HOMOGENISATION IN THE RIBOSOMAL RNA GENES OF AN *EPICHLOË* ENDOPHYTE HYBRID

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

Austen Rawlinson Deldyck Ganley

1999

For Denis and Phil Ganley

ABSTRACT

Homogenisation mechanisms in the ribosomal RNA genes were investigated using *Epichloë* fungal endophyte interspecific hybrid isolate, Lp1. The two progenitor isolates, *Neotyphodium lolii* Lp5 and *E. typhina* E8, were used for comparison. Three areas of homogenisation were examined.

The first area involved characterisation of extraordinary length heterogeneity in the rDNA of Lp1. This was shown by Southern analyses on single-spore isolates to be present intragenomically and localised to the intergenic spacer (IGS). Length heterogeneity is not a feature of either progenitor, suggesting it is a consequence of the hybridisation. The length heterogeneity was shown to result from copy number variation of sub-repeats in the IGS, which is consistent with unequal crossing over occurring in the rDNA, suggesting that unequal crossing over plays a role in homogenisation. Multi-variant repeat PCR mapping of the sub-repeat array revealed that the ends of the array behave differently, and biased initiation of recombination is discussed. Several results are not consistent with homogenisation by unequal crossing over and the potential roles of gene conversion and extrachromosomal rDNA circles in homogenisation are discussed. Finally, evidence is presented that suggests the rate of homogenisation is very rapid. A group I intron is present in the 28S *rrn* gene of Lp1, and is widespread in the *Epichloë* endophytes. Closely-related introns in other fungal 18S *rrn* genes provide evidence for intron transposition.

The second area involved testing the hypothesis that the presence of one type of rDNA sequence in Lp1 is the result of interlocus homogenisation. CHEF gel electrophoresis revealed that Lp1 and Lp5 have at least five rDNA arrays organised as major and minor loci, an unusual situation in fungi. The organisation in E8 could not be determined. One potential rDNA-DNA junction was cloned but has not been analysed.

The final area initially involved testing the hypothesis that interlocus homogenisation of 5S rRNA gene arrays occurs more slowly than that of rDNA arrays in hybrids. However the 5S rRNA genes in the *Epichloë* endophytes were shown to be organised as dispersed copies, not in tandem arrays. Shared polymorphisms between Lp1, Lp5 and E8 may indicate the homogenisation rate of these dispersed repeats is slower, and gene conversion as a homogenisation mechanism is discussed. The 5S rRNA genes are located on the same chromosomal bands as the rDNA in Lp1 and Lp5, and therefore are markers that demonstrate the rDNA-containing Lp5 chromosomes are present in Lp1. This and the CHEF results provide evidence for interlocus homogenisation of the rDNA having occurred in Lp1, and extends observation of this phenomenon to fungi.

ACKNOWLEDGEMENTS

During my years at Massey University (now a decade!) I have come to know many wonderful people who have contributed to this thesis in a myriad of ways. Somehow I am meant to, and want to, distil into a few words all your encouragement, ideas, distractions, patience, forbearance, beers, inspiration, etc., etc. - a nigh-on impossible task! So to everyone who I omit to mention personally - cheers, I'll buy you a beer sometime!

First I want to thank my parents, Janine and Garth, for their unconditional love and support throughout. It was them who instilled in me the belief that I could achieve the goals that I set, whatever they were, and it is that which sees me where I am today. To them and the rest of my family - Beccy, Jules and Karen, Denise and Steve, and the many others, thanks for everything (I couldn't have done it without you guys) - especially for understanding when I have been focussed on work to the exclusion of all else.

Carolyn and David - what can I say? If helping out at all times of the day and night wasn't enough, you then let me live with you, drink your beer, and have parties when you're not there! And that's not even counting work or putting the thesis together... Carolyn, I fail to understand how people in other labs can operate properly without you there - does that express how much I owe you for all your help in the lab? You are a rock. Really. And David - the world would be a poorer place without beer and mushrooms, and who better to enjoy both with.

Rich, Mike and Hale, it was you guys who influenced my impressions on what science and scientists were about and (oddly enough) helped me decide to do this "thing". Our many adventures in places far-and-wide have kept me going throughout adversity, and provided a kind of "reality grounding" for which I am very grateful to have. I hope there will be more!

Over the years I have lived with some wonderful people, who, I suspect, have shaped my life in more ways than I realise. Tania, my longest and dearest flatmate! Living with you was constant fun, and I miss not having you around. Thanks for everything! Justin - you narrowly scrape into this section by virtue of around nine months flatting, but the same couldn't be said as far as friendship is concerned. A Justin in the hand is worth two in the bush, I always say, and have yet to be proved wrong! With Jaki and Lucy, and Steve and Dave I had the unlikely pleasure of having two perfect flats - you guys are great! I also want to thank Bruce and Toshi for letting me take over their place - it was a perfect place for writing. And cheers for the various harvests too!

Anna, I enjoyed living with you when we were together. I guess things change and are not always as we may think or like. I hope we have both learned from our experiences.

I would like to thank many dear friends for helping me see life outside of scientific world. To Damian, Paul and Carolyn, Maria, Cybele, Francesca, Carmel and Tony, Kirsty, Jenny, Jules and Morgan - viva la revolucion!! To Linda, Kate, Peng and Ina, Marty, Mark and Vanessa, Sue, God, Blair, Mike, Marcus, Ian, Roo and Blake I owe thanks for many-and-varied "extra curricular" activities and liberties. On the political front people like the Summer of Resistance rabble, the Waihopai "hippy-type individuals", Edwina, Patrick, Eleven, Ian, Stewart, Chris, Maryanne and Dion, Kevin, Gerard, and Don are a constant source of inspiration. Thanks for your dedication and doing all the stuff that I couldn't do - kia kaha!

I would also like to thank many dear friends in the scientific world. It is people such as Marion, Miranda, Mick and Linda, Paul, Rebecca, Rochelle, Rose, Martin, Ant, Pete, Steve and Basil who make this a great place to work! I would especially like to thank Brendon for all the advice, beers and just plain chatting we shared.

The many people who have passed through the Scott Base lab and surrounds during my time here have all made a mark in their own ways. I would like to thank in particular Christina for our many discussions on endophytes, Lisa for saving me from crises, Emily for beers and rugby, Sirinda, Jo, Bek and Seth. Thanks also go to David Penny for helpful advice and ideas on the evolutionary side of things, Mike Christensen for helpful advice and ideas on the endophyte side of things, and Max Scott as my co-supervisor for trying to tether my flights-of-fancy back to scientific reality!

I would like to thank Massey University for a Doctoral Scholarship that partially supported me financially through this PhD.

And for the dessert course! My most sincere thanks and gratitude go to my supervisor, Barry Scott. I know that the course of study I chose to pursue was not one in your immediate area of interest, nor was one that you would have preferred me to do. But since I made the decision you have provided unstinting input and support of the highest quality, and for your willingness to become familiar with an obscure and at times confusing field to an extent that few would match I am extremely grateful. I have thoroughly enjoyed my time working with you, and I think we got the mix of independence and tutelage about right. I hope this thesis reflects that!

TABLE OF CONTENTS

ABSTRACT iii

ACKNOWLEDGEMENTS iv

TABLE OF CONTENTS vi

LIST OF TABLES xiii

LIST OF FIGURES xiv

CHAPTER 1 INTRODUCTION 1

1.1 BIOLOGY OF THE *EPICHLOË* ENDOPHYTES 2
1.1.1 Interspecific hybridisation of the *Epichloë* endophytes 2
1.1.2 The origin of *Epichloë* endophytes isolate Lp1 3

1.2 RIBOSOMAL RNA GENES 4
1.2.1 The 18S - 5.8S - 28S ribosomal RNA genes 4
1.2.2 The ribosomal DNA in Lp1 5
1.2.3 The 5S ribosomal RNA 7

1.3 CONCERTED EVOLUTION 7
1.3.1 Patterns of concerted evolution 7
1.3.2 Mechanisms of homogenisation 8

1.4 AIMS AND OBJECTIVES OF THIS STUDY 12

CHAPTER 2 MATERIALS AND METHODS 13

2.1 STRAINS AND GROWTH CONDITIONS 14
2.1.1 Fungal and bacterial strains, λ clones and plasmids 14
2.1.2 Fungal growth conditions 14
2.1.3 Single spore fungal purification 14
2.1.4 Bacterial growth conditions 14

2.2 MEDIA AND COMMON SOLUTIONS 14
2.2.1 Potato dextrose media (PD) 18
2.2.2 Luria broth media (LB) 18
2.2.3 SOC media 18
2.2.4 Common buffers and solutions 18

2.3 DNA ISOLATION 21
2.3.1 Isolation of fungal genomic DNA 21
2.3.2 Extraction of plasmid DNA from bacterial cultures by the rapid boil method 21
2.3.3 Extraction of plasmid DNA from bacterial cultures by the alkaline lysis method 22
2.3.4 Extraction of plasmid DNA from bacterial cultures by the caesium chloride method 22

2.4 DNA QUANTIFICATION 23
2.4.1 DNA Quantification by ethidium bromide staining 23
2.4.2 Fluorometric quantification 23
2.4.3 Spectrophotometric quantification 23

2.5 RESTRICTION ENDONUCLEASE DIGESTION OF DNA 24

2.6 PHENOL/CHLOROFORM EXTRACTION 24

2.7 DNA PRECIPITATION 24

2.8 AGAROSE GEL ELECTROPHORESIS 25
2.8.1 Agarose gels 25
2.8.2 Mini gel electrophoresis 25
2.8.3 Overnight gels 25
2.8.4 Detection of DNA 25
2.8.5 Determination of DNA band sizes 25

2.9 SEAPLAQUE DNA EXTRACTIONS 26

2.10 SOUTHERN BLOTTING AND HYBRIDISATION 26
2.10.1 Southern (capillary) blotting 26
2.10.2 Making radiolabelled DNA probes 27
2.10.3 Hybridisation of radiolabelled probes 27
2.10.4 Detection of hybridisation signal 27
2.10.5 Stripping nylon membranes 27

2.11 CAP TREATMENT OF VECTORS 27

2.12 LIGATIONS 28
2.12.1 Ligation into pUC118 28
2.12.2 Ligation into pGEM®-T 28

2.13 TRANSFORMATION OF *E. COLI* 28
2.13.1 Preparation of electrocompetent cells 28
2.13.2 Electroporation and detection of transformants 29

2.14 SUBCLONING DNA FRAGMENTS 29

2.15 GENERATION OF NESTED DELETIONS BY EXONUCLEASE III DIGESTION 29

2.16 DNA SEQUENCING 30
2.16.1 Sequenase™ sequencing 30
2.16.2 Amplicycle™ sequencing 31
2.16.3 Electrophoresis of sequencing reactions 31
2.16.4 Automatic sequencing 32
2.16.5 Sequence analysis 32

2.17 POLYMERASE CHAIN REACTION (PCR) 32
2.17.1 Oligonucleotide primers 32
2.17.2 PCR conditions 32
2.17.3 Stabbed band PCR reamplification 35
2.17.4 Purification of PCR products 35

2.18 MULTI VARIANT REPEAT PCR ANALYSIS 35

2.19 CONTOUR-CLAMPED HOMOGENEOUS ELECTRIC FIELD (CHEF) GEL ELECTROPHORESIS 36
2.19.1 Preparation of fungal protoplasts 36
2.19.2 Preparation of DNA in agarose-embedded plugs 36
2.19.3 Restriction endonuclease digestion of agarose-embedded plugs 37
2.19.4 CHEF gel electrophoresis conditions 37

2.20 PCR-BASED CLONING OF RDNA JUNCTION FRAGMENTS 37
2.20.1 Preparation of genomic (or vector) DNA 37
2.20.2 Preparation of linker DNA 38
2.20.3 Preparation of Dynabeads® 38
2.20.4 Enrichment of junction fragments 38
2.20.5 PCR amplification of junction fragments 39
2.20.6 Cloning of potential junction fragments 39

CHAPTER 3 RESULTS 40

3.1 CHARACTERISATION OF EXTRAORDINARY RIBOSOMAL IGS LENGTH HETEROGENEITY IN THE HYBRID, LP1 41
3.1.1 Generation of single spore isolates 41
3.1.2 Length heterogeneity occurs within the rDNA cluster and is still being generated 41
3.1.3 Length variation of the IGS is not observed in either progenitor of Lp1 46
3.1.4 Length heterogeneity localised to the intergenic spacer 50
3.1.5 Heterogeneity is not a general feature of the Lp1 genome 52
3.1.6 Intergenic spacer contains sub-repeat elements 52
3.1.7 Digestion of the 111/119 bp sub-repeats abolishes heterogeneity 60
3.1.8 Arrangement of the 111/119 bp sub-repeats in the IGS 62
3.1.9 IGS length heterogeneity in other *Epichloë* endophyte hybrids 65
3.1.10 Spread of a deletion in the IGS 65

3.2 GROUP I INTRON IN THE LP1 28S *rrn* GENE 68
3.2.1 Discovery of an insertion element in the Lp1 28S *rrn* gene 68
3.2.2 The insertion element is a group I intron 71
3.2.3 Structure of the Lp1 group I intron 71

3.3 INTERLOCUS HOMOGENISATION OF THE rDNA IN THE HYBRID, LP1 75
3.3.1 Lp1 rDNA is exclusively derived from E8 75
3.3.2 Chromosomal location of the rDNA 77
3.3.3 Separation of the individual rDNA arrays 81
3.3.4 Strategy for cloning rDNA-DNA junction fragments 84
3.3.5 Results of the rDNA junction cloning 86

3.4 CHARACTERISATION OF THE 5S RIBOSOMAL RNA GENES 89
3.4.1 Cloning the 5S rRNA genes 89
3.4.2 5S rRNA genes in the *Epichloë* endophytes are dispersed 91
3.4.3 The rDNA and 5S rRNA genes are located on the same chromosomal bands 94

CHAPTER 4 DISCUSSION 97

4.1 LENGTH HETEROGENEITY IS A RESULT OF UNEQUAL CROSSING OVER IN THE rDNA 98
4.1.1 Unequal crossing over as a mechanism for homogenisation of the rDNA 98
4.1.2 Unequal crossing over in the Lp1 rDNA is mitotic 99

4.2 THE RATE AND NATURE OF TURNOVER IN THE IGS: INCONGRUENCES BETWEEN THE RESULTS AND THE UNEQUAL CROSSING OVER MODEL OF HOMOGENISATION 99
4.2.1 Rapid turnover of repeats in the rDNA 100
4.2.2 Clustering of IGS length variants 100
4.2.3 Restriction of IGS lengths 101
4.2.4 Non-random arrangement of the IGS sub-repeat variants 102

4.3 IMPLICATIONS FOR THE HOMOGENISATION MECHANISM 102
4.3.1 Selection against small spacers 103
4.3.2 Biased initiation of crossing over in the sub-repeats 103
4.3.3 Rapid homogenisation 106

4.4 GENE CONVERSION AS A HOMOGENISATION MECHANISM 109
4.4.1 A rapid homogenisation-like process induced by double strand breaks 109
4.4.2 Potential role of double strand breaks in rDNA homogenisation 111

4.5 EXTRACHROMOSOMAL rDNA CIRCLE AMPLIFICATION AS A MECHANISM FOR HOMOGENISATION 111
4.5.1 Previous models of homogenisation 112
4.5.2 Extrachromosomal rDNA amplification model of homogenisation 114
4.5.3 The nucleolus as a regulatory centre for rDNA homogenisation 116

4.6 THE IGS LENGTH HETEROGENEITY ARISES THROUGH HYBRIDISATION 117

4.7 A GROUP I INTRON IN THE 28S *rrn* GENE 119
4.7.1 Sequence comparison of the Lp1 group I intron 119
4.7.2 Mobility of group I introns 120
4.7.3 Spread of the group I intron by homogenisation 122

4.8 *EPICHLOË* ENDOPHYTES CONTAIN MULTIPLE rDNA ARRAYS 122
4.8.1 There are both major and minor rDNA loci in the *Epichloë* endophytes 123

4.9 CLONING AN rDNA JUNCTION 123

4.10 CHARACTERISATION OF THE 5S rRNA GENES 124
4.10.1 Structure of the 5S rRNA gene 125
4.10.2 The 5S rRNA genes and the rDNA loci are found on the same chromosomal bands 125
4.10.3 Concerted evolution of the 5S rRNA genes 126

4.11 INTERLOCUS HOMOGENISATION IN THE HYBRID, LP1 128

CHAPTER 5 CONCLUSIONS AND FUTURE DIRECTIONS 131

5.1 CONCLUSIONS 132

5.2 FUTURE DIRECTIONS 135
5.2.1 Determination of homogenisation mechanisms 135
5.2.2 Towards an assay for homogenisation 135
5.2.3 Other directions 136

APPENDIX 137

1 PUBLICATION 138

2 SEQUENCE OF THE POTENTIAL LP5 rDNA JUNCTION FRAGMENT 139

BIBLIOGRAPHY 140

LIST OF TABLES

Table 2.1 Fungal and Bacterial Strains, λ Clones and Plasmids 15

Table 2.2 Oligonucleotide Primers 33

Table 2.3 Thermocycling Conditions Used for PCR Amplifications 34

LIST OF FIGURES

Figure 1.1 Restriction map of λPN1 6

Figure 1.2 Unequal crossing over as a mechanism for homogenisation of a tandem array 10

Figure 3.1 *Sal*I genomic digests of Lp1 laboratory cultures and single-spore purified isolates 42

Figure 3.2 Southern analysis of the *Sal*I Lp1 genomic digests from Figure 3.1 with the 4.1 kb *Sal*I IGS probe 44

Figure 3.3 Southern analysis of the *Sal*I Lp1 genomic digests from Figure 3.1 with the 5.6 kb *Sal*I coding region probe 47

Figure 3.4 The progenitors of Lp1 do not display ribosomal length heterogeneity 49

Figure 3.5 Length heterogeneity is specific to the Lp1 ribosomal IGS region 51

Figure 3.6 Length heterogeneity is not a feature of the Lp1 genome 53

Figure 3.7 Generation of nested deletions of the Lp1 IGS clone by exonuclease III digestion 54

Figure 3.8 Sequence of the 4.1 kb *Sal*I IGS clone from pPN50 and surrounding sequence 55

Figure 3.9 Length heterogeneity is the result of copy number variation of the 111/119 bp sub-repeats 61

Figure 3.10 Multi-variant repeat PCR mapping of the 111/119 bp sub-repeats in the Lp1 rDNA IGS 63

Figure 3.11 IGS length heterogeneity is not a general feature of *Epichloë* endophyte hybrids 66

Figure 3.12 Characterisation of the deletion in the Lp1 IGS 67

Figure 3.13 Progression of the deletion through rounds of single-sporing 69

Figure 3.14 Insertion element in the Lp1 28S *rrn* gene 70

Figure 3.15 Group I intron alignment 72

Figure 3.16 Putative internal guide sequence of the Lp1 group I intron 74

Figure 3.17 Comparison of the nts1 - nts2 and nts3 - nts4 PCR products between Lp1, E8 and Lp5 76

Figure 3.18 The *Epichloë* endophytes contain multiple rDNA clusters 78

Figure 3.19 Hybridisation of the 5.6 kb *Sal*I coding region probe is specific for the rDNA in CHEF gels 80

Figure 3.20 CHEF gel separation of intact Lp1 rDNA clusters by *Hin*dIII and *Bam*HI digestion 82

Figure 3.21 CHEF gel separation of intact rDNA clusters in Lp1, Lp5 and E8 83

Figure 3.22 PCR method for cloning rDNA junction fragments 85

Figure 3.23 rDNA junction fragment PCR cloning using *Hin*dIII 87

Figure 3.24 Initial PCR cloning of the 5S rRNA genes 90

Figure 3.25 Hybridisation of the Lp5 5S1 - 5S2 PCR product to Lp1 and progenitors 92

Figure 3.26 Amplification and characterisation of *Epichloë* endophyte 5S rRNA genes 93

Figure 3.27 The 5S rRNA genes are dispersed in the *Epichloë* endophytes 95

Figure 3.28 The 5S rRNA and *rrn* genes are located on the same chromosomal bands 96

Figure 4.1 Model of biased initiation of crossing over based on the Chi system in prokaryotes 105

Figure 4.2 Meiotic recombination disrupts homogenisation 108

Figure 4.3 Extrachromosomal ribosomal DNA circle mechanism for generation of clusters of length variants 115