Chapter 5
Summary and Conclusion

5. Chapter 5: Summary and Conclusion

1. Strains were developed to study chromosome/ nuclear and microtubule organisation during the cell cycle in *M. oryzae*. The nucleus was marked with a histone H1-mCherry tag in the B157 strain using the SRR strategy by ATMT. A separate β-tubulin-sGFP tagged B157 strain was generated by protoplast transformation in order to observe the microtubule network.

2. The hH1-mCherry tagged B157 was used to develop the hH1-mCherry, β-tubulin-sGFP double tagged strain. One round of nuclear division during the process of appressorium formation is critical to pathogenic development. 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.

3. Gene tagging constructs were developed for *DAM1, ASK1* and *MIS12* to replace the native copy of the gene with a GFP/RFP-tagged one. Dam1 was tagged on the N-terminus, while Ask1 and Mis12 were tagged on the C-terminus in the hH1-mCherry strain to give hH1-mCherry;GFP-Dam1, hH1-mCherry;Ask1-GFP and hH1-mCherry;Mis12-GFP strains. These strains were generated by ATMT.

4. All transformants generated in the study were screened by PCR and/or fluorescence microscopy. Southern blotting was used to identify transformants with single integration and confirm locus-specific incorporation. Virulence of strains was assessed by detached leaf spot assay. Strains with phenotype comparable to WT were used for further study.

5. Time-lapse laser-scanning confocal/fluorescence microscopy was used to monitor localisation of the kinetochore proteins both during mitosis and interphase at different stages of fungal development. Mis12-GFP-marked *M. oryzae* kinetochores were clustered during interphase, de-clustered during mitosis and re-clustered during telophase.

6. Mis12 was associated with the nucleus even during interphase while Dam1 and Ask1 showed more dynamic localisation patterns and associated with the nucleus specifically during mitosis. Importantly, multiple GFP-Dam1 and Ask1-GFP spots associated with the chromosomes at the start of mitosis and condensed into one large spot per nucleus during segregation and nuclear migration. This pattern was constant irrespective of developmental stage including, conidia, appressoria and vegetative hyphae.

7. Additional cytoplasmic Dam1 and Ask1 spots were seen in the vegetative hyphae, germ tube and appressorium. Further, Dam1 spots also vary in size.

8. Intense dynamic GFP-Dam1 and Ask1-GFP was also observed as multiple dynamic spots or streaks at the growing tips of vegetative hyphae and germ tubes and in the form of punctae moving back and forth from the hyphal tip cell. The dynamic localisation pattern was dependent on the microtubule network.

9. Conidiation involves three rounds of mitosis. The first mitosis occurs in the conidiophore stalk and the site of septation, which occurs at the base of the conidium, is uncoupled from the site of mitosis.

10. 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. Gene knock-down constructs were generated in pSilentDual-1 (pSD1) to study the role of *CENPC*, *MIS12* and *DAM1* in *M. oryzae*. The pSD1-CenpC, pSD1-Mis12 and pSD1-Dam1 plasmids were moved into *M. oryzae* RGB12 strain (hH1:mCherry, β-tubulin:sGFP) by protoplast transformation. However, most of the transformants were unstable and difficult to revive from stocks and were not used for further study.

11. A *DAM1* knock-out cassette was generated. This construct was transformed into WT strain B157 as well as the nuclear-microtubule dual tagged strain RGB12 by ATMT/ protoplast transformation. The *dam1*Δ mutant in RGB12 was used to study the effect of loss of *DAM1* on nuclear and spindle organisation, mitosis and nuclear migration. Fungal growth, development and virulence were studied in the *dam1*Δ mutant in the B157 background. Similarly, *ask1*Δ mutant was also generated and analysed for fungal development.

12. *dam1*Δ and *ask1*Δ showed reduced hyphal growth. The mutants produced flat colonies with reduced aerial hyphae and melanisation. The vegetative hyphae of *dam1*Δ and *ask1*Δ showed aberrant morphology with smaller and irregular cell compartments and frequent branching pattern with altered branching angles.

13. *dam1*Δ and *ask1*Δ display a decrease in conidium count to ~10% of the WT. Most of the *dam1*Δ and *ask1*Δ spores are 1 or 2 celled and oval-shaped, with only ~10-15% three celled conidia. Most of the *dam1*Δ conidiophores bear only 1-2 conidia, with only 6% conidiophores showing more than 2 conidia.

14. While 81% WT conidia formed appressoria, only 15% of the *dam1*Δ conidia were able to do so. Around half of the *dam1*Δ conidia failed to

germinate while others formed irregular germ tubes or were stuck at the hooking stage. In the rice sheath assay, only 28% *dam1*Δ mutant appressoria showed normal host penetration and invasion compared to 85% of 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.

15. In whole plant infection assay, *dam1*Δ produced smaller and fewer lesions than the WT. Defective development of appressoria and impaired infection in the *dam1*Δ mutant raises the possibility of novel anti-fungal strategies directed towards these fungus-specific Dam1 complex proteins.

16. The length of the mitotic spindle was altered in the absence of Dam1. The nuclear and microtubule organization was affected by the loss of Dam1.

17. In the absence of Dam1, mitosis was prolonged due to delayed anaphase onset, with slow nuclear migration and improper segregation, both during vegetative and pathogenic development. Thus, Dam1 plays a critical role in the poleward segregation of chromosomes during anaphase and in nuclear migration.

Conclusion

The outer kinetochore DASH complex proteins Dam1 and Ask1 display subcellular dynamics different from the inner kinetochore protein Mis12, such that they are associated with the nucleus during mitosis. Further, Dam1 plays a role in proper mitotic progression and chromosome segregation, likely through the establishment of correct spindle structure and KT-MT interactions. Dam1 is important for the development of