Abstract

Magnaporthe oryzae is a causal organism of rice blast disease and is responsible for the loss of millions of tonnes of rice each year worldwide. In addition to being a great threat to food security, it also serves as a model organism to study plant-pathogen interaction. The genome sequence of *M. oryzae* is publicly available and provides opportunity to understand the genetics and functional biology of this fungus. About 80% of the genes in this fungus are still uncharacterised, which opens up an avenue to characterise the gene/s involved in the pathogenic and non-pathogenic developmental processes in this fungus. In order to characterise the gene/s involved in the viability of the fungus a knock-down approach was chosen over knock-out strategy because the gene selected for this study *MoSKP1*- appears to be essential in *M. oryzae*. Knock-down by RNAi and antisense technology using silencing vector pSilent-1 and pSD2 became an important and more appropriate approach in analysing the role of *MoSKP1* in *M. oryzae* B157. Transformants were generated with various levels of *MoSKP1* transcript and the development of the fungus was studied. Integration of transgene construct was confirmed by Southern blot analysis and silencing was confirmed by detecting MoSkp1 siRNA by Northern blot analysis. Ubiquitin mediated protein degradation is a sophisticated process involved in regulation of various kind of cell signalling. A multiprotein complex, E3 ubiquitin ligase, plays a crucial role in the ubiquitin tagging of a specific protein and is found to be indispensable for the fungus. Skp1 (S-phase associated kinase protein 1) is a core component of the SCF E3 ubiquitin ligase complex necessary for protein degradation by the 26S proteasomal pathway. Timely degradation of proteins in a cell is critical to cell

cycle progression, cell signalling and transcriptional regulation. Indispensable in fission yeast, *Skp*1 has a distinct function in a DNA damage checkpoint and interacts with various F-box proteins involved in genome integrity and G2 cell cycle delay.

The rice blast fungus *M. oryzae* has a specialised infection structure, the appressorium; its development is controlled by cell cycle progression. The *SKP1* gene has been shown to have multiple functions; however its function in appressorial development is not known so far. *M. oryzae* has a single *MoSKP1* gene (MGG_04978), required for viability. Various RNAi and antisense transformants of *MoSKP1* showed reduced sporulation, defective spore morphology, less septation, diffused nucleus and were unable to form appressoria on inductive surfaces. Further, they showed an elongated germ tube and did not penetrate host tissue. Initial spore germination of transformants has a similar phenotype to that shown by wild type spores treated with the cell cycle inhibitor Hydroxyurea, suggesting a cell cycle defect. Reduced transcript levels of *MoSKP1* in various knock-down transformants were confirmed by quantitative real time PCR and western blot analysis. Atypical appressoria development correlates with reduction in *MoSKP1* transcript and protein levels in knock-down transformants. Indirect immunolocalisation was performed by generating MoSkp1 antibody in rabbit. MoSkp1 move from spore to germ tube and abundantly expressed in appressoria, indicating its involvement in the early stages of pathogenic development. Reduced *MoSKP1* transcript level further leads to reduced ubiquitination of total proteins in the transformants. Ubiquitination enrichment assay revealed reduction in total protein ubiquitination levels. *MoSKP1* complemented *Skp*1 function in the fission yeast temperature sensitive mutant *skp1 A7* by restoring the normal length of yeast cells at restrictive temperature.

Interaction studies confirmed the ability of MoSkp1 to interact with other proteins and function in complex form. A pull down experiment utilising 6XHis-MoSkp1 protein as bait gave several interacting proteins like RNA polymerase II and the E3 ubiquitin ligase SCF subunit scon3 complex, which further confirms that MoSkp1 is a part of SCF E3 ligase complex and interacts with several regulatory molecules. Yeast two hybrid assay and co-immunoprecipitation showed MoSkp1 interacts with the putative F-box protein (MGG_06351), revealing it's ability to form protein complexes by protein-protein interaction. MoSkp1 Protein levels were also checked in the *MoSKP1* R6 RNAi transformant by performing 2D gel electrophoresis, which showed decreased protein compared to wild type. Bioinformatics prediction was done for the presence of post translational modifications in MoSkp1. Only phosphorylation sites were predicted to be present in the protein, which was further confirmed by phosphorylation and dephosphorylation experiment followed by isoelectric focusing. The shift in the protein band confirms the ability of MoSkp1 to get phosphorylated. This provides evidence to believe that the biological activity of MoSkp1 might be regulated by phosphorylation of MoSkp1 in the cell.

Current investigation on the role of *MoSKP1* suggests that reduction of *MoSKP1* manifests in reduced total protein ubiquitination and consequently defective cell cycle and appressorial development. Thus, *MoSKP1* plays an important role in growth, sporulation, appressorial development and pathogenicity of *M. oryzae*.