Chapter 2:

PRESENT STUDY- Characterisation of genes involved in the development and virulence of pathogenic fungi

Introduction, Objectives and Materials and Methods

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2.1. INTRODUCTION

Fungi form a extremely diverse group of eukaryotic organisms, including unicellular yeasts, dimorphic yeasts that can undergo hyphal transition and multi-cellular filamentous fungi. Fungi are suited to lifestyles in a wide range of 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 substratum attachment are all important for dispersal and virulence in filamentous fungi. The rice blast fungus *Magnaporthe oryzae* is one such filamentous fungus which passes through multiple developmental stages during its infection cycle. Pathogenic development starts when the conidium germinates on the host surface and extends a polarised germ tube that swells at the tip to form the dome-shaped appressorium. The appressorium then extends a penetration peg which ruptures the leaf surface to enter the host and establish infection. These developmental transitions are regulated by the cell cycle. Though a good deal is known about mitosis in *M. oryzae*, as far as kinetochore proteins and the process of chromosome segregation are concerned, little work has been done. *M. oryzae* infects rice and several other cereal crops in India and across the world, negatively impacting global food security. The millions of hectares

of crops lost every year to rice blast necessitate a better understanding of the disease. A detailed investigation of the role of fungus-specific components in development and virulence will suggest ways for overcoming this devastating pathogen without adversely affecting the crop. The microtubule (MT)-associated Dam1/DASH complex proteins are fungus-specific outer kinetochore components whose role has remained relatively unknown in filamentous fungi. Further components of the Dam1 complex are prospective antifungal targets due to their absence from plant genomes. MoDuo1 is the only gene investigated hence far within the group of filamentous fungi, albeit with very little scrutiny of its function at the kinetochore during mitosis or subcellular dynamics through the cell cycle.

The DAM/DASH complex is important for spindle structure and dynamics in yeasts. However, features of the DASH complex vary in *S. cerevisiae*, *S. pombe* and *C. albicans* in sub-cellular localisation, cell-cycle dependent nuclear association, oligomeric states and requirement for survival. As discussed earlier, it has been suggested that the requirement of the DASH complex depends on the number of MTs bound to each kinetochore (Burrack *et al.*, 2011, Thakur and Sanyal, 2011). Whether this is true can only be answered when studies are extended to other fungi beyond the few model yeasts. Therefore, 'Is *DAM1* essential in filamentous fungi?' is still an open question in fungal biology. In the filamentous fungus *N. crassa*, not all DASH complex members may be essential (Freitag, 2016) suggesting an outer KT system in filamentous fungi, different from what has been observed in the various yeasts. A better understanding of the function of Dam1 in filamentous fungi can be obtained by studying its role in poleward movement during anaphase, nuclear transport and microtubule organisation. Understanding Dam1 localisation patterns during the cell cycle with respect to chromosomes and other KT proteins will provide information about kinetochore assembly in fungi. Yeasts

show key differences in KT clustering through the cell cycle as well as in assembly of sub-complexes. In *C. neoformans,* there is hierarchical KT assembly of inner followed by middle and subsequently outer layer, and perturbing the inner KT leads to mis-localisation of the outer proteins (Kozubowski *et al.*, 2013). Interestingly, in *C. albicans* perturbing the outer KT proteins also destabilises the inner layer (Roy *et al.*, 2011). Localisation studies of proteins of different layers of the KT in filamentous fungi will provide information about the interaction and assembly of different layers. Co-localisation studies with other DASH complex members will address whether Dam1 localisation is dependent on other subunits, provide information about DASH complex interactions and functions unique to certain proteins. Further, the localisation with MTs or MT perturbation studies will provide information about the dependence of DASH complex function on MT dynamics. Though early studies have termed the DASH proteins as a kinetochore complex, recent studies are beginning to show a broader spectrum of microtubule association, opening up the possibility that, more than just a kinetochore complex, the DASH complex proteins are likely regulators of microtubule dynamics, while also playing a role at the kinetochore. However, the studies are few and will require more support from studies in diverse organisms. Whether Dam1 localisation is restricted to the mitotic spindle MTs, or extends to the interphase and cytoplasmic MTs, will shed further light on its role in filamentous fungal development. I addressed some of these questions about KT proteins and their roles, in the filamentous fungus *M. oryzae* in the present study.

Here, I particularly investigated the function of components of the DASH complex in chromosome segregation during the developmental switches in *M. oryzae.* Based on protein sequence homology, I was able to identify all 10 members of the DASH complex in *M. oryzae*. Using gene deletion and GFP-tagged strains, I investigated the role of DASH complex proteins in mitosis, particularly spindle structure and dynamics, nuclear migration and

its effect on the development of *M. oryzae*. The role of middle KT protein Mis12 and outer KT proteins Dam1 and Ask1 was addressed in *M. oryzae* development and virulence. I looked at the localisation pattern of the proteins during different developmental stages of the filamentous fungus *M. oryzae*, specifically during mitosis. In order to do so, multiple tagging constructs and tagged fungal strains were developed. Further, I generated mutants of *DAM1* and *ASK1* and studied their effects on fungal development, pathogenicity and nuclear division. I suggest a role for Dam1 in KT-MT attachment, proper poleward movement of chromosomes during anaphase and in nuclear transport. I show that although Dam1 and Ask1 are not essential for viability, they are involved in proper conidiation and polarised hyphal growth and pathogenic development. This work shows that beyond its role in proper segregation of chromosomes during mitosis, DASH complex proteins Dam1 and Ask1 are involved in polarised growth likely through association and regulation of cytoplasmic MT dynamics at the hyphal tip.

2.2. OBJECTIVES

A. 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

2.3. MATERIALS AND METHODS

2.3.1. Fungal Strains and culture conditions

This study was carried out in a rice infection isolate of *Magnaporthe oryzae* from our laboratory (Kachroo et. al., 1994), namely, strain B157 (MTCC accession number 12236, international race IC9). The fungus was

maintained on prune agar (PA). Fungal biomass was cultured in complete medium broth (Dawe et al., 2003) for isolation of nucleic acids, etc. Colony diameter was used as readout of vegetative hyphal growth. Dry weight was determined for fungal biomass grown in liquid CM. For collection of conidia, fungal cultures were maintained on PA for 3 days in the dark and subjected to constant photo-induction till 9-10 days post inoculation when the conidia were collected.

Media composition (1L) for fungal culture

Prune Agar- 40 ml prune juice, 1 g yeast extract, 2.5 g lactose, 2.5 g sucrose, 20 g agar. pH was set to 6.5 with 10M NaOH

YEG Agar- 2 g yeast extract, 10 g dextrose, 20 g agar

Complete medium for *Magnaporthe oryzae*- 10g Dextrose, 5 g Peptone, 1 g Yeast extract,1 g CAA, 6 g $NaNO_3$, 0.5 g KCl, 0.5 g $MgSO_4$, 1.5 g KH_2PO_4, pH 6.5

Basal Medium- 1.7g Yeast Nitrogen Base without amino acids or ammonium sulphate, 2g asparagines, 1g NH_4NO_3, 10g Glucose. pH 6.0 was set with 1M Na_2HPO_4.

2.3.2. Bacterial strains and culture conditions

Escherichia coli (E. coli)

E. coli strain DH5α (F^-, *end*A1, *hsd*R17 (rk^-, mk^+), *sup*E44, *thi*-1, λ^-, *rec*A1, *gyr*A96, *rel*A1Δ (*lacZYA-arg*F) U169 *deo*R (φ80d*lac*Δ (lacz) M15; Bethesda Research Laboratories) was used for bacterial transformation and plasmid propagation. The *E. coli* strain was grown at 37^0C on Luria Bertani (LB) agar medium. *E. coli* transformants carrying plasmid vectors

were grown on LB agar containing either ampicillin or kanamycin at a concentration of 100 µg ml^{-1} or 50 µg ml^{-1}, respectively, as required. Long term preservation was done in 25% glycerol solution at -80^0C.

Agrobacterium tumefaciens (A. tumefaciens)

A. tumefaciens strain LBA4404/pSB1 was used for Agrobacterium tumefaciens mediated transformation (ATMT). The culture was grown at 28/30^0C on LB agar medium. Plasmids were transformed into *Agrobacterium* by triparental mating or electroporation. Transformants carrying plasmid vectors were grown on LB agar containing 50 µg ml^{-1} kanamycin. Long term preservation was done in 25% glycerol solution at -80^0C.

2.3.3. Bioinformatics analysis of DASH Complex proteins

Middle (Mis12) and outer (DASH complex) kinetochore proteins were identified from the *M. oryzae* 70-15 genome sequence, previously provided by the Broad Institute (Cambridge, USA) (http://www.broadinstitute.org/annotation/genome/magnaporthe_grisea/MultiHome.html), by a BLAST_P search using the full length *S. cerevisiae* and *S. pombe* protein sequence. Fungal Dam1 and Ask1 protein sequences were obtained from Uniprot database. The Multiple sequence alignment and phylogenetic analysis was done using COBALT and CLUSTAL OMEGA. The conserved domain prediction was done using Pfam tool. NCOILS was used to determine the coiled coil domains of DASH complex proteins. WoLFPSORT tool was used for predicting localisation of the proteins.

2.3.4. Nucleic acid manipulation

Genomic DNA extraction from *M. oryzae*

M. oryzae B157 was grown in 40 ml complete medium for 2.5 days, harvested on filter paper discs, dried and frozen in liquid nitrogen and

ground to a fine powder. The powdered biomass was transferred to 700 µl DNA extraction buffer (pre-heated at 68^0C) and mixed properly. Next, 70 µl of 20% SDS was added and the contents were mixed properly. The samples were then kept at 68^0C for 10 min. This was followed by addition of 350 µl of 5M potassium acetate and incubation on ice for 30 min. The cell debris was separated by centrifugation at 8,000 rpm for 20 min. The supernatant was transferred to a fresh centrifuge tube and 500 µl of ice cold 100% isopropyl alcohol was added. The solutions were mixed gently and the DNA was precipitated at -20^0C for 30 min. The DNA was collected by centrifugation (10000 rpm, 15 min). The DNA pellet was allowed to air dry at 37^0C and resuspended in 500 µl of TE buffer at 68^0C for 10 min. 20 µl of 1 mg ml^{-1} RNase was added to this solution and further incubated at 68^0C for 30 min. This was mixed with an equal volume of C:I (chloroform:Isoamyl alcohol - 24:1) and centrifuged at 10,000 rpm for 15 min. The upper aqueous phase was collected and to this was added $1/10^{th}$ volume of 3M sodium acetate pH 5.0 and 0.6 volume of ice cold 100% isopropanol and incubated at -20^0C for 1-2 hours. The DNA pellet was recovered by centrifugation and washed once with 70% ethanol. The pellet was then air dried at 37^0C and dissolved in autoclaved milliQ water. The DNA quality of the DNA prep was assessed by agarose gel electrophoresis and concentration was determined using nanodrop spectrophotometer.

DNA extraction buffer for *M. oryzae* (150 ml)- 15 ml 1M Tris HCl pH 8.0, 15 ml 0.5M EDTA pH 8.0, 18.75 ml 4M NaCl. After autoclaving 105 µl β-mercaptoethanol was added.

Isolation of plasmid DNA

Overnight grown culture (1.5 ml) was centrifuged in a microfuge tube and the bacterial pellet was resuspended in 300 µl of STET buffer. To this suspension, 10 µl of 50 mg ml^{-1} lysozyme was added, mixed by vortexing

and incubated for 2 min at room temperature (RT), followed by boiling in a water bath for 45 sec. This was followed by centrifugation at 13,000 rpm for 10 min. The resulting snot was removed with a tooth pick and 20 µl of 5% CTAB was added followed by centrifugation for 10 min. To the pellet, 300 µl of 1.2M NaCl was added and vortexed. To this 180 µl of 100% isopropanol was added. The DNA pellet was obtained by centrifugation for 10 min; washed with 70% ethanol and resuspended in 50 µl of TE.
STET Buffer (100 ml)- 8% Sucrose, 50mM Tris HCl pH 8, 50mM EDTA pH 8.0. After autoclaving 0.1% TritonX-100 was added.

Restriction Digestion

Restriction enzyme digestion of DNA was carried out in reaction buffers, supplied with the corresponding enzymes, as recommended by the manufacturer (New England Biolabs, USA). Ligations were carried out at 22^0C, as recommended (Thermo Fisher, USA).

Southern Hybridization

For Southern blot analysis, 30 µg of genomic DNA was digested with restriction enzymes and the digested products were run on 0.8% agarose gel at 3V/cm. After denaturation and neutralisation of the digested products in the gel, capillary blot transfer was done for 16-20 hours to transfer the DNA fragments onto a nylon membrane (Hybond N+, Amersham, UK). The transferred DNA on the membrane was crosslinked by UV crosslinker (Spectrolinker, XL-1000, USA) at preset optimum cross-linking programme. The labelling of the probes was done according to the manufacturer's instructions (Amersham, Buckinghamshire, UK). The probe was hybridised with DNA on the membrane for 16 hours. The membrane was washed and substrate was added for 5 min. X-ray film was exposed for 4-16 hours with the membrane in Hypercassette (Amersham,

Buckinghamshire, UK). X-ray film was developed and targeted integration in knockout and tagging was confirmed.

2.3.5. Bacterial and fungal transformation

Competent cell preparation and transformation of *E. coli*

A single colony of *E. coli* DH5α was inoculated in 3 ml of LB broth and grown overnight. 100 ml of fresh LB broth was inoculated with 1.0 ml of overnight grown culture and grown at 37^0C till 0.3 to 0.4 O.D $_{\lambda=600}$. The cells were collected by centrifugation at 5,000 rpm for 4 min at 4^0C and resuspended in 10 ml chilled 0.1M $CaCl_2$. The cell suspension was incubated on ice for 30 min. The cells were centrifuged at 4000 rpm for 5 min at 4^0C. The supernatant was discarded and the cells were re-suspended in 1.0 ml of ice cold 0.1M $CaCl_2$ + 20% glycerol. The culture was stored at -80^0C in 100 µl aliquots. For transformation, a 100 µl aliquot of competent cell suspension was mixed with the plasmid DNA (50-100 ng) or ligation mixture and incubated at 4^0C for 30 minutes. Cells were subjected to heat shock at 42^0C for 90 sec, followed by 5 min incubation on ice. 1 ml of LB broth was added to the above suspension and incubated at 37^0C with 200rpm shaking for 45 minutes. 100 µl of transformation mixture was spread on LB agar medium containing either Ampicillin (100 µg ml^{-1}) or kanamycin (50 µg ml^{-1}).

Agrobacterium tumefaciens mediated transformation (ATMT) (Mullins et al., 2001) or protoplast transformation was used for fungal transformation of tagging and gene deletion constructs. The transformants were selected on YEGA or basal medium supplemented with the appropriate antibiotic at concentrations listed below. The antibiotic resistant transformants were screened by locus specific PCR and/or microscopy. Single targeted integration was confirmed by Southern hybridisation. Virulence of transformants was assessed by barley leaf spot inoculation assay. The transformants were stored as filter stocks at -20^0C.

Protoplast transformation of *M. oryzae*

M. oryzae B157 was inoculated in 30 ml of complete medium broth and grown for 2.5 days. The biomass was then filtered with Miracloth (Calbiochem, Darmstadt, Germany), washed with sterile water and the mycelia were resuspended in 30 ml of 1M sorbitol containing 30 mg (1mg ml^{-1}) lysing enzyme (Sigma, St. Louis, USA). The culture was incubated at 28^0C at 100 rpm for 12-16 hours for protoplasting. Next day, the protoplasts were filtered through miracloth, washed and resuspended in 10 ml 1M sorbitol at 4,000 rpm for 5 min at 4^0C twice. The protoplasts were resuspended in 10 ml STC buffer, washed and resuspended again in 1 ml STC buffer. The protoplasts were counted using a haemacytometer (Marienfeld Superior, Lauda-Konigshofen, Germany) and a density of 10^8 cells per ml was maintained. For every transformation 200 µl of protoplasts was mixed with 2-10 µg of DNA and incubated on ice for 15 min. 1 ml of PTC buffer was added to the protoplast-DNA mixture and incubated at 28^0C for 30 min. The whole mixture was transferred to sugar tube containing 3 ml of YEGS and was incubated at 28^0C at 100 rpm for 12-14 hours. After incubation, 10 ml of molten regeneration medium was added, mixed well and poured on YEG agar containing appropriate antibiotics (Hygromycin/ Zeocin/ Geneticin). Appropriate selection antibiotic was also added to the molten medium. Appearance of colonies was monitored within 2-3 days of incubation at 28^0C. *M. oryzae* B157 protoplasts (not treated with DNA) were kept as a control which did not grow on selection medium. The colonies were transferred to selection medium, for secondary selection to eliminate any stray growth. The resistant transformants were then screened by PCR, microscopy and Southern hybridisation.

Media and Reagents for Protoplast transformation

STC Buffer (100 mL)- 18.2 g D-Sorbitol (1M), 5 ml of 1M Tris HCl pH 8.0 (50mM Tris HCl pH 8.0), 0.735 g $CaCl_2.2H_2O$ (50mM)

PTC Buffer (100 mL)- 40 g Polyethylene glycol 3350 (40% w/v), 18.2 g D-Sorbitol (1M), 5 ml of 1M Tris HCl pH 8.0 (50mM Tris HCl pH 8.0), 0.735 g $CaCl_2.2H_2O$ (50mM)

YEGS Solution (100mL)- 0.2g Yeast extract, 1g Dextrose, 18.2 g D-Sorbitol

Molten Regeneration Medium (100mL)- 0.2g Yeast extract, 1g Dextrose, 0.4 g agarose (0.4% w/v)

Antibiotics

Antibiotic	Stock Concentration (mg ml^{-1})	Working Concentration (µg ml^{-1})
Bacterial Selection		
Ampicillin	100	100
Kanamycin	100	50
Rifampicin	5	5
Tetracycline	10	10
Cefotaxime	250	250
Fungal Selection		
Hygromycin B	100	300
Zeocin	100	300
Chlorimuron Ethyl	100 in DMF	100
Glufosinate Ammonium	50	50

Agrobacterium tumefaciens* mediated transformation (ATMT) of *M. oryzae

The *Agrobacterium* strain LBA4404/pSB1 (Komari *et al.*, 1996) was first transformed with respective vectors via triparental mating (Helper plasmid pRK2013; Helper strain *E. coli* DH5α) or electroporation. For triparental mating, a single colony each from the freshly grown recipient *Agrobacterium* LBA4404/pSB1, helper plasmid pRK2013 and the donor strain carrying the respective vector were mixed together on YEP agar (0.2% yeast extract, 0.5% peptone, 2% agar) and incubated at 28^0C for 18-

20 hours. The mixture was scraped and resuspended in 0.9% NaCl. 100 µl of serial dilutions of this bacterial suspension was plated onto selection plates and incubated at 28^0C for 2-3 days till the appearance of colonies. The *Agrobacterium* harbouring T-DNA vector was then used to transform *M. oryzae*. *A. tumefaciens* mediated transformation (ATMT) was performed as described previously (Mullins *et al.*, 2001). Briefly, *M. oryzae* spores were collected from 9-10 days old PA plates and spore concentration was adjusted to $1x10^6$ spores/ml. *Agrobacterium* carrying the *HPT/BAR* gene expression cassette was grown at $28/30^0$C overnight in LB supplemented with appropriate antibiotics. The *A. tumefaciens* cells were diluted to OD_{600} = 0.15 in induction medium (IM) and grown for another 6 hours, both in the presence (IM+ACS) and absence (IM-ACS) of 200µM acetosyringone (ACS) before mixing them with an equal volume of a conidial suspension from *M. oryzae*. This mix (200 µl per plate) was spotted on a 0.45 µm pore size, 45 mm diameter nitrocellulose filter (Millipore, Bangalore, India) placed on co-cultivation medium in presence of 200 µM ACS. Following incubation at 28^0C for 2 days, the filter was transferred to YEG containing Hygromycin B (Invitrogen Life Technologies, CA) to a final concentration of 300 µg ml^{-1} (or any other appropriate antibiotic) as a selection agent for transformants and 250 µg ml^{-1} cefotaxime to eliminate the *A. tumefaciens* cells. Untransformed *M. oryzae* was kept as a control which did not grow on hygromycin containing medium. The transformants were maintained as mono-conidial isolates.

Media and Reagents for ATMT

Induction medium (IM) for *Agrobacterium*

10 ml K salts, 20 ml M salts , 2.5 ml 20% NH_4NO_3, 0.5 ml 1% $CaCl_2$, 10 mM Glucose, 40mM MES, 0.5% Glycerol (v/v), 10 ml 0.01% $FeSO_4$, 5 ml Z salts.

0.01 % $FeSO_4$ and Z-salts solution were prepared separately and autoclaved; they were added after autoclaving the medium, just before use. After autoclaving they were stored at 4°C.

1.8 g or 1.98 g glucose and 7.8 g of MES (2-(N-Morpholino) ethanesulfonic acid) were added to give a final concentration of 10 and 40 mM respectively. 200µM acetosyringone (ACS) was added just before pouring.

Composition for salt solutions for IM

K Salts Solution : K_2HPO_4 20.5%, KH_2PO_4 14.5%

M Salts Solution : $MgSO_4.7H_2O$ 3%, NaCl 1.5% , $(NH_4)_2SO_4$ 2.5%

Z Salts Solution : $ZnSO_4.7H_2O$ 0.01% , $CuSO_4$. $5H_2O$ 0.01% , $ZnSO_4$. H_2O 0.01%, H_3BO_3 0.01%, $Na_2MbO_4.2H_2O$ 0.01%

Co-cultivation medium for *Agrobacterium* (1L)

10 ml K salts, 20 ml M salts, 2.5 ml 20% NH_4NO_3, 0.5 ml 1% $CaCl_2$, 0.5 ml 10mM Glucose, 40mM MES, 0.05% Glycerol (v/v), 2% agar.

1.g or 1.98 g glucose and 7.8 g of MES were added to give a final concentration of 10mM and 40mM respectively. 200µM ACS was added just before pouring.

1X Phosphate Buffer Saline (1L PBS)- 8.0 g NaCl (137mM), 2.68 g $Na_2HPO_4.7H_2O$ (100mM), 0.2 g KCl (2.7mM), 0.27 g KH_2PO_4 (2mM), pH 7.4

2.3.6. Pathogenicity assays

For assessment of appressorium forming ability, freshly prepared conidial suspension (5×10^4 conidia ml^{-1} in sterile water with Streptomycin) obtained from 10-day-old prune agar cultures were applied in aliquots of 20 µl on hydrophobic cover glass. The coverglasses were incubated at room temperature under high humidity for 24 h post inoculation. Conidial germination and appressorium formation were examined by microscopy.

The percentage of conidia that germinated and formed appressoria was calculated.

To determine penetration ability of the fungus, rice leaf sheath inoculation was performed with conidial suspensions (Kankanala et al., 2007) and penetration events and spread of invasive hyphae were assessed 36-40 hpi by microscopy, using a 100X oil immersion lens. For assessment of virulence, detached leaf assay was performed with 8-12 days old barley leaves. The leaves were inoculated with three drops of 10µl each of conidial suspension in 0.2% gelatin and maintained on water agar supplemented with 2 mg l^{-1} kinetin. The disease outcome was recorded 5 days post inoculation.

For *in-planta* infection assays, susceptible, three-four weeks old (3^{rd} – 4^{th} leaf stage) rice seedlings of CO-39 cultivar were sprayed with 10^5 ml^{-1} of WT and mutant conidia in 0.2% gelatin and disease symptoms were recorded 5 dpi.

2.3.7. Microscopy

Brightfield microscopy and widefield fluorescence microscopy were carried out on the Olympus BX51 (Olympus, Japan) with the 40x ELWD or 100x/1.40 oil immersion objective. The long working distance of the 40X ELWD objective lens allowed observation of conidiophores as well as cultures grown on agar medium in their native conditions. This was also useful for live cell imaging. The suitable filter set was selected based on the fluorescent protein or stain baing imaged. The Nikon Eclipse80i (Nikon, Japan) microscope with 20X or 40X objective lens was also used for brightfield microscopy of fungal structures like appressoria and hyphae. The images were captured using a DS-Qi2 camera operated by the Nikon Elements software. Laser scanning confocal microscopy was used to study sub-cellular localisation of tagged proteins (Model: LSM700, Carl

Zeiss Inc., USA) using either the 40X/1.30 EC Plan-Neofluar or a 63X/1.40 Plan-Apochromat oil immersion objective lenses. The microscope was operated using the ZEN 2010 software. Fungal structures were determined by differential interference contrast (DIC) microscopy. GFP and mCherry tagged proteins were imaged in the 488 nm and 555 nm laser channels respectively. For live-cell imaging of fungal structures, cultures were grown on glass-bottom petridishes and viewed with an inverted confocal microscope. To span the entire thickness of fungal structures, images were captured as z-stacks taken at regular intervals ranging from 0.3 - 1µm. To study protein dynamics, time series programs were used with intervals ranging from 5 seconds to 10 minutes, based on the speed of the process being study. Zeiss AxioCam MR Camera was used for acquisition of images. ImageJ tool was used for analysis of images (https://imagej.nih.gov/ij/download.html). Adobe Photoshop CS6 was used for preparation of figures.

2.3.7.1 Conidiation

Conidia were harvested as described previously (Patkar et al., 2012). Briefly, fungal conidia were harvested by scraping the biomass grown on prune agar plates with a sterile loop, resuspending in sterile water and purifying by passing through two layers of Miracloth (Calbiochem, Darmstadt, Germany). Quantification of conidia was done using a haemacytometer (Marienfeld Superior, Lauda-Konigshofen, Germany). Morphology and cell number of the WT, *dam1*Δ and *ask1*Δ conidia was determined by staining with CFW. Cell wall and septa (chitin-based structures) of conidia and vegetative hyphae were stained using 3 µg ml^{-1} Calcofluor White (Whitener 28, Sigma-Aldrich, USA). Arrangement of conidia on conidiophores was assessed in WT, *dam1*Δ and *ask1*Δ strains. A thin mycelial plug was inoculated on 1% agarose and incubated under moist conditions. The conidiophore arrangement was monitored using brightfield microscopy 1 or 2 days post inoculation. Number of conidia

per conidiophore was counted for 100 conidiophores. Bar graph showing the frequency of each cluster size (1 to 8 conidia) was plotted to depict the distribution for a given strain.

2.3.7.2 Hyphal growth

Hyphal morphology of *M. oryzae* was studied by adapting methods developed for analysis of hyphal growth and branching patterns in *N. crassa* (Riquelme et al, 1998, Riquelme and Bartnicki-Garcia, 2004). *M. oryzae* WT and mutant hyphal plugs were inoculated on 35 mm plates filled with 3-5mm thick basal medium (with agarose as the solidifying agent) The fungal colony was allowed to reach a diameter of 2 cm before imaging. The actively growing cells at the colony periphery were then observed using a 40X or 100X objective lens. The hyphal and branch lengths and branching angles were determined using ImageJ. For hyphal morphology studies, 200 images of colony edge (100 hyphae x 2 plates) were captured for WT and *dam1*Δ strain using ProgResC5 Software on Olympus BX51. The distance from the hyphal tip to the point at which branches emerge and branch lengths were analysed for 100 – 130 images using Image J. A scatter plot was used to depict actual distances of 1^{st}, 2^{nd} and 3^{rd} branch form hyphal tip. Branching frequency was plotted as percentage of hyphae that produced a certain number of branches (zero, 1, 2 or 3) within lengths of 300 μm or 500 μm from the growing tip of the primary hypha. Branching angles were measured with reference to the primary hypha from which the branch had emerged in the WT and *dam1*Δ strain for 100 hyphae and frequency of branch angles greater than 60^{o} was determined. All the data shown are from three independent experiments. For fluorescence microscopy of hyphal structures, the fungal strains were inoculated on 2mm thick basal medium with agarose.

To study whether GFP-Dam1 dynamics were dependent on the microtubule network, germinating conidia were treated with 0.5 μM

nocodazole (microtubule inhibitor) and the process was followed for 15 mins capturing images every 5 min.