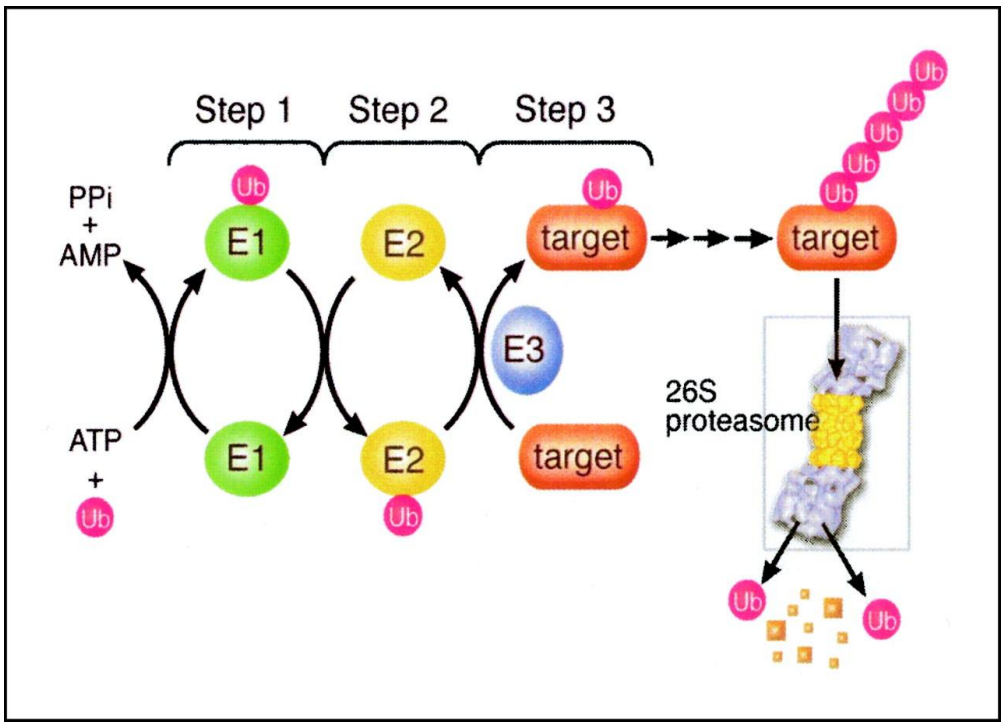
# 1. INTRODUCTION

S-phase kinase-associated protein 1, encoded by *SKP1* gene, is the member of the SCF ubiquitin ligase protein complex. The protein is one of the major components of the SCF-Ubiquitination Complex which facilitates activation of ubiquitination of various substrates. SCF-Ubiquitination Complex belongs to the E3 ubiquitin ligase family which is composed of Skp1p–Cullin–F-box proteins. Skp1 binds to F-box such as cyclin F, S-phase kinase-associated protein 2 (SKP 2) and other regulatory proteins which are involved in ubiquitin dependent proteolysis (Bai *et al*. 1996). The Skp1 protein also interacts with a network of proteins to control β-catenin levels and affects the activity of β-catenin dependent transcription factor **T- C**ell **F**actor (TCF). Specific protein degradation by ubiquitin dependent proteolysis is involved in various cell processes like development in plant (Moon *et al*., 2004), development in *Dictyostelium discoideum* (Pukatzki *et al*. 1998), abiotic stress tolerance in *Arabidopsis thaliana* (Lyzenga and Stone 2011), growth, plant nutrient utilization (Yates and Sadanandom 2013), role in gibberellin signalling (Wang and Dang 2011), cell cycle control and cancer (Nakayama and Nakayama 2006) and DNA damage repair (Tu *et al*. 2012).

Protein degradation in lysosomes is not specific; whereas ubiquitin dependent protein degradation is highly specific to intracellular protein. Proteins have different half life times that vary from a few minutes (e.g. the tumur suppressor p53) to several days (e.g. the muscle proteins; actin and myosin) and up to a few years (e.g. crystallins). Degradation of a protein via the ubiquitin proteasome pathway involves two discrete and successive steps: 1) tagging of the substrate by covalent attachment of multiple ubiquitin molecules and 2) degradation of the tagged protein by the 26S proteasome complex with release of free and reusable

ubiquitin. Conjugation of ubiquitin to the substrate involves ligation of an evolutionary conserved 76 residue polypeptide to the protein substrate via a three step cascade mechanism (Fig. 6). Initially the ubiquitin activating enzyme E1 activates ubiquitin in an ATP-requiring reaction to generate a high energy thiol ester intermediate, E1-S ubiquitin. One of the several E2 enzymes [ubiquitin-carrier proteins or ubiquitin-conjugating enzymes (UBCs)] transfers the activated ubiquitin moiety from E1, via an additional high energy thiol ester intermediate: E2-S ubiquitin, to the substrate that is specifically bound to a member of the ubiquitin protein ligase family, E3. There are a number of different classes of E3 enzymes. For the **H**omologous to the **E**6-AP **C**OOH **T**erminus, (HECT), class domain E3s, the ubiquitin is transferred once again from the E2 enzyme to a Cys residue present at the active site on the E3, to generate a third high energy thiol ester intermediate, ubiquitin S-E3, before it is transferred to the ligase bound substrate. **R**eally **I**nteresting **N**ew **G**ene, (RING), finger containing E3 catalyses direct transfer of the activated ubiquitin moiety to the E3 bound substrate.

E3s catalyse the last step in the conjugation process which involves covalent attachment of ubiquitin to the substrate. The ubiquitin molecule is generally transferred to ε-$NH_2$ group of an internal Lys residue in the substrate to generate a covalent iso-peptide bond. In some cases, however, ubiquitin is conjugated to the $NH_2$-terminal amino group of the substrate. By successively adding activated ubiquitin moieties to internal Lys residues on the previously conjugated ubiquitin molecule, a poly-ubiquitin chain is synthesised. The chain is recognised by the downstream 26S proteasome complex. Thus E3s play a key role in the ubiquitin mediated proteolytic cascade since they serve as the specific recognition factors of the system.




(Mani and Gelmann, 2005)

**Figure 6:** Mechanism of ubiquitin mediated protein degradation

In the SCF complex of budding yeast, Skp1 (S-phase kinase associated protein 1) associates with Roc1-Cul1 heterodimer and bridges it to the substrate adaptor by virtue of its ability to interact with F-boxes. The F-box containing proteins bear additional protein-protein interaction motifs such as WD40 repeats and leucine-rich repeats that recognise sequences in the target protein which is the signal for destruction. Skp1 interacts with a variety of F-box proteins leading to a large range of potential substrates. For example, in budding yeast SCF containing Cdc4 ($SCF1^{cdc4}$) ubiquitinates Sic1, Far1 and Cdc6, whereas additional $SCF1^{\beta\text{-}TrCP}$ targets β-catenin or lκBα (Vodermaier 2004, Hattori *et al*. 2003, Feldman *et al*. 1997, Yam *et al*. 1999). Skp1 also has various functions other than stabilisation of the SCF complex. Skp1 is reported to be associated with a multiple protein complex, (**R**egulator of V ($H^+$)-**A**TPases of the **V**acuolar and **E**ndosomal Membranes) (RAVE), required for V-ATPase assembly. V-ATPases are conserved throughout eukaryotes and have been implicated in metastasis and multidrug resistance (Seol *et al*. 2001). Skp1 is also reported to interact with Rav1p and is not involved in V-ATPase assembly, but in recycling of the complex (Brace *et al*. 2006). In fission yeast, Skp1 interacts with the Pof-6 protein and is essential for cell separation (Hermand *et al*. 2003) as well as spindle morphology and nuclear membrane segregation in anaphase (Lehmann and Toda 2004). The Skp1 of *Arabidopsis* interacts with F-box domain containing effectors of the type III secretion systems (T3SS) of phytopathogenic bacteria *Ralstonia solanacearum* and facilitates the development of disease in the host (Angot *et al*. 2006). It appears that Skp1 is a vital element having varied functions based on the protein partners with which it interacts under various physiological conditions and at diverse locations in each host system.

In *Schizosaccharomyces pombe*, *skp1* mutants have been shown to affect mitosis; specifically, mutants fail to proceed to anaphase and show growth retardation (Lehmann and Toda 2004, Lehmann *et al*. 2004). The ubiquitin proteasome system (UPS) drives the cell cycle in an unidirectional and irreversible manner by targeting key cell cycle regulators for timely degradation. Reversible phosphorylation of cyclin dependent kinases (Cdks) regulates various processes like DNA repair (Ariza *et al*. 1996), protein-protein interaction and chromosome condensation (Siino *et al*. 2003), but not the directionality of the cell cycle. The directionality is determined by the degradation of proteins that are needed in the previous stage of the cell cycle, making it irreversible and driving the cell cycle forward (Vodermaier, 2004). Although cyclins are the activating units of Cdks, the destruction is mainly controlled by a proteasome system wherein the substrate is recognised and tagged with ubiquitin by the E3 ubiquitin ligase enzyme and subsequently degraded by the 26S proteasome (Murray 2004, Renski *et al*. 2008, Yu 2007)**.** In our study with the same mutant background of fission yeast, interspecific gene complementation by Moskp1 showed restoration of normal phenotypes such as cell size and shape, septation patterns and nuclear organisation.

Our analysis of the *M. oryzae* B157 genome data suggested the presence of only one orthologue of *Skp1* and it was of interest to study its functions and interacting partners with regard to the saprophytic and pathogenic life style of the rice blast fungus. *Fusarium oxysporum Skp*1 showed homology with *M. oryzae* accession no. XM_359799 (NCBI) / MG04978.4 (BROAD) 86% at nucleotide level and 76% at protein level. In the present study, we have investigated the involvement of Skp1 in sporulation, growth and under pathogenic conditions for the development of appressoria by knocking down MoSKP1. The knock-down transformants of MoSKP1 in *M. oryzae* showed slow growth, formation of

elongated germ tube, inability to develop appressoria and thus, were unable to penetrate the host.

**Objectives:**

1. Development of disruption, silencing and overexpression vectors for MoSKP1
2. Fungal transformation using MoSKP1 disruption, silencing and overexpression construct
3. Molecular and functional characterisation of the putative transformants
4. Phenotypic analysis and evaluation of the transformants for involvement in pathogenicity