4. *MATERIALS AND METHODS*

4.1 Media and culture conditions

Oat Meal Agar

36.5 g oat meal agar (Hi-Media, India) was added to 800 ml H_2O. The volume was made up to 1L and autoclaved.

YEG

Yeast extract	2 g
Dextrose	10 g
H_2O	to 1L

YEPD

Yeast extract	5 g
Peptone	10 g
Dextrose	10 g
H_2O	to 1L

Complete medium for Magnaporthe oryzae

Dextrose	10 g
Peptone	5 g
Yeast extract	1 g
CAA	1 g
$NaNO_3$	6 g
KCl	0.5 g
$MgSO_4$	0.5 g

KH_2PO_4	1.5 g
H_2O	to 1L
pH	6.5

Minimal medium for Magnaporthe oryzae

Dextrose	10 g
$NaNO_3$	6 g
KCl	0.5 g
$MgSO_4$	0.5 g
KH_2PO_4	1.5 g
H_2O	to 1L
pH	6.5

20X AB Buffer

K_2HPO_4 (anhy.)	6 g
NaH_2PO_4	2 g
H_2O	to 100 ml

Each salt was dissolved separately in ~ 50 ml H_2O and then mixed to obtain 100 ml solution with pH 7.0.

20X AB salts

NH_4Cl	2.0 g
$MgSO_4.7H_2O$	0.80 g
KCl	0.30 g
$CaCl_2.2H_2O$	0.31 g

$FeSO_4.7H_2O$	0.005 g
H_2O	to 100 ml

AB minimal medium for Agrobacterium

AB liquid	90 ml
20X AB buffer	5 ml (1X)
20X AB salts	5 ml (1X)

AB liquid

0.5 g dextrose was added in 90 ml H_2O. 5ml each of 20X AB salts and 20X AB buffer was added.

Induction medium for Agrobacterium

20X AB buffer	5 ml (1X)
20X AB salts	5 ml (1X)
Dextrose	180 mg
Glycerol	0.5 % w/v
H_2O	to 100 ml
pH	5.3 (using ortho-phosphoric acid)

Co-cultivation medium for Agrobacterium

20X AB buffer	5 ml (1X)
20X AB salts	5 ml (1X)
Dextrose	90 mg
Glycerol	0.5 % w/v

H_2O	to 100 ml
pH	5.3 (using ortho-phosphoric acid)

Synthetic Dropout (SD) medium for S. cerevisiae

YNB without amino acids (Hi-Media, India)	1X
Dextrose/Galactose	2 g
H_2O	to 100 ml

STC Buffer

1M D-Sorbitol	18.2 g
50mM Tris HCl pH 8.0	5 ml of 1M Tris HCl pH 8.0
50mM $CaCl_2.2H_2O$	0.735 g
H_2O	to 100 ml

PTC Buffer

40% (w/v) Polyethylene glycol 3350	40 g
1M D-Sorbitol	18.2 g
50mM Tris HCl pH 8.0	5 ml of 1M Tris HCl pH 8.0
50mM $CaCl_2.2H_2O$	0.735 g
H_2O	to 100 ml

YEGS Solution

Yeast extract	0.2 g
Dextrose	1 g
D-Sorbitol	18.2 g

H_2O	to 100 ml

Molten Regeneration Medium

Yeast extract	0.2 g
Dextrose	1 g
Agarose 0.4% (w/v)	0.4 g
H_2O	to 100 ml

4.2 Nutrients

L-Uracil	440 mg/L
L-Leucine	430 mg/L
L-Lysine	430 mg/L
L-Histidine	440 mg/L
L-Tryptophan	440 mg/L

4.3 Buffers

DNA extraction buffer for M. oryzae

1M Tris HCl pH 8.0	15 ml
0.5M EDTA pH 8.0	15 ml
4M NaCl	18.75 ml
After autoclaving add	
β-mercaptoethanol	105 µl
H_2O	to 150 ml

STET Buffer

8% Sucrose	8 g
50mM Tris HCl pH 8.0	5 ml of 1M Tris HCl pH 8.0
50mM EDTA pH 8.0	10 ml of 0.5M EDTA pH 8.0
After autoclaving add	
0.1% TritonX-100	100 µl

Urea Buffer

9.5M Urea	57 g

2% (v/v) NP40	2 ml
5% (v/v) β-mercaptoethanol	5 ml
H_2O	to 100 ml

Sarcosine Buffer for E. coli

1% N-lauryl sarcosine	1 g
25mM Triethanolamine	345 µl
1mM Phenylmethylsulfonyl fluoride (PMSF)	1 ml of 100mM PMSF
0.5% TritonX-100	500 µl
H_2O	to 100 ml

Equilibration Buffer

50mM Tris HCl pH 8.0	5 ml of 1M Tris HCl pH 8.0
300mM NaCl	7.5 ml of 4M NaCl
10mM (v/v) β-mercaptoethanol	70 µl of 14.3M stock
10% (v/v) Glycerol	10 ml
20mM Imidazole	1 ml of 2M Imidazole
1mM PMSF	1 ml of 100mM PMSF
H_2O	to 100 ml

Wash Buffer

50mM Tris HCl pH 8.0	5 ml of 1M Tris HCl pH 8.0
300mM NaCl	7.5 ml of 4M NaCl
10mM (v/v) β-mercaptoethanol	70 µl of 14.3M stock
10% (v/v) Glycerol	10 ml

20mM Imidazole	1 ml of 2M Imidazole
1mM PMSF	1 ml of 100mM PMSF
H_2O	to 100 ml

Elution Buffer

50mM Tris HCl pH 8.0	5 ml of 1M Tris HCl pH 8.0
100mM NaCl	2.5 ml of 4M NaCl
5mM (v/v) β-mercaptoethanol	35 µl of 14.3M stock
10% (v/v) Glycerol	10 ml
200mM Imidazole	10 ml of 2M Imidazole
1mM PMSF	1 ml of 100mM PMSF
H_2O	to 100 ml

1X Phosphate Buffer Saline (PBS)

137mM NaCl	8.0 g
100mM $Na_2HPO_4.7H_2O$	2.68 g
2.7mM KCl	0.2 g
2mM KH_2PO_4	0.27 g
Water	to 1 L
pH	7.4

4.4 Stock Solutions

Antibiotics

Ampicillin	100 mg/ml
Kanamycin	50 mg/ml
Rifampicin	5 mg/ml
Tetracycline	10 mg/ml
Cefotaxime	250 mg/ml
Hygromycin B	200 mg/ml
Zeocin	100 mg/ml
Geneticin	50 mg/ml

Reagents

PMSF	100mM
EDTA pH 8.0	500mM
Tris HCl pH 8.0	1M
Ammonium persulphate (APS)	10% (w/v)
IPTG	100mM

4.5 Bacterial strains

Escherichia coli DH5α (F^-, *end*A1, *hsd*R17 (rk^-, mk^+), *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. The *E. coli* strains were grown at 37^0C 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. Bacterial strains were maintained at 4^0C on LB agar medium. Long term preservation was done in 25% glycerol solution at -80^0C.

4.6 Yeast strains

Saccharomyces cerevisiae strains CM66 (Mata alr1::HIS3, alr2::TRP1, his3-200, ura3-52, leu2-1, lys2-202, trp1-63) and CM52 (Mata his3-200, ura3-52, leu2-1, lys2-202, trp1-63) were used for yeast transformation. The yeast strain CM66 was grown at 28^0C and maintained on YEPD medium containing 500mM $MgSO_4$.

4.7 Fungal cultures

Magnaporthe oryzae B157 strain (MTCC accession number 12236), belonging to the international race IC9 was previously isolated in our laboratory from infected rice leaves (Kachroo *et al.*, 1994). The *Δku80* mutant used in the present study was generated by replacing *MoKU80* ORF with Zeocin selection marker in wild type B157 strain (WT) in our laboratory. The fungus was grown and maintained on YEG medium or Oatmeal agar at 28-30^0C. 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 two layers of Miracloth (Calbiochem, Darmstadt, Germany). The different transformants were maintained as mono-conidal isolates and stored as filter stocks at -20^0C.

4.8 Isolation of plasmid DNA

Overnight grown culture (1.5 ml) was centrifuged in a microfuge tube and the bacterial pellet was resuspended in 300 µl of STET. To this suspension, 10 µl of 50 mg/ml Lysozyme was added, mixed by vortexing and incubated for 2 min at room temperature (RT), followed by boiling in a water bath for 45 sec. This was followed by centrifugation at 13,000 rpm for 10 min. The resulting snot was removed with a tooth pick and 20 µl of 5% CTAB was added followed by centrifugation for 10 min. To the pellet, 300 µl of 1.2M NaCl was added and vortexed. To this 180 µl of 100% isopropanol was added. The DNA pellet was obtained by centrifugation for 10 min; washed with 70% ethanol and resuspended in 50 µl of TE.

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

A single colony of *E. coli* DH5ά was inoculated in 3 ml of LB broth and grown overnight. 100 ml of fresh LB broth was inoculated with 1.0 ml of overnight grown culture and grown at 37^0C till 0.3 to 0.4 O.D $_{\lambda=600}$. The cells were pelleted by centrifugation at 5000 rpm for 4 min at 4^0C. The cells were resuspended in 10.0 ml of ice cold 0.1M $CaCl_2$ and incubated on ice for 30 min. The cells were reharvested by centrifugation at 4000 rpm for 5 min at 4^0C. The cells were resuspended in 1.0 ml of ice cold 0.1M $CaCl_2$ + 20% glycerol. The culture was stored at -80^0C in 100 µl aliquots. For transformation, a 100µl aliquot of competent cell suspension was mixed with the plasmid DNA (50-100 ng) or ligation mixture and incubated at 4^0C for 30 min. Cells were subjected to heat shock at 42^0C for 90 sec, followed by 5 min incubation on ice. One ml of LB broth was added to the above suspension and incubated at 37^0C for 45 min. One hundred µl of transformation mixture was plated on LB agar medium containing either Ampicillin (100 µg/ml) or kanamycin (50 µg/ml).

4.10 Nucleic acid manipulation

Restriction enzyme digestion of DNA was carried out in reaction buffers, supplied with the corresponding enzymes, as recommended (NEB, USA). Ligations were carried out at 22^0C, as recommended (Thermo Fisher, USA).

4.11 Bioinformatics analysis of CorA Mg^{2+} transporters

CorA Magnesium transporters, *MoALR2* and *MoMNR2*, were identified from the *M. oryzae* genome (http://www.broadinstitute.org/annotation/genome/magnaporthe_grisea/MultiHome.html) by a BLAST_P search using the full length *S. cerevisiae* Alr1 protein sequence, while *MoMRS2* (Mgg_02763) and *MoMRS2* (Mgg_06582), were identified using the full length *S. cerevisiae* Mrs2 protein sequence. The conserved domain prediction was done using Pfam tool. ExPASy tool was used to predict the number and orientation of transmembrane helices. WoLFPSORT tool was used for predicting localisation of the CorA proteins.

4.12 Complementation cassette of *MoALR2* and *MoMNR2* in pYES2 vector to give pYES2-*MoALR2*, pYES2-*MoMNR2* and pYES2-*MoMNR2*$_{489\text{-}812}$

The full length gene of *MoALR2* (1.9 kb) was amplified from genomic DNA and cloned at *Pvu*II site in the yeast episomal vector pYES2 (Invitrogen, California, USA) to generate pYES2-*MoALR2*. The full length gene of *MoMNR2* (2.5 kb) was amplified from genomic DNA and cloned first at *Eco*RV site in pBuescript KS (+). The full length gene was moved into pYES2 vector at *Hind*III and *BamH*I site under Gal1 promoter to give pYES2-*MoMNR2*. For *MoMNR2*$_{489\text{-}812}$ cloning, the CorA domain of *MoMNR2* was PCR amplified from genomic DNA and cloned first in pBuescript KS (+) at *Eco*RV site. The CorA domain of *MoMNR2* was moved into pYES2 vector at *Xho*I and *BamH*I site under Gal1 promoter to give pYES2-*MoMNR2*$_{489\text{-}812}$.

4.13 Transformation of *S. cerevisiae* with plasmid DNA

The yeast strain CM66 (Δ*alr1*Δ*alr2* mutant), was transformed with the plasmids pYES2, pYES2-*MoALR2*, pYES2-*MoMNR2* and pYES2-*MoMNR2*$_{489\text{-}812}$ by the 'One-step transformation' method (Chen *et al.*, 1992). *S. cerevisiae* strain CM66 was streaked on YEPD agar containing 500mM $MgSO_4$ and incubated for 36-48 hours at 28^0C. The culture was scraped and resuspended in 1 ml of sterile water. The cells were washed twice in 1 ml of sterile milliQ water. The cells were resuspended in 1 ml of 100mM Lithium Acetate (LiAc) and incubated at 28^0C for 5 min. For every transformation, 100 µl of the above suspension was taken and centrifuged to remove LiAc. This was followed by addition of 240 µl of 50% (w/v) PEG 3350, 36 µl of 1.0M LiAc, 50 µl of Sheared Salmon Sperm DNA (2.0 mg/ml) and 34 µl of plasmid DNA (2 µg) plus sterile water. The whole mixture was thoroughly vortexed and incubated at 28^0C for 12-16 hours. After the incubation, the cells were plated on selection plates. Colonies were selected on SD medium lacking uracil and having lysine, leucine, 2% Galactose and 500mM $MgSO_4$. A negative control was also included containing no plasmid.

4.14 Complementation of *S. cerevisiae* Δ*alr1*Δ*alr2* mutant (CM66)

pYES2, pYES2-*MoALR2*, pYES2-*MoMNR2* and pYES2-*MoMNR2*$_{489\text{-}812}$ transformed colonies obtained were grown in SD media (lysine + leucine + 2% Galactose + 500mM $MgSO_4$) till saturation and cells were spotted on SD media having leucine, lysine, 2% Galactose and 4mM $MgSO_4$/500mM $MgSO_4$. The growth of colonies was observed 4 days post inoculation at 28^0C. For growth curves, overnight grown cultures of CM52_pYES2, CM66_pYES2, CM66_pYES2-*MoALR2* and CM66_pYES2-*MoMNR2* in YPD+500mM $MgSO_4$ were washed with sterile milliQ water twice and the cells were adjusted to 0.01 O.D. in SD media having leucine, lysine, 2% galactose and 4mM $MgSO_4$ (for CM66 transformed cells) and leucine, lysine, histidine, tryptophan, 2% galactose and 4mM $MgSO_4$ (for CM52

transformed cells). The growth of the transformed cells was monitored at intervals of 4 hours up to 40 hours. The experiments were repeated in triplicate.

4.15 Double joint PCR based generation of knockout cassette

A PCR based technique was used to generate knockout cassettes of *MoMNR2* (Mgg_09884), *MoMRS2* (Mgg_02763) and *MoMRS2* (Mgg_06582) (Yu *et al.*, 2004). Primers were designed where the Reverse primer for amplifying the 5' flank had a 23bp overlapping sequence to the promoter (pTrpC) of the Zeocin cassette and the Forward primer for amplifying the 3' flank had a 23bp overlapping sequence to the terminator (tTrpC) of the Zeocin cassette. In the first round PCR, 1.5 kb of 5' Flank and 1.4 kb of 3' flank of *MoMNR2* were amplified from WT B157 genomic DNA. 1.2 kb of Zeocin marker (Zeo^r) was amplified from pBSKS-Zeocin. In the second round PCR, all the three fragments 5' flank, Zeo^r and 3' flank were taken in 1:3:1 molar ratio and a touch-down PCR (60^0C to 50^0C) was performed for fusion of the three fragments with 1 min elongation time. The fusion PCR product was diluted 1:10, 1:50 and 1:100 and PCR was performed for amplification of the full knockout cassette of *MoMNR2* (~4.2 kb) with 5' flank For and 3' flank Rev. In the third round PCR, the specificity of the knockout cassette was achieved by using above round PCR product as template with 1:500 and 1:1000 dilution using internal primers (Nest primers). A ~3 kb knockout cassette of *MoMNR2* was obtained which was gel purified. A similar approach was used for generation of a knockout cassette for Mgg_02763 with Zeocin as selection marker, where the size of the knockout cassette using nested primers was 2.4 kb. A knockout cassette for Mgg_06582 with Hygromycin as selection marker was generated, where the size of knockout cassette using nested primers was 3.3 kb. The gel purified products were used for protoplast transformation of WT fungus.

4.16 Disruption cassette of *MoALR2* in pGKO2 vector

The vector pGKO2-*MoALR2* was constructed for carrying out targeted disruption in WT. The full length gene of *MoALR2* (1.9 kb) was PCR amplified, end filled and cloned at *Eco*RV site in pBluescript KS (+) to give KS-*MoALR2*. The HPT cassette used for disrupting the gene *MoALR2* was excised from the pAN7.1 vector using *Bgl*II and *Hind*III having glyceraldehyde 3 phosphate dehydrogenase (gpdA) promoter, end filled and cloned at *Eco*RV site (present within the *MoALR2* gene). The whole disruption cassette (~6 kb) was moved from pBluescript KS (+) and cloned into a binary vector pGKO2 at *Kpn*I and *Spe*I site. The *A. tumefaciens* strain LBA4404/pSB1 was first transformed with pGKO2-*MoALR2*-HPT via triparental mating (Helper plasmid pRK2013). The transformed *Agrobacterium* was then used to carry out *A. tumefaciens* mediated transformation (Mullins *et al.*, 2001) of *M. oryzae*.

4.17 Disruption cassette of *MoALR2* in pBSKS+ vector

A smaller disruption cassette of *MoALR2* (~4 kb) was also made in the vector pBluescript KS (+) where KS-*MoALR2* was digested with *Eco*RV and HPT cassette of 2 kb was PCR amplified from pSilent and cloned at *Eco*RV site in KS-*MoALR2* to give pBSKS-*MoALR2*-HPT. This cassette was used to carry out transformation of the *Δku80* strain.

4.18 *Agrobacterium tumefaciens* mediated transformation of *M. oryzae*

The *Agrobacterium* strain LBA4404/pSB1 (Komari *et al.*, 1996) was first transformed with respective vectors via triparental mating (Helper plasmid pRK2013; Helper strain *E. coli* DH5α). For triparental mating, a single colony each from the freshly grown recipient *Agrobacterium* LBA4404/pSB1, helper plasmid pRK2013 and the donor strain containing respective vector (pGKO2-*MoALR2*-HPT) were mixed together on YEP agar (Yeast extract 0.2%, Peptone 0.5%, agar 2%) plates and incubated at 28^0C for 18-20 hours. The mixture was

scraped and resuspended in 0.9% NaCl. 100 µl of serial dilutions of this suspension was plated onto selection plates and incubated at 28^0C for 2-3 days till the appearance of colonies. The *Agrobacterium* harbouring T-DNA vector was then used to transform *M. oryzae*. *Agrobacterium tumefaciens* mediated transformation (ATMT) of *M. oryzae* was carried out as described (Mullins *et al.*, 2001). Briefly, *M. oryzae* spores were collected from 7-8 days old oat meal agar plates and spore concentration was adjusted to $1x10^6$ spores/ml. *Agrobacterium* carrying the *hpt* gene expression cassette was grown at 28^0C for 2 days in AB minimal medium supplemented with appropriate antibiotics. The *Agrobacterium tumefaciens* cells were diluted to OD_{600}=0.15 in induction medium (IM) and grown for another 6 hours, both in the presence (IM+AS) and absence (IM-AS) of 200µM acetosyringone (AS). The cells were grown for an additional 6 hours before mixing them with an equal volume of a conidial suspension from *M. oryzae*. This mix (200 µl per plate) was plated on a 0.45 µm pore, 45 mm diameter nitrocellulose filter (Millipore, Bangalore, India) and placed on co-cultivation medium in presence of 200 µM AS. Following incubation at 28^0C for 2 days, the filter was transferred to YEG containing Hygromycin B (Invitrogen Life Technologies, CA) to a final concentration of 400 µg/ml and 100mM/200mM $MgSO_4$ as a selection agent for transformants and cefotaxime (250 µg/ml) to kill the *Agrobacterium tumefaciens* cells. Untransformed *M. oryzae* was kept as a control which did not grow on hygromycin containing medium. The transformants were maintained as mono-conidial isolates.

4.19 Split marker based transformation of *M. oryzae* for *MoALR2*

The split marker products were amplified from the disruption cassette of pGKO2-*MoALR2*-HPT using the primers *MoALR2*_F (5'-ATGTCTGACCACGACGAACACG-3') and HY_R (5'-TGACATCGACACCAACGATCT-3') and YG_F (5'-

TGTCGAGAAGTTTCTGATCGA-3') and *MoALR2*_R (5'-TAAGCTTCCTCGCAG TGCAAAG-3') having an overlap of 1088 bp in the *Hyg* selection marker. Another set of split marker products were also amplified from the disruption cassette of pGKO2-*MoALR2*-HPT using the primers *MoALR2*_F (5'-ATGTCTGACCACGACGAACACG-3') and HY_R (5'-

CGGCCATTGTCCGTCAGGAC-3') and YG_F (5'-GATCGTTATGTTTATCGGCAC-3') and *MoALR2*_R (5'-TAAGCTTCCTCGCAG TGCAAAG-3') having a smaller overlap of 401 bp in the *Hyg* selection marker. Equal molar ratio of both the products was taken and 2µg of total DNA was used for protoplast transformation of *M. oryzae*.

4.20 Antisense cassette generation for *MoALR2* in pSilent-1 and pSilent-Dual2 (pSD2) vectors

For knockdown of *MoALR2* a ~110 bp fragment of *MoALR2*, which is complementary to the 5' UTR (Raman *et al.*, 2013) of *MoALR2*, was amplified and cloned in pSilent-Dual2 vector at *Sma*I site. For simultaneous silencing of *MoALR2* and *MoMNR2*, the full length gene of *MoALR2* cloned in pBluescript KS (+) was digested with *Kpn*I and *BamH*I to give a fragment of 1.4 kb (having a portion of the CorA domain) and was cloned in antisense orientation in pSilent-1 at *Kpn*I and *Bgl*II site. For RNAi constructs (Nakayashiki *et al.*, 2005), the full length gene of *MoALR2* cloned in pBluescript KS (+) was digested with *Hind*III and a 525 bp fragment (spanning CorA domain) was cloned in pSilent Dual 2 vector (having two TrpC promoter in opposite orientations) at *Hind*III site. The knockdown constructs were used for protoplast transformation of WT. Putative knockdown transformants were selected on Hygromycin (200µg ml^{-1}) and Geneticin (1mg ml^{-1}). Untransformed *M. oryzae* was kept as a control which did not grow on either Hygromycin or Geneticin medium. Empty vector

transformation (pSD2) was also done as a control. The transformants were maintained as monoconidial isolates to obtain pure cultures.

4.21 Protoplast transformation of *M. oryzae*

M. oryzae B157 was inoculated in 30 ml of Complete medium broth and grown for 3 days. The biomass was then filtered with Miracloth (Calbiochem, Darmstadt, Germany), washed with sterile water and the mycelia were resuspended in 30 ml of 1M sorbitol containing 30 mg (1mg/ml) lytic enzyme (Sigma, St. Louis, USA). This was incubated at 28^0C at 100 rpm for 12-16 hours for protoplasting. Next day, the protoplasts were filtered, washed and resuspended in 10 ml 1M sorbitol at 4,000 rpm for 5 min at 4^0C twice. The protoplasts were resuspended in 10 ml STC buffer, washed and resuspended again in 1 ml STC buffer. The protoplasts were counted using a hemacytometer (Marienfeld Superior, Lauda-Konigshofen, Germany) and a density of 10^8cells per ml was maintained. For every transformation 200 µl of protoplasts was mixed with 2-5 µg of DNA (dissolved in TE buffer) and incubated on ice for 15 min. To this 1 ml of PTC buffer was added and incubated at 28^0C for 30 min. The whole mixture was transferred to sugar tube containing 3 ml of YEGS and was incubated at 28^0C at 100 rpm for 12-14 hours. After incubation, 10 ml of molten regeneration medium was added, mixed well and poured on YEG agar containing appropriate antibiotics (Hygromycin/Zeocin/Geneticin). Appearance of colonies was monitored within 2-3 days of incubation at 28^0C. Untransformed *M. oryzae* was kept as a control which did not grow on selection medium.

4.22 DNA extraction and Southern blot analysis

M. oryzae B157 grown in 30 ml of Complete medium for 3 days was harvested, frozen in liquid nitrogen and ground to fine powder. The powdered biomass was transferred to 500 µl

pre heated DNA extraction buffer (at 68^0C) and mixed properly. To this 50 µl of 20% SDS was added, mixed properly and was incubated at 68^0C for 10 min. To this 250 µl of 5M CH_3COOK was added and incubated at 4^0C for 30 min. The cell debris was separated by centrifugation at 8000 rpm for 20 min. To the supernatant, 500 µl of ice cold 100% isopropanol was added and incubated at -20^0C for 30 min to precipitate DNA. The precipitated DNA was recovered by centrifugation at 10000 rpm for 15 min. The pellet was air dried and 500 µl of TE buffer was added, DNA was solubilised at 68^0C for 10 min. 20 µl of 1mg/ml RNase was added to this solution and further incubated at 68^0C for 15 min. An equal volume of $CHCl_3$:IAA (24:1) was added, mixed gently and centrifuged at 10000 rpm for 15 min. The upper aqueous phase was separated and to this $1/10^{th}$ volume of 3M CH_3COONa, pH 5.0 and 0.6 volume of ice cold 100% isopropanol was added and incubated at -20^0C for 1 hour. The DNA pellet was precipitated, washed with 70% ethanol, air dried and dissolved in autoclaved milliQ water. The DNA concentration was measured using nanodrop spectrophotometer.

For Southern blot analysis, 20 µg of genomic DNA was digested with restriction enzymes and the digested products were run on 0.8% agarose gel at 50V for 8 hours. After denaturation and neutralisation of the digested products in the gel, capillary blot transfer was done for 16 hours to transfer the DNA fragments onto a nylon membrane (Hybond N+, Amersham, UK). The transferred DNA on the membrane was crosslinked by UV crosslinker (Spectrolinker, XL-1000, USA) at preset optimum cross-linking programme. For *Δmnr2* knockout confirmation, 1 kb Up and 1 kb DN fragments were amplified and used as probe. For knockdown transformants of *MoALR2* a 1.2 kb fragment upstream of *MoALR2* was used as a probe. For simultaneously silenced transformants, ~400 bp TrpcP amplified from pSilent vector was used as probe. The labeling of the probes was done according to the manufacturer's instructions (Amersham, Buckinghamshire, UK). The probe was hybridised

with DNA on the membrane for 16 hours. The membrane was washed and substrate was added for 5 min. X-ray film was exposed for 4 hours with the membrane in Hypercassette (Amersham, Buckinghamshire, UK). X-ray film was developed and targeted integration in knockout and integrations in knockdown was confirmed.

4.23 Generation of antibodies against CorA domain of MoMnr2

The CorA domains of MoAlr2 and MoMnr2 have 50% identity at the protein level and the size of the proteins is 70kDa and 90kDa respectively. The CorA domain from *MoMNR2* was amplified (1 kb) from genomic DNA as it had no intron. The amplified product was cloned in pBluescript KS (+) vector at *EcoR*V site and then was ligated in the bacterial expression vector pET30a (+) vector at *Nde*I and *Kpn*I site translationally in-frame with $(His)_6$ tag at the C terminus. *E. coli* BL21 DE3 cells were transformed with the protein expression construct. The transformed colonies were grown in Luria Bertani (LB) medium O/N. 1% inoculum was used the next day and the culture was grown to an O.D. (λ_{600}) of 0.4 to 0.6 O.D. 1mM IPTG was used for induction of the protein for another 4 hours. The induced protein having Polyhistidine at the carboxyl terminus was purified using Ni-NTA affinity chromatography (Novagen, Darmstadt, Germany) according to manufacturer's protocol. The purified protein was used to raise polyclonal antibodies in New Zealand White Rabbit using 20 µg of purified protein and equal volume of Freund's Complete Adjuvant. Subsequently, after 21 days two booster doses were given at intervals of 7 days with Freund's Incomplete Adjuvant. Antibody titer was estimated by indirect-enzyme linked immunosorbent analysis (ELISA) using HRP conjugated anti-rabbit IgG (Bangalore Genie, Bangalore, India) as the secondary antibody.

4.24 Quantitative Real time PCR (qRT-PCR) analysis for gene expression

M. oryzae B157 was grown in Complete Medium (CM) for 72 hours (when no treatment was given to the biomass), or in CM for 48 hours, followed by two washes of the biomass with sterile milliQ water. The fungal biomass was then transferred into Minimal Medium for 24 hours after which it was given treatments of Mg^{2+} or Ca^{2+} for the given time period. Fungal biomass was harvested and frozen in liquid nitrogen. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, California, USA). 2µg of total RNA was reverse transcribed into first strand cDNA using oligo (dT) primer and M-MuLV Reverse transcriptase (NEB, Massachusetts, USA). Quantitative PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye either on a Light Cycler system for real-time PCR (Roche Applied Science, Mannheim, Germany) or ABI 7900 HT real time PCR (Applied Biosystems, California, USA), according to the manufacturer's instructions. Thermal cycling conditions consisted of 10 minutes at 95^0C 1 cycle, followed by 40 cycles of 10 seconds at 95^0C, 10 seconds at 55^0C and 15 seconds at 72^0C for SYBR chemistry. Also Taqman Probes (Applied Biosystems, California, USA) specific for gene(s) were used to validate and study expression profile quantitatively. Reaction conditions were according to manufacturer's instructions. Thermal cycling conditions consisted of 2 minutes at 50^0C 1 cycle, 10 minutes at 95^0C 1 cycle, 40 cycles of 15 seconds at 95^0C and 1 minute at 60^0C. Each qRT-PCR quantification was carried out in triplicate and every repeat was performed in duplicate. To compare the relative abundance of target gene transcripts, the average threshold cycle (Ct) was normalised to that of GPDH gene for each of the treated samples as $2^{-\Delta Ct}$, where $\Delta Ct = (Ct_{\text{gene of interest}} - Ct_{GPDH})$ and fold changes were calculated by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{\text{gene of interest}} - Ct_{GPDH})_{\text{test condition}} - (Ct_{\text{gene of interest}} - Ct_{GPDH})_{\text{control}}$. The transcript levels were expressed as relative values, with 1 corresponding to the Wild type (WT).

4.25 Protein extraction and Western blot analysis

Total protein was extracted from WT and knockdown transformants grown in CM for 72 hours (when no treatment was given to the biomass); otherwise the fungus was grown in CM for 48 hours, which was followed by two washes of the biomass with sterile milliQ water. The fungal biomass was then transferred into Minimal medium for 24 hours after which the fungus was given treatments of Mg^{2+} or Ca^{2+} for the given time period. Fungal biomass was harvested and frozen in liquid nitrogen. Protein was extracted in Urea Buffer (9.5M urea, 2% v/v NP40, 5% βME) for 1 hour at RT. The concentration of the protein was estimated by Bradford method. The protein samples were electrophoresed on 10% SDS–polyacrylamide gel, followed by electro transfer to nitrocellulose membrane (Hybond ECL, Amersham, Buckinghamshire, England). The immunoblots were developed with primary antibody against MoMnr2 CorA domain and secondary antibody conjugated with HRP using 3'-3'- diamino Benzidin tetrahydrochloride dehydrate (Fluka, Washington DC, USA) detection method (Bangalore Genei, Bangalore, India) and SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Rockford, USA) as per the manufacturer's instructions.

4.26 Indirect immunolocalisation of MoAlr2 and MoMnr2 in *M. oryzae*

The WT and *Δmnr2* spores were fixed with 10% formaldehyde, 5% acetic acid, and 85% ethanol for 30 minutes at room temperature and the fixed sample was incubated in PBS + 0.1% Triton X-100 for 2-5 minutes. The sample was given wash with PBS for 10 to 15 minutes. The fixed samples were further treated as described (Patkar and Chattoo, 2006) with a few modifications. Primary antibodies used were against CorA domain of MoMnr2. Secondary antibody, TRITC-conjugated anti-rabbit IgGs raised in goat, (Sigma Chemical Co, St Louis, MO, USA) diluted to 1:20 in PBS was used for staining for 2 hours. Three washes each of 15 minutes were given with PBST, which was followed by vacuolar staining with

Oregon green 488 carboxylic acid diacetate (cDFFDA) (Molecular Probes, Invitrogen, California, USA) stain for 10 minutes followed by three washes with PBST. The slides were observed under 63X using LSM 700 microscope (Carl Zeiss, LSM 700, Jena, Germany) at 557nm excitation and 576nm emission for TRITC and at 501nm excitation and 526nm emission for Oregon green 488. Image analysis was done using ZEN software.

4.27 Sensitivity to Cobalt (III) Hexaammine

For assaying the sensitivity of the knockdown and knockout transformants towards CorA specific inhibitor (Cobalt (III) hexaammine (Co (III) Hex), concentrations ranging from 300µM to 400µM of Cobalt (III) hexaammine were added to YEG medium. The sensitivity was assessed 5 days post inoculation and radial growth was measured for these transformants.

4.28 X-ray fluorescence (XRF) for element analysis

For determining the levels of elements (mainly Mg^{2+} and Ca^{2+}) in the knockout and knockdown transformants X- ray Fluorescence Analysis (XRF) was performed (Reidinger *et al.*, 2012). WT, knockout and knockdown transformants were grown in CM for 48 hours. The fungal biomass was washed twice with sterile milliQ water and transferred into Minimal media for 24 hours. Then the biomass was grown under different concentrations of Mg^{2+} (4mM, 50mM and 250mM) for another 6 hours. The biomass was harvested, frozen in liquid nitrogen and ground to fine powder and dried completely at 37^0C for 3-4 days, after which the element analysis was done using Energy dispersive X-ray Fluorescence Spectrometer EDX-720/800HS (Shimadzu, Singapore).

4.29 Surface hydrophobicity assay

The knockout and knockdown transformants were tested for defects in surface hydrophobicity with water and detergent solution (0.02% SDS+5mM EDTA). 5 day old fungal culture grown on YEGA was inoculated with water and detergent solutions. The wettability of the transformants was checked by the extent to which water or detergent was retained on mycelia compared to WT.

4.30 Cation sensitivity assay

For cation sensitivity assays, the knockout, knockdown transformants and WT were inoculated on YEGA having Zinc (Zn^{2+} 500µM), Cobalt (Co^{2+} 150µM), Manganese (Mn^{2+} 3mM), Iron (Fe^{3+} 2mM), Copper (Cu^{2+} 750µM), Aluminium (Al^{3+} 800µM), Nickel (Ni^{2+} 200µM). The sensitivity to different cations was studied by comparing the growth of transformants 5 days post inoculation with respect to WT. For all the sensitivity assays the minimal inhibitory concentration (MIC) was determined with respect to WT.

4.31 Phenotypic characterisation of transformants

4.31.1 *Vegetative growth*

Vegetative growth of the knockdown and knockout transformants was measured on OMA and YEGA 5 days post inoculation. The experiments were performed with replicates in three independent experiments.

Aerial hyphal growth of the knockdown and knockout transformants was monitored on 0.8% agarose 2 days post inoculation grown under moist conditions.

Growth in presence of different concentrations of EDTA was tested for WT. 3x3 mm mycelial plugs were inoculated on OMA with and without EDTA and growth was assessed 5

days post inoculation. Restoration of growth on Mg^{2+} supplements in presence of EDTA was assessed. Growth of sectored colonies obtained under stress conditions (EDTA) was checked. Growth of different sectors was assessed at 0.5mM EDTA.

Ability of WT spores to form vegetative hyphal growth following germination was assessed on YEGA and OMA with different concentrations of EDTA. 10 µl of spores with increasing dilution was spotted onto the plates. The ability of Mg^{2+} to restore the germination capability of spores was also checked on Mg^{2+} supplemented medium in presence of EDTA.

4.31.2 *Conidiation*

The ability to produce conidia was measured by counting the numbers of conidia for the knockdown and knockout transformants isolated from the surface. Quantification of conidia was done using a hemacytometer (Marienfeld Superior, Lauda-Konigshofen, Germany).

The ability of WT to sporulate was checked on OMA with different concentrations of EDTA 8 days post inoculation and quantified.

Conidial development on aerial hyphae was monitored after 2 days post inoculation on 0.8% agarose under moist conditions for WT, knockout and knockdown transformants.

4.31.3 *Appressorial assay*

For appressorium formation, equal numbers of spores were inoculated on a hydrophobic surface, Gelbond film (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and incubated under moist conditions for 6 and 12 hours. For non-sporulating knockdown transformants, mycelial plugs from transformants (5^{th} day plate) were inoculated and incubated under moist conditions for 48 hours. Appressorium formation in the knockdown and knockout transformants was checked at 40X magnification (Olympus, Tokyo, Japan). The relative percentage of appressoria formed was calculated for each time interval.

The ability to form appressoria in water, 0.25mM EDTA and 0.25mM EDTA+50mM Mg^{2+} was observed at different time intervals in WT tagged with H1:RFP and Tub:GFP and percentages of spores (ungerminated), germ tubes and appressoria formed were calculated for each time interval and for each condition.

4.32 Detached leaf assay

Leaves of 21 day old rice seedlings of HR-12 cultivar were used for inoculating spores or mycelial plugs (for non-sporulating transformants) of knockout and knockdown transformants and were placed on water agar with kinetin (2 mg l^{-1}). Disease symptoms were recorded after 3–4 days.

4.33 Cell wall integrity assay

For the cell wall integrity assay, WT, knockout and knockdown transformants were inoculated on YEGA containing Congo Red (1.5 mg ml^{-1} and 2 mg ml^{-1}) or Caffeine (2.5mM and 3mM). Sensitivity to these cell wall stress molecules was studied by comparison of growth to that of WT 5 days post inoculation.

YEG and YEG with Congo Red (1.5 mg/ml) and Caffeine (2.5mM) were supplemented with different concentrations of Magnesium. 2X2 mm mycelial plugs of WT and knockdown transformants A2 and A15 were inoculated. Recovery in growth was assessed 5 days post inoculation.

4.34 Quantification of intracellular levels of cAMP

WT, knockout and knockdown transformants were grown in CM for 48 hours. The fungal biomass was washed twice with sterile milliQ water and the biomass was transferred into Minimal media containing 4mM Mg^{2+} for 24 hours. The biomass was harvested and frozen.

The fungal biomass was ground to fine powder in liquid nitrogen and after the liquid nitrogen was evaporated, equal weight of frozen biomass (0.1 gm) was taken and homogenised in 300 µl of 0.1N HCl. The sample was vortexed for 30 minutes, followed by centrifugation at 5000 x g for 15 minutes at room temperature. The supernatant was collected and 100 µl of sample in each case was taken. Quantification of intracellular cAMP levels was carried out by cAMP Direct Immunoassay Kit as per manufacturer's instructions (Calbiochem, Darmstadt, Germany).

4.35 Mg^{2+} limited and pH dependent growth of WT and *Δmnr2* transformant

WT and *Δmnr2* were grown on OMA and YEGA supplemented with 0.5mM EDTA. Growth was assessed 5 dpi.

In order to study pH sensitivity on growth phenotype of WT and *Δmnr2*, strains were grown on YEGA buffered to pH 5.6-8.25 using 20mM HEPES. Sensitivity to different pHs was studied by growth of the fungus 5 days post inoculation.

4.36 Extracellular alkalinisation assay

To assess extracellular alkalinisation in WT and *Δmnr2* on solid media, strains were grown on media containing (0.2% yeast extract+3% glycerol+0.01% bromocresol green, adjusted to pH 4.0 and 4% agar) without glucose (Mayer *et al.*, 2012). The cultures were incubated at 30^0C and change of the medium colour from green to blue was used as a measure of alkalinisation; this was followed daily for 5 days. Experiments were performed at least in duplicate and repeated three times. pH changes were also monitored quantitatively for WT and *Δmnr2*, where the strains were inoculated in liquid broth containing (0.2% yeast extract+3% glycerol, adjusted to pH 4.0 and without glucose) and changes in pH were monitored for 4 days.

4.37 Laccase estimation in WT and *Δmnr2* transformant

Laccase activity was determined in WT and *Δmnr2* as previously described (Iyer and Chattoo, 2003). $1x10^5$ spores/ml of WT and *Δmnr2* were inoculated in 30 ml of complete medium and incubated at 28^0C at 200 rpm. The supernatant was filtered and collected at 36, 48 and 72 hours. Laccase activity was determined at 30^0C using 100 µl of 0.216mM syringaldazine (ε_m=$6.4x10^4$ M^{-1} cm^{-1}; Sigma, USA) in a 1 ml reaction mixture containing 0.05M MES-NaOH buffer (pH 6.0) and 100 µl of culture supernatant. Change in absorbance was monitored for 10 min. One unit of enzyme activity is defined as the amount of enzyme required to oxidise 1 µmole of syringaldazine under standard assay conditions. Units/ml of enzyme was calculated as: {(A_{530} 10 min- A_{530} 0 min) $_{Test}$ - (A_{530} 10 min- A_{530} 0 min) $_{Blank}$}/(0.001x volume of enzyme). Protein concentration was determined by the method of Bradford using bovine serum albumin as standard. Specific laccase activity of WT and *Δmnr2* was expressed as Units/µg. The experiment was performed three times.

4.38 Mating assay in WT and *Δmnr2* transformant

Sexual fertility of the *Δmnr2* transformant was evaluated by crossing with the opposite mating type strain (Chen *et al.*, 2013). WT B157 is MAT1-1 mating type, while Guy11 is MAT1-2 mating type. Perithecia formation was visualised 21 days post inoculation on OMA at 20^0C under constant illumination.

4.39 Generation of localisation construct of *MoALR2* and cloning in pBSKS+ to give KS-Loc*MoALR2*-GFP-Zeocin^r

A PCR based technique was used wherein *MoALR2* was fused to GFP to generate a localisation cassette of *MoALR2*. Briefly, a ~3.5 kb gene product comprising promoter of *MoALR2* and *MoALR2* without stop codon was amplified from genomic DNA. ~750 bp GFP

was amplified and both the products were fused and full length product of ~4.2 kb was amplified using *ALR2* Promoter (F) and GFP (R). To increase the specificity of the product, PCR was kept with *ALR2* Promoter Nest (F) and GFP (R) to obtain a ~4.0 kb product. The PCR product was gel eluted and cloned at *Eco*RV site in pBSKS+ to give KS-Loc*MoALR2*-GFP. Next, Loc*MoALR2*-GFP (~4.0 kb) was amplified from KS-Loc*MoALR2*-GFP and fused with Zeo^r (~1.2 kb) to obtain Loc*MoALR2*-GFP-Zeo^r of ~5.2 kb. The PCR product was gel eluted and cloned at *Eco*RV site in pBSKS+ to give KS-Loc*MoALR2*-GFP-Zeo^r. The gel purified products were used for protoplast transformation of WT fungus and the transformants were selected on YEGA containing Zeocin.

4.40 Statistical Analyses: One-way ANOVA and non-parametric test was performed for all the statistical analyses, wherein the mean of each column was compared with the mean of control column, followed by Fisher's LSD test at 95% confidence interval. ** means P value at <0.0001 and * means significant at P value <0.05.