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 signalling involved in maintaining the mutually beneficial association between *Epichloë festucae* and perennial ryegrass

---

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

Doctor of Philosophy
in
Genetics

at Massey University, Palmerston North,
New Zealand.

Carla Jane Eaton

2009

Abstract

In the mutually beneficial association between the fungal endophyte *Epichloë festucae* and perennial ryegrass, fungal growth is highly regulated and coordinated with that of the host. This implies there must be signalling between the fungus and its host to maintain this close association. Recent work has shown a novel role for reactive oxygen species (ROS) in this symbiotic maintenance, with multiple components of the superoxide-producing NADPH oxidase (Nox) complex being essential for normal association. However, the mechanism by which the Nox complex is regulated is unclear.

To identify potential regulators of the *E. festucae* Nox complex, comparisons were made with well-characterised mammalian systems. This search identified three candidate regulators: a stress activated MAP kinase, *sakA*, and the p21-activated kinases, *pakA* and *pakB*. To investigate if these genes were involved in symbiotic maintenance, replacement mutants were generated by homologous recombination. In culture analysis revealed that the Δ*sakA* mutant was hypersensitive to a range of stresses, whereas the pak mutants were hypersensitive to cell wall stress-inducing agents and displayed altered growth and morphology. Examination of perennial ryegrass infected with these mutants revealed drastically altered plant interaction phenotypes for the Δ*sakA* and Δ*pakA* mutants in comparison to the wild-type strain. Δ*sakA*-infected plants were stunted and displayed striking changes in development, with the base of tillers showing loss of anthocyanin pigmentation and disorganisation of host cells below the meristem, resulting in swollen bases. Plants infected with the Δ*pakA* mutant were severely stunted, had no more than two tillers and senesced soon after planting. In contrast, plants infected with the Δ*pakB* mutant were similar to wild-type, with only slight deregulation of growth *in planta*.

Examination of ROS in culture revealed that Δ*sakA* and Δ*pakA* displayed elevated levels of both superoxide and hydrogen peroxide. ROS levels were also elevated around Δ*sakA* hyphae *in planta*. These results support roles for SakA and PakA in Nox regulation. This work highlights the fine balance between mutualism and antagonism, and provides insight into the molecular basis for mutualism.

Acknowledgments

Firstly, I must thank Barry Scott for his excellent supervision. You really were the best supervisor someone undertaking the trials and tribulations of a PhD could ask for. Thank you for your guidance and patience through the good times and the bad times. Your faith in me kept me going during the times when it felt like nothing was working and I questioned whether I could ever finish. To my co-supervisor Jerry Hyams, thanks for your expert guidance with the *S. pombe* work and for your humour, which was like a breath of fresh air during the trying times of this PhD.

Thanks to the New Zealand Tertiary Education Commission for funding this project through a Top Achievers Doctoral Scholarship, and to the Lincoln Bioprotection CoRE for additional funding. Thanks to Chris Schardl for provision of *E. festucae* 2368 genomic sequence through grant EF-0523661 from the US National Science Foundation.

To Isabelle Jourdain, without your help with the *S. pombe* work and *E. festucae* microscopy much of the results presented in this thesis could not have been achieved. I have valued your help, advice and most importantly your friendship. You were a listening ear when I needed to vent my problems and most of the time could even offer a solution. Without your support all this would not have been possible.

Thanks to all past and present members of the Scottbase lab, you all helped me in so many ways. Special thanks to Michelle Bryant for your support, advice and friendship, especially at the start of my PhD; to Matt Nicholson, for answering my endless questions and helping to 'problem-solve' when things just did not seem to work. Thanks also to Yvonne Rolke for your advice and support, especially during the writing phase, and to Matthias Becker for helping dissect meristems and for the top quality SEM images. To Aiko, Daigo, Sanjay, Kim and Sarah for helpful advice and suggestions. A big thanks to Milena Mitic, Emma Brasell, Gemma Cartwright and Ruth Wrenn for your great friendship throughout my PhD and for helping me to smile during the tough times.

Many thanks to everyone at AgResearch who helped me during this project, especially Mike Christensen for advice and help with analysing the *in planta* phenotypes, Anouck

de Bonth for developing my many immunoblots during the arduous infection experiments, and Wayne Simpson for together with Anouck taking good care of my plants. Thanks to everyone at the Manawatu Microscopy and Imaging Centre, particularly Doug Hopcroft for your top quality sample preparation and advice to help me get the best images possible. Thanks also to Barbara Ambrose for advice and help with plant tissue preparation, sectioning and staining.

Special thanks are due to Ann Truter and Cynthia Cresswell. You two are like the guardian angels of IMBS and I'm sure without you things would fall to pieces – I know I would have, especially during poster printing times! Thanks for all your help with so many things throughout my PhD.

A big thank-you to John O'Sullivan for your support and help during the writing of this thesis. I will forever be grateful to you for introducing me to the wonder of biology all those long years ago. The phrase 'a good teacher makes all the difference' could not be any truer. Your passion for biology inspired me - who knew it would lead me to this point.

Lastly, my deepest thanks must go to my family. Mum, without your support and love none of this would have been possible. Thank you for enduring many hours as an audience for me to practice my presentations to, and for all your advice on the layout and design of presentations and posters. Thanks to my sister and sister-in-law Debbie and Anne-Marie for enduring many "practice presentations" even though you probably have no real interest in grass, fungus or any of those other things I rambled on about. Debbie, your faith in me being able to achieve whatever I set my mind to has meant more to me than you realise. To my brother Shane – thanks for bringing me back to earth when my head gets lost in the world of science! To my nephews and nieces James, Caleb, Tyler, Cassandra, Bailey, Riley and Maia. You have kept me anchored in reality by being able to brighten my mood even on those days when nothing seemed to be going right, and loving me whether or not my experiments were working. Tyler, thank you for always being interested in my work, and for listening to my presentations and asking some pretty good questions – I may make a scientist out of you yet! Dad and Sherree, I love you and miss you and wish you could be here to share this moment with me, but I know you would have been proud.

Table of contents

Abstract ii

Acknowledgments iii

Table of contents v

Abbreviations xiii

List of figures xix

List of tables xxi

1. Introduction 1

1.1. Plant-microbe associations 2
1.2. Plant-fungal associations 2
1.3. Endophyte associations with cool-season grasses 3
1.3.1. Endophyte diversity and lifecycle 3
1.3.2. Benefits of association 5
1.3.3. Endophyte growth in planta 6
1.3.4. Role of reactive oxygen species in maintenance of association 6
1.4. Role of NADPH oxidase (Nox) complexes in fungal symbioses 8
1.4.1. The mammalian Nox complex as a model 8
1.4.2. Roles of the Nox complex in other fungi 9
1.4.3. Possible mechanisms of Nox regulation in fungi 9
1.5. MAP kinase signalling 11
1.5.1. The MAP kinase cascade 11
1.5.2. MAP kinase pathways in fungi 12
1.5.3. Yeast stress-activated MAP kinase pathways 14
1.5.4. Role of the stress-activated MAP kinase pathway in filamentous fungi 14
1.5.4.1. Saprotrophic fungi 15
1.5.4.2. Animal and insect pathogens 15
1.5.4.3. Plant pathogens 17
1.5.4.4. Role in fungicide sensitivity 18
1.6. p21-activated kinase signalling 19
1.6.1. The Rho GTPase molecular switches 19

1.6.1.1. The tripartite role of RhoGDI *21*
1.6.2. The pak conundrum *21*
1.6.3. Role of the p21-activated kinases in yeast *23*
1.6.4. Roles in filamentous fungi *24*
1.7. Aims 25
2. Materials and Methods 28
2.1. Biological material 29
2.2. Medium and growth conditions 33
2.2.1. Escherichia coli *33*
2.2.1.1. Luria-Bertani medium (LB) *33*
2.2.1.2. SOC medium *33*
2.2.2. Schizosaccharomyces pombe *33*
2.2.2.1. Edinburgh minimal medium (EMM) *34*
2.2.2.1.1. Salts solution *34*
2.2.2.1.2. Vitamins solution *34*
2.2.2.1.3. Minerals solution *34*
2.2.2.2. Yeast extract medium (YE) *35*
2.2.2.3. Growth tests *35*
2.2.3. Epichloë festucae *35*
2.2.3.1. Potato dextrose medium (PD) *35*
2.2.3.2. Regeneration medium (RG) *36*
2.2.3.3. Growth tests *36*
2.2.4. Lolium perenne *36*
2.2.4.1. Murashige and Skoog (MSO)-Phytoagar *36*
2.2.4.2. Maintenance of plants in the greenhouse *37*
2.3. DNA isolation 37
2.3.1. Plasmid DNA *37*
2.3.2. Cosmid DNA *37*
2.3.3. Fungal genomic DNA *38*
2.4. DNA manipulation 39
2.4.1. Quantification *39*
2.4.2. Restriction endonuclease digestion *39*
2.4.3. DNA purification *39*
2.4.4. DNA concentration by ethanol precipitation *40*

2.4.5. Sub-cloning ... *40*
2.4.5.1. A-tailing ... *40*
2.4.5.2. Calf intestinal alkaline phosphatase (CIAP) treatment of vectors ... *41*
2.4.5.3. Gel extraction ... *41*
2.4.5.4. PCR product purification ... *41*
2.4.5.5. Ligation ... *41*
2.4.5.6. E. coli transformation ... *42*
2.4.5.6.1. Cracking analysis of transformants ... *42*
2.4.6. Agarose gel electrophoresis ... *42*
2.4.6.1. Electrophoresis of RNA ... *43*
2.4.7. Southern blotting ... *43*
2.4.7.1. Radioactive hybridisation ... *44*
2.4.7.2. Stripping of radioactive membranes ... *45*
2.4.8. Screening the cosmid library ... *45*
2.5. RNA isolation and manipulation ... 45
2.5.1. RNA isolation ... *45*
2.5.2. RT-PCR ... *46*
2.6. DNA sequencing and bioinformatics ... 46
2.6.1. DNA sequencing ... *46*
2.6.2. Sequence comparison and domain characteristics ... *46*
2.6.3. Synteny analysis ... *47*
2.6.4. Bioinformatic analysis of MAP kinase cascades ... *47*
2.6.5. Statistical analysis ... *47*
2.7. PCR analysis ... 48
2.7.1. Standard PCR ... *49*
2.7.2. Extract-N-Amp PCR ... *49*
2.7.3. High fidelity enzymes ... *50*
2.8. Fungal transformations ... 50
2.8.1. E. festucae ... *50*
2.8.1.1. Protoplast preparation ... *50*
2.8.1.2. Transformation ... *51*
2.8.2. S. pombe lithium-acetate/PEG transformation ... *52*
2.9. Plant inoculation and growth analysis ... 52
2.9.1. Seed sterilisation ... *52*

2.9.2. Seedling germination and inoculation ... *52*
2.9.3. Immunoblotting ... *53*
2.9.4. Aniline blue staining ... *53*
2.10. Plant sectioning and staining ... 53
2.10.1. Tissue fixation and wax embedding ... *53*
2.10.2. Alcian blue/safranin O Staining ... *54*
2.11. Microscopy ... 54
2.11.1. Light microscopy ... *55*
2.11.2. Fluorescence microscopy ... *55*
2.11.2.1. GFP ... *55*
2.11.2.2. FM4-64 ... *56*
2.11.2.3. Calcofluor white (CFW) ... *56*
2.11.3. Confocal microscopy ... *56*
2.11.4. Transmission electron microscopy ... *56*
2.11.4.1. Cerium chloride staining for ROS ... *57*
2.11.5. Scanning electron microscopy ... *57*
2.12. Protein isolation and analysis ... 58
2.12.1. Protein isolation from S. pombe ... *58*
2.12.2. Western blotting ... *58*
2.13. Colony staining ... 59
2.13.1. Diaminobenzidine ... *59*
2.13.2. Nitroblue tetrazolium ... *59*
2.14. Host defense response analysis ... 59
2.14.1. Lactophenol-trypan blue staining ... *59*
3. Role of *sakA* in Maintenance of Symbiosis ... 60
3.1. Isolation of the *E. festucae* stress-activated MAP kinase ... 61
3.2. The *sakA* locus displays conserved micro-synteny ... 61
3.3. SakA is a functional orthologue of *S. pombe* Sty1 ... 66
3.3.1. Complementation of stress sensitivity ... *66*
3.3.2. Complementation of cell morphology defect ... *68*
3.3.3. S. pombe is unable to splice E. festucae introns ... *68*
3.4. Translocation of SakA to the nucleus is induced by stress ... 71
3.5. Deletion of *sakA* alters growth and stress responses in culture ... 71
3.5.1. Complementation of the ΔsakA mutant ... *78*

3.6. The *sakA* mutant has increased ROS levels in culture 78

3.7. SakA does not regulate *nox* expression.. 80

3.8. *sakA* is required for normal association with perennial ryegrass 80

3.8.1. Growth in planta... *84*

3.8.2. The ΔsakA mutant does not induce a host defense response.................................... *87*

3.8.3. The ΔsakA mutant induces branching of the host vascular tissues........................... *87*

3.8.4. Epiphyllous growth... *91*

3.9. The Δ*sakA* mutant alters development of its grass host 91

3.10. *In planta* ROS levels are increased in Δ*sakA* mutant associations 94

4. Analysis of the p21-Activated Kinases 98

4.1. Isolation and characterisation of the *E. festucae pak* genes 99

4.1.1. Identification of the E. festucae pak genes.. *99*

4.1.2. Conserved microsynteny and genomic rearrangement at the pak loci........................ *102*

4.2. Targeted replacement of the *pakA* and *pakB* genes....................................... 109

4.2.1. Preparation of pakA and pakB replacement constructs... *109*

4.2.2. Screening for pakA and pakB replacement mutants.. *112*

4.2.3. Complementation of the ΔpakA and ΔpakB mutants.. *114*

4.3. *pakA* and *pakB* are essential for normal growth in culture............................. 115

4.3.1. Growth in culture.. *115*

4.3.2. Changes at the microscopic level.. *115*

4.3.2.1. The ΔpakA mutant displays altered cell compartment size............................ *118*

4.3.2.2. The ΔpakA mutant displays increased vacuole size....................................... *118*

4.3.2.3. Structures at the hyphal tip.. *121*

4.4. The Δ*pakA* mutant displays increased ROS levels in culture......................... 121

4.5. *pakA* is required for symbiotic maintenance.. 124

4.5.1. The ΔpakA mutant reduces host survival and induces severe stunting...................... *124*

4.5.2. The pak mutants display deregulated growth in planta.. *126*

4.5.2.1. The ΔpakA mutant colonises host vascular bundles...................................... *126*

4.5.3. ΔpakA mutant hyphae display altered morphology in planta................................. *129*

5. Bioinformatic Analysis of MAP Kinase Pathways 131

5.1. Analysis of *E. festucae* MAP kinase pathways.. 132

5.1.1. Search approach *132*

5.2. The stress-activated MAP kinase pathway 134

5.2.1. Primary kinases *134*
5.2.2. The MAP kinase cascade *134*
5.2.2.1. Ssk2p/22p *134*
5.2.2.2. Ste11p *139*
5.2.2.3. Pbs2p *139*
5.2.2.4. OSM1 .... *140*
5.2.3. Phosphatases *140*
5.2.3.1. Ptp2p *140*
5.2.3.2. Ptp3p *141*
5.3. The pheromone response pathway 141
5.3.1. Primary kinases *141*
5.3.1.1. Bem1p *141*
5.3.1.2. MST20 .... *145*
5.3.2. The MAP kinase cascade *145*
5.3.2.1. Ste11p *145*
5.3.2.2. Ste7p *145*
5.3.2.3. PMK1 .... *146*
5.3.2.4. Ste50p *146*
5.3.2.5. Ste5p *146*
5.3.3. Phosphatases *147*
5.3.3.1. Ptp2p *147*
5.3.3.2. Ptp3p *147*
5.3.3.3. Msg5 .... *147*
5.4. The cell integrity pathway 147
5.4.1. Primary kinases *150*
5.4.1.1. MPKC *150*
5.4.2. The MAP kinase cascade *150*
5.4.2.1. Bck1p *150*
5.4.2.2. MKK1 .... *151*
5.4.2.3. MPS1 .... *151*
5.4.3. Phosphatases *151*
6. Discussion 152
6.1. *E. festucae sakA* encodes a functional MAP kinase 153
6.1.1. sakA complements S. pombe sty1Δ stress sensitivity and morphology defects *153*

6.1.2. SakA translocates to the nucleus in response to osmotic stress *153*
6.2. Loss of *sakA* induces stress sensitivity and fungicide resistance 154
6.2.1. The ΔsakA mutant displays increased stress sensitivity in culture *154*
6.2.2. sakA is required for sensitivity to the phenylpyrrole fungicide fludioxonil *155*
6.3. Difficulties in generation and complementation of Δ*sakA* 155
6.4. The Δ*sakA* mutant displays altered morphology in culture 156
6.5. Loss of *sakA* induces changes associated with increased aging....................... 158
6.6. Link between *sakA* and ROS signalling 159
6.6.1. The ΔsakA mutant displays increased ROS levels in culture and in planta *159*
6.6.2. Other potential sources of ROS *160*
6.7. *sakA* is required for symbiotic maintenance 162
6.7.1. The ΔsakA mutant has reduced ability to colonise perennial ryegrass *162*
6.7.2. Loss of sakA induces increased tillering *164*
6.7.3. Plants infected with the ΔsakA mutant have poor root systems *164*
6.7.4. The ΔsakA mutant induces host stunting and precocious senescence *165*
6.7.5. The ΔsakA mutant displays reduced epiphyllous growth *166*
6.7.6. Growth of the ΔsakA mutant in planta is deregulated *168*
6.7.7. The ΔsakA mutant shows altered morphology in planta *168*
6.7.8. ΔsakA mutant hyphae are surrounded by an electron dense ECM in planta *169*
6.8. Loss of *sakA* induces changes in host development....................................... 170
6.8.1. ΔsakA mutant-infected tillers display swollen bases *170*
6.8.2. ΔsakA mutant-infected tillers display a loss of anthocyanin pigmentation *171*
6.8.3. ΔsakA mutant-infection induces increased branching of host vasculature *173*
6.9. The *sakA* locus displays conserved micro-synteny 174
6.10. Sequence analysis of the *E. festucae pak* genes 175
6.11. Paks are required for normal growth in culture 175
6.11.1. Paks are required for growth under cell wall-stressing conditions *176*
6.12. The *E. festucae pak* mutants display altered morphology in culture............... 176
6.12.1. The pak mutants display altered branching in culture *176*
6.12.2. The ΔpakA mutant displays altered cell dimensions *178*
6.12.3. The ΔpakA mutant contains large vacuoles in culture *178*
6.13. Loss of *pakA* induces increased ROS levels in culture 179
6.14. *pakA* is likely required for symbiotic maintenance....................................... 180
6.15. Inability of *pakA* and *pakB* to complement the altered symbioses................. 182

6.16. The pak loci display conserved synteny and gene rearrangement 184
6.17. Features of the *E. festucae* MAP kinase pathways 185
6.18. Conclusions 186
7. Appendices 188
7.1. Multiple sequence alignments 189
7.1.1. Alignment of pak sequences for primer design 189
7.2. Vector and construct maps 189
7.2.1. pGEM®-T Easy (Promega) 190
7.2.2. pUC118 190
7.2.3. pPN83 191
7.2.4. pII99 191
7.2.5. pCR4-TOPO 192
7.2.6. pCE1 (sakA complementation construct) 192
7.2.7. pCE12 (sakA replacement construct) 193
7.2.8. pCE22 (S. pombe gDNA complementation construct) 193
7.2.9. pCE23 (S. pombe cDNA complementation construct) 194
7.2.10. pCE36 (pakA replacement construct) 194
7.2.11. pCE38 (pakB replacement construct) 195
7.2.12. pCE42 (pakB complementation construct) 195
7.2.13. pCE43 (pakA complementation construct) 196
7.3. Sequence Data 196
7.4. Statistical Analysis 196
Bibliography 198
Publication - Eaton et al. (2008). Curr Genet. 53: 163-174 220
Publication - Scott and Eaton. (2008). Curr Opin Microbiol. 11: 488-493 232

Abbreviations

A	Adenine
AD	Activation domain
Ade	Adenine
Amp	Ampicillin
Amp^R	Ampicillin resistant
ABC	ATP-binding cassette
ANS	Anthocyanin synthase
ASS	Active site signature
ATP	Adenine triphosphate
BD	Binding domain
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
˚C	Degrees celsius
CCD	Charged couple device
cDNA	Complementary DNA
CDS	Coding sequence
CFW	Calcofluor white
Cgfp	C-terminal green fluorescent protein tag
CHM	Cla4-homologue *Magnaporthe*
CHS	Chalcone synthase
CIAP	Calf intestinal alkaline phosphatase
Comp	Complemented
CRIB	Cdc42/Rac interactive binding
cv	Cultivar
DAB	3-3'Diaminobenzidine
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole

dATP	Deoxyadenine triphosphate
DBD	DND binding domain
dCTP	Deoxycytosine triphosphate
DIC	Differential interference contrast
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide triphosphate
DSPTP	Dual specificity protein tyrosine phosphatase
Ec	Ectopic
EC	Enzyme commission
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol tetraacetic acid
EMM	Edinburgh minimal medium
FAA	Formaldehyde acetic acid
FAD	Flavin adenine dinucleotide
Fg	*Fusarium graminearum*
FGI	Fungal Genome Initiative
Flu	Fludioxonil
g	Gram
GAP	GTPase-activating protein
GDI	Guanine dissociation inhibitor
gDNA	Genomic DNA
GDP	Guanosine Diphosphate
GEF	Guanine nucleotide-exchange factor
Gen^R	Geneticin resistant
GFP	Green fluorescent protein
GO	Gene ontology
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
h	Hour(s)
H	Histidine

H_2O_2	hydrogen peroxide
HA	Hemagglutinin
HCT	Hydroxycinnamoyl transferase
Hepes	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
His	Histidine
HOG	High osmolarity glycerol
Hph	Hygromycin phosphotransferase
HRP	Horse-radish peroxidase
Hyg^R	Hygromycin resistant
IAA	Indole-3-acetic acid
ID	Identity
IDA	Inferred direct assay
IDI	Inferred direct interaction
IGI	Inferred genetic interaction
IMP	Inferred mutant phenotype
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl α-D-1-thiogalactopyranoside
IS	Intercellular space(s)
kb	Kilobases
KIND	Kinase non-catalytic C-lobe domain
KO	Knock-out
LB	Luria-Bertaini broth
L	Leucine
Leu	Leucine
Lys	Lysine
M	Molar
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MFS	Major facilitator superfamily
min	Minute(s)
μg	Micro-gram
mg	Milli-gram
Mg	*Magnaporthe grisea*

µL	Micro-litre
mL	Milli-litre
µM	Micro-molar
mM	Milli-molar
mm	Milli-metre
MOPS	3-(N-morpholino)-propanesulfonic acid
mRNA	Messenger ribonucleic acid
MSO	Murashige and Skoog medium
MST	*Magnaporthe* Ste20
NA	Numerical aperture
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NBT	Nitroblue tetrazolium
Nc	*Neurospora crassa*
NCBI	National centre for biotechnology information
NEB	New England Biolabs
ng	Nano-gram
Nh	*Nectria haematococca*
nmt	No message in thiamine
NO	Nitric oxide
Nox	NADPH oxidase
Npt	Neomycin Phosphotransferase
OD	Optical density
P	Plant cell
pak	p21-activated kinase
PASS	Phosphatase active site signature
PBD	p21-Rho binding domain
PCR	Polymerase chain reaction
pcw	Plant cell wall
PD	Potato dextrose
PEG	Polyethylene glycol
PH	Pleckstrin homology
Phox	Phagocyte oxidase
PKC	Protein kinase C

pmol	Picomole
PMSF	Phenylmethylsulphonyl fluoride
PTP	Protein tyrosine phosphatase
PVP	Polyvinylpyrolidone
PX	Phox homology
RA	Ras-associating
RCA	Reviewed computational analysis
RG	Regeneration
RNA	Ribonucleic acid
RNase	Ribonuclease
RO	Reverse osmosis
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
Sak	Stress-activated kinase
SAM	Sterile α motif
SD	Synthetic dropout
SDS	Sodium dodecyl sulphate
SE	Standard error
SEM	Scanning electron microscopy
Ser	Serine
SH	Src homology
SLS	Sodium lauroyl sarcosine
SOD	Superoxide dismutase
Sorb	Sorbitol
SSC	Sodium-sodium citrate
Sty	Suppressor of tyrosine phosphatase
T	Thiamine
TAS	Traceable author statement
TAT	Tubulin antibody
TBE	Tris-boric acid-EDTA
tBLASTn	Translated nucleotide database search using a protein query

tBLASTx	Translated nucleotide database search using translated nucleotide query
TEM	Transmission electron microscopy
Thi	Thiamine
Tr	*Trichoderma reesei*
tRNA	Transfer RNA
Trp	Tryptophan
Tub	Tubulin
Ura	Uracil
UTR	Untranslated region
UV	Ultra-violet
v	Vacuole
V	Volts
v/v	Volume/volume ratio
WT	Wild-type
w/v	Weight/volume ratio
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YE	Yeast extract
YPAD	Yeast extract-peptone-adenine-dextrose

List of figures

Figure 1.1	Life-cycle of endophyte-grass associations……..…………………	4
Figure 1.2	NADPH oxidase complexes in mammalian phagocytes and fungi	10
Figure 1.3	General scheme of a MAPK pathway………………………………	13
Figure 1.4	Model showing activation of mammalian p21-activated kinases (paks) by the GTPases Cdc42 and Rac……………………………	20
Figure 1.5	Model showing regulation of Rho GTPases by RhoGDI and a p21-activated kinase (pak)……………………………………….	22
Figure 1.6	Hypothesised regulation of the fungal Nox complex………………	26
Figure 3.1	Amino acid sequence alignment of fungal MAP kinases…………..	62
Figure 3.2	Organisation of the *E. festucae sakA* gene and predicted polypeptide	63
Figure 3.3	Conserved micro-synteny at the *sakA* locus………………………...	64
Figure 3.4	Complementation of *S. pombe sty1*Δ stress sensitivity……………….	67
Figure 3.5	Complementation the *sty1*Δ morphology…………………………..	69
Figure 3.6	Agarose gel electrophoresis of *sakA* RT-PCR products…….........	70
Figure 3.7	Fluorescence microscopy showing GFP localisation of SakA in *S. pombe sty1*Δ mutant cells under osmotic stress…………………	72
Figure 3.8	Complementation of *S. pombe sty1*Δ osmosensitivity with a *sakA* C-terminal GFP fusion protein…………………………………..	73
Figure 3.9	Replacement of *sakA* by homologous recombination………………	74
Figure 3.10	Culture phenotype of the Δ*sakA* mutant……………………………	76
Figure 3.11	Examination of structures at the hyphal tip by DIC and fluorescence microscopy…………………………………………	77
Figure 3.12	Calcofluor white (CFW) staining of hyphal growth in culture……..	79
Figure 3.13	Nitroblue-tetrazolium (NBT) detection of superoxide anion production by *E. festucae*……………………………………………	81
Figure 3.14	*nox* expression analysis……………………………………...........	82
Figure 3.15	Symbiotic phenotype of the *E. festucae* Δ*sakA* mutant……………...	83
Figure 3.16	Growth of the Δ*sakA* mutant *in planta*………………………………	85
Figure 3.17	Examination of hyphal morphology and density *in planta* by TEM..	86
Figure 3.18	Fluorescence micrographs of FM4-64 stained vacuoles in culture…	88

Figure 3.19 Analysis of host defense responses by lactophenol trypan blue staining………………………………………………………….. 89
Figure 3.20 Branching of perennial ryegrass vasculature……………………… 90
Figure 3.21 Examination of epiphyllous hyphal growth……………………….. 92
Figure 3.22 Altered development of Δ*sakA*-infected perennial ryegrass……….. 93
Figure 3.23 Alteration of cell organisation below the shoot apical meristem…... 95
Figure 3.24 Elevated ROS levels in the Δ*sakA* mutant association……………... 96
Figure 4.1 Degenerate PCR amplification of the p21-activated kinases……… 100
Figure 4.2 Organisation of the *E. festucae* p21-activated kinase genes and predicted polypeptides……………………………………………... 101
Figure 4.3 Amino acid sequence alignment of fungal Cla4 homologues……… 103
Figure 4.4 Amino acid sequence alignment of fungal Ste20 homologues…….. 104
Figure 4.5 Conserved micro-synteny and genomic rearrangement at the *pak* loci ………………………………………………………………... 105
Figure 4.6 Replacement of *pakA* and *pakB* by homologous recombination…… 111
Figure 4.7 PCR screen to identify *pakA* and *pakB* replacement mutants……… 113
Figure 4.8 Culture phenotype of the pak mutants…………………………….. 116
Figure 4.9 Growth of the pak mutants in culture……………………………... 117
Figure 4.10 Calcofluor white (CFW) staining of the pak mutants in culture…… 119
Figure 4.11 Fluorescence microscopy showing FM4-64 stained vacuoles in culture………………………………………………………………… 120
Figure 4.12 Examination of structures at the hyphal tip by DIC microscopy….. 122
Figure 4.13 Examination of reactive oxygen species (ROS) levels in culture…... 123
Figure 4.14 Symbiotic phenotype of the *E. festucae* pak mutants……………….. 125
Figure 4.15 Growth of the pak mutants *in planta*……………………………..... 127
Figure 4.16 Growth of strain B3 *in planta*……………………………………..... 128
Figure 4.17 Examination of hyphal morphology and density *in planta* by TEM.. 130
Figure 5.1 Comparison of MAP kinase signalling pathways between *S. cerevisiae* and *M. grisea*……………………………………………... 133
Figure 5.2 The *E. festucae* stress-activated MAP kinase pathway……………… 135
Figure 5.3 The *E. festucae* pheromone response pathway……………………… 142
Figure 5.4 The *E. festucae* cell integrity pathway………………………………. 148

List of tables

Table 2.1	Bacterial strains, fungal strains and plant material…………………..	29
Table 2.2	Plasmids and cosmids……………………………………………...	32
Table 2.3	Separation range for agarose gels…………………………………….	43
Table 2.4	Primers used in this study…………………………………………….	48
Table 3.1	Description of genes displaying conserved synteny at the stress-activated MAP kinase locus……………………………………	65
Table 4.1	Description of genes found at the *pakA* loci…………………………..	106
Table 4.2	Description of genes found at the *pakB* loci…………………………..	108
Table 5.1	GO annotations for components of the stress-activated MAP kinase pathway……………………………………………………………….	136
Table 5.2	Amino acid conservation between *E. festucae* MAP kinase pathway components and filamentous fungal homologues…………………….	138
Table 5.3	GO annotations for components of the pheromone response pathway……………………………………………………………….	143
Table 5.4	GO annotations for components of the cell integrity pathway……….	149