

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**Investigation of the molecular basis of symbiosis
between *Epichloë festucae* and perennial ryegrass**

A thesis presented in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Genetics

at Massey University, Palmerston North

New Zealand

Milena Mitić

2011

Abstract

The symbiosis between the endophytic filamentous fungus *Epichloë festucae* and its plant host, perennial ryegrass (*Lolium perenne*), is a highly regulated mutualistic interaction which represents a good model system for the investigation of plant-fungal mutualism. Fungal signalling pathways play a crucial role in regulation of this interaction. While genes involved in the production of reactive oxygen species (ROS), as well as a member of the MAP kinase signalling pathway, have been shown to regulate maintenance of the mutualistic interaction, the signalling pathways responsible for regulation of this symbiosis are still relatively poorly understood.

In pathogenic fungi, members of calcium signalling pathways, such as Ca²⁺/calmodulin-regulated kinases (CaMKs) and phosphatase (calcineurin), are required for normal host-pathogen interactions. Three genes encoding multifunctional CaMKs, *cmkA*, *cmkB* and *cmkC*, were identified in *E. festucae*, as well as one gene encoding the catalytic subunit of calcineurin, *cnaA*. Targeted replacements of these genes have identified a novel role for the fungal *cmkB* in the regulation of ion homeostasis and an important role for calcineurin for both culture growth and symbiosis maintenance. However, unlike the pathogenic fungi, *E. festucae* CaMKs do not appear to have a role in the regulation of the mutualistic interaction.

In order to identify new genes regulating the symbiosis, T-DNA mutagenesis was used to generate symbiotically defective *E. festucae* mutants. Two mutants, Ag51 and Ag212, with both in culture and *in planta* phenotypes, were identified. A detailed molecular analysis showed that Ag51 had a complex T-DNA insertion while Ag212 had a deletion of ten genes. Ag212 failed to establish plant infection and complementation experiments using cosmids identified candidate genes for both the in culture and *in planta* phenotype. Analysis of the colonization process showed that this mutant is defective in establishing a specific interaction between hyphal and plant cell walls, essential for the plant colonization.

This work provides new insights into calcium signalling in fungi and increases our understanding of plant-fungal mutualism.

Acknowledgments

Firstly, I want to thank my supervisor Barry Scott. When I look back on this PhD, to all tough times, all weird or no results years – I honestly believe you were the best supervisor I could imagine. Sometimes, when things were going very, very badly, your guidance, experience and reassurance were the only anchors I was holding onto. Thank you for that. To my co-supervisor Jasna Rakonjac, thank you for providing the “out-of-the-field” view of my work and special thanks for your help when I had just arrived to New Zealand and had no idea of how everything works here.

I am grateful for the funding that made this project possible. It was founded through Marsden Grant by the Royal Society of New Zealand with additional funding from the Lincoln Bioprotection CoRE.

Thank you, to people who, through their work, provided support to this project. Thanks to Chris Schardl for providing the *E. festucae* 2368 genome sequence. Thanks to Mike Christensen, Anouck de Bonth and Wayne Simpson from the AgResearch, for looking after my plants and always helping with a good advice. Thanks to people from the Manawatu Microscopy and Imaging Centre for their help with the microscopy part of the project. Special thanks to Doug and Jianyu for being helpful and patient with my difficult TEM samples. The big ‘Thank you’ to Murray Cox for his help with bioinformatics and for the fantastic work on the cosmid sequence assembly.

To past and present members of the Scottbase lab – I cannot thank you enough for your help and friendship. To Michelle and Ruth, special thanks for your help with the experimental work in the very beginning of my PhD. To Yvonne, Matthias, Matt, Aiko, Sanjay, Kim, Sarah – thank you for listening to me about my experiments and results, and for your advice and suggestions. To Carla, Emma, Gemma, Daniel – thank for your help in the lab, but above all, thank you for being there when I just needed someone to listen to my complaints about how things are going.

To everyone at IMBS who was there when I needed advice or technical help – thank you.

Thanks to Kezia for providing the non-scientific feedback on this thesis.

There are many people who haven't been involved in this project but without whom it would still be impossible for it to happen. To my Mum Zorica, thank you for making me who I am, for supporting me to be who I am and for being happy for me to be who I am (and I know none of this is easy). Thank you for always supporting my choices, from being one of the few who supported my choice of a 'risky' university degree, to supporting my decision to leave for the other side of the world. To my brother Marko, well, thank you for somehow, suddenly, growing up and becoming someone I can talk to about the life, the universe and everything. I have to admit I have no idea how that happened but I'm glad it happened ☺. To my best friend Milena – Misho, it would take too long to thank you for everything I would need to, so you'll have to take just one, general "thank you". Sadly, some people are not with us any more, but I will carry their influence with me for the rest of my life. To a remarkable woman, my grandmother Radica Milenković, thank you for being who you were in a place where it was not easy and for inspiring me in my very young years to pursue my views of life. To Mihajlo Miša Elezović, thank you for your immense support during my undergrad years and during my move to NZ. I wish you were here to see the result and I know you would've been proud.

John, thank you for everything.

Contents

Abstract	i
Acknowledgments.....	ii
Contents	iv
List of figures	xi
List of tables.....	xiv
Abbreviations	xv
1. Introduction.....	1
1.1. Symbiotic associations of plants and fungi	2
1.2. Plant-endophytes association	3
1.2.1. Endophyte lifecycle and associations with plants.....	3
1.2.2. <i>E. festucae</i> as an experimental model system for genetic analysis of plant/endophyte symbiotic associations.....	6
1.2.3. <i>E. festucae</i> growth <i>in planta</i>	6
1.2.4. Role of molecular signaling in the regulation of the <i>E. festucae</i> -plant association.....	7
1.3. Calcium signaling.....	9
1.3.1. Generation and decoding of calcium signals	9
1.3.2. Calcium and polarized cell growth – importance for hyphal growth	12
1.3.3. Calcium signaling in fungi.....	14
1.3.3.1. Calcium channels.....	14
1.3.3.2. Calcium ATPases and exchangers.....	15
1.3.3.3. Phospholipase C.....	16
1.3.3.4. Calmodulin	17
1.3.3.5. Ca ²⁺ /calmodulin-dependent protein kinases	17
1.3.3.6. Calcineurin.....	19

1.4.	Agrobacterium mediated mutagenesis as a tool for understanding the symbiosis.....	20
1.4.1.	Agrobacterium-mediated transformation (AMT) in nature	21
1.4.2.	Adaptation for use in the laboratory.....	21
1.4.3.	Agrobacterium mediated mutagenesis in fungal research	22
1.5.	Aims of this research	24
2.	Materials and methods	25
2.1.	Biological materials.....	26
2.2.	Media and growth conditions	34
2.2.1.	Luria-Bertani medium (LB)	34
2.2.2.	SOC medium.....	34
2.2.3.	Induction medium (IM).....	34
2.2.4.	Potato dextrose (PD) medium	34
2.2.5.	Regeneration medium (RG)	35
2.2.6.	Water agar plates (WA)	35
2.2.7.	<i>E. coli</i> growth conditions	35
2.2.8.	<i>Agrobacterium tumefaciens</i> growth conditions	35
2.2.9.	<i>Epichloë festucae</i> growth conditions	35
2.2.10.	<i>Epichloë festucae</i> growth tests	36
2.2.11.	<i>Lolium perenne</i> growth conditions.....	36
2.3.	DNA isolation and manipulation.....	36
2.3.1.	Plasmid DNA Isolation	36
2.3.2.	Cosmid DNA isolation.....	37
2.3.3.	Fungal genomic DNA isolation	37
2.3.3.1.	Crude DNA isolation	38
2.3.4.	DNA quantification.....	38
2.3.5.	Restriction endonuclease digestion of DNA	39

2.3.6.	DNA purification	39
2.3.7.	DNA concentration by ethanol precipitation	39
2.3.8.	Gel extraction.....	39
2.3.9.	Calf intestinal alkaline-phosphatase (CIAP) treatment of vectors.....	40
2.3.10.	Ligation	40
2.3.11.	A-tailing.....	40
2.3.12.	Agarose gel electrophoresis.....	40
2.3.13.	Southern blotting	41
2.3.13.1.	Radioactive probe labeling and hybridization	42
2.3.13.2.	Stripping of radioactive membranes	42
2.3.14.	DNA sequencing	42
2.3.15.	Cosmid sequencing.....	43
2.3.16.	Plasmid rescue	43
2.3.16.1.	Phenol-chloroform purification of DNA	43
2.3.17.	Screening of <i>E. festucae</i> cosmid library	44
2.4.	RNA isolation and manipulation.....	44
2.4.1.	RNA isolation and quantification	44
2.4.2.	RNA agarose gel electrophoresis.....	44
2.4.3.	DNase treatment.....	45
2.4.4.	RT-PCR.....	45
2.5.	Cell transformation.....	45
2.5.1.	<i>E. coli</i> transformation.....	45
2.5.1.1.	Screening of <i>E. coli</i> colonies for presence of the plasmid.....	46
2.5.2.	<i>E. festucae</i> transformation.....	46
2.5.2.1.	Preparation of protoplasts	46
2.5.2.2.	Transformation of protoplasts.....	46
2.5.3.	<i>A. tumefaciens</i> mediated T-DNA mutagenesis	47

2.6.	PCR reactions	48
2.6.1.	Standard PCR	53
2.6.2.	High fidelity enzymes	53
2.6.3.	Long template.....	53
2.6.4.	cDNA PCR.....	53
2.6.5.	TAIL-PCR.....	54
2.7.	Plant inoculation and growth analysis.....	54
2.7.1.	Seed sterilization	54
2.7.2.	Seedling germination and inoculation.....	54
2.7.3.	Immunoblotting.....	54
2.7.4.	Aniline blue staining	55
2.8.	Bioinformatics analyses	55
2.8.1.	Sequence comparison and domain analysis	55
2.8.2.	Synteny analysis.....	56
2.9.	Microscopy	56
2.9.1.	Light microscopy	56
2.9.2.	Confocal microscopy	56
2.9.3.	Transmission electron microscopy (TEM)	57
2.10.	Colony staining.....	57
2.10.1.	Diaminobenzidine (DAB)	57
2.10.2.	Nitroblue tetrazolium (NBT).....	57
2.11.	Construction of plasmids	58
2.11.1.	pMMI1	58
2.11.2.	pMMI2.....	58
2.11.3.	pMMI3.....	58
2.11.4.	pMMI4.....	59
2.11.5.	pMMI5.....	59

2.11.6.	pMMI6.....	59
2.11.7.	pMMI7.....	60
2.11.8.	pMMI8.....	60
2.11.9.	pMMI9.....	60
3.	Results.....	61
3.1.	Identification of <i>cmkA</i> , <i>cmkB</i> and <i>cmkC</i>	62
3.2.	<i>cmkA</i> , <i>cmkB</i> and <i>cmkC</i> are differentially expressed in culture and <i>in planta</i> ..	62
3.3.	Targeted replacement of <i>cmkA</i> , <i>cmkB</i> and <i>cmkC</i>	67
3.3.1.	Design of replacement constructs and screening for replacement mutants.....	67
3.3.2.	Genes <i>cmkA</i> , <i>cmkB</i> and <i>cmkC</i> are not essential for normal growth in culture.....	74
3.3.3.	Deletion of <i>cmkA</i> , <i>cmkB</i> or <i>cmkC</i> does not affect ROS production in culture.....	79
3.3.4.	<i>cmkA</i> , <i>cmkB</i> and <i>cmkC</i> are not essential for the symbiotic interaction.....	79
3.4.	Complementation of the $\Delta cmkA$, $\Delta cmkB$ and $\Delta cmkC$ mutants	83
3.5.	Generation and analysis of $\Delta cmkAB$ double-deletion mutants.....	87
3.5.1.	Growth of $\Delta cmkAB$ under high extracellular concentrations of Mg^{2+} and Ca^{2+}	96
3.5.2.	Symbiotic phenotype of $\Delta cmkAB$	100
3.6.	Complementation of double-deletion mutants	100
3.7.	Identification <i>E. festucae</i> homologue of calcineurin A.....	104
3.8.	Deletion of <i>cnaA</i> in <i>E. festucae</i> F11	104
3.8.1.	Culture and symbiotic phenotypes of $\Delta cnaA$	107
3.9.	Analysis of two symbiotic mutants of <i>Epichloë festucae</i> generated by Agrobacterium-mediated mutagenesis.....	108
3.9.1.	In culture phenotype of mutants Ag51 and Ag212.....	111
3.9.2.	Symbiotic phenotype of mutants Ag51 and Ag212.....	111

3.10.	Identification of insertion locus in Ag51	115
3.10.1.	Copy number	115
3.10.2.	Identification of LB insertion site in Ag51 by TAIL-PCR.....	115
3.10.3.	Attempts to locate the right border of Ag51.....	118
3.10.4.	Southern blot analysis of Ag51	118
3.11.	Identification of insertion locus in Ag212	121
3.11.1.	Copy number	121
3.11.2.	Identification of LB and RB using plasmid rescue	121
3.11.3.	Identification of the size of the deletion in Ag212.....	125
3.11.3.1.	Synteny analysis	125
3.11.3.2.	Screening of the cosmid library and Southern analysis of deletion in Ag212.....	129
3.12.	Complementation of Ag212 in culture and <i>in planta</i> phenotype.....	129
3.13.	Detailed analysis of <i>Ag212 in planta</i> phenotype	134
4.	Discussion	143
4.1.	Functional analysis of <i>E. festucae cmkA, cmkB and cmkC</i>	144
4.2.	Role of <i>E. festucae</i> calcineurin in fungal growth and symbiosis	149
4.3.	T-DNA mutagenesis generates mutants defective in growth and symbiosis .	152
4.4.	Conclusions	155
5.	Appendices.....	156
5.1.	Appendix 5.1 WT transcriptome data for <i>cmkA</i>	157
5.2.	Appendix 5.2 $\Delta sakA$ transcriptome data for <i>cmkA</i>	158
5.3.	Appendix 5.3 WT transcriptome data for <i>cmkB</i>	159
5.4.	Appendix 5.4 $\Delta sakA$ transcriptome data for <i>cmkB</i>	160
5.5.	Appendix 5.5 WT transcriptome data for <i>cmkC</i>	161
5.6.	Appendix 5.6 $\Delta sakA$ transcriptome data for <i>cmkC</i>	162
5.7.	Appendix 5.7 Southern blot analysis of T-DNA induced deletion in Ag212 – genomic digests.....	163

5.8. Appendix 5.8 Southern blot analysis of T-DNA induced deletion in Ag212 – cosmid digests	164
5.9. Appendix 5.9 Southern blot analysis of T-DNA induced deletion in Ag212	165
5.10. Appendix 5.10 E2368 genome database unique gene identifiers of genes used in this study.....	166
6. Bibliography.....	167

List of figures

Figure 1.1 Schematic representation of sexual and asexual life cycles of epichloë endophytes.....	4
Figure 1.2 Simplified presentation of calcium signaling in eukaryotic cells.....	13
Figure 1.3 Overview of the binary vector T-DNA transfer system from <i>A. tumefaciens</i> into a fungal cell.....	23
Figure 3.1 Amino acid sequence alignment of fungal Ca ²⁺ /Calmodulin-dependent protein kinase A.....	63
Figure 3.2 Amino acid sequence alignment of fungal Ca ²⁺ /Calmodulin-dependent protein kinase B.....	64
Figure 3.3 Amino acid sequence alignment of fungal Ca ²⁺ /Calmodulin-dependent protein kinase C.....	65
Figure 3.4 Exon-intron structure and protein domains of <i>E. festucae</i> CmkA, CmkB and CmkC.....	66
Figure 3.5 Expression analysis of <i>cmkA</i> , <i>cmkB</i> and <i>cmkC</i>	68
Figure 3.6 Design of <i>cmkA</i> replacement construct and screening strategy for identifying gene deletion.....	70
Figure 3.7 Design of <i>cmkB</i> replacement construct and screening strategy for identifying gene deletion.....	71
Figure 3.8 Design of <i>cmkC</i> replacement construct and screening strategy for identifying gene deletion.....	73
Figure 3.9 Culture phenotype of $\Delta cmkA$, $\Delta cmkB$ and $\Delta cmkC$ mutants.....	75
Figure 3.10 Hyphal morphology of $\Delta cmkA$, $\Delta cmkB$ and $\Delta cmkC$	76
Figure 3.11 Growth of $\Delta cmkA$, $\Delta cmkB$ and $\Delta cmkC$ under different stress conditions...	77
Figure 3.12 Growth of $\Delta cmkA$, $\Delta cmkB$ and $\Delta cmkC$ under conditions of osmotic stress.....	78
Figure 3.13 $\Delta cmkA$, $\Delta cmkB$ and $\Delta cmkC$ mutants growing on high Mg ²⁺ concentrations.....	80
Figure 3.14 H ₂ O ₂ production by $\Delta cmkA$, $\Delta cmkB$ and $\Delta cmkC$ mutants.....	81
Figure 3.15 Superoxide production of $\Delta cmkA$, $\Delta cmkB$ and $\Delta cmkC$ mutants.....	82
Figure 3.16 Symbiotic phenotype of perennial ryegrass plants inoculated with $\Delta cmkA$, $\Delta cmkB$ and $\Delta cmkC$	84

Figure 3.17 <i>In planta</i> phenotype of the $\Delta cmkA$, $\Delta cmkB$ and $\Delta cmkC$ mutants.....	85
Figure 3.18 Fragments used in complementation tests of $\Delta cmkA$, $\Delta cmkB$ and $\Delta cmkC$ mutants.....	86
Figure 3.19 PCR analysis of $\Delta cmkA$, $\Delta cmkB$ and $\Delta cmkC$ complementation transformants.....	88
Figure 3.20 Complementation test for temperature-sensitive phenotype of $\Delta cmkA$, $\Delta cmkB$ and $\Delta cmkC$ mutants.....	89
Figure 3.21 Complementation test for Mg^{2+} sensitive phenotype of $cmkB$ mutant.....	90
Figure 3.22 Construction of replacement fragment for generation of $cmkAB$ and screening strategy for identifying gene deletion.....	92
Figure 3.23 Culture phenotype of $\Delta cmkAB$ double mutants under standard growth conditions compared to WT.....	93
Figure 3.24 Growth of $\Delta cmkAB$ mutants under different stress conditions.....	94
Figure 3.25 ROS production by $\Delta cmkAB$	95
Figure 3.26 Colony morphology and growth of double-deletion mutants $\Delta cmkAB$ on high Mg^{2+} concentrations.....	97
Figure 3.27 Growth of $\Delta cmkAB$ in the presence of high concentrations of Ca^{2+} and EGTA.....	98
Figure 3.28 Ca^{2+} Remediation of $\Delta cmkAB$ Mg^{2+} sensitivity	99
Figure 3.29 Plant phenotype of $\Delta cmkAB$ mutants.....	101
Figure 3.30 <i>In planta</i> hyphal morphology of the $\Delta cmkAB$ mutants.....	102
Figure 3.31 Complementation of $\Delta cmkAB$	103
Figure 3.32 <i>E. festucae</i> strain E2368 has two copies of calcineurin A gene.....	105
Figure 3.33 Construction of $cnaA$ replacement mutants.....	106
Figure 3.34 Culture phenotype of $\Delta cnaA$ mutants.....	109
Figure 3.35 Symbiotic phenotype of $\Delta cnaA$ mutants.....	110
Figure 3.36 Colony morphology and growth of T-DNA mutants Ag212 and Ag51 in culture.....	112
Figure 3.37 Hyphal morphology of Ag212 and Ag51.....	113
Figure 3.38 Ag51 induces a hypersensitive response in perennial ryegrass seedlings.....	114
Figure 3.39 Tandem insertion of T-DNA copies in the genome of Ag51.....	116
Figure 3.40 Identification of the T-DNA LB insertion site in Ag51 using TAIL-PCR.....	117

Figure 3.41 Southern blot analysis of the T-DNA insertion locus in Ag51.....	120
Figure 3.42 Analysis of T-DNA copy number in Ag212.....	122
Figure 3.43 Identification of the T-DNA LB insertion site in Ag212 using plasmid rescue.....	123
Figure 3.44 Identification of the RB locus of T-DNA in Ag212 using plasmid rescue.....	124
Figure 3.45 Synteny between regions of <i>E. festucae</i> and <i>F. graminearum</i>	127
Figure 3.46 Confirmation of predicted positions for genes on contig_1006.....	128
Figure 3.47 Southern blot analysis of the deletion in Ag212.....	131
Figure 3.48 Southern blot analysis of genes present on <i>E. festucae</i> cosmids 12G8 and 17G5.....	132
Figure 3.49 Map of deletion locus in Ag212.....	133
Figure 3.50 Complementation of culture phenotype of Ag212 using cosmids 12G8 and 17G5.....	135
Figure 3.51 Complementation of Ag212 plant phenotype with cosmids 12G8 and 17G5.....	136
Figure 3.52 Colonization of host plant by WT and Ag212 – longitudinal sections....	139
Figure 3.53 Colonization of host plant by WT and Ag212: light micrographs of transverse sections.....	140
Figure 3.54 Colonization of host plant by WT and Ag212: confocal microscopy of transverse sections.....	141
Figure 3.55 Colonization of host plant by WT and Ag212: TEM of transverse sections.....	142

List of tables

Table 2.1 Fungal strains, bacterial strains and plant material.....	26
Table 2.2 Plasmids and cosmids.....	32
Table 2.3 Primers used in PCR reactions.....	49
Table 3.1 Synteny between <i>F. graminearum</i> and <i>E. festucae</i> regions affected in Ag212.....	126
Table 3.2 Genes deleted in <i>E. festucae</i> mutant Ag212.....	130
Table 5.1 <i>E. festucae</i> unique gene identifiers of genes mentioned in this study.....	166

Abbreviations

A	Adenine
Amp^R	Ampicillin resistant
AMT	Agrobacterium mediated transformation
ATP	Adenine triphosphate
BLAST	Basic local alignment search tool
BLASTN	Nucleotide database search using a nucleotide query
BLASTP	Protein database search using a protein query
BLASTX	Protein database search using a translated nucleotide query
bp	Base pair(s)
BSA	Bovine serum albumin
Ca²⁺/CaM	Ca ²⁺ /calmodulin
CaMK	Ca ²⁺ /calmodulin-dependent kinase, protein
CaMKK	Ca ²⁺ /calmodulin-dependent kinase kinase
<i>cmk</i>	Ca ²⁺ /calmodulin-dependent kinase, gene
cDNA	Complementary DNA
CDS	Coding sequence
CIAP	Calf intestinal alkaline phosphatase
DAB	3-3'Diaminobenzidine
DAG	Diacylglycerol
DIC	Differential interference contrast
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
EC	Ectopic
EGFP	Enhanced GFP
EGTA	Ethylene glycol tetraacetic acid
FGI	Fungal Genome Initiative
Gen^R	Geneticin resistant
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
HR	Hypersensitive response
Hyg^R	Hygromycin resistant

IM	Induction medium
ip	inoculation point
IP3	Inositol trisphosphate
Kan^R	Kanamycin resistant
Kb	Kilobases
KO	Knock-out
LB	Luria-Bertaini medium
LB	T-DNA left border
MAPK	Mitogen activated protein kinase
Mb	Megabases
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NBT	Nitroblue tetrazolium
NCBI	National centre for biotechnology information
Nox	NADPH oxidase
PCR	Polymerase chain reaction
PD	Potato dextrose
Phl^R	Phleomycin resistant
PKC	Protein kinase C
PLC	Phospholipase C
RB	T-DNA right border
REMI	Restriction enzyme mediated integration
RG	Regeneration medium
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAM	Shoot apical meristem
TAIL-PCR	Thermal asymmetric interlaced-polymerase chain reaction
TBLASTN	Translated nucleotide database search using a protein query
T-DNA	Transfer DNA
TEM	Transmission electron microscopy

tRNA	Transfer RNA
WA	Water agar
WT	Wild-type
w/v	Weight/volume ratio