6. *DISCUSSION*

Magnesium is an essential mineral nutrient with roles in stability of DNA structure, cell membrane maintenance, activity of ATP, and as a cofactor of several enzymes. In spite of the importance of Mg^{2+} in cellular physiology, there is little information about its transport, regulation and storage in fungi. In this study, we show that the CorA Mg^{2+} transporters MoAlr2 and MoMnr2 play important roles in development and virulence of the model fungal pathogen, *Magnaporthe oryzae*. By analysing by the phenotypic defects seen in the knockout and knockdown transformants - *Δmnr2*, WT+siALR2, *Δmnr2*+siALR2 and transformants in which both genes were simultaneously knocked down (A2 and A15) - we show that CorA Mg^{2+} transporters are intimately involved at all stages of the infection cycle of *M. oryzae*. Element analysis in the knockdown transformants showed that decreased levels of MoAlr2 transporter led to low intracellular levels of Mg^{2+}. The knockdown transformant A15, which showed maximum silencing of *MoALR2* and *MoMNR2*, had the lowest intracellular levels of Mg^{2+}. We also used the Mg^{2+} chelator EDTA to study the effect of extracellular magnesium availability in WT. High concentrations of EDTA completely abolish growth in WT, while lower levels inhibit spore germination, and an even lower concentration of EDTA is sufficient to inhibit the process of appressorium formation. A similar gradation of phenotypes is also observed in the different knockdown transformants, where effects of lower levels of silencing are seen on appressorium development. With stronger silencing spore germination is affected, and at the highest level of silencing, we see drastic effects even on hyphal growth, suggesting that during the course of the infection cycle from vegetative hyphal growth to sporulation to appressorium formation, the requirement for Mg^{2+} transport into the cell goes up. The severity of growth defects of transformants was further enhanced in low Mg^{2+} conditions. Chemical inhibition of Mg^{2+} transport by the CorA specific inhibitor Co(III)Hex. in WT also produced growth defects.

The high Mg^{2+} requirement during the infection cycle could be for processes like cell wall remodelling, cell division and maintenance of surface hydrophobicity that form a critical part of the differentiation process. *Δmnr2* and *MoALR2* knockdown transformants showed surface hydrophobicity defects. Knockdown transformants also showed lower expression of *MoMPG1* and *MoPMK1*. Decrease in hydrophobicity at low levels of Mg^{2+} has been previously observed in *S. cerevisiae* (Smit *et al.*, 1992). The Mpg1 protein at the cell surface detects surface hydrophobicity and conveys the signal to activate adenylate cyclase, which in turn via increased cAMP levels activates Pka and Pmk1 dependent MAP kinase, vital for appressorium development and maturation (Choi and Dean, 1997). The expression of *MoMPG1* is regulated in response to diverse morphogenetic and environmental signals. While MoPmk1 acts downstream of MoMpg1, it is also known in turn to regulate the expression of the *MoMPG1* gene (Soanes *et al.*, 2002). We demonstrate that a reduction in Mg^{2+} levels, either by knockdown of transporter levels or by using EDTA in the WT, affects expression of *MoMPG1*. We also found that the intracellular levels of cAMP are lower in the *MoALR2* knockdown transformants. cAMP is an important secondary messenger in the cell and its levels regulate appressorium formation in *M. oryzae* (Choi and Dean, 1997; Lee and Dean, 1993). MoMpg1 is essential for conidiogenesis and appressorium formation and it has been proposed that MoMpg1 may exert positive feedback on its own expression through the cAMP response pathway (Soanes *et al.*, 2002). Decrease in intracellular cAMP reduces the flux through the Pmk1 pathway and in turn reduces the expression of *MoMPG1*, thus possibly contributing to the defects in conidiation, appressorium formation and infection seen in *MoALR2* knockdown transformants.

We found that silencing of Mg^{2+} transporters led to a loss of cell wall integrity. *MoMPS1*-dependent CWI signalling is important for development and infection in *M. oryzae* (Dean *et al.*, 2005). The cell wall integrity (CWI) cascade in *S. cerevisiae* involves activation of MAP

kinase to regulate cell wall biogenesis, cell wall remodelling, reorganisation of actin cytoskeleton, and secretory vesicle targeting (Levin, 2005). We show that a decrease in Mg^{2+} levels in knockdown transformants affects cell wall structure by alteration of expression of CWI signalling genes. The decreased expression of hydrophobins, *PMK1* and genes of the CWI pathway, and low levels of cAMP in *MoALR2* knockdown transformants are among the major factors contributing to their decreased ability to conidiate, form appressoria and cause infection. Given this requirement for Mg^{2+} transport at all stages of the *M. oryzae* infection cycle, CorA transporters may be good targets for the development of antifungal agents. Ion channel blockers, agents that sequester Mg^{2+} from the fungal environment and rice lines expressing RNAi targeting Mg^{2+} transporter could prove effective against fungal proliferation. Regulation of Mg^{2+} involves localisation, compartmentalisation, and sequestration (Walker, 2004). Higher expression of *MoALR2* was observed in *Δmnr2* transformants. The maximum sensitivity of *Δmnr2* to Co(III)Hex. and higher intracellular levels of Mg^{2+} in *Δmnr2* correlated with the increased levels of *MoALR2*. We suggest that absence of organellar transporter MoMnr2 in *Δmnr2* may lead to up-regulation of expression of the plasma membrane transporter *MoALR2*, possibly to maintain adequate cytosolic Mg^{2+} levels when transport from vacuolar stores is compromised. In *S. cerevisiae*, under Mg^{2+} limiting conditions, vacuolar Mg^{2+} contributes towards the maintenance of cytosolic Mg^{2+} levels through the activity of *MNR2* (Pisat *et al.*, 2009). *Δmnr2* showed less growth than WT in presence of EDTA, suggesting a requirement for MoMnr2 function in regulating Mg^{2+} levels under Mg^{2+} deficient conditions in *M. oryzae* too. We also found that a loss of MoMnr2 protein function led to a defect in the ability to maintain pH in *Δmnr2* compared to WT, as indicated by growth defects on media at different pHs and alteration in the ability to alkalinise the media. Immunostaining in WT and *Δmnr2* suggested vacuolar localisation of MoMnr2. Vacuolar localisation of MoMnr2 and alteration in pH regulation in *Δmnr2* led us to look at

laccase activity as laccase has previously been shown to be a marker for vacuolar function in a *Δvph1* mutant in *C. neoformans* and vacuolar acidification has been shown to be essential for post-translational modification of laccase (Panepinto and Williamson, 2006). The laccase gene *CNLAC1* from *C. neoformans var. neoformans* has been shown to be important for virulence (Salas *et al.*, 1996). Elevated levels of laccase have been shown to cause chestnut blight disease in *Cryphonectria parasitica* (Choi *et al.*, 1992). We found 238.7 fold decreased activity of laccase in *Δmnr2*. Defect in copper assembly into laccase is one of the factors which has been shown in the *C. neoformans Δvph1* mutant responsible for decreased laccase activity, which is restorable by addition of extracellular copper (100µM) (Zhu *et al.*, 2003).

We cloned MoAlr2 in frame with GFP for localisation of MoAlr2, but failed to see any GFP fluorescence in the MoAlr2 transformants despite the fusion construct being transcribed (RT-PCR) and even translated (Western blot using anti-GFP Antibodies). We also studied the localisation of CorA transporters by immunostaining using anti-CorA domain antibodies. There was intracellular as well as membrane staining in the WT spores, while in *Δmnr2*, in which only MoAlr2 is present, there is no intracellular staining, but the membrane shows staining, indicating the presence of MoAlr2 only on the membrane. Precise localisation of MoAlr2 and MoMnr2 with GFP/RFP tagging will help shed light on the differential expression and intracellular fates of these proteins in *Δmnr2* and *MoALR2* knockdown transformants and also at different developmental stages of *M. oryzae*.

In future, the role of CorA transporters in sub-cellular Mg^{2+} distribution and dynamics of cations between the organelles and the cytoplasm need to be addressed in greater detail. The external environment of the fungus is dynamic with respect to the levels of divalent cations. We show that CorA transporters are regulated in response to changes in extracellular Mg^{2+} and Ca^{2+}. *MoALR2* and *MoMNR2* are induced by depletion of extracellular Mg^{2+}, while their levels decreased at higher concentration of Mg^{2+}. *MoMNR2* may be down-regulated to reduce

the efflux of Mg^{2+} from organellar stores to prevent cytoplasmic Mg^{2+} toxicity. With increasing concentration of Ca^{2+}, both the transcript and protein levels of *MoALR2* increased. The severity of panicle blast is positively correlated with Mg^{2+} levels and negatively affected by Ca^{2+} concentration in the plant tissue (Filippi and Prabhu, 1998). Determination of the ionic composition of the leaf and transporter levels in the fungus during invasion and proliferation will shed further light on the significance of Mg^{2+} uptake by CorA transporters at the site of infection. Recently, it has been shown that *MoALR2* is down-regulated during *in planta* growth in barley and rice at 72 hours post inoculation (hpi) (Mathioni *et al.*, 2011). Spore germination and appressorium formation, where we have shown a requirement for MoAlr2, are part of the early events of infection on the plant host and are completed well before 72 hpi. While sufficient intracellular Mg^{2+} is clearly essential for the early developmental events, the requirement for high *MoALR2* levels could be due to low extracellular Mg^{2+} availability before infection, a situation that may not prevail once infection has been established.

Δmnr2 showed an early autolysis phenotype that was aggravated by Mg^{2+} deprivation. The autolysis occurred even earlier in the *MoALR2* knockdown transformants. *Δmnr2* formed fewer aerial hyphae compared to WT and was also shown to impair male fertility.

Due to failure to obtain a true knockout for *MoALR2* in spite the use of various approaches, and from the drastic defects observed in the knockdown transformants, we conclude that *MoALR2* is likely essential for viability of the rice blast fungus. Further, we did not obtain any knockdown transformants with less than 30% expression of *MoALR2*, indicating that a critical level of *MoALR2* expression is essential for viability (although it cannot be ruled out that our silencing procedure is unable to achieve a higher level of silencing). Previous large scale random mutagenesis screens for pathogenicity have also not uncovered any mutants of this

gene. We also tried to generate knockouts of *MoMRS2* (Mgg_06582 and Mgg_02763) and screened over 80 transformants, but failed to get any true knockouts. Study of CorA Mg^{2+} transporters, *MoMNR2*, *MoALR2* and *MoMRS2*, and their localisation will help to understand organellar distribution of these transporters and their contribution to cytosolic homeostasis. This will aid in a better understanding of the intracellular regulation of Mg^{2+} and provide more insight into the contribution of CorA Mg^{2+} transporters towards pathogenesis and development in the rice blast fungus *M. oryzae*.

In summary, we have shown that *MoALR2* is predominantly responsible for maintenance of Mg^{2+} homeostasis and modulates key signalling pathways to affect development and pathogenicity in *M. oryzae*. Knockdown of CorA Mg^{2+} transporters below a critical level makes the pathogen avirulent and hence these transporters are potential targets for anti-fungals.