3. MATERIALS AND METHODS

3.1 Media and culture conditions

All the fungal cultures were maintained on YEG medium (g/l in H_2O, Yeast extract, 2g; Glucose, 10g) and Oatmeal agar (36.5g/l) (Hi-Media, Mumbai, India) at 28^0C. For genetic manipulation fungal cultures were grown broth of complete medium (CM) (g/l in H_2O, Glucose, 10g; Peptone, 5g; Yeast extract, 1g; Casein hydrolysate, 1g; $NaNO_3$, 0.6g; KCL, 0.5g; $MgSO_4$, 0.5g; KH_2PO_4, 1.5g; pH 6.5) The yeast strains were maintained on YEPD medium (g/l in H_2O, Yeast extract, 5g; Peptone, 10g, Glucose, 10g) and for genetic manipulations, synthetic dropout (SD) medium (YNB without amino acids 6.7g/l, Glucose, 20g/l in H_2O) was used.

3.2 Bacterial strains

Escherichia coli DH5α (F^-, *end*A1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *thi*-1, λ^-, *rec*A1, *gyr*A96, relA1 Δ(*lacZYA-arg*F) U169 *deo*R (ϕ80d *lac*Δ (lacz) M15; Bethesda Research Laboratories) was used for bacterial transformation and plasmid propagation. *Escherichia coli* BL21 (DE3) pLysS strain *E. coli* (F^-, *dcm ompT hsdS* (r_B^- m_B^-) *gal* λ (DE3) (pLysS Camr) was used for heterologous protein expression. The *E. coli* strains were grown at 37°C 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 or 50 μg/ml, respectively, as required. *E. coli* BL21 (DE3) strain requires chloramphenicol at a

concentration of 34 μg/ml to maintain pLysS along with the required antibiotic. Bacterial strains were maintained at 4°C as slants or stab cultures on LB agar medium. Long-term preservation was under 25% glycerol solution at -70°C.

3.3 Yeast strains

All the yeast strains are listed in Table 1.A. The yeast strains were grown at 22°C and 37°C as per the requirement of strain and maintained on YEPD medium. Ampicillin was added to the autoclaved medium to avoid bacterial contamination at final concentration of 200 μg/ml. The yeast *smt3* mutant SBY331 and SBY214 of *Saccharomyces cerevisiae* were kind gift from Sue Biggins, Division of Basic Science, Fred Hutchinson Cancer Research Center, Seattle, WA.

3.4 Fungal cultures

Magnaporthe oryzae B157, belonging to the international race IC9 was isolated in our laboratory from infected rice leaves (Kachroo et al., 1994). The fungus was grown on either YEG agar or Oat meal agar, at 28-30°C. Fungal mycelia harvested from 2 days old cultures shaken at 150 rpm in CM broth were used for isolation of genomic DNA and protoplasts transformation was performed as described (Shi et al., 1995). Fungal conidia were harvested by scraping the biomass grown on Oatmeal agar plates with a sterile surgical blade, resuspended in sterile water, and purified by passing through glass wool column. The different transformants of

fungi are mentioned in Table 1B and maintained as monoconidial isolates and stored as 25% spore glycerol stocks at -70°C.

3.5 Competent cell preparation and transformation of *E. coli* with plasmid DNA

Single colony of *E. coli* DH5α was inoculated in 3 ml of Luria-Bertani broth and grown overnight. Hundred ml of fresh LB broth was inoculated with 1 ml of overnight grown culture and grown at 37°C up to 0.3 to 0.4 $O.D_{550}$; chilled on ice and the cells were pelleted by centrifugation at 2012 x g for 5 min at 4°C. The cells were resuspended in ice-cold 0.1 M $CaCl_2$ and incubated for 30 min. The cells were reharvested by centrifugation and resuspended in ice-cold 0.1 M $CaCl_2$ with 20% glycerol. The culture was stored at -70°C in 100µl aliquots. For transformation, a 0.2 ml aliquot of this cell suspension was transferred to a 1.5 ml chilled microfuge tube; mixed with the plasmid DNA (usually 50 ng) or ligation mixture and incubated at 4°C for 30 min. Cells were subjected to heat shock at 42°C for 90 sec, followed by 5 min. incubation on ice. One ml of LB broth was added to the above suspension and incubated at 37°C for 30 min. About 0.1 ml aliquot of transformation mixture was plated on LB agar medium containing either ampicillin (100 µg/ml) or kanamycin (50 µg/ml).

Table 1. Strains used in present study. A. yeast strains and B. fungal strains

A.

Alias (Haploids)	Genotype Description	References
SBY214 (Wild-type)	*MATa ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ*	Biggins et al. (2001)
SBY331 (*smt3-331*)	*MATa ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ smt3-331*	Biggins et al. (2001)
SBY331-pYes-MoSUMO	pYes2-MoSUMO transformant and complementation strain of *smt3-331*	Present work
SBY331-pYes-MoSUMO*gg*	pYes2-*MoSUMOgg* transformant and diglycine mutant of *MoSUMO* strain of SBY214	Present work

B.

Fungal Strains	Genotype Description	References
B157 (WT)	Wild Type	This laboratory (Kachroo et al.,1994, 1995)
ΔMosumo	*MoSUMO* deletion mutant of B157	Present work
ΔMosumo/*MoSUMO* Strain	*ΔMosumo/MoSUMO* Complementation strain of *sumo*	Present work
GFP::SUMO Strain	Localization transformant of MoSUMO	Present work

Table 2. Primers used in present study

Name	Oligonucleotide Sequences
MoSF	CCCTCGAGGGATGATGGATCGCGAGAACGCGGCAG
MoSR	GAAGATCTTCTTAAGCACCACCACCAACCTGCTC
MoSumoFH	AAGCTTATAATGTCGGATCGCGAGAACGGCGCCA
MoSumoAAR	GAATTCAGCAGCAGCAACCTGCT
MoS5F	GGTACC AGGATGACAAGGTGGATCAGT
MoS5R	CCCGGGTTGACGGTGCAGGATTCAGAGG
MoS3F	TGTGAATTCCGAGACATTCAGTCAGTTTGG
MoS3R	TGCCTCGAGTTGTTGGCCGTAGTGGTGGTTT
HPR	TGACATCGACACCAACGATCT
PTF	TGTCGAGAAGTTTCTGATCGA
MoS5NR	GTCGCCATGGTATGGAAGTGGATTGTCTCG
MoS3HF	TGCAAGCTTTTTACGAGACATTCAGTC
MoS3KR	ACTGGTACCTTGTTGGCCGTAGTGGTG
TSF	AGACCATGGACTACAAGGACCACGACGGTGACTATAAGGACCA
	CGACATCGACTACAAGGACGACGACGACAAGATCCACCATCAT
	CATCATCATATGTCGGATCGCGAGAAC
TSR	GATCGAATTCTTAAGCACCACCAACCTGCT
SGF	TCGCATATGTCGGATCGCGAGAAC
GFPF	CCACCATGGTGAGCAAGGGCGAGGAGC
GFPR	ACTCATATGCTTGTACAGCTCGTCCAT
GXSumoF	CGAGGGATCCATGTCGGATCGCGAGAACGGC
GXSumoR	CGATCTTAAGTTAAGCACCACCAACCTGCTCCT

3.6 Transformation of *S. cerevisiae* with plasmid DNA

Yeast strain SBY331, was transformed with the plasmid or yeast expression construct by 'One-step transformation' method (Chen et al., 1992). The yeast culture was spread on a YEPD plate and incubated for 20-24 h. A small part of the growing culture was transferred using a sterile toothpick to a microfuge tube containing 100 µl of 45% PEG 4000 (solution in 0.1 M Lithium acetate, ph. 6.0, 0.1 M DTT), 5 µl of 10 µg/ml of calf thymus DNA as carrier, which was sheared to give molecules in the 500 bp range. One µg of transforming DNA was added to each tube. The whole mixture was thoroughly vortexed and incubated overnight instead of heat shock at 42^0C for 1h as SBY331 was temperature sensitive strain. The cells were then gently spread on pre dried SD drop out medium plates with required amino acids.

3.7 Isolation of plasmid DNA

Overnight grown culture (1.5 ml) was centrifuged in a microfuge tube and the bacterial pellet was resuspended in 200 µl of STET (8% Sucrose, 5% TritonX-100, 50 mM Tris pH 8.0, 50 mM EDTA pH 8.0). Lysozyme (25 µl of 10 mg/ml) was added to it, mixed by vortexing and boiled in a water bath for 45 sec followed by centrifugation at 13414 x g for 10 min. The resulting snot was removed with a tooth pick and 20 µl of 5% CTAB was added followed by centrifugation for 5 min. To the pellet, 300 µl of 1.2 M NaCl was and 750 µl of 100% ethanol was added.

The DNA pellet was obtained by centrifugation for 10 min; washed with 70% ethanol and resuspended in 50 µl of TE (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0).

3.8 Nucleic acid manipulations

Restriction enzyme digestion of DNA was carried out in reaction buffers, supplied with the corresponding enzymes, as recommended by the suppliers. Blunt end ligations were carried out at 20°C, overnight, and sticky end fragments were ligated at 16°C for 3-4 h.

3.9 Blastp, multiple sequence alignment and phylogram generation

The Blastp search tool was used against *Magnaporthe oryzae* genome database (http://www.broad.mit.edu) using corresponding SMT3 protein from *Saccharomyces cerevisiae* as query sequence. The assignment of orthology was based on the number of the E-values in the BLAST output (E value-$1e^{-20}$). In the presence of key amino acid residues of MoSUMO along with the SUMO of *Homo sapiens, Arabidopsis thaliana* and yeasts like *S.cerevisiae, Schizosaccharomyces pombe, Candida albicans* and some ascomycetes such as *Neurospora crassa*, *Colleotrichum fioriniae, Fusarium graminearum were* aligned using MUSCLE program and the phylogenetic tree was constructed using MEGA6 program (Tamura et al., 2013). Sumoylation site prediction was carried out using SUMOsp 2.0 program (Xue et al., 2006) as well as GROMO database (Thakur et al., 2009).

3.10 Protoplast transformation

The protoplast transformation protocol was same as described (Leung et al., 1990) and (Shi et al., 1995) with minor modification. In brief, protoplast were prepared by digesting mycelial blocks (2 days plod culture, grown in CM) in 30 ml of 1M Sorbitol containing lysing enzyme (30mg/ml) for 4h at 80 rpm. Protoplast were washed and maintained in STC buffer containing 20% sucrose, 25 mM Tris-HCl pH 7.5, 25mM $CaCl_2$ until transformation. 1 µg of linear DNA was added to 200 µl of protoplast suspension ($1X10^8$ protoplast/ml) in 50 ml centrifuge tube, placed on ice for 20 min. After 20 min incubation, 1ml of PTC buffer (PEG 3350 60% in 25mM $CaCl_2$ was added in DNA suspension and mixed gently followed by incubation at room temperature for 30 min. The protoplast were regenerated in 3ml regeneration medium (CM broth containing 1M Sorbitol) and incubated at 28^0C with 80 rpm shaking for12-14h. The protoplast suspension was mixed with 6 ml of top agar (CM containing 1% agarose and required antibiotic for selection) and poured immediately on predried CM plates supplemented with preferred antibiotic used for selection. Plates were incubated for 3 days at 28^0C. The transformants were selected after monoconidial isolation.

3.11 Generation of yeast expression vectors of *MoSUMO* and mutated diglycine *MoSUMOgg* for expression study in *S. cerevisiae*

Inter-species genetic complementation was carried out by expressing the *MoSUMO* in *smt3 (sumo)* mutant of *S.cerevisiae*. Primers are listed in Table 2. A 350bp ORF of *MoSUMO* gene was amplified from cDNA of *M. oryzae* using MoSF and MoSR primers and cloned at *Xho*I and *Xba*I site of pYES2.0 yeast expression vector under the GAL1 inducible promoter which was induced by 2% galactose, comprising uracil as auxotrophic marker resulted in pYes-MoSUMO clone. The diglycine motif GG of MoSUMO was mutated by site directed mutagenesis approach. For this, a 330bp of *MoSUMO* ORF was amplified from cDNA using MoSumoHF and MoSumoAAR primers, cloned at *Hind*III-*EcoR*I site of pYES2.0 vector. The resulting vectors pYes-MoSUMO and pYes-MoSUMOgg were confirmed by DNA sequence analysis and introduced into the yeast *smt3* mutant. Putative transformants were selected on drop out media containing 2% galactose, without uracil. Transformants were confirmed with PCR using gene specific primer. To visualize sister chromatids, copper sulfate was added to media at a final concentration of 0.25 mg/ml to induce the GFP-lacI fusion protein which is under the control of the copper promoter. Growth conditions of yeast were performed as described (Biggins et al., 2001). Complementation of *MoSUMO* in *S. cerevisiae smt3* mutant strain was assessed by observing the ability of transformants to

restore growth of *smt3* mutant strain at 37^{0}C and defect of chromosome segregation in *smt3* mutant strain.

3.12 Generation of knock-out, complementation and localization construct of *MoSUMO*

Targeted gene deletion or knock-out construct was generated using split marker strategy (Catlett et al., 2002) (Figure 4). In brief, 1.2kb 5' flanking (5'F) and 1.1kb 3'flanking region (3'F) were amplified using MoS5F/MoS5R and MoS3F/MoS3R primer set respectively and hygromycin phosphotransferase (HPT) gene with TrpC promoter and TrpT terminator were amplified, cloned separately in pBluescript KS+ at *Eco*RV site, resulted in KS-5'F, KS-3'F and KS-HPT subclones (Figure 9A,B,C). A *Bam*HI-*Bgl*II ˜850bp released fragment from KS-5'F was subcloned in KS-HPT at *Bam*HI site in sense orientation resulted in KS-5'F-HPT (Figure 9D). Similarly, *Xho*I digested 1.1kb fragment from KS-3'F was subcloned in KS-HPT at *Xho*I site in sense orientation resulted in KS-HPT-3'F (Figure 9E). A 1.9 kb ligated fragment of 5'F and HPT was amplified using MoS5F/HPR primers from KS-5'F-HPT clone and 2kb ligated fragment of HPT and 3'F was amplified using PTF/MoS3'F. These two fused PCR products had 320bp of overlapping region of HPT gene (Figure 4 and 9F). 1µg of these PCR products were mixed in 1:1 ratio and *M. oryzae* B157 was transformed with it by protoplast transformation. The resulting transformants were selected on CM media

containing 250 µg/mL hygromycin B (Sigma Chemical, St. Louis, MO, USA). The hygromycin resistant transformants were screened with PCR using *MoSUMO* gene specific primers and β-tubulin gene amplification was used for genomic DNA control. Positive transformants were further confirmed with Southern blot hybridization using different a, b and c probes and referred as *ΔMosumo* mutants.

The complementation construct for *MoSUMO* was generated under the control of native promoter. A 1.2 kb promoter region (amplified using MoS5F/MoS5NR) and 1.1kb 3'flanking region (amplified using MoS3HF/MoS3KR) of gene subcloned in pBluescript KS+ named as KS-PRT and KS-3RT, respectively. To generate the complementation construct, a 535bp SUMO coding region was amplified with TSF/TSR primers and ligated to *Sac*I-*Nco*I ~800 bp digested fragment of KS-PRT. The TAP sequence which consists of 3XFLAG and 6XHIS was inserted in TSF primer. The fused product (Promoter-*MoSUMO*) was cloned in KS-Zeocin vector which was developed by cloning zeocin gene at *Eco*RV site of pBluescript KS+ resulted in KS-Prom-TAP-SUMO-ZEO construct. Similarly, ~800bp *Sal*I digested fragment from KS-3RT was cloned downstream of KS-Prom-SUMO-ZEO at *Sal*I site in sense orientation resulted in complementation construct KS-Prom-SUMO-ZEO-3'F. This complementation construct was transformed in *ΔMosumo* mutant strain of *M. oryzae* so that selection of transformant could be easy (Figure 5). The positive complemented transformants were screened on CM plates containing 200

µg/ml zeocin (Invitrogen Life Technologies, California, USA) as well as hygromycin, separately. Transformants were selected on the basis of resistance towards zeocin and sensitivity to hygromycin which further confirmed the replacement of hygromycin cassette with TAP-SUMO-zeocin cassette by Southern blot analysis using different probes.

The localization construct of MoSUMO was generated using GFP as reporter gene. For GFP-*MoSUMO* fusion, *MoSUMO* gene was amplified with primer SGF/TSR and GFP gene was amplified with GFPF/GFPR primers from ZsGreen vector (Invitrogen, Life Technologies, California, USA). The GFP was fused at the N-terminus of MoSUMO. The GFP and *MoSUMO* genes were ligated and cloned in complementation construct in such a way that TAP-*MoSUMO* was replaced with GFP-*MoSUMO* fused product, having zeocin gene as selection marker. The wild type B157 strain was transformed with GFP-*MoSUMO* fusion construct by protoplast transformation. The transformants were selected on CM media supplemented with 200 µg/mL zeocin and confirmed the number of integration in genomic DNA of transformants with Southern blot analysis.

3.13 DNA extraction and Southern blot analysis

Total DNA from *M. oryzae* was extracted as described (Dellaporta et al., 1983). Approximately 5g biomass was taken from culture grown in CM for 3-4 days, frozen in liquid nitrogen and ground into a fine powder in a pre chilled mortar

and pestle. The powder was transferred to a 50 ml centrifuge tube containing 15 ml of extraction buffer (0.1 M Tris, pH 8.0; 0.05 M EDTA, pH 8.0; 0.5 M NaCl; 0.01 M β-mercaptoethanol). To this mixture, 1 ml of 20 % SDS was added, mixed and the tubes were incubated at 65°C for 10 min. Thereafter, 5 ml of 5 M potassium acetate was added, the tubes were shaken gently and were incubated on ice for 20 min followed by centrifugation at 2012 x g for 30 min. The supernatant was collected in a clean 50 ml centrifuge tube containing 10 ml of isopropanol, mixed and the tubes were incubated at -20°C for 30 min. The DNA was pelleted down at 2012 x g for 30 min

and the supernatant was gently poured off. Remaining liquid was removed by keeping the tubes inverted and the pellets were then air dried. The DNA was redissolved in 700 µl of sterile distilled water, transferred to 1.5 ml microfuge tube and centrifuged to remove all the insoluble debris. Five µl of RNase (10 mg/ml) was added to the DNA solution and was incubated at 68°C for 10 min. Further, 400 µl of chloroform: isoamylalcohol (24: 1) was added to these tubes, mixed well and spun for 1 min in microcentrifuge. The top aqueous phase was transferred to another 1.5 ml tube. To this solution, 75 µl of 3 M sodium acetate, pH 5.2, and 500 µl of isopropanol was added, mixed well, and DNA was pelleted by centrifugation in a microfuge. The pellet was washed with 70 % ethanol, dried and redissolved in 100 µl of sterile distilled water.

Figure 4: Schematic representation of split-marker system

Substitution of a wild-type *MoSUMO* gene is only feasible when three cross over events occur to generate a functional marker gene HPT. This one is flanked by homologous gene sequences (5'F and 3'F) that allow homologous recombination between genomic DNA and the split-marker DNAs to substitute the *MoSUMO* gene by the selectable marker.

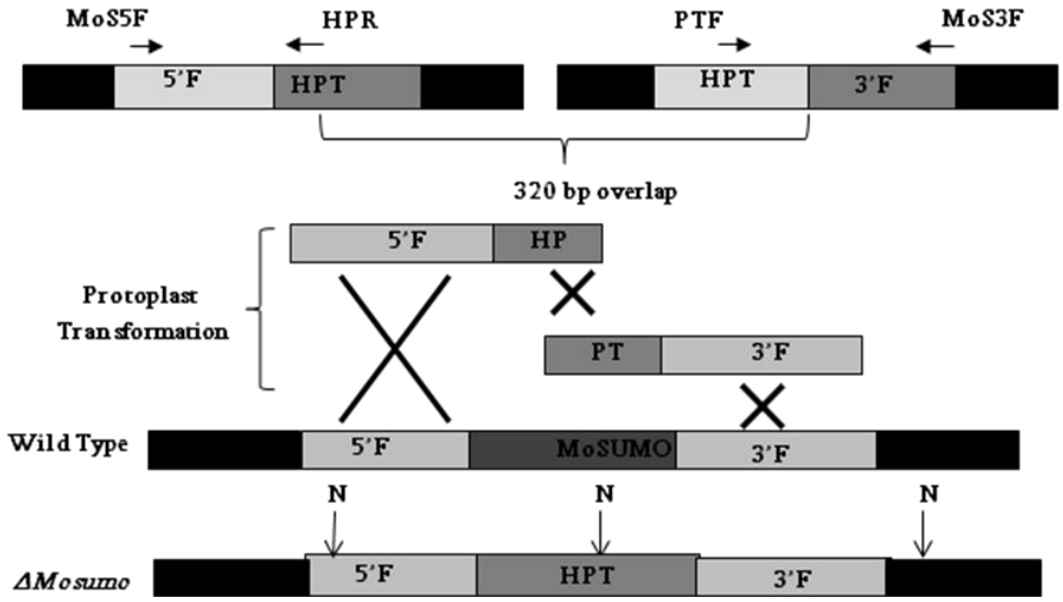
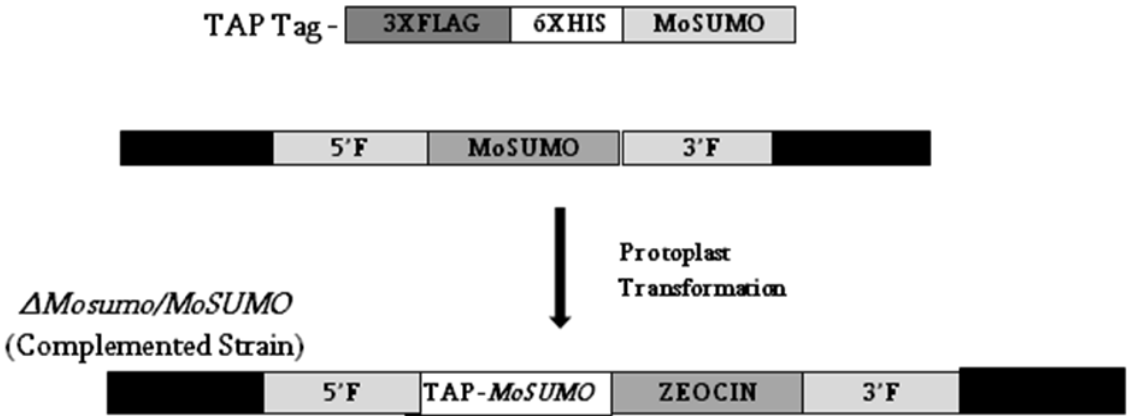

MoS5F
HPR
PTF
MoS3F
5'F
HPT
HPT
3'F
320 bp overlap
5'F
HP
Protoplast
Transformation
PT
3'F
Wild Type
5'F
MoSUMO
3'F
N
N
N
ΔMosumo
5'F
HPT
3'F

Figure 5: Scheme to illustrate strategy for complementation construct

Substitution of a HPT gene with TAP-*MoSUMO*-Zeocin constructs, flanked by 5'flanking (5'F) and 3'flanking (3'F) sequence which facilitate the homologous recombination between genomic DNA of *ΔMosumo* mutant strain.


TAP Tag -
3XFLAG
6XHIS
MoSUMO
5'F
MoSUMO
3'F
Protoplast
Transformation
ΔMosumo/MoSUMO
(Complemented Strain)
5'F
TAP-MoSUMO
ZEOCIN
3'F

Southern blot was prepared as described (Sambrook et al., 1989). The genomic DNA (10 µg) from untransformed and different transformants was digested with restriction enzymes as recommend by the supplier; electrophoresed on 0.8% agarose gels and blotted onto nylon membrane (Hybond N^+, Amersham Biosciences, Buckinghamshire, England). DNA was fixed to the membranes by UV crosslinking (12 x 10^4 µJ/cm^2) using UV cross-linker (Spetrolinker, Spectronics Corporation, USA).

Hybridisation probe was labeled nonradioactively using Gene Images AlkPhos Direct Labelling and Detection System as per manufacturer's instructions (Amersham Biosciences, Buckinghamshire, England). Filters were exposed to X-ray films with intensifying screen for appropriate time at -70°C. Standard procedures were followed for autoradiography.

3.14 Total RNA extraction and RT-PCR (Reverse Transcriptase Polymerase Chain Reaction)

Fungal biomass grown in liquid media was frozen in liquid nitrogen. Total RNA was isolated using TRIZOL reagent (Invitrogen Life Technologies, California, USA). The quality of isolated RNA was checked by electrophoresis on formaldehyde gels and quantified by UV spectrophotometry. 2 µg of total RNA was used to synthesize the first strand cDNA using MuMLV reverse transcriptase

(Fermentas GmBH, St. Leon-Rot, Germany) and oligo $(dT)_{12}$ in 20 µl reaction system. Five microlitre of this RT product was used to carry out the RT-PCR.

3.15 Heterologous expression of recombinant MoSUMO Protein in *E. coli*

A 443bp ORF of *MoSUMO* gene was amplified using GXSumoF/GXSumoR from cDNA and cloned at *Bam*HI/*Eco*RI site of pGEX-3X vector (Amersham Bioscience, USA) to obtain pGEX-MoSUMO. The GST tag was in frame to MoSUMO, separated by Factor Xa cleavage site, resulted in pGEX-*MoSUMO* clone. This plasmid was transformed into *Escherichia coli* BL21 (DE3) pLysS. The recombinant GST-MoSUMO fusion protein was extracted and purified using glutathione sepharose 4B (GE Healthcare) according to the manufacturer's guideline. The purified GST-MoSUMO fusion protein was cleaved with Factor Xa (Merck) and MoSUMO protein was purified again with glutathione sepharose 4B.

3.16 Production of polyclonal antibodies against MoSUMO

To generate the antibody against MoSUMO protein, 100 µg of purified MoSUMO protein was injected in New Zealand White Rabbit along with equal volume of Freund's Complete adjuent. Subsequently, two booster doses (50 µg) were given to the rabbit with Freund's Incomplete adjuent after 2-3 weeks interval. After 7 days, the rabbit was bled and antiserum was collected. The serum was separated carefully and centrifuged at 10,000 rpm for 10 min at 4^0C. The antibody IgG was

purified with Protein A sepharose CL-4B (GE Healthcare Life sciences) by affinity purification according to the manufacturer's instructions and checked for the titer by ELISA as well as with western blot.

3.17 Protein extraction and western hybridization analysis

The vegetative hyphae of B157 were frozen in liquid N2. Frozen tissue was ground in equal volumes (w/V) of PBS buffer (pH-7.4) containing 140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4 and centrifuged at 13,000g and 4°C for 30 min to pellet debris. Supernatant was diluted 1:1 in 23 Laemmli buffer containing 2.5% β-mercaptoethanol, heated to 95°C for 5 min, and separated by 12% SDS-PAGE. Protein gel was transferred onto PVDF membrane (Hybond-P, GE Healthcare lifesciences) with a Banglore Genei electroblotting apparatus. Ponceau S-stained bands were used as loading standards for the samples. Anti-MoSUMO antibodies (Rabbit) (1:3000) raised against purified MoSUMO protein was used for the immunodetection of MoSUMO and sumoylated proteins. The Anti-rabbit IgG, horseradish peroxidase conjugate produced in goat (Sigma Chemicals, MO, USA) was used as secondary antibody. Immunoblots was developed with super Signal West Pico chemiluminescent substrate (Pierce, Rockford, USA).

3.18 Immunostaining

Immunostaining was performed with purified polyclonal MoSUMO antibody in hyphae and conidia. The samples were fixed with 10% formaldehyde, 5% acetic acid, and 85% ethanol for 30 min at room temperature and incubated in 0.1% toluidine blue for several hours to overnight. The fixed slides were further treated as described (Patkar and Chattoo, 2006). TRITC conjugated seconday antibody was as secondary antibody and observed with microscopy.

3.19 Assays of growth, conidiation and appressorium development

For evaluating the growth of wild type and transformants, mycelial blocks (5 mm) of culture were grown on OMA and growth diameter was measured up to 10 days. Conidia were collected from 7-8 days old OMA plates with 1 ml of distilled water and counted using a haemocytometer. Conidia harvested from 8-d-old cultures were resuspended to 1×10^4 conidia/ml in water and kept on hydrophobic coverslip under humid environment at 28^0C for 12h. To develop appressorial structure from hyphae, hyphal blocks from 7 days old OMA cultures were placed on artificial hydrophobic surface and incubated within a moist chamber at 28^0C for 48h followed by microscopy. Each experiment was repeated at least three times.

3.20 Statistical analysis

Data are shown as mean ± SEM for no. of times experiment was repeated. Comparisons of groups were performed using student t-test for repeated measurements to determine the levels of significance for each group. The number of germinated conidia was counted and analyzed using one way ANOVA. The experiments were performed minimum three times independently and $p < 0.05$ was considered as statistically significant. GraphPad Prism was used to perform all the statistical analysis.

3.21 Plant infection assay and penetration assay

In planta infection assays was carried out as described (Bonman and Mackill, 1988). Conidial suspension (1×10^5/ ml in 0.2% gelatin) of wild type and transformants were sprayed with an artist's airbrush onto the 21 days old rice cultivar CO39. Inoculated plants were placed under controlled-environment chambers (light 14 h at 25°C and dark 10 h at 20°C and 90% relative humidity) 6 days for full symptoms to become apparent. The disease severity was assessed as described (Yara et al., 2008) at 6 days after leaf inoculation for 3-4 leaves per assay. For drop-inoculation assays with detached barley leaves, conidia were diluted to 10^5/ml. A 15 µl drop of diluted conidia was pipetted onto rice leaves placed over 2% (w/v) water agar containing kinetin (2mg/ml) and kept in a sealed moisture chamber. Symptom development was examined after incubating at 28^0C (12 h

light/12 h dark) for 5 dpi. The invasive hyphae of *M. oryzae* were observed at 12hpi with aniline blue (0.05%) staining in host tissue.

3.22 Microscopy Methods

All fungal images of hyphal growth, mature conidia, and developmental stages of appressorium were recorded with 63 X Zeiss LSM 700 confocal laser microscope systems (Carl Zeiss, LSM 700, Germany). The microscopic observation of invasive hyphae and appressorial development from hyphae were examined with 20X Olympus BX51 fluorescent microscopes by epifluorescent illumination. Slides were prepared by sealing the cover slip with adhered conidia. Image analysis was performed using the ZEN image software (Zeiss). Calcofluor white (CFW) (10 µg/ml) (Sigma Chemical, MO, USA) was used to stain chitin. Analysis of sister chromatids was performed as described (Biggins et al., 2001). 4', 6-diamidino-2-phenylindole (DAPI) (Sigma Chemical, MO, USA) was used at 1 µg/ml final concentration and aniline blue (Hi-Media, Mumbai, India) (0.05%) was used to stain invasive hyphae.

3.23 Two dimensional polyacrylamide gel electrophoresis (2DGE)

Mycelial proteins were disrupted using liquid nitrogen in UTE buffer (7M Urea, 2M Thiourea, 1% Triton X-100, 2% DTT, 4% CHAPS, 2% IPG buffer (3-10 pH), 1mM EDTA and 1X protease inhibitor cocktail) and incubated on ice for 1 h. After

repeated vortexing, cell debris were removed by centrifugation (20 min, 13,000 rpm, 4°C), and protein extracts were stored at –20°C. Multiphor II (Amersham Pharmacia Biotech Uppsala, Sweden) was used for isoelectric focusing with the immobilized pH gradient (IPG) strips (pH 3-10, non-linear gradient, 18 cm, Amersham Pharmacia Biotech). The IPG strips were rehydrated with 350 µl of protein sample (2mg) for 10h at room temperature using homemade Immobiline dry strip IEF focusing tray. 3ml of mineral oil was added on to the IPG strips to avoid evaporation. The IEF tray was covered with parafilm and incubated overnight for rehydration of IPG strips.

In gel, rehydration of the strips was used for loading of the sampkes on IPG strips. After complete rehydration, excess mineral oil was drained and strips were placed aligned in the grooves of a clear sheet in the immobiline strip tray, adjacent to each other. Filter paper wicks were hydrated in distilled water, dabbed to remove excess water, cut to size and placed at the two ends of the aligned strips. The electrodes are then placed in position over the paper wicks. Dry strip cover fluid was then added to prevent evaporation during the focusing and dissipate heat produced during the run. The temperature of the unit was maintained by circulating water at 20^0C. The voltage settings for isoelectric focusing were 50V for 1h, 500V for 3h, and 3500V for 24h, to a total of 86 KVh for a pH gradient 3-10 non-linear strip.

Equilibration and second dimension

After the first dimensional separation, the strips were incubated in equilibration buffer (50mM Tris-HCl, pH 6.8, 6M urea, 30%v/v glycerol, 2% w/v SDS, 20mM DTT) for 15 min followed by an additional 15 min incubation using equilibration buffer with 0.2M iodoacetamide instead of DTT. Two dimensional seperations were performed using 12% polyacrylamide gels according to Laemmli. The gels were cast (gel size: 140 X 150 X 1.5mm) and a filter paper label was placed at the corner of cathodic end. Electrophoresis was carried out at room temperature using Biorad vertical electrophoresis unit, at a constant current of 40mA/gel for 6h. After second dimensional separation, gels were stained with 0.1% Coomassie Brilliant Blue R-250 in fixing solution (40% v/v ethanol, 10% acetic acid in water), and destained with 5% ethanol, 7% acetic acid in water. Finally the gels were analysed using PDquest Advance software 2.0. and the gels were stored wet in sealed covers in cold.

3.24 Isolation and identification of protein spots from 2DGE

The differentially expressed protein spots were cut manually and stored in -80^{0}C. The spot identification with MALDI-TOF/TOF was outsourced by Sandor Proteomics Pvt. Ltd., Hydrabad, India. The peptide sequencing of protein spots were done with MALDI TOF/TOF MS Bruker Daltonics ULTRAFLEX III instrument and further analysis was done with FLEX ANALYSIS SOFTWARE for

obtaining the PEPTIDE MASS FINGERPRINT. The masses obtained in the peptide mass fingerprint were submitted for Mascot search in "CONCERNED" database for identification of the protein.

3.25 Isolation of enriched sumoylated proteins

The sumoylated proteins of mycelia were isolated with SUMO Qapture-T kit (Enzo Life Science, Farmingdale, New York) followed by the manufacturer's instructions and were detected with western blot using MoSUMO antibody (1: 2000). In brief, total mycelial proteins were extracted in the binding buffer (50mM Tris-HCl, pH 7.5) containing 1X protease inhibitor. 100µl of total protein (1µg/µl) was added to 40µl of matrix suspension (previously equilibrated with binding buffer) to a fresh tube containing 200µL binding Buffer and allowed binding of sumoylated conjugates to the affinity matrix overnight at 4°C on a rotor mixer. The unbound fraction was removed by centrifuge for 30 seconds at a speed of 5000g. Matrix was washed with 200µL Binding Buffer followed by mixing gently by inverting tube and discarded the supernatant by centrifuge samples for 30 seconds at a speed of 5000 g. Matrix was washed once more with binding buffer. The SUMO-protein conjugates were eluted by addition of 100µL binding buffer and 25µL 5 × SDS-PAGE gel loading buffer to the sample and quenched by mixing at room temperature for 5 minutes, followed by heating to 95^0C for 10 minutes.

Elution Fraction was clarified by centrifugation prior to SDS-PAGE and western blot analysis.