as a signal for proper kinetochore-microtubule attachment on which the spindle assembly checkpoint intersects through kinases like Mps1, Plo1 and Ipl1/Aurora kinase B (Buttrick, Lancaster et al. 2012). In case of incorrect kinetochore-spindle attachment, phosphorylation by

these kinases reduces the affinity of the Dam complex to microtubules, leading to dissociation and allowing for new correct bipolar attachment (Courtwright and He 2002).

Localisation of DASH complex members has been studied in all 3 yeasts using GFP fusion proteins. They often appear as a distinct spot in the nucleus that co-localises with other kinetochore proteins like Mtw1 and Ndc80 (Sanchez-Perez, Renwick et al. 2005). Dam1 is known to localise to the kinetochore during mitosis in *S. pombe*, while Dad1, another DASH member, localises to the kinetochore throughout the cell cycle and its localisation is dependent on Mis6. Dad1 appears to have some additional role at the kinetochore not shared by other members of the complex. In *S. pombe*, Dam1 seems to be recruited to the centromere in early mitosis, where it is observed as a single spot/dot/puncta that segregates into several spots at metaphase. In addition to these stable spots some transient spots are also seen, which the authors suggest may be microtubule associated dynamic Dam1. The monomers have been shown to bind and move along the microtubules and occasionally merge into larger oligomers, or cross over to neighbouring microtubule tracks. The complex shows great affinity for microtubules and is known to be associated with the (+) ends of polymerising as well as depolymerising microtubules, and are categorised as a Microtubule (+) end tracking proteins (TIPs). The *S. pombe* microtubules show intensity most likely derived from oligomers of 2-5 monomers, referred to as patches (Gao, Courtheoux et al. 2010).

In *S. cerevisiae*, Dam1 is present during all stages of the cell cycle. It is initially present as a single spot that divides into two spots closely associated with the spindle pole body. It is also observed all along the spindle. Kinetochore association of Ask1 is dependent on Ndc10 and microtubule spindle. Further, DASH complex functions as oligomers. *in vitro* analysis, using *S. cerevisiae* proteins, has shown 16-25 member oligomeric rings that encircle the microtubule. However, the rings are not essential for microtubule attachment and evidence for formation of such rings *in vivo* is lacking. Several members of the complex show a coiled-coil domain, a domain that is frequently present in proteins that form oligomers and therefore, common among cytoskeletal proteins.

Though there are several similarities in how Dam1 functions in the different yeasts, there are key differences in the role and localisation. All members of the DASH complex including Dam1 are essential for viability in *S. cerevisiae* and *C. albicans*, while this is not the case in *S. pombe*, where none of the members are essential for survival. Earlier it was believed that whether Dam1 is essential or not depends on the type of centromere, with Dam1 being essential for 'point' centromeres and not essential for 'regional' centromeres. Centromeric DNA shows high sequence variation across species and even among chromosomes of a given species in some cases. While the yeasts have shown a sequence-dependent determination of chromosomal sites, the higher eukaryotes suggest no specific centromeric DNA and the centromeres rather seem to be defined by epigenetic marks and chromatin status. The short *S. cerevisae* DNA-dependent centromeres that are upto 400bp are defined as 'point' centromeres while the centromeres of *S. pombe* and higher eukaryotes, which often encompass large stretches of DNA that may extend to several 1000kbs, are termed 'regional' centromeres. But with information from *C. albicans* where Dam1 is essential in spite of having 'regional' centromeres, the hypothesis is that the requirement of the DASH Complex depends on the number of MTs attached to the KT. The point centromeres of *S. cerevisiae* show one MT compared to the 2-4 MTs observed at the regional centromeres of *S. pombe* and therefore DASH is essential in the first (Burrack, Applen et al. 2011, Thakur and Sanyal 2011). Whether this is true can only be answered when studies are extended to other fungi. Though DASH has been extensively studied in yeasts, very little has

been done in this regard in filamentous fungi. ‘Is DAM1 essential in filamentous fungi?’ is still an open question. A recent review hints that in *Neurospora crassa,* a filamentous fungus, not all DASH complex members may be essential (Freitag 2016) suggesting an outer kinetochore system in filamentous fungi, different from what has already been observed in the various yeasts. Within eukaryotes, the kinetochore can vary in structure and composition comprising of 50-80 proteins (Van Hooff et al. 2017). In *S. cerevisiae* the DASH complex is roughly a 200 kDa complex while in fission yeast it is roughly around 150 kDa. There is great variation in sequence and size of these proteins across different fungal species. A better understanding of the functional nature of Dam1 in filamentous fungi can be obtained by studying its role in specific key processes like poleward movement during anaphase, nuclear transport and microtubule organisation. Dam1 localisation patterns during the cell cycle with respect to chromosomes and other KT proteins and whether Dam1 localisation is only associated with mitotic spindle MTs or also with other interphase and cMTs will shed further light on its role in fungal development. Co-localisation with other DASH complex members as well as also addressing whether Dam1 localisation is dependent on other subunits will provide information about DASH complex interactions. Though the *M. oryzae* genome sequence is available, the centromeres still remain unknown. As M. oryzae most likely has sequence independent centromeres like *S. pombe*, once kinetochore protein function is established, these proteins can be used to identify centromere regions. Further, yeasts show key differences in kinetochore clustering across the cell cycle and also in assembly of sub-complexes. In *C. neoformans*, hierarchical kinetochore assembly of inner followed by middle and subsequently outer kinetochore is observed, and perturbing the inner kinetochore leads to mislocalisation of the outer proteins (Kozubowski, Yadav et al. 2013). Interestingly, in *C. albicans* perturbing the outer KT proteins also destabilises the inner layer (Roy, Burrack et al. 2011). Co-localisation of proteins of different layers of the kinetochore in filamentous fungi will provide information about the interaction and assembly of different layers. Filamentous fungi are an extremely diverse group in terms of their life cycle and developmental stages. It will be interesting to know the role of kinetochore proteins in the different developmental stages of filamentous fungi. Going by the diversity observed within the several model yeasts, there is a possibility of diverse kinetochore structures within the highly specialised and diversified filamentous fungi, especially pathogens that carry large amounts of repetitive DNA sequences, and where variants with the presence of additional chromosomes are often observed in the wild. *M. oryzae* will be an excellent system to investigate centromere biology and fill this gap.

We decided to address some of the questions raised earlier, about kinetochore proteins and their roles, in the filamentous fungus *Magnaporthe oryzae*. Based on protein sequence homology, we were able to identify all 10 members of the DASH complex in *M. oryzae*. All members of the complex, when compared with other fungi show great variation in size and sequence. In terms of sequence the filamentous fungi seem to cluster together, and in size lie somewhere in between the great diversity observed in yeasts. Ask1 is the largest member in *M. oryzae* as opposed to Dam1 which is largest in *S. cerevisiae*.

In this study, we addressed the role of kinetochore proteins in *M. oryzae* development and virulence. We studied *MIS12* from the middle kinetochore and *DAM1* and *ASK1* from the outer kinetochore. We further looked at the localisation pattern of the proteins during different developmental stages of the *M. oryzae* infection cycle, and specifically during nuclear division. In order to do so, multiple tagging constructs and tagged fungal strains were developed as tools.

Further, we generated a mutant of *DAM1* and studied its effects on fungal development, pathogenicity and nuclear division.
Phenotypic characterisation of mutants and localisation studies using tagged strains are key tools in studying gene function. While gene knock-out generally involves replacement of the gene of interest by a selectable marker, gene tagging involves the addition of a tag at the locus of interest. Both these processes require targeted gene integration. Gene (DNA) integration requires the function of the inherent DNA repair mechanisms - non-homologous end joining (NHEJ) or homologous recombination (HR). While NHEJ is a non-specific, sequence independent repair system that ligates the ends produced by double stranded breaks, HR involves recombination with a homologous template sequence. Like most other filamentous fungi, the predominant repair mechanism in *M. oryzae* is NHEJ and as a result the average frequency of targeted gene integration is around 7%. This low efficiency of gene targeting means screening a large number of transformants to identify true integrants, making the process laborious.

Ku80 and Ku70 are DNA binding proteins that form a heterodimer that recognises the double stranded breaks and recruits the NHEJ machinery. Inactivation of Ku80/Ku70 has been shown to increase the efficiency of site specific integration without compromising the wild type phenotype in *Arabidopsis thaliana* and *Aspergillus nidulans*. A study in *M. oryzae* strains Guy11 and P1.2 where KU80 (MGG_10157) was knocked-out, also reported an increase in targeted integration upto 80% without any defects in pathogenecity (Villalba, Collemare *et al.*, 2008). Since the present work was carried out in the Indian *M. oryzae* strain B157, it was essential that a NHEJ deficient version of this strain be developed as a convenient tool for subsequent work.

Objectives

A: Development of a strain for improved gene targeting in *M. oryzae*
1. *Ku80* deletion in *M. oryzae* strain B157
2. Phenotypic characterisation of the Ku80 deletion strain

B: Characterisation of kinetochore genes in *M. oryzae*
1. Study of localisation of kinetochore proteins
2. Generation of mutants of selected kinetochore genes
3. Phenotypic characterisation of mutants

Results

A: Development of a strain for improved gene targeting in *M. oryzae*

1. *Ku80* deletion in *M. oryzae* strain B157
The upstream and downstream 1-1.5kb flanking regions of *KU80* ORF were amplified from *M. oryzae* genomic DNA by PCR and cloned on either side of the Zeocin resistance cassette in pBS-KS$^+$ vector backbone. The KS-Moku80Δ::Zeo construct thus generated was transferred into *M. oryzae* strain B157 by protoplast transformation. The transformants were screened for targeted integration by PCR and confirmed by Southern Hybridisation. The confirmed *KU80* deletion strain, *ku80*Δ, was then used for further phenotypic characterisation.

2. Phenotypic characterisation of the Ku80 deletion strain, *ku80*Δ

The hyphal growth of the *M. oryzae* wild type B157 strain (WT) and *ku80*Δ was compared on OMA and they showed comparable colony size. Conidiation of *ku80*Δ was normal both in terms of morphology and number. Appressorium formation was assessed on *in-vitro* hydrophobic surface and morphology of spores, germ tube and appressorium was found similar to WT. Pathogenicity of WT and *ku80*Δ was determined by carrying out infection assay on 15 – 21 day old rice seedlings and both strains produced typical disease lesions 5 days post inoculation (dpi).

The *ku80*Δ strain was analysed with respect to a number of pathogenicity related traits like hyphal growth, pigmentation, conidiation, appressorium formation and rice infection and was found similar to WT. Thus, the *ku80*Δ strain can be used as a background strain for subsequent studies to aid targeted integration by homologous recombination and reduce the number of transformants to be screened in *M. oryzae* B157 strain.

This strain was used to generate knock-outs of the Mg transporter *ALR2*, as part of a separate study in the lab, after multiple attempts to generate the mutant in the WT strain had failed. The gene is most likely essential for viability and we were also not able to generate mutants in the *ku80*Δ strain. (Reza *et al*., 2016)

B: Characterisation of kinetochore genes in *M. oryzae*

1. Study of localisation of kinetochore proteins

Generation of background strains for analysis of mitosis:

Strains were developed to study nuclear and microtubule organisation throughout cell cycle and particularly during mitosis in *M. oryzae*.

To mark the *M. oryzae* nucleus, histone H1-mCherry tagged B157 strain was generated using the Sulfonylurea Resistance Reconstitution (SRR) strategy by *Agrobacterium tumefaciens* mediated transformation (ATMT). The tagged strain was confirmed by PCR, fluorescence microscopy and Southern hybridisation. Similarly, a separate β-tubulin-sGFP tagged B157 strain was also generated by protoplast transformation to observe the microtubule network. As kinetochore proteins sit at the chromosome–microtubule interface, these single-tagged strains were later used to develop dual-tagged strains in combination with different kinetochore proteins. The hH1-mCherry tagged B157 was also used to develop the hH1-mCherry, β-tubulin-sGFP double tagged strain. The hygromycin resistant transformants, RGB3, RGB12 and RGB14, were confirmed by PCR, fluorescence microscopy and Southern hybridisation. The double tagged RGB3, RGB12 and RGB14 strains were analysed for growth, sporulation, spore germination and appressorium formation. RGB12 showed comparable phenotype with WT and was selected for further study.

The process of appressorium formation is critical to pathogenic development and involves one round of nuclear division. This process was observed by time-lapse laser-scanning confocal/ fluorescence microscopy using the above RGB12 strain to obtain a WT reference for the subsequent analysis of kinetochore mutants. hH1-mCherry was observed as a single round structure indicative of the nucleus. Each of the three cells of the conidium showed one round nucleus. Nuclear division during appressorium formation is preceded by S-phase indicated by the larger size of the nucleus in the germinating cell as compared to the remaining two nuclei of the conidium. β-tubulin-sGFP was observed in the form of a network of filaments throughout the cells of the conidium with more intense network at the cell periphery. Upon conidium germination and during appressorium formation, the microtubules appeared to be concentrated in

the germ tube and reduced in the conidium. Spindle formation is characterised by concentration of the microtubules at the mitotic site and weakening of the β-tubulin-sGFP signal in the rest of the cell. Mitosis is generally preceded by nuclear migration into the germ tube. This is followed by nuclear segregation in the germ tube, and migration of one daughter nucleus to the newly formed appressorium and the other to the original germinating cell of the conidium. Thus, in *M. oryzae* strain B157, mitosis is similar to other previously studied strains. Nuclear migration and mitosis is an extremely fast process that occurs within minutes and regulates the formation of functional mature appressoria. Any negative effect on proper completion of mitosis due to defects in kinetochore function would delay or stall the process of appressorial development.

Generation of kinetochore tagged strains:

In order to study localisation of kinetochore proteins, gene tagging constructs were developed for *DAM1*, *ASK1* and *MIS12* to replace the native copy of the gene with a GFP/RFP-tagged one by in-locus integration. While Dam1 was tagged on the N-terminus, Ask1 and Mis12 were tagged on the C-terminus. Dam1 and Ask1 constructs were based on the marker fusion tagging strategy. In order to study localisation of the proteins with respect to nucleus, these proteins were tagged in the hH1-mCherry strain developed earlier to give hH1-mCherry;GFP-Dam1, hH1-mCherry;Ask1-GFP and hH1-mCherry;Mis12-GFP strains. These strains were generated by ATMT, and screened by PCR. Correct integration was confirmed by Southern Hybridisation. Further efforts are under way to tag kinetochore proteins in the β-tubulin-sGFP tagged strain to study their localisation with reference to the microtubule network as well as with other kinetochore proteins study interaction between different kinetochore layers.

Microscopic analysis of tagged strains:

Time-lapse laser-scanning confocal/fluorescence microscopy was used to study the above strains at different stages of fungal development. Importantly, during appressorium formation multiple GFP-Dam1 spots localise to the nucleus at the onset of mitosis. With mitotic progression, these spots became more intense and condensed into one large spot per nucleus during segregation and nuclear migration. Additional intense spots were also seen in the appressorium during segregation. GFP-Dam1 was also observed as multiple dynamic spots or streaks at the growing tips of vegetative hyphae and germ tubes. Some of the spots occasionally also travel away from the growing tips. Further it was observed to be associated with nuclei in vegetative hyphae. Not all nuclei and growing tips show Dam1 spots. Further, Dam1 spots also vary in size. Similar localisation patterns were also observed for the other DASH complex member Ask1-GFP. Mis12-GFP was observed as a single spot per hH1-mCherry tagged nucleus, indicating that *M. oryzae* kinetochores are clustered during interphase. Mis12 was associated with the nucleus even in conidia, which is not the case for Dam1 and Ask1. Mis12 seems to be associated with the nucleus even during interphase while Dam1 and Ask1 show a more dynamic localisation patterns and associate with the nucleus during specifically during mitosis.

2. Generation of mutants of selected kinetochore genes

The kinetochore is a multiprotein complex divided into the inner, middle and outer kinetochore. The inner kinetochore associates directly with the DNA, the middle kinetochore is made up of sub-complexes that act as a scaffold for assembly of the outer kinetochore complex that associates with the microtubules. Representative genes, *CENPC*/*MIF2* from the inner, *MIS12*/*MTW1* from the middle and *DAM1* and *ASK1* from the outer kinetochore complex were

selected for study. Kinetochore proteins play a role in chromosome segregation during cell division and their absence can often have catastrophic effects on cell survival. The core kinetochore proteins are essential in many systems studied so far. Functional analysis of these proteins has been previously done through the use of temperature sensitive mutants and gene knock-down.

Gene knock-down approach by RNA interference was used to study the role of *CENPC*, *MIS12* and *DAM1* in *M. oryzae*. *DAM1* is a member of the DASH complex and has been shown to have different roles in different fungal species. While it is essential in *S. cerevisiae*, it is not required for viability in *S. pombe.* It is not known whether Dam1 is essential in *M. oryzae*, therefore, generation of knock-out transformants was also attempted.

Generation of knock-down transformants for *CENPC, MIS12 and DAM1*

Knock-down constructs were generated in pSilentDual-1 (pSD1) where a fragment of the gene of interest was cloned in between two promoters (TrpC and gpdA) placed in opposite orientation that allow the expression of both the strands and hence provide a system for generation of double- stranded RNA. Recombinant plasmids were confirmed by PCR and restriction digestion. The pSD1-CenpC, pSD1-Mis12 and pSD1-Dam1 plasmids were moved into *M. oryzae* RGB12 strain (hH1:mCherry, β-tubulin:sGFP) by protoplast transformation. Transformants were selected for Geneticin resistance. However, most of the transformants were unstable with sectoring and cultural heterogeneity and difficult to revive from stocks. As a result these transformants were not used for further study. Next we focused on generation of the *DAM1* knock-out mutant.

Generation of *DAM1* knock-out mutant

For this, a *DAM1* knock-out cassette was generated. Up-stream and down-stream flanking sequences of *DAM1* were amplified from genomic DNA by PCR and fused with the Zeocin resistance cassette by double-joint PCR. The fusion product was cloned into pBSKS^{+} and ATMT based vector. This construct was transformed into WT strain B157 as well as the nuclear-microtubule dual tagged strain RGB12 by ATMT/ protoplast transformation. Zeocin resistant transformants were selected and screened by PCR. A *DAM1* deletion (*dam1*Δ) strain with single integration was confirmed by Southern hybridisation. The *dam1*Δ mutant in RGB12 was used to study the effect of loss of *DAM1* on nuclear and spindle organization, mitosis and nuclear migration. To ensure that the phenotypes being studied were only a result of *dam1*Δ, and not due to any complication from hH1-mCherry, β-tubulin-sGFP tagging, fungal growth, development and virulence were also studied in the *dam1*Δ mutant in the B157 background.

3. Phenotypic characterisation of mutants

Characterisation of development and pathogenicity

Vegetative Growth:

WT and *dam1*Δ cultures were inoculated on YBE/Prune agar and colony diameter was measured 8-10 dpi. *dam1*Δ vegetative hyphal growth was reduced to ~60% of WT. Hyphal growth was also reduced in liquid culture to ~33% dry weight of WT biomass. *dam1*Δ also produced flat colonies that lacked the fluffy aerial hyphae observed in the WT and showed reduced melanisation. When stained with Calcofluor White (CFW) to view cell walls and septa, the vegetative hyphae of *dam1*Δ showed aberrant morphology with a different branching pattern.

*dam1*Δ showed crooked hyphae of variable diameter rather than the fairly straight uniform hyphae produced by the WT. It is likely that Dam1 is required for defining hyphal cell polarity and this is in accordance with the GFP-Dam1 signal observed at the growing tips.

Asexual differentiation - Conidiation:

Conidiation, or formation of three celled asexual spores, is an important stage of the infection cycle and is critical to both initiation of infection and spread. Conidiation was monitored in WT and *dam1Δ* from day 8 post inoculation to day 14. First of all, *dam1*Δ displays a considerable decrease in conidium count to ~10% compared to WT. Further, the spores lack the typical drop shaped three celled structure seen by CFW staining in the WT. Most of the *dam1*Δ spores are 1- or 2-celled and oval-shaped, with only ~10-15% 3-celled conidia. Along with reduced cell number, the *dam1*Δ conidia showed decrease in average length and sometimes also displayed incomplete septa. Conidium viability is also affected. Further, while above 80% of WT conidiophores show more than 2 sympodial conidia, most of the *dam1*Δ conidiophores bear only 1-2 conidia, with only 6% conidiophores showing more than 2 conidia.

Pathogenic Development - Appressorium formation:

Appressorium formation from conidia was studied on hydrophobic surface *in vitro*. While above 80% WT conidia formed appressoria, only ~20% of the *dam1*Δ conidia were able to do so. Around half of the *dam1*Δ conidia failed to germinate. Some of the conidia formed irregular germ tubes. In some cases, the appressoria were stuck at the hooking stage while some formed normal appressoria. Further, many conidia showed more than one branched germ tubes, indicating shift to vegetative growth rather than appressorial development.

Infection:

Barley spot inoculation and rice sheath assay were carried out to study host penetration and host invasion. In the rice sheath assay, only ~20% *dam1*Δ mutant appressoria showed normal host penetration and invasion compared to ~85% WT appressoria. Most of the conidia failed to form normal functional appressoria even on the host surface, either failing to show penetration or displaying restricted invasive hyphae. The *dam1*Δ mutant also showed a higher frequency of aberrant appressoria that appeared either heart- or hand-shaped, unlike the normal dome-shaped appressoria of the WT. Many conidia diverted to vegetative growth instead of forming appressoria, similar to the situation seen on *in vitro* hydrophobic surface.

Characterisation of Nuclear division:

Mitosis was studied in the *dam1*Δ mutant during appressorium formation. Some conidia show mitosis similar to WT, where mitosis occurs within 3-6 hpi and is completed within a few minutes. However, several conidia were delayed in the onset of mitosis. Mitosis was also prolonged, extending over an hour in some cases, with slow nuclear migration characterised by the nucleus being stretched across the germ tube and finally ending in improper segregation. Thus, Dam1 seems to play a critical role in the pole-ward segregation of nuclei during anaphase and in nuclear migration. This correlates well with the Dam1 localisation in association with the segregating nuclei in the germ tube as well as in the appressoria during mitosis.

Nuclear distribution was also affected in the *dam1*Δ mutant. Many conidia showed cells with disorganised chromatin unlike the proper round nucleus observed in the WT, but more as an irregular mass. Sometimes the conidia also appeared to have undergone degeneration with hH1-

mCherry signal distributed all over the cytoplasm. Conidia with more than one nucleus per cell were also observed.

Conclusions: Dam1 is required for fungal development. DAM1 is not essential for viability in *M. oryzae*. Dam1 associates with both mitotic as well as interphase microtubules. Dam1 localises to the kinetochore at the onset of mitosis while Mis12 is present throughout the cell cycle.

Publications:

Reza MH, **Shah H**, Manjrekar J, and Chattoo BB. (2016) Magnesium Uptake by CorA Transporters is Essential for Growth, Development and Infection in the Rice Blast Fungus *Magnaporthe oryzae*. *PLoS ONE*, 11(7): e0159244.

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