## 7. *SUMMARY*

a) We identified two putative orthologues of the *Saccharomyces cerevisiae* CorA Magnesium transporter gene ALR1 in the *M. oryzae* genome, which were named as MoAlr2 (Mgg_08843) and MoMnr2 (Mgg_09884). Two further putative CorA family genes were identified, namely *MoMRS2* (Mgg_02763) and *MoMRS2* (Mgg_06582).

b) The coding sequence of *MoALR2* and *MoMNR2* were cloned in a yeast episomal vector, pYES2 for functional complementation studies in a *S. cerevisiae* double mutant, CM66 (Δ*alr1*Δ*alr2*), which is a haploid disruptant for both *ALR1* and *ALR2* genes. Both *MoALR2* and *MoMMR2* individually could rescue the $Mg^{2+}$ uptake defect of the *S. cerevisiae* double mutant CM66 and hence have a definite role in $Mg^{2+}$ transport.

c) A *MoMNR2* knockout cassette was generated by a Double joint PCR based approach using Zeocin as selection marker. The *MoMNR2* knockout was confirmed in the transformants by Southern blot analysis.

d) Double joint PCR was also used to generate a knockout cassette of *MoMRS2* (Mgg_02763 using Zeocin as selectable marker) and *MoMRS2* (Mgg_06582 using Hygromycin B as selectable marker). No true knockout could be generated for either of the *MoMRS2* genes, Mgg_02763 and Mgg_06582.

e) We attempted disruption of *MoALR2* by ATMT, with dual selection vector pGKO2, using F2DU for negative selection. Protoplast transformation was done with the full disruption cassette and by split marker technique. Further, high $Mg^{2+}$ concentration was also used during selection to overcome the selective disadvantage facing slow-growing mutants. We also attempted knockout of *MoALR2* in the *Δku80* background (which aids homologous integration), but all methods yielded only ectopic integrants, suggesting that *MoALR2* is likely to be essential for viability of *M. oryzae*, as seen in

*S. cerevisiae* and *H. pylori*. Subsequently, an alternative approach of gene silencing was carried out where *MoALR2* was silenced in WT and *Δmnr2* backgrounds. Simultaneous silencing of both *MoALR2* and *MoMNR2* genes was also carried out in WT B157.

f) The knockdowns were validated by analysis of relative expression of *MoALR2* and *MoMNR2* by quantitative Real Time PCR (qRT-PCR). Transformants in the WT background (WT+siALR2) showed transcript levels of *MoALR2* ranging from 48% to 85%, while those in the background of *Δmnr2* (*Δmnr2*+siALR2) showed transcript levels ranging from 66% to 88% compared to WT. The double knockdown transformants for *MoALR2* and *MoMNR2* showed transcript levels ranging from 30% to 80% and 37% to 90% for *MoALR2* and *MoMNR2* respectively, compared to WT.

g) Co(III) Hexaammine sensitivity test was done to ensure that reduced transcript levels translated into decline in transporter levels. Two independent knockdown transformants from each category, namely Alr2 silencing (WT+siALR2), *Δmnr2*+siALR2 and simultaneously silenced for *MoALR2* and *MoMNR2*, which were least sensitive to Co(III)Hex., were selected for further characterisation of CorA $Mg^{2+}$ transporters.

h) Southern blot analysis confirmed single copy integration of the knockdown cassette in the selected knockdown transformants.

i) The CorA domain of *MoMNR2* was expressed in *E. coli* BL21 DE3 cells, purified using Ni-NTA affinity chromatography and was used to raise polyclonal antibodies. Western blot analysis confirmed that both CorA proteins, MoAlr2 and MoMnr2, are expressed simultaneously in *M. oryzae*. The knockdown transformants studied also showed lower protein levels of these CorA $Mg^{2+}$ transporters than WT.

j) The subcellular localisation of CorA transporters in *M. oryzae* using polyclonal antibodies against the MoMnr2 CorA domain showed that in wild type, the CorA transporters localised to plasma membrane and vacuole, while immunostaining of *Δmnr2* with CorA antibodies showed staining only of plasma membrane due to MoAlr2.

k) *Δmnr2* showed more growth inhibition than WT on $Mg^{2+}$ limiting conditions, had slower growth at pH 6.5, 7.2, 7.8 and 8.25 and an alteration in pH regulation different from WT suggesting that MoMnr2 has a role in the maintenance of pH within the cell. Laccase activity decreased 238.7 fold and 12.39 folds in *Δmnr2* at 48 and 72 hours respectively compared to WT. The *Δmnr2* mutant failed to induce perithecia formation in an opposite mating type strain, Guy11 suggesting that *MoMNR2* is required for male fertility.

l) A localisation cassette for *MoALR2* was generated by Double joint PCR wherein first the *MoALR2* gene with 1.5 kb promoter sequence (Loc*MoALR2*) was fused in frame with GFP. This fusion product was further used to fuse $Zeocin^{r}$ thus giving Loc*MoALR2*-GFP-Zeo. The selected transformants failed to show any GFP fluorescence, even though GFP was expressed at transcript and protein levels.

m) The knockdown transformants showed decreased intracellular $Mg^{2+}$ levels, while *Δmnr2* showed increased levels as determined by XRF analysis. A change in $Mg^{2+}$ levels led to alteration in cation sensitivity in these knockdown transformants towards various cations.

n) Extracellular $Mg^{2+}$ levels were shown to affect the expression of *MoALR2* and *MoMNR2* both at transcript and protein levels, while extracellular $Ca^{2+}$ affected the expression of only *MoALR2*.

o) The *MoALR2* knockdown transformants showed reduction in growth compared to WT. Similar growth defects were also observed in situations of low $Mg^{2+}$ availability in WT.

p) *Δmnr2* and *MoALR2* knockdown transformants displayed autolysis at the centre of colonies, with autolysis being more severe in transformants with *MoALR2* expression below 50%.

q) Compared to WT, the knockdown transformants showed an easily wettable phenotype. *Δmnr2* also showed a wettable phenotype. This wettable phenotype was mediated through hydrophobins, as the expression of *MoMPG1* decreased significantly in the knockdown transformants.

r) The knockdown transformants showed a decrease in conidiogenesis. A2 and A15 which had lowest expression of *MoALR2* were non-sporulating. The percentage of spores that failed to germinate and form appressoria increased with the increase in level of silencing in the other knockdown transformants. Detached leaf infection tests showed that the severity of infection also decreased in the knockdown transformants.

s) The levels of cAMP in *Δmnr2* and the knockdown transformants varied from 97 to 20 fmol $mg^{-1}$ as compared to 105 fmol $mg^{-1}$ seen in WT. The expression of *MoMAC1* and *MoPMK1* decreased in the knockdown transformants.

t) The degree of sensitivity towards cell wall stressors like Congo red and caffeine increased in proportion to silencing of *MoALR2* in the knockdown transformants. There was decreased expression of two chitin synthase genes *MoCHS1* and *MoCHS4* in the knockdown transformants and alteration in the expression of the genes involved in the regulation of cell wall integrity signalling.