농학석사학위논문

Functional conservation of *MoCRZ1* in *Magnaporthe oryzae* and its orthologs in *Fusarium graminearum* and *Neurospora crassa*

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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Functional conservation of *MoCRZ1* in *Magnaporthe oryzae* and its orthologs in *Fusarium graminearum* and *Neurospora crassa*

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Functional Conservation of *MoCRZ1* in *Magnaporthe oryzae* and its orthologs in *Fusarium graminearum* and *Neurospora crassa*

UNDER THE DIRECTION OF DR. YONG-HWAN LEE

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

BY
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APPROVED AS A QUALIFIED THESIS OF DA-YOUNG LEE
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ABSTRACT

Functional conservation of *MoCRZ1* in *Magnaporthe oryzae* and its orthologs in *Fusarium graminearum* and *Neurospora crassa*

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Calcium signaling is one of the most common and important signal transduction cascades present in any living organism and is said to be highly conserved in throughout evolution. In *Magnaporthe oryzae*, a rice blast pathogen, calcium signaling pathway is required for conidiation and infection-related development, which contributes to the pathogenicity of the

fungus. *M. oryzae* calcineurin-responsive zinc finger 1 (*MoCRZ1*) is involved in the calcium-dependent signaling pathway of *M. oryzae*, which acts as a transcription factor. Deletion mutants of *MoCRZ1* (Δ*Mocrz1*) showed drastic reduction in conidiation, hypersensitivity to calcium and cell membrane destabilizing agents as well as dramatic reduction in virulence. In this study, the orthologs of *MoCRZ1* in *Fusarium graminearum* (*FgCRZ1*) and *Neurospora crassa* (*NcCRZ1*) were identified using BLASTP. Domain analyses showed that *FgCRZ1* and *NcCRZ1*, possess one calcineurin-docking domain and two C_2H_2 zinc finger domains, similar to *MoCRZ1*, suggesting that the orthologs interact with calcineurin and that they are transcription factors involved in calcium signaling. Transformations of *FgCRZ1* or *NcCRZ1* into Δ*Mocrz1* complemented Δ*Mocrz1* defects in conidiation, appresorial turgor generation and eventually restored the virulence of nearly non-pathogenic Δ*Mocrz1*. Both *FgCRZ1* and *NcCRZ1* were able to suppress the calcium sensitivity of Δ*Mocrz1*, however, only Δ*Mocrz1*::*NcCRZ1* was able to resist the cell wall disturbing agent (Congo red) and only Δ*Mocrz1*::*FgCRZ1* was tolerant to cell membrane destabilizing agent (SDS), showing selective complementation of cell wall and membrane stress intolerant phenotype of Δ*Mocrz1*. Taken altogether, these results suggest that *FgCRZ1* and *NcCRZ1* are calcineurin-responsive

zinc finger transcription factors in *F. graminearum* and *N. crassa*, respectively, which are highly conserved despite of the differences of in lifestyle from which they belong, and could substitute the major roles of *MoCRZ1* in conidiation, calcium sensitivity and virulence.

KEYWORDS: Functional conservation *Magnaporthe oryzae CRZ1*, *Fusarium graminearum CRZ1*, *Neurospora crassa CRZ1*, Calcium signaling,

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INTRODUCTION

All living cells must be able to perceive signals from their internal and external environments that act as stimuli to regulate their proliferation, differentiation, arrest or death (Dickman and Yarden, 1999) and be able to react to various environmental as well as developmental signals (Sanders *et al.*, 2002).

Signal transduction networks are utilized by all living cells in order to conduct processes involved in nutrient acquisition, development, metabolism and adaptation (Sanders *et al*., 1999). Fungi sense and respond to the immediate environment via signal transduction cascades, which transduce external information and effect appropriate responses (Soriani *et al.*, 2008). Various signaling networks have evolved allowing cells to produce appropriate cellular responses, mediated by receptors, non-protein messenger, transcription factors and more. (Sanders *et al.*, 2002). Among the numerous signal transduction cascades, calcium signal transduction pathway is utilized by all eukaryotic cells to control gene expression and cellular processes (Matheos *et al.*, 1997). Ca^{2+}-mediated signaling has been less investigated in filamentous fungi, than in yeasts although some notable

work was performed on calcineurin (Stie and Fox, 2008).

Various cellular processes in organisms are under the control of Ca^{2+}, a ubiquitous and versatile intracellular signal (Berridge *et al*., 2000). This molecule regulates various pathways, such as in cell growth and development in organisms (Berridge *et al*., 2003). Transduction of abiotic and biotic signals lead to changes in cytosolic free calcium (Sanders *et al.*, 2002). For instance, in *S. cerevisiae,* Ca^{2+} levels rise as a response to stress (Cyert, 2003) and this increase in cytosolic calcium concentration activates serine/threonine phosphatases such as calcineurin, in which its activity is dependent to intracellular calcium concentration changes (Cyert *et al.*, 1991; Stie and Fox, 2008). The Ca^{2+}/calmodulin-regulated protein phosphatase, calcineurin, is highly conserved throughout evolution and is an essential component of the Ca^{2+}- mediated signaling in eukaryotes (Cyert, 2003). This phosphatase is essential for controlling cell morphology as well as virulence in fungi (Cyert, 2003; Fox and Heitman, 2002; Steinbach *et al.*, 2007). Furthermore, calcinerurin-dependent signaling mechanisms mediates fungal stress responses and pathogenesis (Stie and Fox, 2008).

Activated calcineruin targets key substrates that support cell survival, such as the calcineurin-responsive transcription factor *Crz1* of *S. cerevisiae*

(Cyert, 2003; Matheos *et al.*, 1997; Stathopoulos-Gerontides *et al.*, 1999; Stathopoulos and Cyert, 1997) and mediates the dephosphorylation of the cytoplasmically localized transcription factor, *Crz1*, resulting to its translocation into the nucleus (Viladevall *et al.*, 2004). Calcineurin allows fungi to genetically and biochemically adapt to specific environmental cues (Stie and Fox, 2008) and in some fungi, including a few filamentous fungi, Ca^{2+} known to be involved in physiological processes such as spore germination, hyphal tip growth and branching (Osheror and May, 2001; Gadd 1994; Shaw and Hoch, 2001) (Gadd *et al.*, 1994; Osherov and May, 2001; Stie and Fox, 2008). In addition, calcineurin/Crz1p regulated genes are said to be involved in cell survival under stress conditions (Cyert, 2003).

In the non-pathogenic model yeast *S. cerevisiae*, calcineurin activity regulates stress activated transcription, Ca^{2+} homeostasis (Cyert, 2001; Fox and Heitman, 2002). Genes responsible for cell wall integrity and for ion transport ATPases, which contribute to yeast's alkaline cation homeostasis, were found to be essential for the maintenance of cell wall integrity (Cunningham and Fink, 1996; Mazur *et al.*, 1995; Mendoza *et al.*, 1994).

Several studies have reported the existence of the orthologs in *S. cerevisiae Crz1* in numerous fungi (Choi *et al.*, 2009; Matheos *et al.*, 1997;

Mendizabal *et al.*, 1998; Schumacher *et al.*, 2008; Soriani *et al.*, 2008; Stathopoulos and Cyert, 1997; Zhang *et al.*, 2013). In addition, orthologs of *Crz1*, including *MoCRZ1*, have been were reported to contain two protein domains, the zinc finger motifs as well as a putative calcineurin-docking domain (Choi *et al.*, 2009).

Recent studies of *Crz1* orthologs in have shown sensitivities to various ions. In *S. cerevisiae*, Δ*crz1* showed sensitivity to cations such as Ca^{2+}, Na^{+}, Li^{+}, Mn^{2+} and hydroxyl anions (Matheos *et al.*, 1997; Stathopoulos and Cyert, 1997) while in fission yeast *Schizosaccharomyce pombe*, *Prz1* showed hypersensitivity to Ca^{2+}. Yeast *Torulaspora delbrueckii TdCRZ1*, sensitivie to high Na^{+} (Hernandez-Lopez *et al.*, 2006). Human pathogenic yeast-like pathogens, *Candida albicans* exhibited sensitivity to alkaline cations (Santos and de Larrinoa, 2005). In the case for human pathogenic filamentous fungi, *CrzA*, *Crz1* ortholog in *A. fumigatus* showed acute sensitivity to increased concentrations of Ca^{2+} and Mn^{+} (Soriani *et al.*, 2008), while *A. nidulans crzA* exhibited extreme sensitivity to Ca^{2+} and alkaline pH (Spielvogel *et al.*, 2008). For plant pathogenic filamentous pathogens, *Botrytis cinerea* and *Penicillium digitatum*, *BcCRZ1* was identified to be sensitive to extreme pH, Ca^{2+}, Li^{+}, H_2O_2 and *TdCRZ1* to be sensitive to Ca^{2+}

and H_2O_2 (Zhang *et al.*, 2013).

Crz1 orthologs were also identified to be involved in various developmental processes as well as in virulence. *Crz1* orthologs in *C. glabarata*, *T. delbruecki*, *S. pombe* were found to be involve in virulence and stress responses (Hirayama et al., 2003; Miyazaki et al., 2010; Hernandez-Lopez et al., 2006), while filamentous ascomycetes such as *B. cinerea*, *A. fumigatus*, *A. nidulans* and *P. digitatum* were identified to play a role in mycelial growth and virulence (Cramer *et al.*, 2008; Schumacher *et al.*, 2008; Soriani *et al.*, 2008; Zhang *et al.*, 2013).

In *Magnaporthe oryzae*, a pathogenic filamentous ascomycete utilizing appressorium to penetrate the host cells, *M. oryzae* calcineurin-responsive transcription factor 1 (*MoCRZ1*), was reported to be involved in conidiation, calcium stress, cell membrane stress and virulence of the said pathogen (Choi *et al.*, 2009).

Fusarium graminearum is a filamentous plant pathogenic ascomycete which causes the *Fusarium* head blight of wheat. This pathogen does not produce an appressorium, however, penetrates the host through the spaces between lemma and palea (Guenther and Trail, 2005; Jansen *et al.*, 2005) and eventually gets in contact with the stomatal openings of the plant.

Another filamentous ascomycete, yet does not exhibit pathogenic lifestyle, *N. crassa*, is saprophyte which is often abundantly found after forest fires (Xie *et al.*, 2004).

In the present study, the *MoCRZ1* orthologs in ascomycetes of different lifestyles, *F. graminearum* (*FgCRZ1*) and *N. crassa* (*NcCRZ1*), were identified and characterized and these orthologs were found to play a role in conidiation, calcium stress tolerance, cell membrane stresses and virulence.

MATERIALS AND METHODS

I. Fungal strains, media and growth conditions

M. oryzae wild-type strain KJ201, the *MoCRZ1* deletion mutant strain (Δ*Mocrz1-2*), *F. graminearum* and *N. crassa* were obtained from the Center for Fungal Genetic Resources (CFGR, http://genebank.snu.ac.kr). *M. oryzae* wild-type strain KJ201 was maintained in V8 juice agar media (8% V8 juice (w/v), 1.5% agar powder (w/v), pH6.7) at 25℃ under the constant fluorescent light, while Δ*Mocrz1* mutant was maintained in hygromycin B amended V8 juice agar media (200 ppm final concentration). Fungal transformants generated in this study, Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*, were maintained in V8 juice agar media supplemented with hygromycin B (200 ppm final concentration) and geneticin (200 ppm final concentration). The inoculum sources for all the experiments performed in this study were grown on V8 juice agar media for 7 days at 25℃ under the continuous light condition, except for the ion sensitivity and cell wall and membrane stability experiments, which were grown on V8 agar media then transferred onto minimal agar media (1% glucose (w/v), 0.1% trace element, 0.1% vitamin supplement, 0.6% $NaNo_3$ (w/v), 0.05%

KCl (w/v), 0.05% $MgSO_4$ (w/v), 0.15% KH_2PO_4 (w/v), pH 6.5) at 25℃ under the continuous light condition.

II. Nucleic acid manipulations

Isolation of genomic DNA was done using the standard protocols (Rogers and Bendich, 1985). Genomic DNA was isolated from mycelia collected from 5 day-old cultures grown in liquid complete medium (LCM) (0.6% yeast extract (w/v), 0.6% casamino acid (w/v) and 1% sucrose (w/v)) exposed to shaking at 25C. $CaCl_2$ (0.4M), NaCl (0.5M), SDS (0.1%), CongoRed (400ppm) were supplemented to CM agar, respectively, to determine the effects of these compounds on the fungal growth of the strains of interest. Agarose gel separation, restriction enzyme digestion were performed following standard procedures (Sambrook *et al.*, 1989). DNA fragments for DNA hybridization probes were labeled with ^{32}P by using Rediprime™ II Random Prime Labeling System kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's manuals. Easy-Spin™ total RNA extraction kits (Intron Biotechnology, Seongnam, Korea) were used for the isolation of total RNAs and followed the manufacturer's instructions. To obtain the cDNAs of *F. graminearum* and *N.*

crassa, 5μg of total RNA was used and cDNA synthesis was performed using the oligo dT primers with the using the ImProm-II™ Reverse Transcription System kit (Promega, Madison, WI, USA) following the manufacturer's instructions.

III. Identification of *FgCRZ1* and *NcCRZ1*

Nucleotide and proteins sequences were retrieved and analyzed using the softwares provided by the Comparative Fungal Genomics Platform (Park *et al.*, 2008; http://cfgp.snu.ac.kr/) and National Center for Biotechnology Information (http://www.ncbi.nlm. nih.gov/blast/) (McGinnis and Madden, 2004). To find the putative *MoCRZ1* orthologs in *F. graminearum* and *N. crassa*, 726 amino acid sequence of *MoCRZ1* was used as the query sequence against the *F. graminearum* 3A protein and *N. crassa* 10 protein datasets, using the BLASTP, respectively. Reciprocal BLASTP was also done to add confidence for the BLASTP searches. Nucleotide and amino acid sequences were aligned using the ClustalW algorithm (Thompson *et al.*, 1994) and domain structures were identified using InterProScan (Mulder *et al.*, 2005).

IV. Generation of complementation constructs and fungal transform

Protocols for protoplast preparation and fungal transformations were adapted from a previous study (Leung *et al.*, 1990). Complementation constructs used for fungal transformations were primarily generated by double joint PCR. Each complementation construct was composed of a promoter fragment, open reading frame (ORF) fragment and a terminator fragment. The promoter fragment, 1.2 kb upstream of *MoCRZ1* open reading frame (ORF), was amplified with primers *MoCRZ1* promoter 5F and 3R, using the genomic DNA of KJ201. The *FgCRZ1* ORF fragment (2177 bp) was amplified with *FgCRZ1*-cDNA-fusion-5F and 3R using the cDNA of *F. graminearum*. This fragment contained *FgCRZ1* ORF while for the *NcCRZ1* fragment (2165 bp), *NcCRZ1*-cDNA-fusion_5F and 3R primers were used to amplify NcCRZ1 ORF from the cDNA of *N. crassa*. The terminator fragment (720 bp) of the constructs was amplified using eGFP_5F and Ncterm_3R primers. The final single PCR products, consisting of *MoCRZ1* promoter-*FgCRZ1* ORF-terminator (4097 bp) and *MoCRZ1* promoter-*NcCRZ1* ORF- terminator (4085 bp), to be used for the complementation of *FgCRZ1* and *NcCRZ1* were amplified with primers *MoCRZ1*promoter-5F-nested and Ncterm_3R_nested (Table 1). The

complementation constructs were co-transformed with pII99 containing the geneticin resistance gene, into the protoplasts of Δ*Mocrz1*. Hygromycin and geneticin-resistant fungal transformants were selected using TB3 agar media (0.3 % yeast extract (w/v), 0.3% casamino acids (w/v), 1% glucose (w/v), 20% sucrose (w/v) and 0.8% agar powder (w/v)) supplemented with hygromycin B (200ppm final concentration) and geneticin (200ppm final concentration). Hygromycin B and geneticin-resistant Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* transformants were screened by PCR using primer sets that amplified their respecting ORFs, *FgCRZ1*-ORF_5F and 3R and *NcCRZ1*-ORF_5F and 3R, respectively.

V. Southern hybridization analysis

Southern blot analysis was conducted using standard procedures (Sambrook *et al.*, 1989). To confirm the successful ectopic insertion of *FgCRZ1*, genomic DNA of Δ*Mocrz1*::*FgCRZ1* was isolated from mycelia and digested using *SmaI*. The 500 bp-*FgCRZ1* probe was generated with primers *FgCRZ1*-ORF_5F and *FgCRZ1*-ORF_3R-southern probe, amplified using the gDNA of Δ*Mocrz1*::*FgCRZ1* as template. As for *NcCRZ1*, genomic DNA of Δ*Mocrz1*::*NcCRZ1* was isolated and digested using *XhoI*.

A 591 bp-probe was generated with primers *NcCRZ1*-ORF_5F and *NcCRZ1*-ORF_3R-southern probe using the gDNA of Δ*Mocrz1*::*NcCRZ1* as template. The *FgCRZ1* probe hybridized to a 1249 bp-fragment in the gDNA of Δ*Mocrz1*::*FgCRZ1* while the *NcCRZ1* probe hybridized with a 1440 bp-fragment in the gDNA of Δ*Mocrz1*::*NcCRZ1*, confirming the successful single ectopic insertion of *FgCRZ1* and *NcCRZ1* into the protoplasts of Δ*Mocrz1*.

VI. Conidiation and conidiophore production assays

Conidia for conidiation assays were harvested from strains inoculated on V8 agar which were grown for 7 days at 25℃. Sterilized distilled water was used to harvest the conidia of all the strains and the conidial suspensions produced were filtered through 2 layers of Miracloth. The filtered conidia suspensions were placed in 15ml falcon tubes and were adjusted to a final volume of 5ml using sterilized distilled water. The falcon tubes containing the conidia suspension were briefly vortexed. 10μl was pipetted out and the number of conidia was counted using a hemacytometer. This experiment was done triplicates with three experimental repeats. To microscopically observe the conidiophore and conidia production, the surface of the actively

growing mycelia was scraped off and cut into 1cm x 1cm agar block. The agar block was placed on a slide glass, and the scraped mycelial surface was covered with a coverslip. The set up was placed in a moistened box, incubated for 24hours at 25℃.

VII. Sensitivity to ions, cell wall and cell membrane disturbing agents

Sensitivity to ions, cell wall and membrane degrading agents was measured by taking the diameter (in centimeters) of the mycelial colony grown on different stress media. Strains were grown on minimal media and inoculum (6mm in diameter) was taken from actively growing mycelia portion of the colony. 6mm diameter inoculum was inoculated on CM supplemented with 0.4M $CaCl_2$, 0.5M NaCl, 400ppm Congo red and 0.1% SDS then incubated for 12 days at 25℃.

VIII. Appressorium formation, cytorrhysis, host penetration and infiltration assays

Conidial germination and appressorium formation assays were measured by observing conidia suspension dropped on hydrophobic coverslips. 40μl of conidia suspension ($3x10^4$ conidia per milliliter) was dropped on top of

hydrophobic coverslips. The experiment was done in triplicates. Conidia were harvested using sterilized distilled water from 7-day-old mycelia inoculated on V8 agar media. The coverslips were supported by a slide glass and were placed in a moistened box. Incubation was done at 25℃ for 24 hours. Microscopic observations were performed to determine the number of conidia germinated as well as the percentage of germinated conidia which produced appressorium. To test for the turgor generation ability of the appressorium formed, cytorrhysis technique was used as previously described (Odenbach *et al.,* 2007). To determine the ability of the strains to penetrate the host plant cells, rice sheath assays were performed as previously described (Viaud *et al.*, 2002). Conidia suspension adjusted to $3x10^4$ conidia/ml were introduced to rice sheaths from 4-6 leaf stage rice plant (Koga *et al.*, 2004). The rice sheaths containing the conidia suspensions were placed in a moistened box and incubated at 25℃ for 48 hours. After incubation, the chlorophyll enriched portions of the rice sheathes were trimmed off and a single layer of epidermal cells of the mid vein were microscopically observed. As for the infiltration assay, 3x10 conidia/ml conidia suspensions were introduced to wounds, made by piercing the leaf using a syringe, on the surface of rice leaves. The samples

were placed in a moistened box and were incubated at 25℃ for 5 days.

IX. Pathogenicity assay

Conidia were collected from 7-day-old mycelial colony grown on V8 juice agar for 7 days at 25℃. The conidia suspensions were prepared and adjusted to a $5x10^5$ conidia/ml, adjusting the final volume to 10ml with 250ppm Tween 20. Spray inoculation of the suspensions were done onto the 3 or 4-week-old susceptible rice plant cultivar Nakdongbyeo. The inoculated rice plants were placed in a plastic bag for 24 hours to allow sufficient humid environment then transferred into a rice growth incubator. The rice incubator used was programmed to provide 80% humidity at 35℃, with a photoperiod of 16hours (fluorescent lamps) for days (Valent *et al*, 1991). Then the rice plant was incubated at 30℃ for 6 days.

RESULTS

I. Identification of *MoCRZ1* orthologs in *F. graminearum* and *N. crassa*

Orthologs of *M. oryzae* calcineurin-responsive transcription factor (*MoCRZ1*), were identified in the genomes of *F. graminearum* and *N. crassa* using BLASTP searches (www.cfgp.riceblast.snu.ac.kr). Predicted amino acid sequences of *MoCRZ1* (MGG_05133.7) showed highest homology to FGSG01341.3 (*FgCRZ1*) in *Fusarium graminearum* dataset 3A and NCU07952.3 (*NcCRZ1*) in *N. crassa*. MoCRZ1p encodes a transcription factor of 726 amino acids and contains two C_2H_2 zinc finger motifs and showing high levels of similarity with its orthologs in *F. graminearum* and *N. crassa*.

FgCRZ1p and encodes 714 amino acids and shares 61.17% homology to MoCRZ1p while NcCRZ1p is comprised of 710 amino acids and 54.75% homology with respect to MoCRZ1p. The two C_2H_2 zinc finger domains (IPR007087) located at the C-terminus of MoCRZ1p were also identified in FgCRZ1p and NcCRZ1p (Figure 1A), and found to be highly conserved in terms of amino acid sequence. The two zinc finger motifs located near the carboxyl-terminus of MoCRZ1p were located at the amino acid positions

500~526 and 528~555. In FgCRZ1p, they were present at the amino acid positions 493~519 and 521~548 while in NcCRZ1p was identified to be at the amino acid positions 487~513 and 515~542. FgCRZ1p zinc finger motif sequences were 96% identical to that of the MoCRZ1p except at the 4^{th} and 21^{th} amino acid of the first and second zinc finger domains, respectively (Figure 1B). As for NcCRZ1p zinc finger motif sequences, they were 95% identical to that of the MoCRZ1p, except at the 4^{th} and 5^{th} amino acid of the first zinc finger motif and at the 2^{nd} and 7^{th} amino acid of the second zinc finger. C-terminal zinc finger motifs are known to act as the mediator for *Crz1* to bind to the DNA (Hirayama *et al.*, 2003) and together with the observations suggests that *FgCRZ1* and *NcCRZ1* are transcription factors.

In addition to the zinc finger motifs, another protein domain, the calcineruin docking domain (CDD) found in *MoCRZ1*, was found also present in both *FgCRZ1* and *NcCRZ1* (Figure 1C). CDD is the site for calcineurin to directly interact with *Crz1* in *S. cerevisiae* and this specific recognition sequence is characterized by the amino acid sequence PIISIQ (Boustany and Cyert, 2002). This sequence is essential for the dephosphorylation of Crz1p by calcineurin and leads to the subsequent

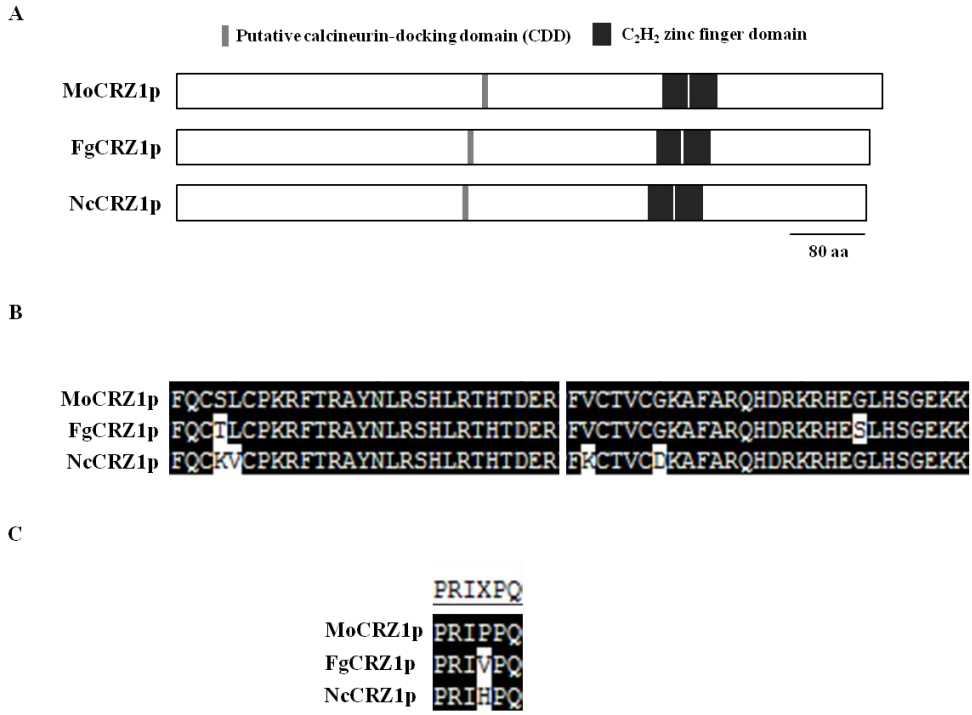

Figure 1. Protein structure and amino acid sequence alignments of MoCRZ1p, FgCRZ1p and NcCRZ1p. (A) A putative calcineurin-docking domain (CDD) and two C_2H_2 zinc finger motifs are present in MoCRZ1p, FgCRZ1p and NcCRZ1p. (B) Amino acid sequence alignments of the two zinc finger motifs found in *MoCRZ1*, *FgCRZ1* and *NcCRZ1*, showing high degree of conservation. (C) Conserved CDD among the *MoCRZ1* orthologs in *F. graminearum*, *N. crassa* together with *MoCRZ1*.

translocation of the said transcription factor into the nucleus (Boustany and Cyert, 2002). CDDs were found to be well-conserved in various filamentous ascomycetes as well as in *MoCRZ1* (Choi *et al.*, 2009), and this site is located prior to the two zinc finger motifs was found to be located in the same manner in FgCRZ1p and NcCRZ1p. Furthermore, Putative CDD in MoCRZ1p, was composed of six amino acids, PRIPPQ. This domain was also identified in FgCRZ1p as PRIVPQ and in NcCRZ1p as PRIHPQ, with only a single amino acid modification on the 4th amino acid along the CDD sequence.

II. *FgCRZ1* and *NcCRZ1* restores conidiation defect of Δ*Mocrz1*

Fungal transformants Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*, were generated using PEG-mediated fungal co-transformation method. Each complementation constructs were composed of the following fragments amplified with primers previously mentioned (Table 1): the promoter (1.2 kb upstream of the *MoCRZ1* ORF), ORF (*FgCRZ1* ORF or *NcCRZ1* ORF) and eGFP-tagged terminator (Figure 2A), and were co-transformed together with the geneticin resistance gene-containing pII99, into hygromycin-resistant Δ*Mocrz1* protoplasts. The putative fungal transformants were

primarily screened on TB3 agar plates amended with hygromycin B (200 ppm) and geneticin (200 ppm), verified using PCR and confirmed by Southern hybridization analysis (Figure 2B).

To observe the effect of *FgCRZ1* and *NcCRZ1* on the ability to restore the conidia-producing ability of Δ*Mocrz1*, conidiophore formation and conidia production (asexual reproduction) were observed microscopically. KJ201 (wild type) produced three conidia per conidiophore in a sympodial manner, while Δ*Mocrz1* mutants produced dramatically reduced number of conidiophores with almost no conidia on their tips (Figure 3). In contrast, both Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* showed restoration similar to that of the wild type phenotype. Conidia produced by the fungal transformants Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* both produced three-celled, pointed blunt apex conidia, in a sympodial manner, similar to that of the wild type. Comparison of the extent of restoration of coindia between the two fungal transformants showed that Δ*Mocrz1*::*FgCRZ1* produced greater amount of conidia than Δ*Mocrz1*, however, less conidiophores and conidia than Δ*Mocrz1*::*NcCRZ1*. In the case of Δ*Mocrz1*::*NcCRZ1*, it was able to produce abundant conidia and conidiophores, nearly as of the wild type.

Table 1. Primers used in this study.

Primer name	Sequence (5'-3')
*MoCRZ1*_Promoter_5F	TTGACAAGCTGTGTAGTAGCGAGAAC
*MoCRZ1*_Promoter_3R	GGCGAAAGCTATGCGGTG
FgCRZ1-cDNA-fusion_5F	CGCATAGCTTTCGCCATGGATCAACAAGCTCAGGCTC
FgCRZ1-cDNA-fusion_3R	TCGCCCTTGCTCACCATCCGGCCACCAAAGTCACTG
NcCRZ1-cDNA-fusion_5F	CGCATAGCTTTCGCCATGGACCAACAGTACACCGA
NcCRZ1-cDNA-fusion_3R	TCGCCCTTGCTCACCATACGGCCGCCGTAGTCACTC
eGFP_5F	ATGGTGAGCAAGGGCGAGGAG
Ncterm_3R	ATCATCATGCAACATGCA
MoCRZ1 promoter_5F_nested	GTGCCAGTTGAGAGGGATCAAAATA
Ncterm_3R_nested	GATGTATTAAGAGTATAGGGGTC
FgCRZ1-ORF-5F	ATGGATCAACAAGCTCAGGCTC
FgCRZ1-ORF-3R	CCGGCCACCAAAGTCACTG
NcCRZ1-ORF_5F	ATGGACCAACAGTACACCGACG
NcCRZ1-ORF_3R	ACGGCCGCCGTAGTCACT
FgCRZ1-ORF_3R-southern probe	TGG AAC AAG TGA GGA GGG GTG
NcCRZ1-ORF-3R-southern probe	ATTGCGCGAATGCCTTCCTGTT

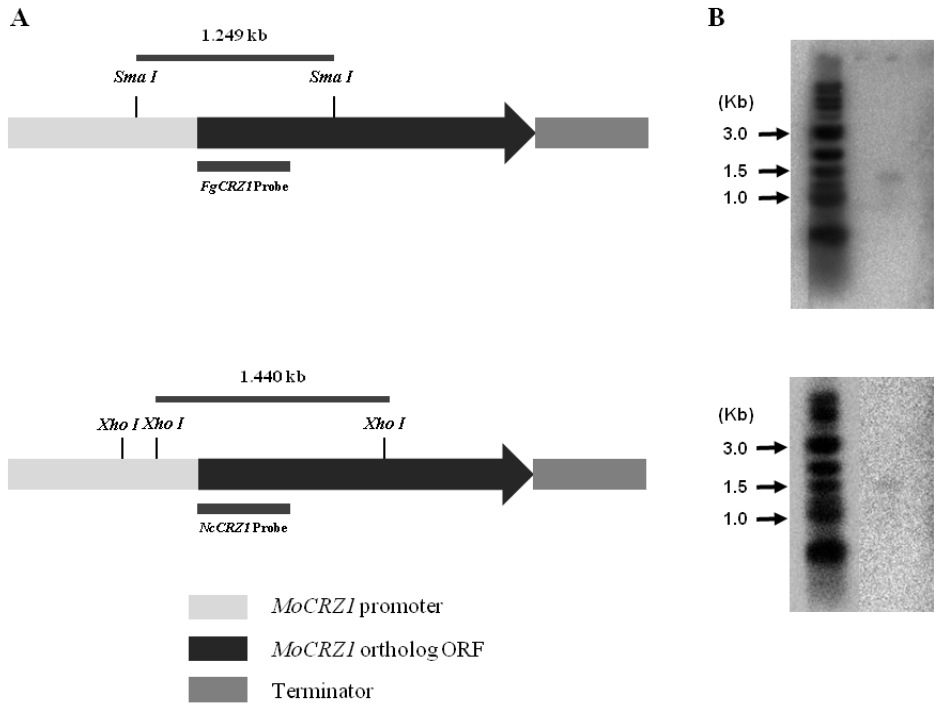

Figure 2. Complementation constructs and Southern hybridization of Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*.(A) Complementation constructs of *FgCRZ1* and *NcCRZ1* mainly containing 3 fragments; *MoCRZ1* promoter, ORFs sequence (*FgCRZ1* or *NcCRZ1*) and the terminator. (B) Southern hybridization blots confirming that Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* fungal transformants contain single insertion of the complementation constructs, respectively. gDNA from Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* was digested with *SmaI* and *XhoI*, respectively. The dotted lines indicate the size of the expected band fragment.

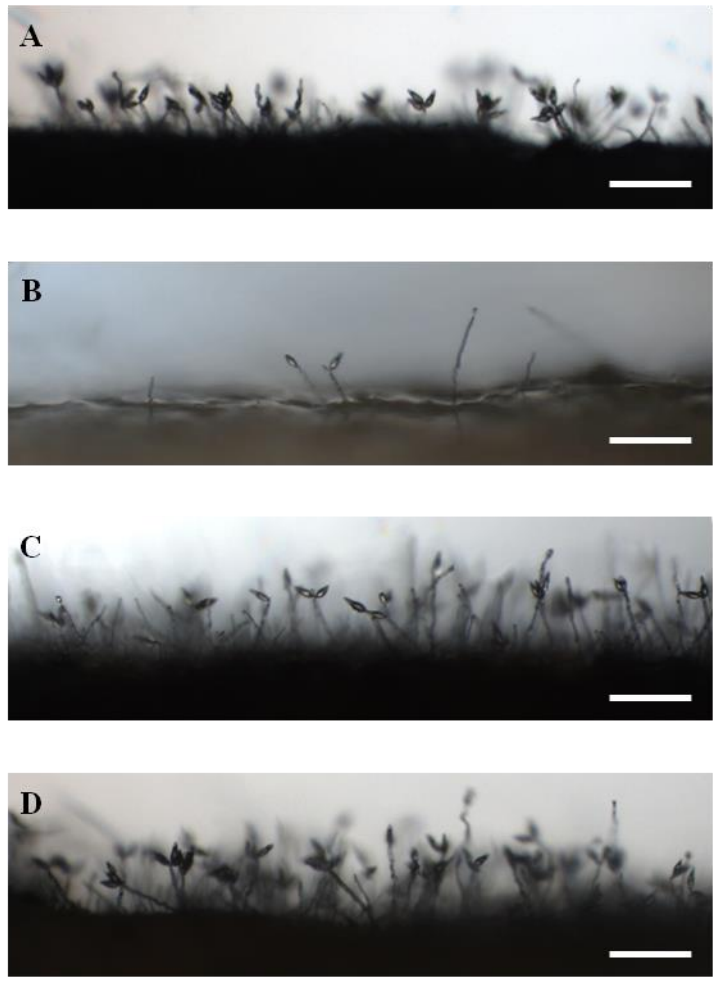

Figure 3. Conidiophore development and conidia of Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*. (A) KJ201 (wild type) (B) Δ*Mocrz1* (C) Δ*Mocrz1*::*FgCRZ1* and (D) Δ*Mocrz1*::*NcCRZ1* strains. Mycelial agar blocks were taken from actively growing portion of the mycelia grown in V8 agar plates. Incubation was done 24 hrs at 25℃ Scale bar indicates 100 μm.

To determine whether *FgCRZ1* and *NcCRZ1* could restore the decrease in conidia production of Δ*Mocrz1* on it was able to produce abundant conidia and conidiophores, nearly as of the wild type. Behalf of *MoCRZ1*, conidiation assays were conducted. According to Choi and colleagues, Δ*Mocrz1* exhibited significant decrease in conidia and conidiophore production (Choi *et al.*, 2009) and this was also observed in this study. Significant reduction in the production of conidia in Δ*Mocrz1* mutants were observed (20% of the wild type), relative to the amount of conidia produced by the wild type (Figure 4). However, this defect was restored upon the introduction of *FgCRZ1* or *NcCRZ1* into Δ*Mocrz1*, respectively. Δ*Mocrz1*::*FgCRZ1* showed restoration of conidia production rate of up to 76% compared to the wild type, while 91% of the wild type conidiation was observed for Δ*Mocrz1*::*NcCRZ1*. In addition, Δ*Mocrz1*::*MoCRZ1* (Choi *et al.*, 2009) was observed that *MoCRZ1* has restored conidiation upto 126% of the conidiation exhibited by the wild type. Thus, the capability *MoCRZ1* orthologs in *F. graminearum* and *N. crassa* to rescue the conidiation defect of Δ*Mocrz1* suggest that *FgCRZ1* and *NcCRZ1* play a role in conidiation. These observations are further supported by previous studies of *Crz1* orthologs in *A. fumigatus* , *B. cinerea* and *P. digitatum*, which were reported

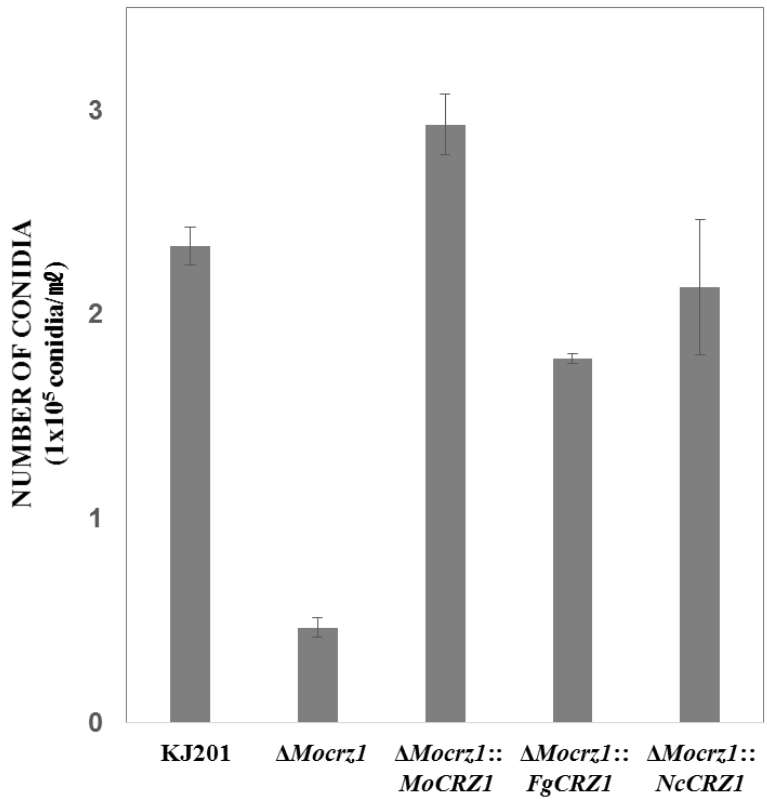

Figure 4. Conidiation of of Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*. Quantification of conidiogenesis KJ201, Δ*Mocrz1*, Δ*Mocrz1*::*MoCRZ1*, Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*; Conidia were harvested with 5ml sterile water, filtered through 2 layers of Miracloth and counted with a hemacytometer under a light microscope.

to play a role in conidiation as well (Schumacher *et al.*, 2008; Soriani *et al.*, 2008; Zhang *et al.*, 2013).

To observe whether *FgCRZ1* and *NcCRZ1* affect conidial germination and appressorium formation of Δ*Mocrz1*, one hundred randomly selected conidia were observed using the microscope. Germination rate was determined by counting the number of conidia germinated out of the one hundred conidia counted, while the appressorium formation rate was calculated by counting the number of appressoria produced by germinated conidia. The rates of these two developmental processes showed that both Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*, showed similar germination and appressoria formation rates as of the wild type, Δ*Mocrz1* and Δ*Mocrz1*::*MoCRZ1*, showing no significant difference among all the tested strains, in terms of their germination and appressorium formation rates (Figure 5).

III. *FgCRZ1* and *NcCRZ1* can rescue Δ*Mocrz1* from hypersensitivity to calcium stress

To determine whether *FgCRZ1* and *NcCRZ1* could replace the role of *MoCRZ1* in tolerating ionic stresses, Δ*Mocrz1*::*FgCRZ1* and

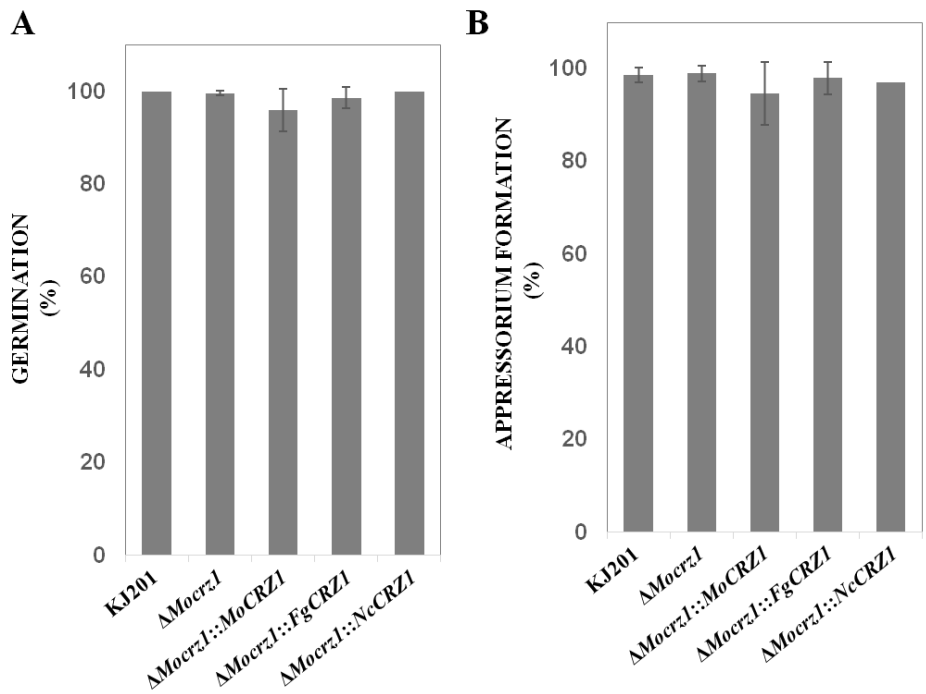

Figure 5. Germination and appressorium formation rates of Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*. (A) Germinated conidia were counted from randomly counted one hundred conidia to determine the germination rates of the strains were counted. (B) Appressoirum formation rate was determined by counting the number of appressorium formed out of the number of germinated conidia.

Δ*Mocrz1*::*NcCRZ1* were grown on complete media (CM)supplemented with different ions, $CaCl_2$ (0.4M) and NaCl (0.5M). Mycelial colony diameters on the 12th day of incubation were measured (Table 2). Growths of mycelial colonies of the control strains (KJ201, Δ*Mocrz1* and Δ*Mocrz1*::*MoCRZ1*) and Δ*Mocrz1*::*NcCRZ1* showed similar mycelial growth on the CM (control conidition) except for Δ*Mocrz1*::*FgCRZ1*, which showed slight decrease in mycelial diameter compared to that of the wild-type. On CM supplemented with $CaCl_2$ (0.4M), mycelial growth of Δ*Mocrz1* was strongly inhibited compared to that of the wild type while Δ*Mocrz1*::*FgCRZ1*, Δ*Mocrz1*::*NcCRZ1* and Δ*Mocrz1*::*MoCRZ1* were able to resist calcium stress and grow similar to the wild type phenotype (Figure 6). As for NaCl-supplemented CM, mycelial growth of Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* did not show significant difference from the wild type. In some filamentous fungi, such as *M. oryzae*, *A. nidulans*, C. *albcans*, *P. digitatum* and *A. fumigatus*, deletion of *Crz1* orthologs in these fungi were shown to exhibit hypersensitivity to calcium (Choi *et al.*, 2009; Hernandez-Lopez *et al.*, 2006; Santos and de Larrinoa, 2005; Spielvogel *et al.*, 2008). Altogether, this suggests that *FgCRZ1* and *NcCRZ1* were able to substitute *MoCRZ1* and function in tolerating calcium stress in *MoCRZ1* deletion

Table 2. Mycelial growths of Δ*Mocrz1*:: *FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* under ionic, cell wall and membrane stress condition

Strains	Mycelial growth (cm)				
	CM	$CaCl_2$ (0.4M)	NaCl (0.5M)	SDS (0.1%)	CongoRed (400 ppm)
KJ201	7.3 ± 0.1a	4.6 ± 0.1a	6.3 ± 0.1a	4.4 ± 0.2a	4.9 ± 0.3a
Δ*Mocrz1*	6.9 ± 0.6ab	0.9 ± 0.3b	6.0 ± 0.1b	1.8 ± 0.4b	4.5 ± 0.2ab
Δ*Mocrz1*::*MoCRZ1*	7.0 ± 0.3ab	4.2 ± 0.4a	6.4 ± 0.1a	4.5 ± 0.2a	5.1 ± 0.3a
Δ*Mocrz1*::*FgCRZ1*	6.0 ± 0.8b	4.4 ± 0.2a	5.9 ± 0.0b	4.2 ± 0.4a	4.2 ± 0.3b
Δ*Mocrz1*::*NcCRZ1*	7.4 ± 0.1a	4.2 ± 0.1b	5.9 ± 0.1b	1.7 ± 0.4b	4.5 ± 0.1ab

Figure 6. Mycelial growth of under different cation stress conditions. KJ201 (wild-type), Δ*Mocrz1*, Δ*Mocrz1*::*MoCRZ1*, Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* strains were grown 12 days on CM and CM supplemented with $CaCl_2$ (0.4M) and NaCl (0.5). 6mm-diameter mycelial agar blocks were used as inoculum onto different media and incubated for 12 days at 25℃.

mutant and suggests that these genes function in regulating calcium homeostasis.

IV. *FgCRZ1* and *NcCRZ1* selectively complements sensitivity of Δ*Mocrz1* to cell wall and membrane stress

To test whether *FgCRZ1* and *NcCRZ1* could replace *MoCRZ1* in withstanding cell wall and membrane stresses in Δ*Mocrz1*, cell wall perturbing agent (Congo red), and cell membrane destabilizing agent (SDS) were supplemented in CM. On Congo red supplemented CM (400 ppm), Δ*Mocrz1*::*FgCRZ1* exhibited decrease in mycelial growth, while Δ*Mocrz1*::*NcCRZ1* showed growth similar to that of the wild type (Figure 7). Furthermore, Wild type, together with *Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*MoCRZ1* did not show significant difference in terms of mycelial growth on CM supplemented with SDS (0.1%), however, mycelial growths of Δ*Mocrz1* and Δ*Mocrz1*::*NcCRZ1* were severely compromised.

Upon the introduction of *FgCRZ1* to *MoCRZ1* deletion mutants, the fungal transformant was still sensitive to Congo red, similar to the *MoCRZ1* deletion mutant phenotype, however was able to overcome the effects of SDS. In contrast, *NcCRZ1* was able to restore the sensitivity of *MoCRZ1*

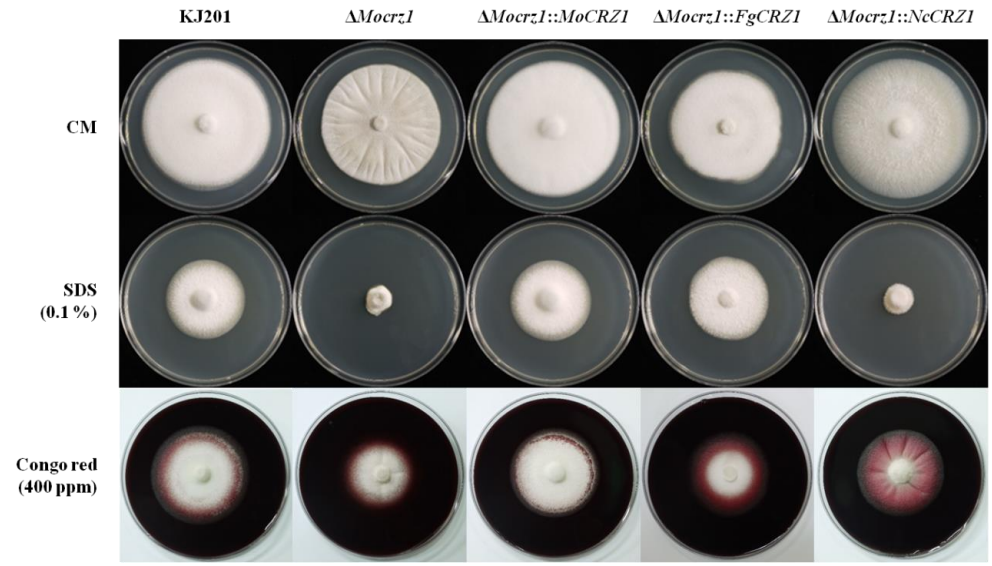

Figure 7. Mycelial growth of under cell wall and cell membrane destabilizing conditions. KJ201 (wild-type), Δ*Mocrz1*, Δ*Mocrz1*::*MoCRZ1*,Δ*Mocrz1*::*FgCRZ1*,Δ*Mocrz1*::*NcCRZ1* strains were grown 12 dyas on CM and CM supplemented with Congo red (400 ppm) and SDS (0.1%). 6mm-diameter mycelial agar blocks were used as inoculum onto different media and incubated for 12 days at 25℃.

deletion mutants to Congo red, but the mycelial growth of the transformant growth was strongly inhibited by the effects of SDS. This selective restoration of the mutant phenotype to cell wall and membrane destabilizing suggest that the transcription factors *FgCRZ1* and *NcCRZ1* play different roles and perform general functions in filamentous ascomycetes.

V. *FgCRZ1* and *NcCRZ1* restores full virulence in Δ*Mocrz1*

The *Crz1* orthologs in *A. fumigatus*, *A. nidulans*, *B. cinerea*, *C. glabarata* and *P. digitatum* were identified to be involved in pathogenicity (Miyazaki *et al.*, 2010; Soriani *et al.*, 2008; Spielvogel *et al.*, 2008; Zhang *et al.*, 2013). Likewise, Δ*Mocrz1* were reported to produce appressoria, however, only few, small disease lesions were observed on the leaves of the susceptible rice cultivar Nakdongbyeo (Choi *et al.*, 2009). The significant decrease in appressorial turgor generation exhibited by the Δ*Mocrz1* lead to the mutant's exhibit appressorial-mediated penetration to gain entry into host cells.

To investigate the restoration of appressorial turgor generation defect of Δ*Mocrz1* by *FgCRZ1* and *NcCRZ1*, cytorrhysis assays were performed to compare appressorial turgor of the wild type against of that of

Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*. Varying concentrations of glycerol (3M and 5M) were used and the proportion of collapsed appressoria after exposure to glycerol was calculated to estimate appressorial turgor. The number of collapsed appressoria for every glycerol solution concentration was measured and used as an indirect way of estimating the possible appressorial turgor generated within the appressoria produced by germinated conidia of the tested strains.

Wild type was observed to have the lowest number of collapsed appressoria under 3M and 5M concentrations of glycerol solution, while Δ*Mocrz1* mutants showed the highest rate of collapsed appressoria (Figure 8). Appressoria of Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* were observed to have lower rates of collapsed appressoria compared to Δ*Mocrz1*, but higher rates compared to the wild type. Between the two fungal transformants, the rate of collapsed appressoria for Δ*Mocrz1*::*NcCRZ1* is higher than Δ*Mocrz1*::*FgCRZ1*. These results indicate that *FgCRZ1* and *NcCRZ1* was able tolerate osmotic stress and restore the defects of appressorial turgor implying that these genes are involved in regulating osmostability of Δ*Mocrz1*.

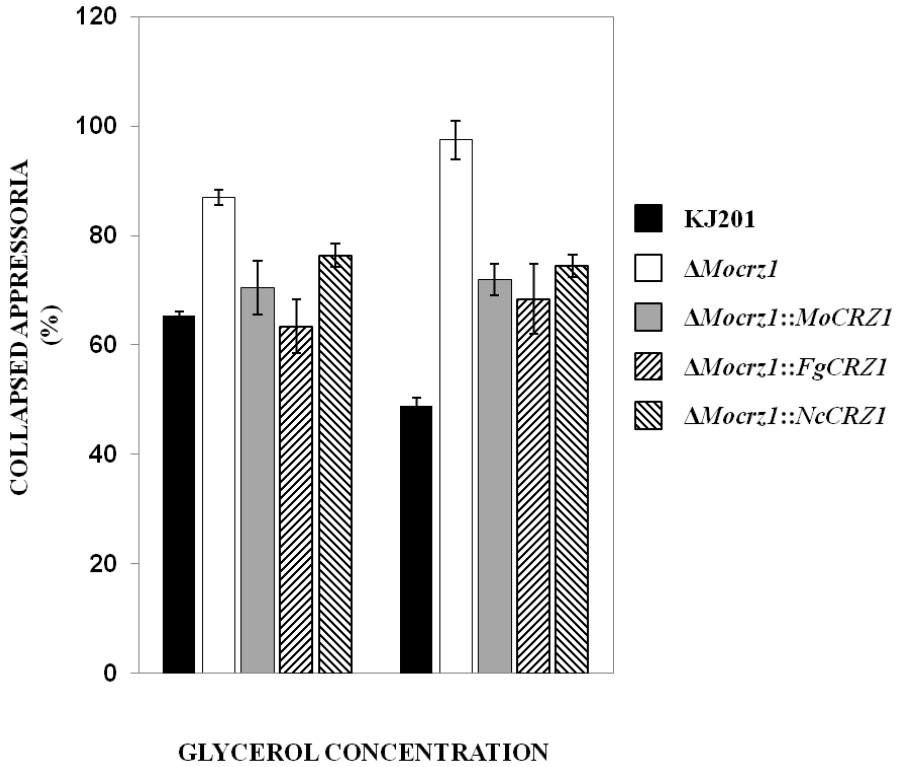

Figure 8. Cytorrhysis of Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*. Ability of appressorial turgor generation was measured via cytorrhysis assay. Matured appressoria were formed for 36 hours on hydrophobic coverslips. And the water droplet was replaced with 3 and 5 M glycerol. The percentage of collapsed appressoria was determined with three replicates

To determine whether *FgCRZ1* or *NcCRZ1* can substitute the role of *MoCRZ1* in terms of pathogenicity and to verify whether the previous observations of the restored appressorial turgor of *Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* would be enough to penetrate and produce disease on susceptible host, pathogenicity assays were performed. Spray inoculations using conidial suspensions were done onto the leaves of the susceptible rice cultivar, Nakdongbyeo. Small or nearly no necrotic lesions were observed on the rice leaves inoculated with the conidia of Δ*Mocrz1* mutants while the wild type produced typical greyish black lesions on the rice leaves (Figure 9). As for the Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* fungal trasnsformants, they both exhibited similar disease symptoms and severity as of the wild-type. In addition, to observe whether Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* could show invasive growth within the host cell, without having to penetrate from the surface of the rice leaves, wound inoculation assays were performed by introducing conidia suspensions on purposely wounded rice leaves. Wild type and Δ*Mocrz1*, together with all the fungal transformants were able to produce one disease lesion per infiltration site (Figure 10). Rice sheath assays were done to test whether Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* fungal transformants possess the

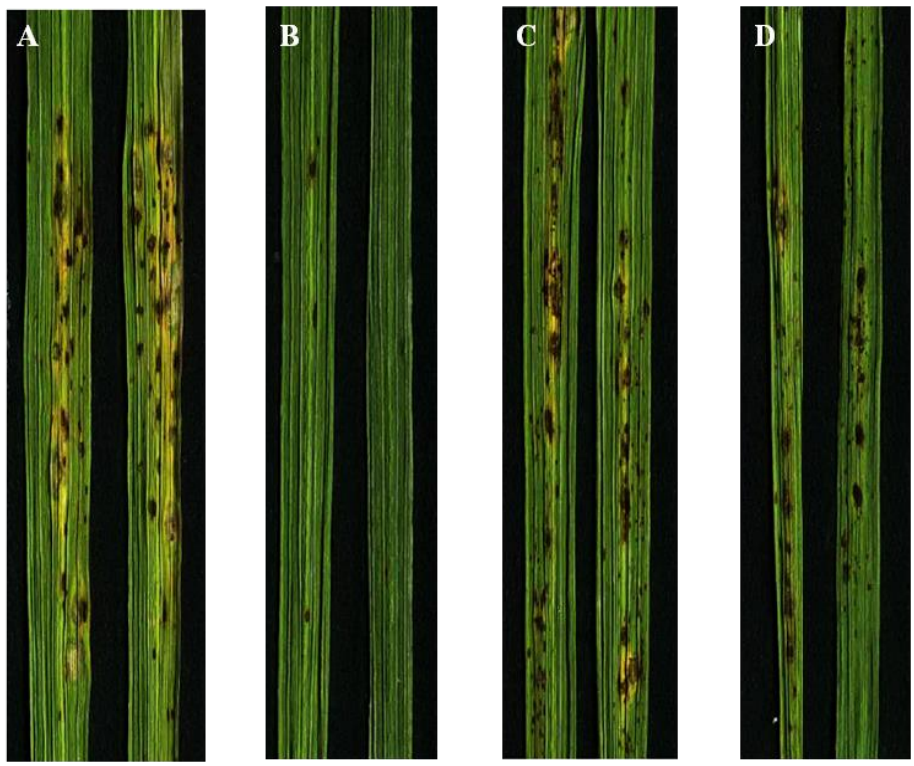

Figure 9. Pathogenicity of Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*. Spray inoculation for pathogenicity assay was performed with conidia suspension ($5x10^4$ conidia/ml) of each strain from 7-day-old V8 agar media. Susceptible rice seedlings were sprayed with conidia suspension and incubated in the growth chamber for 6 days after one day under the dark condition at 25℃. Photographs were taken 7 days after inoculation. (A) KJ201 (B) Δ*Mocrz1* (C) Δ*Mocrz1*::*FgCRZ1* (D) Δ*Mocrz1*::*NcCRZ1*

Figure 10. Infiltration infection assay of Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*. Infiltration infection assay was performed with 7-day-old conidia suspensions which were cultured on V8 agar media. 20 μl of adjusted conidia suspensions ($3x10^4$ conidia/ml) were dropped on wounded sites of susceptible rice leaves. Infected rice leaves were incubated in moistened box at 25℃. Photographs were taken 5 days after inoculation.

ability to invasively colonize healthy, susceptible rice leaf cells. Δ*Mocrz1* mutants failed to penetrate the host cells and perform invasive growth (Figure 11). Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* fungal transformants, the just as the wild type, which was observed to penetrate a single host cell and aggressively invade the neighboring adjacent rice leaf cells.

Taken altogether, Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*, were able to produce disease on susceptible rice plant leaves nearly identical to that of the wild-type, showing that the introduction of *FgCRZ1* and *NcCRZ1*, respectively, substantially complement the nearly non-pathogenic phenotype expressed by the *MoCRZ1* mutants. Furthermore, significant increase in appressorial turgor generation was exhibited by the appressoria produced by Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*, implying that *FgCRZ1* and *NcCRZ1* were able to rescue the appresorial turgor generation defect of Δ*Mocrz1*. This observation could further support the observation of Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* being able to produce disease lesions on rice leaves, that restoration of appressorial turgor by *FgCRZ1* and Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1* through rice sheath assays and infiltration assays further supports these observations, that the

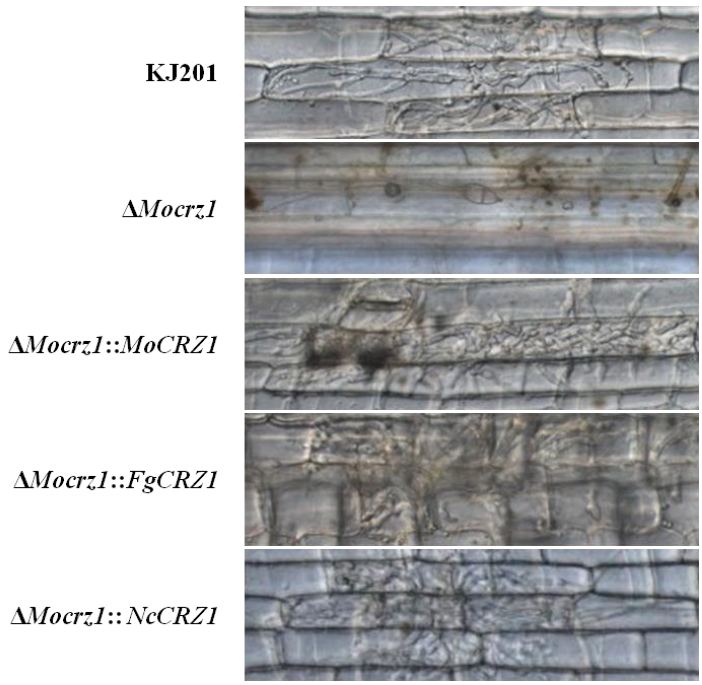

Figure 11. Penetration assay of Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*. Invasive growth of each strain was performed by penetration assay. Conidia suspension ($3x10^4$ conidia/ml) was injected into the excised rice leaves and infected rice leaves were incubated in moistened box at 25℃. Microscopic observation was performed at 48 hours after inoculation.

introduction of *FgCRZ1* and *NcCRZ1* enables the *Mocrz1* deletion mutant to perform invasive growth and produce disease lesions on rice leaves.

DISCUSSION

In eukaryotes, calcineurin is responsible for calcium signal transduction (Aramburu *et al.*, 2004) and transcription factor-mediated regulation of gene expression is one of the essential roles performed by calcineurin-dependent signaling.(Matheos *et al.*, 1997; Mendizabal *et al.*, 1998; Stathopoulos and Cyert, 1997).

MoCRZ1, calcineurin-responsive transcription factor in the rice blast-causing, plant pathogenic ascomycete *Magnaporthe oryzae*, is the ortholog of *Crz1* of *S. cerevisiae*, and have been reported to be involved in numerous processes related to calcium sensitivity, cell membrane stability and pathogenicity of the said pathogen (Choi *et al.*, 2009). The aim of this study was to identify *MoCRZ1* orthologs in filamentous ascomycetes *F. graminearum*, a phytopathogen which penetrates host through stomatal openings and *N. crassa* which is a saprophytic filamentous ascomycete, and to determine their functional conservation with respect to *MoCRZ1* of the appressorium-producing phytopathogen *M. oryzae*.

In this study, orthologs of *MoCRZ1* in *F. graminearum* (*FgCRZ1*) and *N. crassa* (*NcCRZ1*) were identified, respectively. Both orthologs encoded two

zinc finger motifs near the carboxyl terminus, similar to that of *MoCRZ1*. In addition, these orthologs contained CDDs, which is the site for calcineurin-recognition of *Crz1* (Boustany and Cyert, 2002). These observations, together with the high degree conservation of amino acid sequences observed, suggest that these orthologs encode transcription factors which interact with calcineurin. Upon the introduction of *FgCRZ1* and *NcCRZ1* into Δ*Mocrz1* (Δ*Mocrz1*::*FgCRZ1* and Δ*Mocrz1*::*NcCRZ1*, respectively), defects of Δ*Mocrz1* in conidiation, conidiophore production were rescued, suggesting that these genes play a role in conidiation. In addition, *FgCRZ1* was able to tolerate cell membrane stress but not to cell wall stress. In contrast, *NcCRZ1* was able tolerate cell wall stress, however, was sensitive to cell membrane destabilizing agents. This selective complementation of *MoCRZ1* functions suggest that the transcription factors *FgCRZ1* and *NcCRZ1* play different roles and perform general functions in filamentous ascomycetes of different lifestyles.

Several studies showing intra and inter-kingdom functional conservation of various signal transduction components were reported. For instance, mammalian phospholipase C-δ (PL-δ1) of mouse was able to functionally substitute the *M. oryzae* phospholipase C 1 (*MoPLC1*) by restoring restore

calcium flux within *M. oryzae* (Rho *et al.*, 2009). In addition, *M. grisea* cpkA homolog in the obligate phytopathogen *Blumeria graminis*, was expressed in a cpkA mutants of the non-obligate pathogen, *M. grisea* and heterologous complementation was observed by the restoration of appressorium kinetics as well as pathogenicity of *M. grisea* (Bindslev *et al.*, 2001). Furthermore, human pathogen *C. albicans CRZ1* (*CaCRZ1*) introduced into *S. cerevisiae CRZ1* defective strain (Δ*crz1*) was able to control the expression of several $Ca^{2+/}$calcineurin-responsive genes acting on the calcineurin-dependent regulatory element (CDRE) sequence in promoters of the genes.

In summary, orthologs of *M. oryzae* calcineurin-responsive transcription factor 1 were identified in *F. graminearum* (*FgCRZ1*) and *N. crassa* (*NcCRZ1*) and these transcription factors are suggested to be involved in conidiation, calcium homeostasis, cell membrane integrity and virulence. Taken all together, these results show the remarkable evolutionary functional conservation of signaling components involved in $Ca^{2+/}$calcineurin-dependent conidiation, calcium homeostasis and infection-related development among filamentous ascomycetes of different lifestyles.

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요약(국문초록)

칼슘신호전달체계는 모든 생명체에 있어서 가장 중요한 신호전달체계 중 하나이며, 진화 과정 동안 잘 보존되어왔다. 칼슘신호전달체계는 식물 병원균인 벼 도열병균에서 병 발생과 관련하여 필수적인 발달단계 중 분생포자 생성 및 침입과 관련한 발달과정에 관여함으로써 중요한 영향을 미친다. 벼 도열병균의 칼슘신호전달체계에서 *M. oryzae* calcineurin-responsive zinc finger 1 (*MoCRZ1*) 은 칼슘에 의해 영향을 받는 하위전사조절유전자로, *MoCRZ1* 의 결손 변이체는 병원성이 심각히 줄어들었을 뿐만 아니라 분생포자 생성과 칼슘 첨가 조건 및 세포막을 불안정하게 하는 조건에서의 균사생장이 매우 감소하였다. 본 연구에서는 *MoCRZ1* 의 이종상동성 유전자를 벼 도열병균과 서로 다른 생활사를 가진 자낭균문 곰팡이 중 침입 관련 구조를 만들지 않는 병원균 붉은곰팡이병균(*Fusarium graminearum*)과 비병원균 붉은빵곰팡이균(*Neurospora crassa*)에서 BLASTP 를 이용하여 알아보고, 각각을 *FgCRZ1* 과 *NcCRZ1* 으로 명명하였다. 또한, 벼 도열병균 내에서 *MoCRZ1* 의 이종상동성 유전자(*FgCRZ1* 및 *NcCRZ1*)가 기능적으로 상호보완이

가능한지 여부에 대해 밝히고자 하였다. 도메인 분석에 따르면, *FgCRZ1* 과 *NcCRZ1* 은 *MoCRZ1* 과 유사하게 한 개의 calcineurin-docking domain (CDD)과 두 개의 C_2H_2 zinc finger domain 을 갖는다. 이는 *FgCRZ1* 과 *NcCRZ1* 이 칼시뉴린과 상호작용하며, 칼슘신호전달체계에서 전사조절인자로써 역할을 할 것임을 시사한다. 이들의 기능적 상호보완 여부를 규명하기 위하여, *FgCRZ1* 과 *NcCRZ1* 이 *MoCRZ1* 결손 변이체(Δ*Mocrz1*)에 형질전환된 형질전환체들 (Δ*Mocrz1*::*FgCRZ1* 및 Δ*Mocrz1*::*NcCRZ1*) 을 확보하였다. Δ*Mocrz1*::*FgCRZ1* 및 Δ*Mocrz1*::*NcCRZ1* 는 Δ*Mocrz1* 의 분생포자 생성 감소 및 부착기 팽압 형성 결함을 회복함으로써 병원성을 회복하였고, 칼슘에 대한 민감도 또한 정상적으로 회복하였다. 그러나, 세포벽 불안정 조건인 CongoRed 첨가 조건에서는 Δ*Mocrz1*::*NcCRZ1* 만이 균사생장을 회복하는 반면, 세포막 불안정 조건인 SDS 첨가 조건에서는 Δ*Mocrz1*::*FgCRZ1* 만이 균사생장을 회복하였다. 이는 *FgCRZ1* 과 *NcCRZ1* 이 *MoCRZ1* 의 기능을 부분적으로 회복한다는 것을 시사한다. 본 연구결과를 종합해보면, 두 자낭균문 곰팡이 *F. graminearum* 과 *N. crassa* 의 *MoCRZ1* 이종상동성 유전자인 *FgCRZ1* 과 *NcCRZ1* 은 서로 다른 생활사를 가졌음에도 불구하고 각각 칼시뉴린과 상호작용하는 zinc finger 전사조절인자로써 벼 도열병균 내에서 분생포자 생성과 칼슘에 대한 민감도,

병원성에 있어 *MoCRZ1* 의 주요 기능을 대체할 수 있다는 것을 시사한다. 따라서, 본 연구는 선택된 사상성 자낭균들에서 생활사와 칼슘신호전달체계에 관련된 전사유전자의 기능 보존에 대한 연관성 여부를 제시하고 있으므로 충분한 가치가 있다고 판단된다.

주요어 : 진화적 기능 보존, 벼 도열병균 *CRZ1*, 칼슘신호전달체계, 붉은곰팡이병균 *CRZ1*, 붉은빵곰팡이균 *CRZ1*, 칼슘전달체계

학 번 : 2012-21173

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제일 먼저 이용환 교수님께 많은 감사의 마음을 드리고 싶습니다. 학생으로써 공부뿐만이 무엇이 진정 중요한것인지, 과학자가 되려면 꼭 가지고 있어야 하는 생각들이 무엇인지를 다시 한번 생각해 볼 수 있게 해주시고 균병학 연구실에서 공부할 수 있는 기회를 주셔서 감사합니다. 제가 앞으로 살아가는데 참 많은 도움이 될 것 같습니다. 항상 잊지 않고 처음에 시작하던 열정을 기억하며 공부하겠습니다. 그리고 저의 논문 위원님들이신 가종억 교수님, 김국형 교수님께 감사드리고 식물 병리가 무엇인지 깊이 다시 생각해볼수 있는 기회와 지식을 가르쳐주신 이인원 교수님, 황인규 교수님, 김영호 교수님 그리고 박은우 교수님께 감사드립니다. 도열병을 이라는 인연을 만나게 해주시고 항상 저를 믿어주시고 응원해주시는 한성숙 박사님, 감사합니다. 무엇보다 많이 서툴고 부족한 저를 이해해주시고 도와주신 우리 식물 균병학 연구실의 모든 구성원분들께 감사드립니다. 맨 처음에 제가 실험실에 들어온 후 여러가지 실험들을 가르쳐주시고 저의 논문 초창기에 함께 고민하고 저를 도와주셨던 박숙영 박사님, 여성 과학자의 길로 걸어나가고 있는 제가 본받고 싶은 점들이 많습니다. 감사합니다. 저의 안부와 연구상황에 대해 항상 적극적으로 관심 보여주시고 도와주시고 저의 질문에 친절하게 답해주신

최재혁 박사님께도 깊은 감사합니다. 때때로 궁금한 질문이 생겨서 물어보면 논리적으로 풀어서 성의껏 답해주시는 전준현 박사님, 항상 안부를 물어봐주셔서 마음을 따뜻하게 해주신 졸업하신 고재덕 박사님께도 감사드립니다. 항상 옆에서 저희 연구실 식구들을 챙겨주시는 윤여경 선생님, 그리고 수줍게 항상 인사해주는 사닷. 알베리 에게도 감사드립니다. 그리고 아람이, 저의 동기 아람이에게 고마운 마음을 전합니다, 너가 있어서 다행이였고 너가 있어서 고마웠어! 그리고 처음 SRC에 도착하고 친절하게 실험실 물품이나 연구관련 질문에 대해 답해주고 때때로 깨알웃음을 선사했던 성범오빠, 무뚝뚝하고 말은 없어보이지만 배려해주던 종범, 열정적으로 자신감있게 연구를 하는 재영오빠, 얘기하면 재밌는 경채오빠, 만나면 항상 스쿠버 다이빙 얘기나 요리 얘기를 할수 있어 즐겁게 대화하는 경용오빠, 믿음안에 형제님이여서 그런지 마음을 편하게 해주는 조언을 해주던 성형오빠, 차분하게 성실하게연구하면서 컴퓨터로 artwork도 너무 잘하는 재진오빠, 친동생처럼 항상 응원해주는 서문이, 허무개그여도 항상 웃음을 선물해주는 기태오빠, 그리고 많은 도움, 응원을 아까지 않고 옆에서 힘이 되어주었던 현정이에게 진심으로 감사드립니다.

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2014년 2월

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