# 2. REVIEW OF LITERATURE

## 2.1 Rice blast disease

Worldwide attempts are being made to study the host pathogen interactions using *Magnaporthe oryzae*, the causal agent of rice blast disease. Rice is one of the major nutritional sources, being the staple for more than half the population of the world. Rice is grown in over 100 countries and more than 3 billion people derive their one fifth calorific intakes from rice consumption (Maclean *et al.*, 2002). Rice blast epidemics have occurred in between 2001 and 2005 due to which 5.7 million hectares of rice were destroyed. The requirement for a better understanding of the disease becomes obvious if poor durability of many blast-resistant cultivars of rice is considered. The rice-infecting strains of *M. oryzae* are presumed to have originated in the Middle Yangtze Valley of China around 7,000 years ago and then spread throughout the world (Couch, 2005).

## 2.2 Rice blast fungus as a model system to study plant pathogen interaction

Filamentous fungi represent a diverse and economically important class of organisms including pathogens of humans, animals, and crop plants and also industrially important organisms. *M. oryzae* is a haploid ascomycete, heterothallic filamentous fungus. A number of attributes have allowed *M. oryzae* to emerge as a model

phytopathogen and has become an excellent model organism for investigation of fungal phytopathogenicity and host–pathogen interactions, largely because of its economic importance, but also due to the experimental tractability of the fungus (Valent, 1990; Tucker and Talbot, 2001). Notably, *M. oryzae* shares many characteristic features associated with other important cereal pathogens, like appressorium formation and intracellular tissue invasion (Foster, 2003; Ebbole, 2007; Caracuel *et al*., 2007). This opens up the possibility of finding generic methods and disease determinants, which can be targeted for broad-spectrum crop disease intervention. *M. oryzae* can be cultured on defined media, various genomic and cDNA libraries are available and genetic maps of the fungus have been developed (Nitta *et al*., 1997; Zhu *et al*., 1997; Nishimura *et al*., 1998; Farman and Leung 1998; Zhu *et al*.1999). Transformation protocols have been developed and various auxotrophic and drug resistance markers are available (Parsons *et al*. 1987; Valent and Chumley, 1991; Kachroo *et al*., 1997). Enhanced transformation frequencies can be attained using *Agrobacterium tumefaciens*–mediated transformation (Rho *et al*., 2001). Around 20% frequency of homologous recombination replaces a gene of interest of transformants in *M. oryzae*, although the process is highly locus dependent. The high throughput efficient gene disruption can be carried out using *in vitro* transposon mutagenesis with larger flanking regions (Backer *et al*., 2001; Hamer

*et al.*, 2001). The early stages of the plant infection process that includes germination, appressorium formation, and penetration can be studied *in vitro* using artificial surfaces (Bourett and Howard 1990). Infections can be carried out in a variety of hosts (Lau *et al.*, 1996) and the infection process can be followed biochemically or cytologically, allowing the analysis of infection deficient mutant phenotypes (Bourett and Howard 1990; Heath *et al.*, 1992).

## 2.3 The sexual reproduction of *M. oryzae*

*M. oryzae* is a heterothallic (self-incompatible) ascomycete. The sexual cycle of *M. oryzae* has not been seen in nature, but some strains undergo sexual crosses in the laboratory (Yaegashi and Udagawa, 1978). Among isolates of *M. oryzae*, fertile strains are rarely observed, rice pathogenic strains are predominantly infertile in the field (Kato, 1982). The high levels of sexual fertility have been found in wheat blasts and crossing to produce abundant viable ascospores (Urashima *et al.*, 1993). In *M. oryzae*, two mating types of the fungus are present, *MAT1-1* and *MAT1-2*. When fertile isolates having opposite mating types are paired together on suitable growth medium like oatmeal agar at 20$^{o}$C, they form sexual fruiting bodies called perithecia within 21 days (Valent and Chumley, 1991). The creation of such strain has facilitates the genetic analysis of *M. oryzae*. Sexually fertile strains produce two forms of spores,

ascospores and microconidia. Large number of ascospores is produced in unordered asci within flask shaped perithecia with long necks. (Yaegashi and Udagawa, 1978). Asci can be break downed to liberate the ascospores, which are arranged as unordered octads (four pairs of spores representing the products of meiosis that have undergone a subsequent mitotic division). Ascospores are hyaline and crescent-shaped, with four cells, each containing a single mitotically derived nucleus. Mature asci are released through the perithecial necks and ascospores are liberated into a viscous liquid. Appressoria are also produced by ascospores for plant penetration. Sexually fertile isolates also produce small, crescent-shaped microconidia from phialides, 6 micrometers in length and 0.7 micrometers in width which are hypothesized to function as spermatia (Chuma *et al.*, 2009).

## 2.4 The asexual reproduction and infection cycle of rice blast

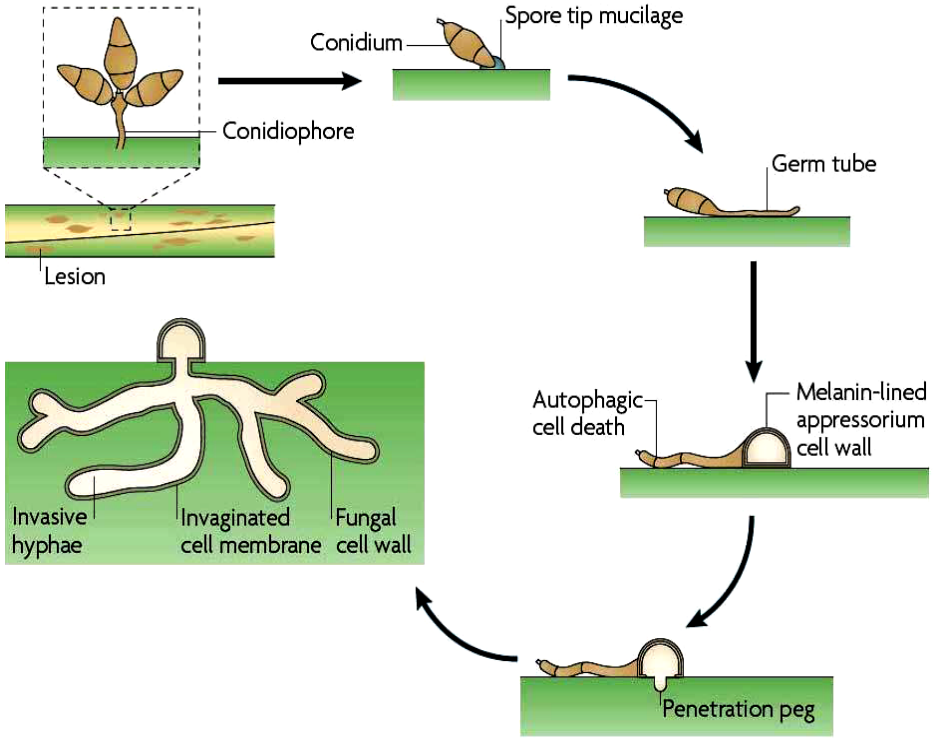
The infection cycle is well studied for rice isolates (Wang and Valent, 2009; Wilson and Talbot, 2009; Valent and Khang, 2010) however wheat and ryegrass isolates appear to execute the same infection cycle (Tufan *et al.*, 2009). Rice blast infections are initiated with asexual conidia. Conidia are produced on aerial conidiophores with sympodial arrangement and liberation of the conidia is primarily by air and splash dispersal. The variability of the fungus and the propensity for new races in the field, make its

control and management difficult. The foliar infection is initiated by attachment of a three-celled conidium of *M. oryzae* to the rice leaf surface. The tear drop-shaped conidium adheres tightly to the leaf surface in presence of high humidity by means of an adhesive (mucilage), which is released from an apical compartment in the spore tip during hydration. This adhesive facilitates the conidia to attach itself tightly to the hydrophobic rice surface and allows germination to occur (Hamer *et al*. 1988). Once attached, the germ tube normally emerges from one of the apical cells of conidium and extends for only a short time and grows across the leaf surface. The tip of the elongating germ tube enlarges and forms a dome-shaped, melanin-pigmented infection structure called the appressorium within 4–6 hours (Figure 3). The word appressoria is derived from the latin "apprimere" meaning "to press against". Appressorium development can be stimulated by various signals, such as presence of cutin monomers such as 9, 10-epoxy-18-hydroxyoctadecanoic acid or lipid monomers, such as 1,16-hexadecanediol (Gilbert *et al*. 1996), wax compounds (Uchiyama *et al*., 1979; DeZwaan *et al*., 1999) starvation stress (Jelitto *et al*., 1994; Talbot *et al*., 1997), hydrophobicity of the leaf surface and addition of external cAMP (Lee and Dean 1993; 1994). Appressoria anchor firmly to the leaf surface and then produces enormous turgor pressure (8 MPa) used to breach the cutical layer (Howard *et al*., 1991). *CPKA* (catalytic subunit of protein kinase A) and *PMK1* (mitogen

activated protein kinase 1) accompanied appressorial turgor generation, dependent on mobilisation of carbohydrate and lipid reserves, which accumulate in developing appressoria in mature cells prior to plant infection (Thines *et al.*, 2000; Weber *et al.*, 2001). Carbohydrates and lipids are converted into glycerol in the appressorium. The melanin layer within the appressorium maintains the glycerol content, retarding its efflux during turgor development (Howard and Ferrari, 1989; De Jong *et al.*, 1997). Therefore, melanin deficient mutants do not generate appressorial turgor and are non pathogenic (Chumley and Valent, 1990). Cellular turgor is translated into mechanical force, which is exerted by the emerging penetration peg, forcing it through the leaf cuticle. The polarization of the cytoskeleton to the point of infection is also initiated by breaching of the cell wall (Bourett and Howard, 1990; Bechinger *et al.*, 1999). After entry into the plant, host epidermal cells are occupied by hyphae of the fungus within 24 hours. Neighboring epidermal or parenchymal cells are penetrated within 48 hours. Fungal hyphae grow rapidly through the plant tissue, resulting in the disease lesions after 4 days after inoculation that are symptomatic of rice blast disease.

**Figure 3: Infection cycle of *Magnaporthe oryzae***

Steps of infection cycle include the landing of the three-celled conidia are borne sympodially on aerial conidiophores. Establishment of conidia on a host leaf, germination from one of the three cells with the extension of germ tube, hooking and swelling at the tip of the germ tube to form melanized appressorium, emergence of penetration peg from appressorium, invasion of the host tissue, and spread of infection hyphae within the host (Wilson and Talbot, 2009).


Conidium
Spore tip mucilage
Conidiophore
Germ tube
Lesion
Autophagic cell death
Melanin-lined appressorium cell wall
Invasive hyphae
Invaginated cell membrane
Fungal cell wall
Penetration peg

## 2.5 Root infection by *M. oryzae*

Phytopathogenic fungi can be divided broadly into two types, leaves and stems infecting plants and root infecting fungi (Agrios, 1997). *M. oryzae* can not only infect the foliar region of the plant but also infects cereal roots indicating that it maintains the capacity to infect distinct plant tissue types and develop a range of infection structures (Dufresne and Osbourn, 2001). The mode of root infection is the same as most root infecting fungi; it grows as long hyphae that form an infection layer to gain entry to the root's tissue. The melanised appressoria associated with classical foliar infection are not observed on the surface of rice roots when either conidia or mycelium are used as inoculum. In contrast, hyphal swellings resembling the hyphopodia of root-infecting fungi are evident at infection sites (Sesma and Osbourn, 2004). Once embedded in the root, the fungus can produce resting structures. The blast fungus can also invade the vascular system of plants, growing inside the xylem and phloem and blocking the transport of nutrients and water from the roots, and produce lesions on aerial plant parts.

## 2.6 Signaling pathways in *M. oryzae*

The role of mitogen-activated protein kinases (MAPKs) has been investigated well in the development of dimorphic and filamentous fungi. The MAPK cascade is one of

the most widespread and best studied signaling systems. MAPKs operate in association with upstream kinases in order to convey an environmental or developmental signal from the cell periphery to the nucleus to bring about gene expression. The appressorium development, penetration peg formation and adaptation to hyper-osmotic stress are regulated by MAPK cascades in *M. oryzae,* involving a series of sequentially acting protein kinases.. This kinase signaling module comprised of an upstream MAPK kinase kinase (MAPKKK) that is activated to phosphorylate its target, a MAPK kinase (MAPKK). MAPKKK phosphorylation of MAPKK then permits this kinase to phosphorylate the ultimate kinase target in the pathway, the MAPK, leading to activation of this key signaling molecule. Phosphorylated and active MAPK can then regulate its downstream effector molecules, leading to the appropriate regulatory effects. Three MAPK pathway, *PMK1*, *MSP1*, and *OSM1*, have been identified in *M. oryzae* that play a key role in appressorium formation and function. The gene *PMK1* is highly homologous to the yeast MAPK genes *FUS3* and *KSS1.* The core of the Pmk1 pathway regulates both the pheromone signalling and filamentation pathways in *S. cerevisiae*. In *M. oryzae*, the *pmk1* mutants are unable to make appressoria, and when germlings growing on glass surfaces are stimulated with cAMP they can start appressorium differentiation, but this does not proceed beyond the swelling and hooking of the germ tube apex. Thus, *Pmk1* is required for appressorium

formation and invasive growth but is dispensable for other aspects of growth and development. However, Pmk1 pathway components such as the MAPK are involved in mating and pathogenesis, whereas Mst12, the Ste12-related transcription factor, is dispensable for mating altogether. *Pmk1* may act downstream of the cAMP dependent pathway (Xu and Hamer, 1996). *MPS1* is functionally related to *SLT2*, yeast MAPK which is part of a signaling pathway in yeast. *MPS1* is required for the maintenance of cell wall integrity during various stages of fungal development and is required for the appropriate function of the appressorium (Xu *et al*., 1998). *OSM1* in *M. oryzae* is the functional homolog of *HOG1* in yeast, deletion of *OSM1* did not result in apparent defects in vegetative growth, but reduced conidition was detected. The response of hypha to hyperosmotic stress is impaired in *osm1* mutants and this defect is related to a failure in the accumulation of arabitol in the mycelium. The accumulation of glycerol and turgor pressure is not affected in *osm1* appressoria. Thus, it is possible that the OSM1 mediated MAPK pathway acts independently of the signal transduction pathway that leads to appressorium turgor (Dixon *et al*., 1999). Both CPKA and PMK1 signaling pathways have been shown to be necessary for carbohydrate mobilisation during appressorium formation, and thus perhaps enzymes in these pathways may be good candidates for genes regulated directly or indirectly by these pathways (Thines *et al*., 2000). The role of signal transduction pathways have

been implicated in appressorial development and function. The regulatory pathway for appressoria formation in *M. oryzae* require cAMP signal (Lee and Dean, 1993). A cyclic AMP (cAMP) response pathway is believed to be triggered at an early stage of *M. oryzae* germ tube elongation because *mac1* mutants, which lack the enzyme adenylate cyclase, required for synthesis of cAMP, are unable to form appressoria and are consequently nonpathogenic cAMP exerts its effect principally through the activation of cAMP-dependent protein kinase (PKA) (Adachi *et al.*, 1998; Dean, 1997) The cAMP signal may be produced in response to surface hydrophobicity or germ tube contact with a hard surface. The catalytic subunit of PKA (CPKA) releases by the binding of cAMP to the regulatory subunit of an inactive enzyme. The active catalytic subunit may activate phosphorylation cascades or migrate to the nucleus to phosphorylate target proteins (Rosen, 1995). *cpka* mutants are delayed in appressorium formation and they are unable to penetrate into the rice plant. The possibility is that CPKA is playing a important role in the accumulation of glycerol, necessary for the generation of turgor pressure within the melanised appressoria (Xu *et al.*, 1997). During germ tube extension, *MPG1* hydrophobin encoding gene is highly expressed, and it secures the attachment to the hydrophobic leaf cuticle by the secretion of the hydrophobin (Talbot *et al.*, 1993; 1996). The MPG1 mutants are inefficient in appressorium production and poorly pathogenic; indicating that surface

attachment is a necessary for the signaling pathways that regulate appressorium formation (Talbot *et al.*, 1993). A Heterotrimeric G protein activate secondary messengers and regulate gene expression by transducing environmental signals using a novel receptor, G-protein-coupled receptors with seven transmembrane helices (GPCR) (DeZwaan *et al.*, 1999), and is also involved in transmission of inductive signals (Liu and Dean, 1997). The Pth11 GPCR also operates upstream of the cAMP signalling pathway in *M. oryzae*, suggesting that the CFEM-GPCR family could provide a number of distinct and unforeseen inputs into this pathway (DeZwaan *et al.*, 1999). Addition to this, calcium signaling plays a role in germination and the induction of appressorium development (Lee, 1998; Liu, 1999). Calmodulin gene expression is dependent on spore attachment to the host surface and is induced along with appressorium formation. Diacylglycerol also serves as an inducer of appressorium formation, involved in surface sensing mechanism for calcium and protein kinase C (Thines *et al.*, 1997).

## 2.7 Genome architecture of *M. oryzae*

In 1998, the international rice blast genome (IRBG) consortium (http://www.riceblast.org) was established with the goal of obtaining the complete genome sequence of *M. oryzae.* The *M. oryzae* genome is 39 Mb in size and organized

in seven chromosomes. The fully annotated *Magnaporthe* genome has now been publically released (http://www.genome.wi.mit.edu/annotation/fungi/Magnaporthe /index.html). *M. oryzae* genome sequence provides a detailed insight into its genome with a prediction of over 11000 genes (Dean *et al.*, 2005). The genome of *M. oryzae* contains more genes than the genomes of its closely related fungi, *Neurospora crassa* and *Aspergillus nidulans*.

*M. oryzae* sequence was the first to be generated from a plant-pathogenic fungus, and together with the recent release of the rice genome sequence, it obviously represents the first real opportunity to dissect host–pathogen relationships at the whole-genome or systems level. In order to study the detailed mechanism of the interaction of the fungus with the host, it becomes essential to study the genes involved in the process. The development of various transformation systems and techniques for random mutagenesis and targeted gene mutation have been adapted and developed as efficient tools for investigating gene function in fungi. High throughput approaches like expressed sequence tags (EST) sequencing (Kamakura *et al.*, 1999; Kim *et al.*, 2001; Rauyaree *et al.*, 2001), serial analysis of gene expression (SAGE) (Irie *et al.*, 2003), microarray (Takano *et al.*, 2003) and protein analysis (Kim *et al.*, 2004) have helped in analyzing the genes responsible for pathogenicity at large scale. In addition,

individual genes playing a role in the infection mechanism of the fungus can be studied by targeted or random gene disruptions (Idnurm and Howlett, 2001).

## 2.8 Posttranslational modifications (PTMs)

The changes in protein abundance have provided valuable clues about the cellular processes that contribute to fungal pathogenesis. However, measurements of protein abundances alone are not sufficient to understand the regulation of such processes, because the activities of many eukaryotic proteins are modulated at posttranslational levels. The complexity and diversity of a proteome are greatly increased by reversible covalent post-translational modifications, which compensate for the surprisingly low number of genes in vertebrate genomes. Most proteins undergo some form of PTMs, which can alter their physicochemical properties and conformation. PTMs are particularly suitable for prompt cellular response to external and internal factors since their kinetics are much faster than the regulation of protein expression levels. A complex interplay of these modifications regulates fundamental protein properties, such as stability, localization, activity and interaction with other proteins. Functions of several proteins are regulated by the covalent attachment of polypeptide modifications, the well known examples are ubiquitin and ubiquitin-related modifiers. Unlike small molecule modifiers such as acetylation, glycosylation or

methylation, conjugation by ubiquitin and ubiquitin-related modifiers provides much larger and chemically diverse surfaces. One such modification of substrate proteins, covalent attachment of the SUMO protein, is a well characterized process that targets substrates resulting in altered structure, function, activities or interaction of intracellular targets.

## 2.9 Sumoylation Pathway

The protein Small Ubiquitin-like Modifier (SUMO) was first identified in the yeast, *S. cerevisiae*, as a high copy suppressor of a temperature-sensitive mutation in a centromeric protein (MIF2) and given the name SMT3 (Meluh and Koshland, 1995). The first protein identified as a target for sumoylation was the RanGTPase activating protein RanGAP1 (Matunis *et al.*, 1996; Mahajan *et al.*, 1997) which had been implicated in both nuclear transport and the control of mitosis. The initial report describing SUMO as a post-translational modification named the protein Gap Modifying Protein 1 (GMP1) because the RanGAP1 protein was modified (Matunis *et al.*, 1996), but afterward it was named as small-ubiquitin-like-modifier (SUMO) based on sequence similarity to ubiquitin (Mahajan *et al.*, 1997). The revelation that unmodified RanGAP1 localized to the cytoplasm and SUMO-modified RanGAP1 localized at the nuclear envelope demonstrated that SUMO was a posttranslational

modification and provided an initial assumed function for sumoylation (Matunis *et al.*, 1996; Mahajan *et al.*, 1997). Afterward, multiple proteins within the cell are targeted for sumoylation, and the majority of sumoylated proteins localize to the nucleus (Kamitani *et al.*, 1997). The Small ubiquitin-like modifier (SUMO) is the Ubl that seems to modify the largest pool of proteins and it shares approximately 20% sequence identity with ubiquitin (Johnson, 2004). SUMO and ubiquitin have similar protein size, tertiary structure and a C-terminal di-glycine motif. Despite the similarity in the three-dimensional structures between ubiquitin and SUMO, surface charges are very different. In contrast to ubiquitin, whose surface electrostatic potential is positive, the corresponding region of SUMO is negatively charged. However, SUMO contains a long, flexible N-terminal extension that extends away from the rest of the protein, and also has several differences in surface charge distribution (Bayer *et al.*, 1998). In addition, the ubiquitin pathway has a large number of E2 s and E3 s, whereas the SUMO pathway only uses a single E2 and a few E3s (Muller *et al.*, 2001). Importantly, in several SUMO proteins this N-terminal extension contains a lysine residue that can be sumoylated, resulting in the formation of poly-SUMO chains on a target protein (Tatham *et al.*, 2001) Ubiquitination is well known for targeting substrates for degradation, whereas sumoylation regulates a substrate's functions mainly by altering the intracellular localization, protein-protein

interaction and transcription factor activity. The number of SUMO proteins for a given organism can vary widely. The yeast contains a single SUMO gene (Meluh and Koshland, 1995), three separate SUMO proteins are known to express in human cells that all become covalently attached to target proteins (Kamitani *et al.*, 1998), while eight different SUMO proteins are predicted in *Arabidopsis* (Kurepa *et al.*, 2003).

## 2.10 Substrate specificity in sumoylation

Most of the substrates has been identified as SUMO targets modulated by SUMO covalent conjugation pathway having consensus motif ΨKXE/D where Ψ is any large, hydrophobic amino acid (such as Val, Ile, Leu, Met, or Phe), K is the target lysine, X is any amino acid, E is a glutamic acid and D is aspartic acid. (Zhao *et al.*, 2007; Rodriguez *et al.*, 2001). Most SUMO substrates localize to the nucleus, and many, including Sp100, HDAC4, Mdm2, and Smad4, require their NLSs for sumoylation (Lin *et al.*, 2003; Kirsh *et al.*, 2002; Miyauchi *et al.*, 2002; Sternsdorf *et al.*, 1999). Around 75% of known substrates are modified within consensus motif (Xu *et al.*, 2008). However, sumoylation can also occur at lysine residues without this consensus motif, such as nonconsensus sumoylation sites. In addition, although not all lysine residues within the ΨKXE/D motif are sumoylated (Wilkinson, 2010). Sumoylation consensus sequence ΨKXE/D is still generally believed to be helpful for predicting sumoylation

sites. Moreover, some sumoylated proteins, such as Mdm2, Daxx, CREB, and CTBP-2, do not contain a ΨKXE sequence; others are still sumoylated. (Kagey *et al.*, 2003; Miyauchi Y *et al.*, 2002; Jang *et al.*, 2002; Rangasamy, 2000). In addition to covalent attachment of SUMO to substrates, specific motifs has been identified that mediate non-covalent interactions with SUMO. The most recognized motif consists of hydrophobic core, which is often flanked by acidic residues. The best characterized of SUMO interaction motif (SIM) have consensus sequence, V/I-X-V/I-V/I or V/I-V/I-X-V/I/L where X can be any amino acid. Several proteins are sumoylated non-covalently via short SUMO interacting motifs (SIMs) (Song *et al.*, 2004; Song *et al.*, 2005). This has been also involved in various cellular processes including host-pathogen interaction (Gareau *et al.*, 2010, Wimmer *et al.*, 2012).

## 2.11 Functional consequences of SUMO modification

Initial study as to the function of SUMO dealt largely with sub cellular localization. The aforementioned relocalization of the protein RanGAP1 from the cytoplasm to the nuclear pore complex upon modification by SUMO was the first example of subcellular re-localization of a protein directed by sumoylation (Matunis *et al.*, 1996; Mahajan *et al.*, 1998). DRP1 is a GTPase protein required for mitochondrial fission. Sumoylation of DRP1 facilitates its recruitment from the cytosol to the mitochondrial outer membrane (Harder *et al.*, 2004). SUMO also was shown to co-localize with the protein PML to large

multi-protein complexes in the nucleus called PML nuclear bodies, and subsequent identification of PML as a target for sumoylation led to the suggestion that sumoylation of proteins might play a role in sub-nuclear localization of target proteins (Howe *et al.*, 1998; Kamitani *et al.*, 1998; Muller *et al.*, 1998). This analysis was further strengthened by the revelation that another protein associated with nuclear dots, Sp100, might also be a target for sumoylation (Sternsdorf *et al.*, 1997). Components of the SUMO system have been shown to play critical roles in regulation of gene expression (Girdwood *et al*, 2004), RNA metabolism, DNA replication and repair (Baek, 2006), nucleo-cytoplasmic transport, neuronal survival (Lieberman, 2004), cancer development and cell cycle regulation, including roles in division, mitotic chromosome structure, cell cycle progression, kinetochore function and cytokinesis (Dasso, 2008; Geiss-Friedlander & Melchior, 2007; Meulmeester and Melchior, 2008). Cellular stresses, such as heat shock, hibernation and osmotic stress induce SUMO conjugation (Kurepa *et al*, 2003; Saitoh and Hinchey, 2000). In most cases, sumoylation negatively regulates gene expression by either enhancing the function of transcription repressors or inhibiting the function of transcription activators (Verger *et al.*, 2003; Gill, 2005; Hay, 2006). However, the opposite occurs occasionally. For example, heat shock factor 1 (HSF1) is sumoylated in response to stress and HSF1 sumoylation often leads to activation of its target genes (Marx, 2005; Hong, 2001). In these cases, sumoylation is induced globally with many target proteins being affected,

although the cellular mechanisms responsible for this global change of the SUMO conjugation pattern remain to be discovered (Tempe *et al*, 2008).

## 2.12 SUMO in signal transduction pathways

NFkB transcription factor activation is induced by inflammatory response pathway via promoting ubiquitin-dependent degradation of NFkB inhibitor IkB. SUMO conjugation to IkBα can inhibit this step because SUMO is conjugated to the same Lys where ubiquitin would be attached, thereby preventing IkBα degradation (Desterro *et al.*, 1998). Consistent with a role for SUMO in stabilizing IkBα, SUMO overexpression inhibits NFkB-dependent transcription in mammalian cells (Desterro *et al.*, 1998). Interestingly, SUMO has the opposite effect on the orthologous pathway in *Drosophila*, where sumoylation of the NFkB ortholog Dorsal apparently promotes its import into the nucleus and transcriptional activity (Bhaskar *et al.*, 2000). Another example of a role for SUMO in signal transduction is found in *Dictyostelium*, where extracellular cAMP signals chemotaxis and aggregation controlled by MAP kinase pathway (Sobko, 2002). Within 15 s after cAMP addition, sumoylation of MAPK kinase MEK1 occurs, and the initially nuclear MEK1 and SUMO localize to the plasma membrane. It is not known whether nuclear export is enhanced by SUMO or MEK1 association with plasma membrane. Simultaneously, the relocalization of downstream MAPK ERK1occurs from the cytoplasm to the plasma membrane, suggesting that ERK1 activation takes place in the plasma

membrane. Noticeably, after pathway activation by 3 min, MEK1 has been desumoylated, and MEK1 and SUMO both become undetectable from the plasma membrane. The SUMO pathway also affects signaling dependent on axin, a protein that acts as a scaffold for enzymes in the Wnt pathway and participates in activation of the JNK MAP kinase. The extreme C terminus of auxin is sumoylated at two sites (Rui *et al*, 2002), and deletion of these sites eliminates MEKK1-dependent JNK activation but Wnt signaling has unaffected.

### 2.13 Sumoylation and diseases

Sumoylation is a dynamic process that could be reversed by deconjugating enzymes. A delicate balance between sumoylation and desumoylation is essential to normal cell functions. Growing evidence has shown that the loss of this balance in sumoylation and desumoylation can lead to diseases including cancer, diabetes and neurodegenerative diseases such as Parkinson's disease, Alzeimer's disease and Huntington's disease (Zhao, 2007; Sarge and Park-Sarge, 2009; 2011). It has been shown that sumoylation of amyloid precursor protein (APP) close to the β-secretase cleavage site is associated with a decrease of Aβ aggregates, which is generally believed a probable cause of Alzeimer's disease (Sarge, and Park-Sarge, 2009; Gocke, 2005; Zhang, 2008). The causative relationships between the deregulation of sumoylation and pathogeneses of the diseases are still not known and under active investigation. Studies so far have suggested that SUMO target

proteins might be therapeutic targets for treating these diseases (Sarge and Park-Sarge, 2009; 2011).

## 2.14 Role for sumoylation in abiotic stress

The notion that sumoylation played a role in stress response came from experiments aimed at determining the roles of the various SUMO proteins in mammalian cells. SUMO-1 is predominantly conjugated to proteins under non-stress conditions, while SUMO-2 and SUMO-3 are conjugated to proteins in response to a variety of abiotic stresses, including salt stress, heat stress and oxidative stress (Saitoh and Hinchey, 2000; Sramko *et al.*, 2006). Similar observations have been observed in regard to the use of the proteins AtSUMO1 and AtSUMO2 for sumoylation in response to a variety of stresses in *Arabidopsis* (Kurepa *et al.*, 2003). The contribution of SUMO during stress has perhaps, best been studied in response to heat stress. The heat shock factors, HSF1, HSF2, and HSF4b, are modified by SUMO in mammalian cells (Goodson *et al.*, 2001; Hong *et al.*, 2001; Hietakangas *et al.*, 2006). The DNA binding activity of these transcription factors is increased by sumoylation (Goodson *et al.*, 2001; Hong *et al.*, 2001). Additionally, HSF1, modulates the induction of heat shock protein (HSP) expression in response to elevated stress, is sumoylated upon heat stress and this sumoylation correlates with a localization of HSF1 with nuclear granules (Hong *et al.*, 2001). In *Arabidopsis* HSFA2 is targeted for sumoylation during extensive exposure to heat stress and during recovery (Cohen-Peer *et*

*al.*, 2010). In this case, sumoylation negative regulation of sumoylation regulates the activity of this transcription factor is regulated by negative regulation of sumoylation . Another *Arabidopsis* transcription factor, c-Myb, is modified by SUMO in response to various stress conditions, including heat stress, although the role of this modification is still unclear (Sramko *et al.*, 2006). Several proteomic studies have attempted to identify what proteins globally are changed in response to various stress treatments. One of the most comprehensive and quantitative reports to date identified several hundred proteins that changed sumoylation state in response to heat stress (Golebiowski *et al.*, 2009). Proteins directly involved in stress response, such as HSF1 and HSP proteins (HSP40, -60, and -70), were identified as SUMO targets in response to heat stress (Golebiowski *et al.*, 2009). Proteins involved in transcription, translation, DNA repair, and cell cycle regulation are also frequently identified targets for sumoylation in response to heat stress (Zhou *et al.*, 2004; Golebiowski *et al.*, 2009; Miller *et al.*, 2010; Bruderer *et al.*, 2011). Around 31 sumoylated proteins has been identified in *C. albicans* as sumoylated proteins in response to stress (e.g., Hsp60, Hsp70 family members, Hsp104), the cytoskeleton and polarized growth (e.g., Cct7, Tub1,Mlc1), secretion, and endocytosis (e.g., Lsp1, Sec24, Sec7) (Leach *et al.*, 2010).

## 2.15 Role of SUMO in mitosis

The linkage between sumoylation and mitosis was established earlier even before the discovery of SUMO protein; it was well known that Ubc9p is essential for degradation of B-type Cyclins in budding yeast (Seufert *et al.*, 1995), which is a key mitotic regulator that are degraded via ubiquitination at anaphase onset. Moreover, SMT3 was isolated in yeast screens for temperature-sensitive mutants which were defective in chromosome segregation (Biggins *et al.*, 2001), and for suppressors of mutations of *mif2*, a homologue of the vertebrate centromeric CENP-C protein (Meluh and Koshland, 1995). *S. pombe* strains lacking SUMO conjugation is viable, slow growing, and sensitive to DNA damaging agents, have a high frequency of chromosome loss and defective mitosis, and develop elongated telomeres (Tanaka *et al.*, 1999; Khodairy *et al.*, 1995; Shayeghi *et al.*, 1997). Further, *dpias* gene was isolated as a suppressor of position effect variegation, an effect in which transcriptional silencing of adjacent loci was induced by heterochromatin in the *D. melanogaster* (Hari, 2001). *dpias* mutants also have chromosome condensation defects, chromosome segregation defects, high frequency of chromosome loss, and defects in telomere clustering (Hari, 2001). The *S. cerevisiae ulp2* mutant also has a number of phenotypes showing genomic instability and also defective in targeting the condensin complex, which is required for

chromosome condensation, to rDNA repeats (Strunnikov *et al.*, 2001; Schwienhorst *et al*, 2000; Li, 2000).

A number of lines of evidence implicate a role of SUMO in kinetochore function. Overexpression of SUMO suppresses CENP-C mutant phenotype (Fukagawa *et al.*, 2001). Additionally, SUMO localizes to the kinetochore in mammalian cells, and associated proteins with both centromeres and PML, emerging the possibility of a SUMO-related mechanism (Pluta *et al.*, 1998, Joseph *et al.*, 2002). The well characterized centromere function includes *S. cerevisiae* strains lacking the SUMO isopeptidase Ulp2, which exhibit separation of chromosome near the centromere prior to mitosis (Bachant J *et al.*, 2002). The *ulp2* mutant strains contain elevated levels of sumoylated topoisomerase II (Top2), and the chromosome separation phenotype was suppressed by mutating the SUMO attachment sites in Top2 and temperature sensitivity of *ulp2* mutants, indicating that these phenotypes resulted from excess SUMO conjugation to Top2. In *C. albicans*, many daughter cells remained joined to their mother cell and some were multinucleate suggesting the cell cycle defects. Furthermore, the growth and cell division related sumoylation targets were identified (Leach MD *et al.*, 2011). The deletion of sumoylation sites of Construction triple mutant of Cdc3p, Cdc11p and Shs1p abolished almost all mitotic sumoylation at the bud neck, and dramatically decreased the overall level of sumoylation within

G2/M phase budding yeast cells. This triple mutant failed to correctly disassemble of septin filaments, and thus retained persistent septin rings from prior divisions. This phenotype showed that SUMO conjugation is important for septin ring dynamics during the cell cycle. In addition to these drastic changes in sumoylation patterns, however, the triple sumoylation mutant grew without defect, with no sensitivity to stress conditions, and did not show the phenotype of *uba2-ts10* strain (Johnson and Blobel, 1999). The septins expressed during vegetative growth, Cdc10p (Panse VG *et al.*, 2004) and Cdc12p (Denison C *et al.*, 2005), have consequently been found as sumoylation targets in proteomic analysis. It is possible that decreased level of mitotic sumoylation of septins may have compensated for the absence of Cdc3p, Cdc11p and Shs1p modification in the triple mutant. This might be consistent with the fact that absence of any overt cell cycle defects in the triple mutants cells.

### 2.16 Cross-talk between sumoylation and other post-translational modifications

Protein functions are not regulated separately by different PTMs, but rather by an intricate crosstalk, in which PTMs may have an agonistic or antagonistic effect on each other (Hunter, 2007). There appears to be significant cross-talk and overlap between sumoylation and other post-translational modifications, particularly phosphorylation. The sumoylation of IκBα occurs on the same lysine residue as

targeted for ubiquitination and acts to prevent ubiquitination of this protein (Desterro *et al.*, 1998). In addition, IκBα is also a substrate for phosphorylation and this modification appears to act to control whether or not IκBα is sumoylated or ubiquitinated. Phosphorylation of IκBα marks it for ubiquitination, and phosphorylated IκBα cannot be sumoylated (Desterro *et al.*, 1998). Negative regulation of sumoylation by phosphorylation occurs for other proteins as well, as phosphorylation appears to negatively regulate the sumoylation of PML (Muller *et al.*, 1998). The MEK1 protein from *D. discoideum* that is translocated from the nucleus to the plasma membrane upon sumoylation is both itself a protein kinase and a substrate for phosphorylation. It was shown that phosphorylation of MEK1 regulates sumoylation, as a non-phosphorylatable mutant cannot be sumoylated. Furthermore, a constitutively active mutant in which the phosphorylation sites were mutated to glutamate residues was constitutively sumoylated (Sobko *et al.*, 2002). An additional case of the cross-talk between SUMO and phosphorylation is the phosphorylation-dependent sumoylation motif (PDSM) motif which is present in heat shock factor-1 among others, consists of the classical consensus motif followed by a phosphorylated serine and proline: ΨKXEXXpSP (Hietakangas *et al.*, 2006). However, there are other examples of phosphorylated residues which enhances sumoylation of a target protein that do not belong to the PDSM motif (Vanhatupa *et al.*, 2008), indicating that,

phosphorylation can act to both positively and negatively regulate sumoylation of proteins, depending on the target protein.

Sumoylation regulates multiple biological processes including regulation of growth, cell cycle, nuclear transport, genome integrity, stress resistance, signal transduction by modifying a variety of different substrates in eukaryotic cells. It is not completely clear how sumoylation affects the various cellular processes and its effects on the cell and its virulence. The consequences of sumoylation are unknown in plant pathogen. This reasoned the sumoylation may be imperative in different biological processes that are critical for the pathogenicity of *M. oryzae*.

In this study, attempts were made to characterize SUMO in *Magnaporthe oryzae* in a global manner by combination of molecular and proteomic approach particularly to know the contribution of SUMO in development and pathogenicity of rice blast fungus. The *in silico* analysis of MoSUMO predicted 6,000 sumoylation sites for 4,494 protein sequencesat a high cut-off value using GROMO database in *M. oryzae* (Thakur *et al.*,2009). Since MoSUMO contain single small ubiquitin like modifier domain, the investigation of ubiquitin domain containing proteins revealed that *M. oryzae* contain 12 ubiquitin related proteins (Oh *et al*, 2012). The UEP1 and UEP3

were expressed abundantly during plant colonization in host tissue but were down regulated by starvation stress whereas PUB4 was highly expressed after starvation stress (Oh *et al*, 2012; McCafferty and Talbot, 1998). Subsequently, polyubiquitin found a role in growth, conidiation, appressorium formation, virulence and sexual development of *M. oryzae* (Oh *et al*., 2012).