Molecular characterisation of the EAS gene cluster for ergot alkaloid biosynthesis in epichloë endophytes of grasses

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

Doctor of Philosophy
in
Molecular Genetics

at Massey University, Palmerston North,
New Zealand

Damien James Fleetwood

2007
Abstract

Clavicipitaceous fungal endophytes of the genera *Epichloë* and *Neotyphodium* form symbioses with grasses of the family Pooidae in which they can synthesise an array of bioprotective alkaloids. Some strains produce the ergot alkaloid ergovaline, which is implicated in livestock toxicoses caused by ingestion of endophyte-infected grasses.

Cloning and analysis of a plant-induced non-ribosomal peptide synthetase (NRPS) gene from *Neotyphodium lolii* and analysis of the *E. festucae* E2368 genome sequence revealed a complex gene cluster for ergot alkaloid biosynthesis. The *EAS* cluster contained a single-module NRPS gene, *lpsB*, and other genes orthologous to genes in the ergopeptine gene cluster of *Claviceps purpurea* and the clavine cluster of *Aspergillus fumigatus*. Functional analysis of *lpsB* confirmed its role in ergovaline synthesis and bioassays with the *lpsB* mutant unexpectedly suggested that ergovaline was not required for black beetle (*Heteronychus arator*) feeding deterrence from epichloë-infected grasses. Southern analysis showed the cluster was linked with previously identified ergot alkaloid biosynthetic genes, *dmaW* and *lpsA*, at a subtelomeric location. The ergovaline genes are closely associated with transposon relics, including retrotransposons, autonomous DNA transposons and miniature inverted-repeat transposable elements (MITEs), which are very rare in other fungi.

All genes in the cluster were highly expressed *in planta* but expression was very low or undetectable in mycelia from axenic culture, including under nitrogen-, carbon- or phosphate-limited conditions. Comparative analysis of the *EAS* gene cluster in four different epichloë strains showed marked differences in gene expression and ergot alkaloid synthesis. Gene order is conserved in each strain although evidence for recombination between two MITEs and expansion or reduction of a simple sequence repeat (SSR) at a single intergenic region was observed. Heterologous expression of a candidate regulatory gene, *laeA*, from *Aspergillus nidulans*, which is a global regulator of secondary metabolism in aspergilli, did not affect *eas* gene
expression. This, along with phylogeny and microsynteny analysis, suggests there is not an orthologue of this gene in epichloë.

This work provides a genetic foundation for elucidating biochemical steps in the ergovaline pathway, the ecological role of individual ergot alkaloid compounds, and the regulation of their synthesis in planta.
Acknowledgements

Like raising a child, so I am told, it takes a community to complete a PhD. Thank you so much to everyone that had input into this thesis, whether intellectual, experimental or emotional.

Thank you to my supervisors, Richard Johnson and Barry Scott. You made a great team and you were, in different ways, wonderful supervisors: rigorous, supportive and astute. Thank you both.

Thank you to everyone at AgResearch for a fun, stimulating environment. Special thanks to Tina Voisey for your support and many, many laughs. Thank you Greg Bryan for doing all the boss stuff I don’t know much about but know to be important. Thanks Mike Christensen for imparting a measure of your immense knowledge and passion for epichloë endophyte biology. Thanks Wayne Simpson for showing me what to do with dirt and other very interesting stuff. Thanks Geoff Lane and Brian Tapper for several enjoyable discussions about chemistry and biology. Thanks Anar Khan for superlative bioinformatics support from afar. Thank you to members of the lab: Shalome, Linda, Charlotte, Steven, Kurtis, Lee, Jasmin, Jen, Anu and Melle, for a supportive, occasionally hilarious, environment. Thank you Helal Ansari, Michelle Thornton and Kim Richardson, for failed, but fun, attempted experiments. Thank you Sue, Julie, Nadia, Maree, Kathy, Dale and everyone else that keeps the world going around without names on papers.

Thanks to the members of ScottBase, Massey University, past and present, for being so inclusive. Special thanks to my science heroes Brendon Monahan and Carolyn Young for many stimulating discussions. Thank you Ruth Wrenn for running around after me from 2 km; and always with a smile.

Thank you to my parents for your support and encouragement from across the sea.

Several people need acknowledgement for technical and/or intellectual input into the data presented in this thesis. Aiko Tanaka provided the ps12 sequence that started the ball rolling. Chris Schardl, University of Kentucky, allowed access to the
pre-release of the *E. festucae* E2368 genome sequence, which added immensely to this work. Geoff Lane, Karl Fraser and Albert Koulman performed or interpreted LC-MS/MS analyses. Andrew Griffiths provided some of the genomic DNA samples and Jennifer Pratt performed some of the diagnostic PCRs described in Table 6.1. Anar Khan prepared the phylogenetic tree in Figure 4.5. Mike Christensen, Wayne Simpson and Anouck De Bonth provided biological material and took special care of my plants. Alison Popay provided expertise and materials for design and interpretation of the insect feeding choice test, Lisa Evans scored the feeding damage and John Koolard performed statistical analysis.

Finally, Lisa Paton. Above all else your love and support has kept me going. Thank you.

This PhD was truly a community effort and I have had a wonderful time taking part in it. To everyone that helped along the way, named and unnamed, family, friends, workmates, thank you.

This research was supported by a grant (C10X0203) from the New Zealand Foundation for Research, Science and Technology (FRST). The *E. festucae* E2368 genome sequencing was supported by a grant (EF-0523661) to Chris Schardl, Mark Farman (University of Kentucky) and Bruce Roe (University of Oklahoma) from the US National Science Foundation (NSF), and a grant (2005-35319-16141) to Chris Schardl from the US Department of Agriculture National Research Initiative (NRI).
Table of Contents

Abstract ........................................................................................................................................... iii
Acknowledgements ......................................................................................................................... v
Table of Contents .............................................................................................................................. vii
List of Figures ..................................................................................................................................... x
List of Tables ..................................................................................................................................... xi
List of Abbreviations ......................................................................................................................... xii

1. Introduction ................................................................................................................................. 1

1.1 Introduction................................................................................................................................ 2

1.2 The Epichloë Endophyte/Grass Symbiosis ............................................................................... 3
  1.2.1 Endophyte Life Cycle ........................................................................................................... 3
  1.2.2 Endophyte Taxonomy .......................................................................................................... 6
  1.2.3 Epichloë Bioprotective Alkaloids......................................................................................... 7

1.3 Fungal Secondary Metabolism – Natural Products ................................................................ 11
  1.3.1 Classes of Fungal Natural Products ..................................................................................... 12
  1.3.2 Biosynthetic Gene Clusters .................................................................................................. 14
  1.3.3 Gene Cluster Evolution ....................................................................................................... 15
  1.3.4 Regulation of Secondary Metabolite Genes ....................................................................... 18

1.4 Non-Ribosomal Peptide Synthetases ....................................................................................... 22
  1.4.1 Domain Structure of NRPSs ................................................................................................. 23

1.5 Ergot Alkaloids .......................................................................................................................... 27
  1.5.1 Historical Overview ............................................................................................................ 27
  1.5.2 Ergot Alkaloids in Endophyte-Infected Pasture ................................................................... 28
  1.5.3 Ergot Alkaloid Biosynthesis ................................................................................................ 28
  1.5.4 Ergot Alkaloid Genetics ...................................................................................................... 30

1.6 Background and Aims of this Study .......................................................................................... 33

2. Materials and Methods ............................................................................................................... 35

2.1 Biological Material .................................................................................................................... 36

2.2 Fungal Growth and Media ......................................................................................................... 38

2.3 Bacterial Growth and Media ...................................................................................................... 38

2.4 Enzymes and Chemicals ............................................................................................................ 38

2.5 DNA Isolation ............................................................................................................................ 38
  2.5.1 Plasmid DNA extraction ....................................................................................................... 38
  2.5.2 Genomic DNA Isolation ..................................................................................................... 39
  2.5.3 Chromosomal DNA Isolation in Agarose Plugs ................................................................ 41
  2.5.4 λ DNA Isolation .................................................................................................................. 41

2.6 DNA Manipulation ..................................................................................................................... 42
  2.6.1 DNA Quantification ............................................................................................................ 42
  2.6.2 Restriction Digests .............................................................................................................. 42
  2.6.3 DNA Purification and Precipitation ....................................................................................... 42
  2.6.4 Agarose Gel Electrophoresis ............................................................................................... 43
  2.6.5 Southern Analysis ................................................................................................................ 43
  2.6.6 Subcloning .......................................................................................................................... 45

2.7 Library Screening ....................................................................................................................... 47
  2.7.1 λ Library Plating and Blotting ............................................................................................. 47
  2.7.2 DIG Hybridisation of Filters and identification of positive clones ..................................... 48
  2.7.3 Phagemid Excision .............................................................................................................. 49
3. Results - Isolation and Characterisation of a Gene Cluster for Ergovaline Biosynthesis

3.1 Isolation and Analysis of the NRPS Gene PS12 and Upstream Sequence
   3.1.1 The lpsB Gene
   3.1.2 LpsB, a Single-Module NRPS
   3.1.3 The easE Gene and Predicted Oxidoreductase EasE

3.2 Creating an *E. festucae* lpsB Mutant
   3.2.1 Preparation of an *lpsB* Deletion Construct
   3.2.2 Transformation and Screening of *E. festucae*
   3.2.3 Transformation and Screening of *E. festucae*

3.3 Phenotypic Characterisation of DFM3
   3.3.1 Growth in Culture and *In Planta*
   3.3.2 Alkaloid Analysis of the *lpsB* Mutant
   3.3.3 Complementation of DFM3

3.4 Insect Bioassays with the *lpsB* Mutant

3.5 Extension of the EAS Cluster
   3.5.1 *Analysis of easA, easF and easG*

3.6 Linkage of the EAS Gene Cluster with *dmaW* and *lpsA* and Identification of *cloA*

3.7 The EAS Cluster is Located Near a Telomere

3.8 Genome Sequence Analysis – Completion of the Cluster
   3.8.1 Bioinformatics Analysis of *E. festucae* easC, easD, and Full-Length easH and *cloA*

4. Results - Expression and Promoter Analysis
   4.1 EAS Cluster Expression Analysis
   4.1.1 *In Planta* Expression
   4.1.2 Expression Analysis of *lpsB* in Axenic Culture

4.2 EAS Cluster Promoter Analysis

4.3 *laeA*, a Candidate Regulatory Gene
   4.3.1 Cloning Strategies
   4.3.2 Heterologous Expression of *laeA* in *E. festucae*
## List of Figures

1.1. Epichloë endophyte growth *in planta* .............................................................................. 4
1.2. The epichloë endophyte lifecycle ...................................................................................... 5
1.3. Relationship of gene, protein modules and domains ......................................................... 23
1.4. Simplified epichloë ergot alkaloid biosynthetic pathway ................................................. 30
3.1. Physical map of pDF1 and pDF2 .................................................................................. 60
3.2. 3’ RACE analysis of *lpsB* ........................................................................................ 62
3.3. Domain structure of LpsB .............................................................................................. 63
3.4. COM domain Multiple sequence alignments ..................................................................... 65
3.5. Schematic describing preparation of pDF6 ...................................................................... 69
3.6. Targeted gene replacement of *lpsB* .............................................................................. 70
3.7. Analysis of *lpsB*-mutant-infected ryegrass symbiota ...................................................... 72
3.8. Extracted ion chromatograms from LC-MS/MS ............................................................. 73
3.9. Feeding preference of black beetle .................................................................................. 76
3.10. Physical map and %AT composition of the *N. lolii* EAS locus ........................................ 77
3.11. ADF1 is a chimeric clone .............................................................................................. 78
3.12. Comparative *eas* gene order ...................................................................................... 80
3.13. Cloning the 5’ end of *lpsA* ........................................................................................ 84
3.15. Southern blot analysis indicating linkage of *lpsB*, *lpsA* and *dmaW* ......................... 86
3.16. Southern blot analysis indicating linkage with the telomere ........................................ 87
3.17. Physical map and AT% of *E. festucae* E2368 Contig 1139 ........................................... 89
3.18. Physical map and AT% of *E. festucae* E2368 Contig 2048 ........................................... 90
4.1. RT-PCR analysis .............................................................................................................. 95
4.2. Real time RT-PCR analysis of selected genes from the *eas* cluster ................................. 95
4.3. Distribution of motifs 1 and 2 ....................................................................................... 99
4.4. Distribution of motifs 3, 4 and 5 .................................................................................... 100
4.5. Phylogenetic analysis of selected protein methyl-transferases ....................................... 101
4.6. Conserved microsynteny at the *A. nidulans laeA* locus ................................................. 102
4.7. Preparation of an *laeA* expression construct and screening of transformants ................. 103
4.8. Real time RT-PCR analysis of *laeA*-expressing strains *in planta* ................................. 105
5.1. Location of transposons within the EAS cluster .............................................................. 109
5.2. Sequence diversity at *dmaW* loci ................................................................................ 111
5.3. Toru structure ................................................................................................................ 111
5.4. Rima structure .............................................................................................................. 115
5.5. Iwa structure ................................................................................................................ 117
5.6. Schematic of DNA transposon structure ....................................................................... 119
5.7. TSDs are shared between some Wha end sequences ....................................................... 120
5.8. Schematic of retrotransposon structure ....................................................................... 123
5.9. Assembly of a full-length White .................................................................................... 125
5.10. Alignment of White and Tah LTRs .............................................................................. 126
6.1. LC-MS/MS analysis of *Neotyphodium* strains .............................................................. 135
6.2. Expression of *eas* genes in different epichloë strains .................................................... 137
6.3. Gene order in different epichloë EAS clusters ............................................................... 138
6.4. Schematic showing repeat-mediated polymorphism at the *easA*-*easG* locus ............... 140
6.5. EAS gene homologues in aspergillus gene clusters ....................................................... 143
List of Tables

1.1. Variation of amino acids in postions one and two of ergopeptines.................................29
2.1. Biological material...........................................................................................................36
2.2. PCR primers used in this study.......................................................................................50
3.1. Conserved motifs found in LpsB....................................................................................63
3.2. Substrate-specificity determining amino acids..............................................................64
3.3. LC-MS/MS analysis of mutant/grass symbiota...............................................................75
3.4. Similarity of N. lolii eas genes with C. purpurea and A. fumigatus orthologues............79
3.5. Bioinformatic analysis of genes within the N. lolii EAS cluster......................................83
3.6. N. lolii EAS genes in the E. festucae E2368 genome.......................................................88
5.1. Insertion sites of Toru elements in 12 contigs ...............................................................114
5.2. Insertion sites of Rima elements.....................................................................................116
5.3. Insertion site of Iwa elements.........................................................................................118
5.4. Insertion sites of Wha elements.....................................................................................121
5.5. RIP indices for transposons associated with the EAS cluster.........................................128
5.6. RIP sequence context preference summed for both strands.........................................129
5.7. Ascomycete RIP sequence context preference..............................................................129
6.1. Taxonomic Distribution of eas Genes.............................................................................133