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**The role of the G protein and cAMP/PKA
signalling pathway in establishment and
maintenance of the mutualistic *Epichloë
festucae* – ryegrass association**

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requirements for the degree of

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“Never Give Up, Never Surrender!”

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(Galaxy Quest)

ABSTRACT

Growth of the fungal endophyte *Epichloë festucae* in mutualistic symbiotic association with *Lolium perenne* (perennial ryegrass) is highly regulated and synchronised with the growth of the host plant leaf. To maintain this pattern of fungal growth *in planta*, specific signalling between symbiont and its host grass is required. To sense the extracellular environment and respond to changes, filamentous fungi rely on G protein-coupled receptors (GPCRs), which transmit signals predominantly via heterotrimeric G proteins to downstream pathways such as the cAMP/Protein Kinase A (PKA) and MAP kinase signalling pathways. In phytopathogenic fungi, G protein signalling and the associated cAMP/PKA pathways are often essential for a normal host interaction. Signal transduction using the second messenger cAMP to activate the PKA activity is finely balanced through a regulatory feedback loop for signal attenuation regulated by 3'-5'-cyclic nucleotide phosphodiesterases (PDE).

Using a blast-based bioinformatic approach, a total of 40 genes encoding putative GPCRs were identified in the genome of *E. festucae*, grouping into 13 of 14 classes of the recent classification system for fungal GPCRs. Among genes encoding components of the G protein signalling cascade, nine of these GPCRs including the two cAMP receptor-like GPCRs, *Gpr1b* and *Gpr2*, show significant transcriptional up-regulation in association with the host compared to the respective expression level when grown in axenic culture. A reverse genetics approach was used to functionally characterise the identified cAMP receptor-like GPCRs (*Gpr1a*, *Gpr1b* and *Gpr2*). While deletion of *gpr1a* was unsuccessful, plants infected with an *E. festucae* Δ *gpr1b* mutant showed a severe breakdown of the *E. festucae*-ryegrass association, whilst no effects were observed for Δ *gpr2*-inoculated plants. Among numerous other genes putatively involved in G-protein and cAMP/PKA signalling, two putative PDEs involved in regulation of the cAMP-mediated signal were also identified. Deletion of *pdeH* (Δ *pdeH*), a gene encoding a PDE with high affinity towards cAMP, had a dramatic effect on the endophyte-plant association. In contrast, deletion of *pdeL* (Δ *pdeL*), a gene encoding a PDE with low affinity towards cAMP, had no effect on the host interaction phenotype, while primarily modulating the intracellular cAMP level during nutrient-induced activation of the cAMP/PKA

signalling pathway in axenic culture. Finely balanced cAMP levels are crucial for various cellular processes including hyphal growth, cellular differentiation of asexual development and conidiogenesis.

This research identified Gpr1b as an important receptor involved in maintenance of the highly restricted endophytic growth pattern of *E. festucae* in association with the host, potentially regulated by plant-derived molecules. These signals are mediated by the cAMP/PKA signalling pathway for an appropriate alteration in gene expression and subsequent cellular adaptation. Intracellular cAMP levels triggering these concentration-dependent processes are regulated by PdeH, which specifically acts *in planta*. This study shows that G-protein and cAMP/PKA signalling play an important role in regulating environmental signalling for establishment and maintenance of the mutualistic association between *E. festucae* and *L. perenne*.

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TABLE OF CONTENTS

Chapter 1 Introduction	1
1.1 <i>Epichloë festucae</i> forms a mutualistic association with perennial ryegrass	2
1.1.1 Mutual benefits of symbiotic interaction	2
1.1.2 The lifecycle of <i>E. festucae</i> includes both asexual and sexual stages	3
1.1.3 <i>E. festucae</i> shows a host-synchronised growth pattern in association with perennial ryegrass.....	6
1.2 Specific signalling is necessary for symbiosis between fungus and host.....	9
1.2.1 G protein signalling pathways transduce extracellular signals to downstream pathways.....	9
1.2.2 The mitogen-activated protein kinase pathway	13
1.2.3 The G protein cyclic AMP/PKA signalling pathway	16
1.2.4 G protein coupled receptors sense environmental changes and transduce signals for cellular response.....	19
1.2.5 Role of fungal GPCRs in pathogenicity through activating the cAMP-dependent PKA signalling cascade.....	22
1.2.6 cAMP-phosphodiesterases terminate cAMP/PKA signalling	24
1.3 Aims	27
Chapter 2 Materials and Methods.....	29
2.1 Bioinformatics.....	30
2.1.1 Nucleotide and protein sequences	30
2.1.2 tBLASTn analysis.....	30
2.1.3 Multiple sequence alignment	30
2.1.4 Phylogenetic analysis	31
2.1.5 Protein prediction	31
2.1.6 GC profile	31
2.2 Molecular and biological material.....	31
2.3 Growth conditions and media	40
2.3.1 <i>Escherichia coli</i>	40
2.3.1.1 Luria-Bertani growth (LB) medium	40
2.3.1.2 SOC medium.....	40
2.3.2 <i>Saccharomyces cerevisiae</i>	40
2.3.2.1 Yeast Peptone Dextrose (YPD) medium	41

2.3.2.2	SD uracil dropout (SD ura ⁻) medium	41
2.3.3	<i>Epichloë festucae</i>	41
2.3.3.1	Potato dextrose (PD) medium	41
2.3.3.2	Regeneration (RG) medium	41
2.3.3.3	Water (H ₂ O) medium	42
2.3.3.4	Modified Czapek Dox (CDGN) medium.....	42
2.3.3.5	Czapek Dox salts (CD) medium	42
2.3.3.6	Czapek Dox (CZ) medium	42
2.3.3.7	Modified Blankenship medium.....	42
2.4	Colony growth in axenic culture	46
2.4.1	Analysis of hyphal growth.....	46
2.4.1.1	Non-transfer experiment.....	46
2.4.1.2	Transfer experiment	47
2.4.2	Determination of hyphal growth rate	47
2.4.2.1	Hyphal growth rate for non-transfer experiment.....	47
2.4.2.2	Hyphal growth rate for transfer experiment	48
2.4.3	Fungal growth under stress conditions	48
2.4.4	Fungal growth in presence of plant-derived molecules	49
2.4.5	Chemical treatment of fungal colonies	50
2.5	Molecular techniques.....	51
2.5.1	DNA isolation	51
2.5.1.1	Plasmid DNA isolation from <i>E. coli</i>	51
2.5.1.2	Cosmid DNA isolation from <i>E. coli</i>	51
2.5.1.3	Plasmid DNA isolation from <i>S. cerevisiae</i>	51
2.5.1.4	Genomic DNA from <i>E. festucae</i>	52
2.5.1.5	Crude genomic DNA from <i>E. festucae</i>	53
2.5.2	DNA purification	53
2.5.3	Restriction enzyme (RE) digestions	53
2.5.4	Ethanol precipitation	54
2.5.5	Gel electrophoresis.....	54
2.5.6	DNA quantification	54
2.5.7	Polymerase chain reaction (PCR).....	55
2.5.7.1	Standard PCR	55
2.5.7.2	High-Fidelity PCR.....	55
2.5.7.3	PCR for GC-rich DNA sequences	56
2.5.8	TOPO-cloning.....	57
2.5.9	DNA sequencing.....	57
2.5.10	Preparation of constructs	58

2.5.10.1	<i>E. festucae gpr1a</i> gene replacement construct	58
2.5.10.2	<i>E. festucae gpr1b</i> gene replacement construct	58
2.5.10.3	<i>E. festucae gpr2</i> gene replacement construct	58
2.5.10.4	<i>E. festucae pdeH</i> gene replacement construct	59
2.5.10.5	<i>E. festucae pdeL</i> gene replacement construct	59
2.5.10.6	<i>E. festucae gpr1b</i> complementation construct.....	59
2.5.10.7	<i>E. festucae pdeH</i> complementation construct.....	59
2.5.10.8	<i>E. festucae pdeL</i> complementation construct.....	60
2.5.10.9	Yeast recombinational cloning (YRC)	60
2.5.10.10	Gibson assembly (GA)	61
2.5.11	Bacterial Transformation	61
2.5.11.1	Transformation of electrocompetent <i>E. coli</i> cells	61
2.5.11.2	Transformation of chemically competent <i>E. coli</i> cells.....	62
2.5.11.3	<i>E. coli</i> - transformation plasmid screen (CloneChecker™)	62
2.5.12	Fungal transformation	63
2.5.12.1	Preparation of fungal protoplasts	63
2.5.12.2	Transformation of fungal protoplasts	63
2.5.13	Single spore isolation	64
2.5.14	Southern blotting	64
2.5.14.1	DNA probe-labelling.....	65
2.5.14.2	Hybridisation and detection.....	66
2.5.15	Identification of cosmids from <i>E. festucae</i> cosmid library	67
2.6	Quantification of intracellular cAMP.....	67
2.6.1	Growth and harvest of fungal mycelia and extraction of cAMP.....	67
2.6.2	Quantification of cAMP by ELISA assay.....	68
2.7	Plant methods.....	68
2.7.1	Seed sterilisation	68
2.7.2	Seedlings growth conditions	69
2.7.3	Plant inoculation	69
2.7.4	Standard plant growth conditions	69
2.7.5	Axenic culture plant growth conditions	69
2.7.5.1	Murashige and Skoog (MSO) medium	70
2.7.6	Detection of endophyte infected plants	70
2.7.7	Extraction of apoplastic fluid (APF) from <i>L. perenne</i> plants.....	70
2.8	Microscopic analysis.....	71
2.8.1	In culture analysis.....	71
2.8.1.1	Determination of hyphal fusion frequency	71
2.8.2	<i>In planta</i> analysis.....	71

2.8.2.1	Confocal laser scanning microscopy (CLSM).....	71
2.8.2.2	Transmission electron microscopy (TEM).....	72
2.9	Statistical analyses	72
2.9.1	Carbon sources	73
2.9.2	Amino acids.....	73
2.9.3	Environmental pH stress.....	73
2.9.4	Plant-derived molecules	74
Chapter 3	Bioinformatic analysis of the cAMP G protein-coupled receptors and phosphodiesterases in the <i>E. festucae</i> genome.....	75
3.1	Identification of G protein-coupled receptors in <i>E. festucae</i> genome	76
3.2	Bioinformatic approach to characterize cAMP-receptor like GPCRs in <i>Epichloë</i> spp.	84
3.3	Cyclic nucleotide AMP phosphodiesterases (PDEs) in the <i>E. festucae</i> genome	100
3.4	Transcriptional difference of <i>E. festucae</i> wild-type growth in culture and in association with the host	110
Chapter 4	Functional analysis of cAMP receptor-like G protein-coupled receptors.....	115
4.1	Functional analysis of the Gpr1a.....	116
4.1.1	Attempted deletion of the <i>E. festucae gpr1a</i> gene	116
4.2	Functional analysis of the Gpr1b.....	122
4.2.1	Deletion of the <i>E. festucae gpr1b</i> gene	122
4.2.2	Deletion of <i>gpr1b</i> does not alter fungal development in axenic culture	124
4.2.3	Deletion of <i>gpr1b</i> caused a dramatic breakdown of the symbiotic interaction	126
4.2.3.1	Symbiotic interaction phenotype of $\Delta gpr1b$ strains.....	126
4.2.3.2	Microscopic analysis reveals unrestricted growth and increase of fungal biomass <i>in planta</i>	130
4.2.3.3	$\Delta gpr1b$ strains display an increased number of epiphyllous hyphae	135
4.3	Functional analysis of the Gpr2.....	136
4.3.1	Deletion of the <i>E. festucae gpr2</i> gene	136
4.3.2	Deletion of <i>gpr2</i> had no effect on fungal development in axenic culture	138
4.3.3	Gpr2 does not show a functional role in the plant symbiotic interaction....	140
4.3.3.1	Symbiotic interaction phenotype of $\Delta gpr2$ strains.....	140

4.3.3.2	Microscopic analysis of $\Delta gpr2$ revealed wild-type like fungal colonisation of host tissue	142
4.4	Analysis of hyphal growth for $\Delta gpr1b$ and $\Delta gpr2$ mutant strains.....	147
4.4.1	<i>E. festucae</i> cAMP-receptor like GPCRs are not directly involved in nutrient sensing.....	148
4.4.2	Gpr1b or Gpr2 may have an accessory role in sensing specific amino acids	151
4.4.3	Deletion of <i>gpr1b</i> may increase sensitivity of <i>E. festucae</i> to oxidative stress	155
4.4.4	<i>E. festucae</i> Gpr1b is potentially involved in sensing of an unknown component in apoplastic fluid.....	157
4.4.5	Gpr1b and Gpr2 are not directly involved in any processes in culture potentially mediated by the cAMP signalling pathway.....	160
4.4.6	Nutrient rich medium triggers increase in intracellular cAMP levels, but Gpr1b or Gpr2 are not directly responsible	162
Chapter 5	Functional analysis of cAMP-phosphodiesterases	165
5.1	Functional analysis of PdeH	166
5.1.1	Deletion of the <i>E. festucae pdeH</i> gene.....	166
5.1.2	Deletion of <i>pdeH</i> does not alter fungal development in axenic culture	168
5.1.3	Deletion of <i>pdeH</i> caused a dramatic breakdown of the symbiotic interaction.....	170
5.1.3.1	Symbiotic interaction phenotype of $\Delta pdeH$ strains	170
5.1.3.2	Microscopic analysis reveals unrestricted growth and increase of fungal biomass	174
5.1.3.3	Microscopic analysis of $\Delta pdeH$ revealed wild-type like fungal colonisation of host tissue	179
5.2	Functional analysis of PdeL.....	180
5.2.1	Deletion of the <i>E. festucae pdeL</i> gene	180
5.2.2	Deletion of <i>pdeL</i> resulted in a significant reduction in hyphal growth in axenic culture	182
5.2.3	PdeL does not show a functional role in the plant symbiotic interaction	187
5.2.3.1	Symbiotic interaction phenotype of the $\Delta pdeL$ strain	187
5.2.3.2	Microscopic analysis of $\Delta pdeL$ revealed wild-type like fungal colonisation of host tissue	189
5.3	Analysis of hyphal growth for the $\Delta pdeH$ and $\Delta pdeL$ mutant strains	191

5.3.1	<i>E. festucae</i> low affinity phosphodiesterase is important for hyphal growth especially on nutrient-rich media	191
5.3.2	Hyphal growth is negatively affected by the presence of various amino acids when PdeH or PdeL are absent	195
5.3.3	Cell-wall stress and oxidative stress masks the hyphal growth defect of the $\Delta pdeL$ mutant strain in culture	199
5.3.4	Plant derived molecules have no obvious effect on the hyphal growth for the mutant strains, $\Delta pdeH$ and $\Delta pdeL$, in axenic culture	201
5.3.5	Chemical alteration of activity for key-enzymes of the cAMP signalling pathway in the PDE mutants	203
5.3.6	Nutrient-dependent increase of intracellular cAMP levels in culture is regulated mainly by the low affinity phosphodiesterase in culture.....	206
Chapter 6 Summary discussion and conclusions with recommendations for future work.....		209
6.1	Fungal GPCRs and the role of cAMP receptor-like GPCRs in the mutualistic <i>E. festucae</i> – <i>L. perenne</i> association.....	210
6.1.1	Phylogenetic diversity of cAMP receptor-like GPCRs	212
6.1.2	Role of class V GPCRs in <i>E. festucae</i> and other fungi.....	213
6.1.3	Role of cAMP receptor-like GPCRs in nutrient and amino acid sensing.	216
6.1.4	Role in association with the host.....	217
6.2	The role of the cAMP-phosphodiesterases in <i>E. festucae</i>	229
6.2.1	PDEs in <i>E. festucae</i>	230
6.2.2	The potential role of cAMP signalling in expressoria development.....	239
6.3	Conclusion	241
Chapter 7 Appendices.....		247
Chapter 8 Supplementary data files in accompanying digital media		277
Chapter 9 References.....		283

LIST OF FIGURES

Figure 1-1 Lifecycle of <i>Epichloë</i> endophyte.....	5
Figure 1-2 <i>E. festucae</i> shows a synchronised growth <i>in planta</i>	8
Figure 1-3. The heterotrimeric G-protein cycle.....	11
Figure 1-4 The G-protein cAMP/PKA signalling pathway	18
Figure 1-5 Structure of G-protein coupled receptors.....	20
Figure 1-6 cAMP synthesis and hydrolysis.....	25
Figure 3-1 Classification of fungal G protein-coupled receptors	82
Figure 3-2 Multiple sequence alignment of <i>E. festucae</i> Gpr1a homologues	84
Figure 3-3 Multiple sequence alignment of <i>E. festucae</i> Gpr1b homologues.....	87
Figure 3-4 Multiple sequence alignment of <i>E. festucae</i> Gpr2 homologues.....	88
Figure 3-5 Phylogenetic analysis of fungal class V cAMP receptor-like GPCRs.....	91
Figure 3-6 Phylogenetic analysis of class V GPCRs within the <i>Epichloë</i> species.....	92
Figure 3-7 Organisation of the <i>E. festucae gpr1a</i> gene and predicted polypeptide .	94
Figure 3-8 Organisation of <i>E. festucae gpr1b</i> gene and predicted polypeptide.....	96
Figure 3-9 Organisation of <i>E. festucae gpr2</i> gene and predicted polypeptide	98
Figure 3-10 Multiple sequence alignment of fungal PdeH homologues.....	102
Figure 3-11 Multiple sequence alignment of fungal PdeL homologues	103
Figure 3-12 Maximum-likelihood dendrogram of fungal 3'-5' cyclic phosphodiesterases	104
Figure 3-13 Organisation of the <i>E. festucae pdeH</i> gene and predicted polypeptide	106
Figure 3-14 Organisation of the <i>E. festucae pdeL</i> gene and predicted polypeptide	108
Figure 3-15 Gene expression change of GPCR encoding genes of <i>E. festucae</i> wild-type in association with the host plant.....	110
Figure 3-16 Transcriptional change of genes encoding components of the G protein and cAMP/PKA signalling pathway of the <i>E. festucae</i> wild-type in association with the host plant.....	113
Figure 4-1 Strategy for deletion of <i>E. festucae gpr1a</i>	117
Figure 4-2 DNA GC content of <i>gpr1a</i> locus	118
Figure 4-3 Molecular analysis of <i>E. festucae gpr1a</i> -containing cosmids.....	120

Figure 4-4 Physical map of <i>E. festucae gpr1a</i> locus and PCR amplification of non-specific products	121
Figure 4-5 Strategy for deletion of <i>gpr1b</i> and identification of $\Delta gpr1b$ mutant strains	122
Figure 4-6 Culture phenotype analysis of the $\Delta gpr1b$ mutant strain	125
Figure 4-7 Hyphal fusion event in $\Delta gpr1b$ mutant strains.....	125
Figure 4-8 Symbiosis phenotype of <i>Lolium perenne</i> infected with $\Delta gpr1b$ mutant strain.....	127
Figure 4-9 Complementation of $\Delta gpr1b$ mutant strain.....	128
Figure 4-10 Light microscopy of the $\Delta gpr1b$ mutant strain <i>in planta</i>	131
Figure 4-11 Transmission electron microscopy of the $\Delta gpr1b$ mutant strain <i>in planta</i>	132
Figure 4-12 Hyphae per intercellular space of $\Delta gpr1b$ mutant strain	133
Figure 4-13 Confocal laser-scanning microscopy of the $\Delta gpr1b$ mutant strain <i>in planta</i>	135
Figure 4-14 Strategy for deletion of and identification of $\Delta gpr2$ mutant strains ..	136
Figure 4-15 Culture phenotype analysis of $\Delta gpr2$ mutant strain.....	139
Figure 4-16 Hyphal fusion event in $\Delta gpr2$ mutant strains	140
Figure 4-17 Symbiosis phenotype of <i>Lolium perenne</i> infected with $\Delta gpr2$ mutant strain.....	141
Figure 4-18 Light microscopy of the $\Delta gpr2$ mutant strain <i>in planta</i>	143
Figure 4-19 Transmission electron microscopy of the $\Delta gpr2$ mutant strain <i>in planta</i>	144
Figure 4-20 Hyphae per intercellular space of $\Delta gpr2$ mutant strain.....	145
Figure 4-21 Confocal laser-scanning microscopy of the $\Delta gpr2$ mutant strain <i>in planta</i>	147
Figure 4-22 Colony morphology of the GPCR mutant strains in response to nutrient limitation.....	149
Figure 4-23 Variation in hyphal growth rate of the GPCR mutant strains in response to nutrient starvation and to growth on various carbon sources.....	151
Figure 4-24 Variation in hyphal growth-rate of the GPCR mutant strains in response to amino acids	152
Figure 4-25 Variation in colony growth of the GPCR mutant strains under various stress conditions	157

Figure 4-26 Variation in hyphal growth rate of the GPCR mutants in response to plant extracts	158
Figure 4-27 Effect of inhibition/activation of key enzymes in the cAMP signalling pathway on growth of wild-type and GPCR mutants	161
Figure 4-28 Quantification of the intracellular cAMP levels in the <i>Δgpr1b</i> and <i>Δgpr2</i> mutants under nutrient limitation	163
Figure 5-1 Strategy for deletion of <i>pdeH</i> and identification of <i>ΔpdeH</i> mutant strains	166
Figure 5-2 Culture phenotype analysis of <i>ΔpdeH</i> mutant strain	169
Figure 5-3 Hyphal fusion event in <i>ΔpdeH</i> mutant strains	170
Figure 5-4 Symbiosis phenotype of <i>Lolium perenne</i> infected with <i>ΔpdeH</i> mutant strain	171
Figure 5-5 Complementation of <i>ΔpdeH</i> mutant strain	172
Figure 5-6 Light microscopy of the <i>ΔpdeH</i> mutant strain <i>in planta</i>	175
Figure 5-7 Transmission electron microscopy of the <i>ΔpdeH</i> mutant strain <i>in planta</i>	176
Figure 5-8 Hyphae per intercellular space of <i>ΔpdeH</i> mutant and complementation strains	177
Figure 5-9 Confocal laser-scanning microscopy of the <i>ΔpdeH</i> mutant strain <i>in planta</i>	179
Figure 5-10 Strategy for deletion of and identification of <i>ΔpdeL</i> mutant strains ..	180
Figure 5-11 Culture phenotype analysis of <i>ΔpdeL</i> mutant strain	183
Figure 5-12 Hyphal fusion event in <i>ΔpdeL</i> mutant strains	184
Figure 5-13 Sporulation assay for the <i>ΔpdeL</i> mutant strains	184
Figure 5-14 Complementation of <i>ΔpdeL</i> mutant strain	187
Figure 5-15 Symbiosis phenotype of <i>Lolium perenne</i> infected with <i>ΔpdeL</i> mutant strain	188
Figure 5-16 Confocal laser-scanning microscopy of the <i>ΔpdeL</i> mutant strain <i>in planta</i>	191
Figure 5-17 Colony morphology of the PDE mutant strains in response to nutrient limitation	193
Figure 5-18 Variation in hyphal growth rate of the PDE mutant strains in response to nutrient starvation and to growth on various carbon sources	195

Figure 5-19 Variation in hyphal growth-rate of the PDE mutant strains in response to amino acids	196
Figure 5-20 Variation in colony growth of the PDE mutant strains under various stress conditions	200
Figure 5-21 Variation in hyphal growth rate of the PDE mutants in response to plant extracts	203
Figure 5-22 Effect of inhibition/activation of key enzymes in the cAMP signalling pathway on growth of wild-type and GPCR mutants	204
Figure 5-23 Quantification of intracellular cAMP concentration in $\Delta pdeH$ and $\Delta pdeL$ mutants under nutrient limitation.....	207
Figure 6-1 The fungal G protein and cAMP pathway in association with the host.....	244

LIST OF TABLES

Table 2-1	Organisms and plasmids used.....	32
Table 2-2	List of primers used.....	35
Table 2-3	Table of growth media and supplements.....	43
Table 2-4	Table of growth media and amino acid supplements	45
Table 3-1	Table of putative <i>Epichloë</i> GPCRs.....	76
Table 3-2	<i>Epichloë</i> Class XIV - Pth11-like GPCRs.....	81
Table 3-3	<i>Epichloë</i> PDEs.....	100
Table 3-4	Components of G-protein and cAMP/PKA signalling pathway	112

LIST OF APPENDICES

Appendix 1: Table of genes used in this study	248
Appendix 2: Plasmid map of pRS426.....	257
Appendix 3: Plasmid map of pSF15.15.....	258
Appendix 4: Plasmid map of pSF16.17.....	259
Appendix 5: Plasmid map of pAB01	260
Appendix 6: Plasmid map of pAB02	261
Appendix 7: Plasmid map of pAB03	262
Appendix 8: Plasmid map of pAB04.....	263
Appendix 9: Plasmid map of pAB05.....	264
Appendix 10: Plasmid map of pAB06	265
Appendix 11: Plasmid map of pAB08	266
Appendix 12: Plasmid map of pCR2.1-TOPO®	267
Appendix 13: Non-transfer experiment design (A) and measurement (B)	268
Appendix 14: Transfer experiment design (A) and measurement (B).....	269
Appendix 15: Table of <i>E. festucae</i> GPCR homologues.....	2670
Appendix 16: Rooted phylogenetic dendrogram of class V GPCRs among <i>Epichloë</i> species	272
Appendix 17: Table of <i>E. festucae</i> homologues for components of the G protein and cAMP/PKA signalling pathway.....	2673
Appendix 18: Table of transcriptional difference of <i>E. festucae</i> wild-type growth in culture and in association with the host.....	2674

LIST OF SUPPLEMENTARY FILES

Supplementary 1: Multiple sequence alignment of <i>E. festucae</i> Pre2 homologues	278
Supplementary 2: Predicted protein topology of <i>E. festucae</i> Pre2.....	278
Supplementary 3: Multiple sequence alignment of <i>E. festucae</i> Pre1 homologues	278
Supplementary 4: Predicted protein topology of <i>E. festucae</i> Pre1.....	278
Supplementary 5: Multiple sequence alignment of <i>E. festucae</i> Gpr4 homologues	278
Supplementary 6: Predicted protein topology of <i>E. festucae</i> Gpr4	278
Supplementary 7: Multiple sequence alignment of <i>E. festucae</i> Gpr5 homologues	278
Supplementary 8: Predicted protein topology of <i>E. festucae</i> Gpr5	278
Supplementary 9: Multiple sequence alignment of <i>E. festucae</i> Gpr6 homologues	278
Supplementary 10: Predicted protein topology of <i>E. festucae</i> Gpr6.....	278
Supplementary 11: Multiple sequence alignment of <i>E. festucae</i> Gpr7a homologues	278
Supplementary 12: Predicted protein topology of <i>E. festucae</i> Gpr7a	278
Supplementary 13: Multiple sequence alignment of <i>E. festucae</i> Gpr7b homologues	278
Supplementary 14: Predicted protein topology of <i>E. festucae</i> Gpr7b	279
Supplementary 15: Multiple sequence alignment of <i>E. festucae</i> Gpr7c homologues	279
Supplementary 16: Predicted protein topology of <i>E. festucae</i> Gpr7c.....	279
Supplementary 17: Multiple sequence alignment of <i>E. festucae</i> Gpr8 homologues	279
Supplementary 18: Predicted protein topology of <i>E. festucae</i> Gpr8.....	279
Supplementary 19: Multiple sequence alignment of <i>E. festucae</i> Gpr9 homologues	279
Supplementary 20: Predicted protein topology of <i>E. festucae</i> Gpr9.....	279
Supplementary 21: Multiple sequence alignment of <i>E. festucae</i> Gpr10 homologues	279
Supplementary 22: Predicted protein topology of <i>E. festucae</i> Gpr10	279
Supplementary 23: Multiple sequence alignment of <i>E. festucae</i> Gpr10b homologues	279
Supplementary 24: Predicted protein topology of <i>E. festucae</i> Gpr10b.....	279

Supplementary 25: Multiple sequence alignment of <i>E. festucae</i> Gpr9b homologues	279
Supplementary 26: Predicted protein topology of <i>E. festucae</i> Gpr9b.....	279
Supplementary 27: Multiple sequence alignment of <i>E. festucae</i> Ops1 homologues	280
Supplementary 28: Predicted protein topology of <i>E. festucae</i> Ops1.....	280
Supplementary 29: Multiple sequence alignment of <i>E. festucae</i> Gpr11 homologues	280
Supplementary 30: Predicted protein topology of <i>E. festucae</i> Gpr11.....	280
Supplementary 31: Multiple sequence alignment of <i>E. festucae</i> Gpr13 homologues	280
Supplementary 32: Predicted protein topology of <i>E. festucae</i> Gpr13.....	280
Supplementary 33: Multiple sequence alignment of <i>E. festucae</i> Gpr14a homologues	280
Supplementary 34: Predicted protein topology of <i>E. festucae</i> Gpr14a.....	280
Supplementary 35: Multiple sequence alignment of <i>E. festucae</i> Gpr14b homologues	280
Supplementary 36: Predicted protein topology of <i>E. festucae</i> Gpr14b.....	280
Supplementary 37: Predicted protein topology of <i>E. festucae</i> Pth11-1.....	280
Supplementary 38: Predicted protein topology of <i>E. festucae</i> Pth11-2.....	280
Supplementary 39: Predicted protein topology of <i>E. festucae</i> Pth11-3.....	280
Supplementary 40: Predicted protein topology of <i>E. festucae</i> Pth11-4.....	280
Supplementary 41: Predicted protein topology of <i>E. festucae</i> Pth11-5.....	281
Supplementary 42: Predicted protein topology of <i>E. festucae</i> Pth11-6.....	281
Supplementary 43: Predicted protein topology of <i>E. festucae</i> Pth11-7.....	281
Supplementary 44: Predicted protein topology of <i>E. festucae</i> Pth11-8.....	281
Supplementary 45: Predicted protein topology of <i>E. festucae</i> Pth11-9.....	281
Supplementary 46: Predicted protein topology of <i>E. festucae</i> Pth11-10.....	281
Supplementary 47: Predicted protein topology of <i>E. festucae</i> Pth11-11.....	281
Supplementary 48: Predicted protein topology of <i>E. festucae</i> Pth11-12.....	281
Supplementary 49: Predicted protein topology of <i>E. festucae</i> Pth11-13.....	281
Supplementary 50: Predicted protein topology of <i>E. festucae</i> Pth11-14.....	281
Supplementary 51: Predicted protein topology of <i>E. festucae</i> Pth11-15.....	281
Supplementary 52: Predicted protein topology of <i>E. festucae</i> Pth11-16.....	281

Supplementary 53: Predicted protein topology of <i>E. festucae</i> Pth11-17.....	281
Supplementary 54: Predicted protein topology of <i>E. festucae</i> Pth11-18.....	281
Supplementary 55: Predicted protein topology of <i>E. festucae</i> Pth11-19.....	281
Supplementary 56: Multiple sequence alignment of <i>E. festucae</i> Gpa1 homologues	281
Supplementary 57: Multiple sequence alignment of <i>E. festucae</i> Gpa2 homologues	282
Supplementary 58: Multiple sequence alignment of <i>E. festucae</i> Gpa3 homologues	282
Supplementary 59: Multiple sequence alignment of <i>E. festucae</i> Gpb1 homologues	282
Supplementary 60: Multiple sequence alignment of <i>E. festucae</i> Gpg1 homologues	282
Supplementary 61: Multiple sequence alignment of <i>E. festucae</i> Ric8 homologues	282
Supplementary 62: Multiple sequence alignment of <i>E. festucae</i> AcyA homologues	282
Supplementary 63: Multiple sequence alignment of <i>E. festucae</i> Pkac1 homologues	282
Supplementary 64: Multiple sequence alignment of <i>E. festucae</i> Pkar1 homologues	282

ABBREVIATIONS

aa, AA	amino acid(s)
AC	adenylate cyclase
Amp	Ampicillin
AMP	Adenosine monophosphate
AmpR	Ampicillin resistance
APF	Apoplastic fluid
APS	Apoplastic space
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
BLASTn	BLAST search against a nucleotide sequence database with a nucleotide sequence query
BLASTp	BLAST search against a protein sequence database with a protein sequence query
bp	Base pairs
C	Plant cuticle
C-terminus	carboxyl terminal sequence (COOH-terminus)
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COOH	Carboxy terminal sequence
CD	Czapek Dox salts medium
CDGN	Czapek Dox salts medium, modified
cDNA	Complementary DNA
CDS	Coding sequence
CLSM	Confocal laser-scanning (fluorescence) microscopy
cm	Centimeter
conc.	Concentration
CR	Congo Red
CRISPR	Clustered regularly-interspaced short palindromic repeats
CFW	Calcofluor white
CWI	Cell wall integrity

CZ	Czapek Dox medium
cPKA/Pkac	Protein kinase A catalytic subunit
DAB	3,3'-Diaminobenzidine
DIC	Differential interference contrast
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribose nucleoside triphosphate
ECL	Extracellular loop
EDTA	Ethylene diamine tetra-acetic acid
Epi	Epiphyllous/Epiphytic (hyphae)
<i>E</i> value	Expect value
Exp	Expressoria structure (hyphae)
FRET	Förster resonance energy transfer
g	Gram
x g	Acceleration due to gravity on Earth (9.8 m s ²)
gDNA	Genomic DNA
Gen	Geneticin
Gen ^R	Geneticin resistance
GPCR	G protein-coupled receptor
G protein	Guanine nucleotide-binding protein
G α	Guanine nucleotide-binding protein alpha (α) subunit
G β	Guanine nucleotide-binding protein beta (β) subunit
G γ	Guanine nucleotide-binding protein gamma (γ) subunit
GEF	Guanine nucleotide exchange factor
GAP	GTPase-activating proteins
GTP	guanosine triphosphate
GDP	guanosine diphosphate
GTPase	hydrolase enzymes that bind and hydrolyze guanosine triphosphate (GTP)
GMP	Guanosine monophosphate
h	Hour(s) or hyphae according to context
His	Histamine
<i>hph</i>	Hygromycin resistance-conferring gene

Hyg	Hygromycin
Hyg ^R	Hygromycin resistance
ICS	Intracellular space
ICL	Intracellular loop
IMBX	3-iso-1-methylxanthine
Kb	kilobase
LA	Linoleic acid
LB	Luria-Bertani
l	Litre
M	Molar (Moles per liter)
MAPK	Mitogen-activated protein kinases
MAPKK	Mitogen-activated protein kinases kinases
MAPKKK	Mitogen-activated protein kinases kinases kinases
mRNA	Messenger RNA
mg	Milligram
min	Minute(s)
mm	Millimeter
mM	millimole
ml	millilitre
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NCM	Nitrocellulose membrane
ng	Nanograms
N-terminus	Amino terminal sequence (NH ₂ -terminus)
<i>nptII</i>	Geneticin resistance-conferring gene
°C	Degrees Celsius
ORF	Open reading frame
PC	Plant cell
PCR	Polymerase chain reaction
PCW	Plant cell wall
PD	Potato dextrose
PEG	Polyethylene glycol
PKA	Protein kinase A
Ppi	Pyrophosphate

PR	pheromone response pathway
PDE	phosphodiesterase
PAQR	progestin adipoQ Receptor
qPCR	Quantitative polymerase chain reaction
Qui	Quinine
RE	Restriction enzyme
RG	Regeneration (medium)
RGS	Regulator of G protein signalling
RNA	Ribonucleic acid
RNAseq	RNA sequencing
Rnase	Ribonuclease
ROS	reactive oxygen species
RPM	Revolution per minute
RPKM	Reads Per Kilobase Million
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
rPKA/Pkar	Protein kinase A regulatory subunit
s	Second(s)
SAM	Shoot apical meristem
SDS	Sodium dodecyl sulfate
SM	Secondary metabolism OR secondary metabolite
SD	Standard deviation
SE	Standard error
Sub	Sub-cuticular (hyphae)
TBE	Tris-boric acid-EDTA
tBLASTn	BLAST search against a translated nucleotide sequence database with a protein sequence query
TE	Trace elements
TF	Transcription factor
TM	Transmembrane
Tm	Melting temperature
V	vacuole
v/v	Volume:volume ratio

w/v	Weight:volume ratio
WGA	Wheat germ agglutinin
WT	Wild type
μg	microgram
μl	microliters
μm	micrometer
μM	micromolar

