http://researchspace.auckland.ac.nz

ResearchSpace@Auckland

Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage.
http://researchspace.auckland.ac.nz/feedback

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form and Deposit Licence.

Structure-Function Relationships in Plant Terpene Synthases

Sol Alexander Green

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy in Biological Sciences,
The University of Auckland, 2009.

ABSTRACT

Terpene synthases (TPS) are a family of enzymes responsible for synthesising the vast array of terpenoid compounds known to exist in nature. In plants these compounds serve to increase ecological fitness, while commercially they represent one of the key compound classes in determining the quality of food products, including flavour and aroma in fruit crops.

Preliminary functional characterisation of an apple (*Malus x domestic*a) TPS responsible for the production of α-farnesene (MdAFS1), which constitutes the major terpene volatile in apple, showed novel features not previously reported for a TPS enzyme. These included the apparent ability of MdAFS1 to generate α-farnesene by a prenyltransferase reaction and being the first angiosperm TPS with a dependence on potassium for activity. The focus of this PhD study was to extend the preliminary functional analysis of MdAFS1 and provide definitive proof for this prenyltransferase activity and also the location and nature of the MdAFS1 potassium binding region. In the research presented here, mutagenesis of the MdAFS1 aspartate-rich divalent metal ion binding motif removed TPS and prenyltransferase activities, showing not only that MdAFS1 possessed intrinsic prenyltransferase function but that both TPS and prenyltransferase catalysis were occurring through the same active site. Protein modelling also revealed a surface-exposed loop (H-αl loop) in MdAFS1 that fulfilled the necessary requirements for a potassium binding region. Site-directed mutagenesis analysis of specific residues within this loop then revealed their crucial importance to this potassium response and strongly implicated specific residues in direct potassium binding. The role of the H-αl loop in terpene synthase potassium coordination was confirmed in a conifer pinene synthase also using site-directed mutagenesis. These findings provide the first direct evidence for a specific potassium binding region in two functionally and phylogenetically divergent terpene synthases. They also provide a basis for understanding potassium activation in other TPS enzymes and establish a new role for the H-αl loop region in TPS catalysis.

Structural insights were also sought for how MdAFS1 might have evolved from an ancestral mono-TPS enzyme. A combination of contact mapping and mutagenesis analysis identified a single non-synonymous nucleotide substitution that could explain one route for the requisite shift in substrate specificity necessary for α-farnesene synthesis.

Taken together, the work presented in this thesis provides the foundation for future investigation into the catalytic mechanisms and evolution of plant terpene synthases. Broadening the scope of this and related studies so as to begin unravelling the complex interplay between plant ecological fitness and TPS catalytic specificity still remains an enormous future challenge.

.

ACKNOWLEDGEMENTS

Firstly I would like to sincerely thank my supervisors Professor Ted Baker and Dr William Laing for their support, guidance, enthusiasm and constructive criticism during the course of this study. I count myself privileged to have had the opportunity to learn the necessary scientific disciplines for the completion of a PhD study from two scientists whom I admire and respect immensely.

I am also indebted to my associate supervisor and former manger at Plant & Food Research, Dr Elspeth McRae. Elspeth's support was not only crucial in initiating this study but also in instilling in me the belief that I could successfully complete it. I would like to thank my associate supervisor Dr Jo Putterill for agreeing to act as the bridge between plant science at Plant & Food Research and Auckland University and the encouragement she has given me throughout the course of this study.

Numerous people at Plant & Food Research and Auckland University are owed thanks for the support, advice and technical assistance they have given me. In particular, thanks to Dr Ellen Friel for teaching me the basics of GC-MS analysis and Mindy Wang for the continuing support she has given me in using the GC-MS facility. To Dr Adam Matich for his considerable help with running numerous GC-MS analyses and synthesis of precursors and also, being party to numerous discussions regarding the baffling nature of terpene chemistry. To Dr Daryl Rowan who, by choice or otherwise, was more often than not included in these same discussions and for his help in getting the first MdAFS1 paper to publication. To the members of the Flavour Biotechnologies team at Plant & Food Research for their support and patience. In particular I would like to thank Niels Nieuwenhuizen for his ideas, technical expertise, and help in finishing the potassium work for publication. To Dr Cyril Hamiaux for his help with the technical and theoretical aspects of protein crystallisation and crystallography and to Dr Ian Ferguson for his continued support and help in reviewing draft chapters. To Janine Cooney and Dr Dave Greenwood their help with the various LC/MS analyses. To Martin Heffer, Tim Holmes and Minna Pesonen for providing help with high quality images and photographs and to Michele Napier for all the Endnote help.

I would also like to thank the members of the structural biology team at Auckland, both past and present, for their support. In particular, to Dr Chris Squire for his time, patience and help in

protein modelling, ligand docking and generally putting up with someone who had little background in structural biology or computational methods for protein structure prediction. To Heather Baker for her support, advice and technical assistance in the protein crystallisation work. To Dr Tom Caradoc-Davies for his advice on the finer points of protein purification and crystallisation and to Ivan Ivanovic for his help in the crystallisation screening. I would also like to thank Dr Jörg Bohlmann and Karen Reid at the Michael Smith Laboratories, University of British Columbia, Vancouver, Canada, for making available the various conifer terpene synthases expression constructs.

I am grateful to Plant & Food Research for giving me the opportunity to pursue my scientific goals. This work was supported by the New Zealand Foundation for Research, Science, and Technology (C06X0403) and Plant & Food Internal Investment funding. I am also grateful to Plant & Food Research and the University of Auckland for providing the financial assistance that has allowed me the opportunity to present my work at The International Terpnet Conference in Tokyo, Japan (2009).

Finally I would like to thank my wife Emma and my children, Lana, Jacob and Finn who have survived the ups and downs of a preoccupied and often absent husband and father. The completion of this challenging and highly rewarding study would not have been possible without their unconditional love, support and patience.

TABLE OF CONTENTS

ABSTRACT .... **I**

ACKNOWLEDGEMENTS .... **III**

ABBREVIATIONS .... **XVI**

1 GENERAL INTRODUCTION **1**

1.1 Introduction to terpenes 1
1.1.1 Role of terpenes in essential metabolite production *1*
1.1.2 Secondary metabolic functions of terpenes *2*
1.1.2.1 Floral scents 2
1.1.2.2 Defence compounds 2
1.1.3 Commercial uses of terpenes *3*
1.2 Overview of terpene biosynthesis 3
1.2.1 IDP biosynthesis *4*
1.2.2 Mevalonate pathway *4*
1.2.3 Mevalonate independent pathway *5*
1.2.4 Terpenoid biosynthesis in plants *6*
1.3 Terpene synthases 8
1.3.1 Classification of terpene synthase genes *8*
1.3.1.1 Subgroups. 8
1.3.1.2 Terpene synthase classes 9
1.4 Terpene synthase structure 9
1.4.1 Primary structural features *9*
1.4.2 Crystal structures *10*
1.4.2.1 Terpene synthase loop regions 11
1.5 Terpene synthase chemistry 12
1.5.1 Monoterpene cyclisation *13*
1.5.2 Formation of sesquiterpenes *15*
1.5.3 Formation of diterpenes *17*
1.6 Regulation of terpene synthesis 17
1.6.1 Transcriptional regulation *18*
1.6.2 Post transcriptional / translational regulation *19*
1.7 Terpene synthase evolution 19
1.8 Local context for the research 22
1.9 Project aims 22

2 MATERIALS AND METHODS **24**

2.1 *In silico* analysis 24
2.1.1 Amino acid sequence alignments *24*
2.1.2 BLAST analysis *24*
2.1.3 Chloroplast targeting peptide (CTP) prediction *24*
2.1.4 Open reading frame (ORF) prediction *25*
2.1.4.1 Phylogenetic analysis 25
2.1.5 Protein homology modelling *25*
2.1.5.1 MdAFS1 modelling 25
2.1.5.2 FDP substrate docking in MdAFS1 25
2.1.5.3 Lotus corniculatus ocimene synthase (LjEβOS) modelling 26
2.1.5.4 Modelling of other terpene synthases 26
2.1.6 Viewing and manipulating 3D protein models and structures *26*
2.2 Enzymes and Chemicals 27
2.3 DNA methods 27

2.3.1 Media and buffers ... *27*
2.3.2 Antibiotic final concentrations ... *27*
2.3.3 Bacterial strains ... *28*
2.3.4 Bacterial genotypes ... *28*
2.3.5 Storage of bacteria ... *28*
2.3.6 Isolation of plasmid DNA ... *28*
2.3.7 Agarose gel electrophoresis ... *29*
2.3.8 DNA quantification ... *29*
2.3.9 DNA ligation ... *29*
2.3.10 General cloning ... *29*
2.3.11 TOPO Cloning ... *30*
2.3.12 AcTPS1 cloning ... *30*
2.3.12.1 pET-30 expression vector construction ... 30
2.3.12.2 TEV protease linker construction ... 31
2.3.13 Primer design ... *31*
2.3.14 PCR amplification of DNA sequences ... *32*
2.3.15 QuikChange™ mutagenesis ... *32*
2.3.16 PCR Product Purification ... *34*
2.3.17 Transformation of cells ... *34*
2.3.18 Restriction analysis ... *35*
2.3.19 Dephosphorylation of DNA ... *35*
2.3.20 DNA sequencing ... *36*
2.4 Protein methods ... 36
2.4.1 Production of recombinant proteins in E. coli: Autoinduction method ... *36*
2.4.2 Extraction of soluble proteins from E. coli cells ... *37*
2.4.3 Purification of recombinant terpene synthase proteins ... *37*
2.4.3.1 Purification of His_6-tagged recombinant proteins ... 38
2.4.3.2 Anion exchange chromatography ... 38
2.4.3.3 Ammonium sulphate precipitation ... 39
2.4.3.4 Hydrophobic interaction chromatography ... 39
2.4.3.5 Size exclusion chromatography ... 40
2.4.4 Protease treatment ... *41*
2.4.5 Storage of proteins ... *41*
2.4.6 Denaturing gel electrophoresis ... *41*
2.4.6.1 Sample preparation ... 42
2.4.6.2 SDS-PAGE materials ... 42
2.4.6.3 Electrophoresis and staining ... 42
2.4.7 Western Blotting ... *43*
2.4.8 Automated electrophoresis ... *43*
2.4.9 Protein quantification ... *44*
2.4.10 Dynamic light scattering (DLS) analysis ... *44*
2.5 Protein crystallisation ... 44
2.5.1 Crystallisation screening ... *44*
2.5.2 Crystal scoring ... *45*
2.5.3 Optimisation of TPS crystals obtained from initial screens ... *45*
2.5.3.1 Fine screening ... 45
2.5.3.2 Use of inhibitors ... 46
2.6 Volatile collection ... 46
2.6.1 Solid phase microextraction (SPME) ... *46*
2.6.2 Solvent extractions ... *47*
2.7 GCMS ... 47
2.7.1 SPME analysis (general) ... *47*
2.7.2 GCMS analysis for AcTPS1 ... *48*
2.7.2.1 Non-chiral analysis of the AcTPS1 products ... 48
2.7.2.2 Enantioselective GCMS ... 48

2.7.3 LC MS/MS analysis of DDXXD mutants 48
2.8 Radiometric enzyme assays 49
2.8.1 FDP activity assays 49
2.8.2 GDP activity assays 50

3 BIOINFORMATICS ANALYSES OF TERPENE SYNTHASE GENES 51

3.1 Introduction 51
3.1.1 Plant & Food Research EST and compound databases 52
3.1.2 Summary of terpene synthase EST analysis carried out prior to this PhD study 53
3.1.3 Summary of terpene synthases analysed prior to this PhD study 55
3.1.3.1 EST 57400 (MdAFS1) 55
3.1.3.2 EST 75565 55
3.1.3.3 EST 21063 55
3.1.3.4 EST 36941 56
3.2 Terpene synthase bioinformatics 56
3.2.1 Shorthand naming convention for terpene synthase genes and enzymes 56
3.2.2 Sequence analysis of putative terpene synthase cDNAs 57
3.2.3 Subgroup assignments of Plant & Food Research terpene synthases 60
3.2.4 Sequence analysis and phylogenetics summary 62
3.2.4.1 New TPS-a and TPS- b members 62
3.2.4.2 New TPS-g members 62
3.2.4.3 A novel TPS-f member 63
3.3 Final conclusions 64
3.4 Summary 66

4 PRENYLTRANSFERASE ACTIVITY IN AN APPLE α-FARNESENE SYNTHASE 67

4.1 Introduction 67
4.1.1 Summary of MdAFS1 functional analysis carried out prior to this PhD study 68
4.2 Biosynthesis of (*E,E*)-α-farnesene from GDP 69
4.2.1 A potential prenyltransferase activity 69
4.2.1.1 MdAFS1 activity analysis using deuterated precursors 71
4.2.1.2 MdAFS1 passively carries FDP or α-farnesene 73
4.2.2 Labelled precursor analysis conclusions 74
4.3 Peptide analysis of MdAFS1 75
4.3.1 Testing for prenyltransferase activity in a germacrene-D synthase 76
4.4 Mutagenesis analysis of MdAFS1 78
4.4.1 Mutagenesis strategy 78
4.4.2 Production of DDXXD mutant enzymes 79
4.4.3 Terpene synthase activity associated with DDXXD mutations 80
4.4.4 Prenytransferase activity in response to DDXXD mutations 82
4.4.5 Investigation of the importance of the second DDXXD aspartate in MdAFS1 85
4.5 Discussion 86
4.5.1 Bacterial contamination and retention of substrate or product 86
4.5.2 DDXXD mutagenesis 87
4.5.3 A common ancestral origin of TPS-b enzymes and prenyltransferases 87
4.5.3.1 Structure-function similarities in Class I isoprenoid biosynthetic enzymes 88
4.5.3.2 Substrate specificity in MdAFS1 and other Class I isoprenoid enzymes 89
4.5.3.3 Covalent attachment of FDP in MdAFS1 90
4.6 Summary 91

5 DEFINING THE POTASSIUM BINDING DOMAIN IN AN APPLE α-FARNESENE SYNTHASE 92

5.1 Introduction 92
5.1.1 Terpene synthase monovalent cation dependency 93
5.2 MdAFS1 protein homology modelling 94

5.2.1 The H-α1 loop is identified as a probable K^+ binding region *95*
5.3 MdAFS1 H-α1 loop Mutagenesis 97
5.3.1 Generation of mutant recombinant enzymes *97*
5.3.2 D484 and S487 residues are crucial for MdAFS1 activity *98*
5.3.3 Kinetic analysis of the MdAFS1 potassium response *100*
5.3.4 H-α1 loop residue conservation in TPS-b enzymes *101*
5.3.5 Introduction of a conserved TPS-b lysine into MdAFS1. ..... *102*
5.3.6 Predicted coordination of potassium in MdAFS1 ..... *105*
5.3.7 Mutagenesis of H-α1 loop residues in other terpene synthases *107*
5.3.7.1 H-α1 loop mutagenesis in the potassium-independent TPS-b enzymes 107
5.3.7.2 The role of the H-α1 loop in the potassium-dependent TPS-d enzymes 107
5.3.8 Construction of the TPS-b and TPS-d mutants *108*
5.3.9 Activity analysis for TPS-b and TPS-d monoterpene synthase mutants *110*
5.4 Discussion 113
5.4.1 AcTPS2 and PsTPS2 H-α1 loop residue analysis *115*
5.5 Summary 118

6 EVOLUTION OF FDP SPECIFICITY IN AN APPLE α-FARNESENE SYNTHASE 119

6.1 Introduction 119
6.2 Contact mapping 120
6.2.1 Overview of contact mapping *120*
6.2.2 MdAFS1 contact mapping *121*
6.2.3 The active site Trp in BPPS is highly conserved in TPS-b synthases *123*
6.2.4 C298W mutagenesis in MdAFS1 supports the proposed emergence of FDP catalysis in TPS-b synthases *125*
6.2.5 Loss of monoterpene synthase activity in the MdAFS1 ancestor *127*
6.2.5.1 Final considerations for the MdAFS1 protein engineering 127
6.2.6 MdAFS1 active site aromatic and basic residue comparisons to LjEβOS *130*
6.2.7 LjEβOS homology modelling *131*
6.2.7.1 Aromatic residue differences between MdAFS1 and LjEβOS 131
6.2.7.2 Differences in basic residues between MdAFS1 and LjEβOS 132
6.2.7.3 Conservation of active site aromatic and basic residues in TPS-b synthases 133
6.2.8 Final selection of mutable residue targets *135*
6.2.9 Construction and analysis of the MdAFS1 aromatic mutants *135*
6.3 Evolution of FDP specificity in the TPS-d subgroup 137
6.3.1 Norway spruce terpene synthase contact mapping *138*
6.3.2 Contribution of mapped P. abies residues to active site topology *139*
6.3.3 PaTPS-Far mutagenesis strategy *143*
6.4 Discussion 146
6.4.1 Evolution of FDP catalysis in the TPS-b synthases *147*
6.4.2 Implications for a conserved tryptophan on TPS-b mono synthase activity *148*
6.4.3 Modulating monoterpene synthase activity in MdAFS1 by protein engineering *149*
6.4.4 Evolution of FDP specificity in the TPS-d subgroup *150*
6.5 Summary 152

7 PURIFICATION AND CRYSTALLISATION OF AN APPLE α-FARNESENE SYNTHASE 153

7.1 Introduction 153
7.1.1 Terpene synthase crystallography *153*
7.2 Production of MdAFS1 for crystallisation 154
7.2.1 Purification of His_6-tagged MdAFS1 protein for crystallisation *155*
7.2.2 His_6-tagged MdAFS1 anion exchange chromatography *156*
7.2.3 Final purification of the His_6-tagged MdAFS1 by SEC *157*
7.2.4 His_6-tagged MdAFS1 crystallisation screening *158*

7.2.5 Removal of the His_6-tag region ... *160*
7.2.6 Strategy for obtaining a crystallisation-optimal MdAFS1 protein ... *160*
7.2.6.1 Surface entropy in proteins ... 161
7.2.6.2 A surface entropy reduction approach for MdAFS1 crystallisation ... 161
7.2.7 Purification optimisation for recombinant ΔR33 protein ... *163*
7.2.7.1 Hydrophobic interaction chromatography (HIC) ... 164
7.2.7.2 Size exclusion chromatography (SEC) ... 165
7.2.8 Purity and monodispersity of the MdAFS1ΔR33 preparations ... *167*
7.2.9 Initial MdAFS1ΔR33 crystallisation screening ... *167*
7.2.10 Fine crystallisation screening results for ΔR33 ... *167*
7.2.11 Further attempts to crystallise MdAFS1ΔR33 ... *168*
7.2.11.1 Crystallisation in the presence of a substrate analogue ... 168
7.2.11.2 Crystallisation results for MdAFS1ΔR33 appear to be largely batch dependent ... 169
7.3 Final conclusions and future directions ... 170

8 CHARACTERISATION OF A NOVEL KIWIFRUIT TERPENE SYNTHASE ... 172

8.1 Introduction ... 172
8.2 Functional characterisation of AcTPS1 ... 173
8.2.1 Expression and purification of recombinant AcTPS1 ... *173*
8.2.2 AcTPS1 headspace volatile analysis ... *176*
8.2.3 AcTPS1 product artefacts ... *178*
8.2.4 Confirmation of the AcTPS1 terpene profile ... *179*
8.2.5 Enantiomeric analysis of AcTPS1 ... *180*
8.2.6 AcTPS1 kinetics ... *182*
8.3 Final conclusions for AcTPS1 functional analysis ... 182
8.4 Crystallisation of AcTPS1 ... 183
8.4.1 Removal of the His_6-tag from AcTPS1 ... *183*
8.4.2 Preparation of cleaved AcTPS1-TEV protein for crystallisation screening ... *184*
8.4.3 Non-tagged AcTPS1 crystallisation screening ... *187*
8.4.4 Future strategies for crystallising AcTPS1 ... *188*
8.5 Final conclusions regarding AcTPS1 crystallisation ... 189

9 CONCLUDING DISCUSSION ... 190

9.1 Introduction ... 190
9.2 MdAFS1 prenyltransferase activity ... 190
9.3 Potassium binding ... 192
9.3.1 The H-α1 loop in terpene synthase catalysis ... *194*
9.3.2 The H-α1 loop in K^+-dependent TPS-d enzymes ... *195*
9.3.3 Proposed geometry for K^+ coordination in MdAFS1 ... *196*
9.4 Evolution of FDP specificity in MdAFS1 ... 197
9.5 Evolution of FDP catalysis in the TPS-d subgroup ... 198
9.6 MdAFS1 crystallisation ... 199
9.7 Characterisation of AcTPS1 ... 200
9.8 Future work ... 201
9.8.1 MdAFS1 crystallisation ... *201*
9.8.2 Crystallisation of other fruit and flower terpene synthases ... *202*
9.8.3 MdAFS1 evolution ... *202*
9.9 Significance of this research ... 203

10 LIST OF REFERENCES ... 204

11 APPENDICES ... 224

11.1 MdAFS1 Ramachandran analysis ... 224
11.2 TPS-b enzyme H-α1 loop modelling ... 225
11.3 Rare codon analysis for PaTPS-Far and MdAFS1 ... 226

11.4 MdAFS1 surface entropy prediction 226
11.5 MdAFS1ΔR33 crystallisation cube 227
11.6 MdAFS1ΔR33 fine crystallisation screen matrix 228
11.7 Publications 228

LIST OF FIGURES

Figure 1-1: Examples of primary metabolites synthesised from terpene precursors.................................. 2
Figure 1-2: Mevalonate pathway.. 5
Figure 1-3: MEP pathway steps to the formation of IDP... 6
Figure 1-4: Subcellular compartmentation of terpenoid biosynthesis in plants..................................... 7
Figure 1-5: Structural similarity of terpene synthases.. 11
Figure 1-6: Structure of a *Salvia officinalis* monoterpene synthase.. 12
Figure 1-7: Geranyl diphosphate cyclisation mechanism... 14
Figure 1-8: The cyclisation of geranyl diphosphate by monoterpene synthases...................................... 14
Figure 1-9: Farnesyl diphosphate reactions... 15
Figure 1-10: Farnesyl diphosphate cyclisation mechanisms... 16
Figure 1-11: Schematic of a peltate glandular trichome of peppermint.. 18
Figure 1-12: Schematic proposal for evolution of the terpene synthase gene family in plants................. 21
Figure 3-1: Amino acid alignment of Plant & Food Research terpene synthase sequences..................... 58
Figure 3-2: Terpene synthase phylogenetics.. 61
Figure 3-3: TPS-g Neighbour Joining tree.. 63
Figure 3-4: N-terminal sequence alignments for TPS-f synthases... 64
Figure 4-1: Mechanism for farnesyl diphosphate formation.. 70
Figure 4-2: Purification of recombinant MdAFS1... 70
Figure 4-3: α-farnesene production from MdAFS1 activity assays with GDP and IDP precursors.......... 71
Figure 4-4: Deuterated precursor chemical structures... 72
Figure 4-5: GCMS profiles of deuterated and non-deuterated α-farnesene products.............................. 73
Figure 4-6: Release with time of α-farnesene from purified MdAFS1 in the absence of FDP precursor.. 74
Figure 4-7: MdAFS1 peptide fragments identified by LC MS/MS analysis.. 76
Figure 4-8: Germacrene-D enantiomers produced from FDP 77
Figure 4-9: Chiral GCMS analysis of the *A. deliciosa* germacrene-D synthase AdGDS1........................ 77
Figure 4-10: Purification of MdAFS1 DDXXD mutant enzymes.. 80
Figure 4-11: Relative activities of non-mutated (WT) and DDXXD mutant MdAFS1 enzymes............. 81
Figure 4-12: Headspace GCMS analysis of the different MdAFS1 recombinant enzymes...................... 81
Figure 4-13: Hydrolysis of FDP to farnesol.. 82
Figure 4-14: Acidic hydrolysis of the GDP/IDP activity assays for the DDXXD mutants....................... 83
Figure 4-15: Analysis of FDP production from MdAFS1 mutants... 84
Figure 4-16: Loss of sesquiterpene activity in the D327A mutant.. 85
Figure 4-17: Active site comparisons between a tobacco epi-aristolochene and an avian FDP synthase.. 90
Figure 5-1: MdAFS1 homology model.. 95

Figure 5-2: The H-α1 loop region in MdAFS1. ... 96
Figure 5-3: Production of MdAFS1 H-α1 loop mutant recombinant enzymes. ... 98
Figure 5-4: Potassium responses for WT and H-α1 loop mutated MdAFS1. ... 99
Figure 5-5: Monoterpene volatile analysis of H-α1 loop mutants. ... 100
Figure 5-6: Kinetic evaluation of MdAFS1 potassium dependence. ... 101
Figure 5-7: Phylogenetic and sequence analysis of TPS-b subgroup enzymes. ... 102
Figure 5-8: S487K mutant Experion™ analysis. ... 103
Figure 5-9: Introduced H-α1 loop lysine effects on the activation of MdAFS1 by potassium. ... 104
Figure 5-10: MdAFS1-S487K mutant FDP kinetics. ... 105
Figure 5-11: Stereoview showing proposed octahedral coordination of K^+ in MdAFS1. ... 106
Figure 5-12: TPS-d enzyme H-α1 loop region. ... 108
Figure 5-13: SDS-PAGE analysis of recombinant AcTPS2 proteins. ... 109
Figure 5-14: PsTPS2 protein Experion™ analysis. ... 110
Figure 5-15: Potassium responses for wild-type and mutated PsTPS2 and AcTPS2 enzymes. ... 112
Figure 5-16: PsTPS2 volatile analysis. ... 112
Figure 5-17: AcTPS2 volatile analysis. ... 113
Figure 5-18: Stereoview of the MdAFS1 substrate binding pocket. ... 115
Figure 6-1: MdAFS1 active site model with docked FDP substrate. ... 123
Figure 6-2: Conservation of a TPS-b active site tryptophan. ... 124
Figure 6-3: Purification of the recombinant C298W MdAFS1 enzyme. ... 125
Figure 6-4: Comparative activity analysis for WT and C298W mutant enzymes. ... 126
Figure 6-5: Non-quantitative activity analyses of MdAFS1 (WT) and C298W enzymes. ... 126
Figure 6-6: Catalytic scheme for ocimene formation from GDP. ... 128
Figure 6-7: TPS-b phylogenetics. ... 129
Figure 6-8: Active site aromatic residue comparisons between MdAFS1 and LjEβOS. ... 132
Figure 6-9: Active site aromatic and basic residue configurations in the *L. corniculatus* (*E*)-β-ocimene (LjEβOS) homology model and the *M. spicata* limonene (LS) structure. ... 133
Figure 6-10: Western blot analysis of MdAFS1 active site aromatic residue mutants. ... 136
Figure 6-11: Major terpene products from terpene synthases in Norway spruce. ... 138
Figure 6-12: Active site modelling in TPS-d enzymes. ... 141
Figure 6-13: Amino acid basis for the differences in *P. abies* terpene synthase active site topologies. .. 142
Figure 7-1: IMAC Purification of His_6-Tagged MdAFS1 ... 155
Figure 7-2: SDS-PAGE analysis of His_6-tagged MdAFS1 IMAC fractions. The ... 156
Figure 7-3: Anion exchange purification of His_6-tagged MdAFS1 ... 157
Figure 7-4: Final purification of His_6-tagged MdAFS1 for crystallisation screening. Peak ... 158
Figure 7-5: SDS-PAGE analysis of His_6-tagged MdAFS1 SEC fractions. ... 158
Figure 7-6: Strategy to reduce entropy shielding in MdAFS1. ... 162

Figure 7-7: Activity results for MdAFS1 ΔR33 ammonium sulphate precipitation fractions. 163
Figure 7-8: Elution profile and activity analysis of HIC purified ΔR33 protein. 164
Figure 7-9: SDS-Page analysis of peak 1 HIC eluted protein fractions. 165
Figure 7-10: Final purification steps for ΔR33 crystallisation screening preparations. 166
Figure 7-11: MdAFS1ΔR33 F-FDP inhibition kinetics. 169
Figure 8-1: IMAC elution profile for recombinant AcTPS1. 174
Figure 8-2: IMAC purification of His_6-tagged AcTPS1. 174
Figure 8-3: Semi-quantitative size exclusion chromatography for AcTPS1. 175
Figure 8-4: SDS-PAGE analysis of AcTPS1 size exclusion chromatography purification fractions. 176
Figure 8-5:AcTPS1 headspace volatiles from FDP. 177
Figure 8-6: AcTPS1 headspace volatiles from GDP. 177
Figure 8-7: Thermal rearrangement of nerolidol. 179
Figure 8-8: AcTPS1 Terpene synthase analysis. 180
Figure 8-9: Enantioselective GCMS analysis of AcTPS1 sesquiterpene synthase activity. 181
Figure 8-10: Enantioselective GCMS analysis of AcTPS1 monoterpene synthase activity. 181
Figure 8-11: AcTPS1 substrate kinetics. 182
Figure 8-12: Removal of AcTPS1-TEV His_6-Tag. 184
Figure 8-13: IMAC purification elution chromatograph for the recombinant AcTPS1-TEV protein...... 185
Figure 8-14: SDS-PAGE analysis of cleaved AcTPS1-TEV following the second IMAC step. 185
Figure 8-15: Final purifications for AcTPS1-TEV protein. 186
Figure 8-16: SDS-PAGE analysis of the final non-tagged AcTPS1 purification step. 186
Figure 8-17: Crystals seen in the initial AcTPS1 screens. 187
Figure 8-18: X-ray diffraction patterns for the initial AcTPS1 screening crystals.................................. 188
Figure 8-19: AcTPS1 surface entropy prediction analysis. 189
Figure 9-1: MdAFS1 K^+ and Mg^{2+} binding regions. 194

LIST OF TABLES

Table 2-1: Terpene synthase primers for Zero Blunt and pET-TOPO cloning. 30
Table 2-2: TEV linker reaction components. 31
Table 2-3: TEV linker reaction conditions. 31
Table 2-4: Reaction component and conditions for non-mutagenic PCR amplifications. 32
Table 2-5: MdAFS1 mutagenic primers. 33
Table 2-6: PsTPS2 mutagenic primers. 34
Table 2-7: AcTPS2 mutagenic primers. 34
Table 2-8: Restriction digest set-up. 35
Table 2-9: Terpene synthase sequencing primers. 36
Table 2-10: Recipe for two 10% (w/v) separating, 4% (w/v) stacking SDS-PAGE gels 42
Table 2-11: Crystallisation screen scoring 45
Table 3-1: Summary of Plant & Food Research EST databases 53
Table 3-2: Plant & Food Research terpene synthases. 54
Table 3-3: Shorthand naming of Plant & Food Research terpene synthase ESTs 57
Table 3-4: Sequence characteristics and initial assignment of terpene synthase types 60
Table 3-5: Amino acid identity comparisons of selected TPS-g synthases. 63
Table 3-6: Amino acid sequence identity comparisons of TPS-f synthases. 64
Table 3-7: Final subgroup classification of Plant & Food Research terpene synthase genes 66
Table 4-1: Precursor and activity buffer terpene contamination. 68
Table 4-2: Peptide matches to *M. domestica* α-farnesene synthase and known *E. coli* proteins. 75
Table 5-1: Terpene synthase activities of MdAFS1 WT and H-α1 loop mutant enzymes. 99
Table 5-2: Farnesene isomer composition from MdAFS1 WT and H-α1 loop mutant enzymes. 99
Table 5-3: Farnesene isomer composition for WT and S487K MdAFS1 enzymes. 104
Table 5-4: Kinetics summary for WT and S487K mutated MdAFS1 recombinant enzymes. 105
Table 5-5: Summary of MdAFS1, AcTPS2 and PsTPS2 enzyme responses to potassium. 116
Table 6-1: Active site residue comparisons for BPPS and MdAFS1. 122
Table 6-2 LjEβOS amino acid matches to the MdAFS1 contact map amino acid residues. 130
Table 6-3: Relative activities of MdAFS1 active site aromatic residue mutants. 137
Table 6-4: Norway spruce terpene synthase contact map residue comparisons. 140
Table 6-5: Pairwise sequence comparisons of PaTPS-Far with other *P. abies* terpene synthases. 143
Table 6-6: Codon analysis of selected first-tier contact residues in *P. abies* terpene synthases. 144
Table 6-7: Minimal mutational routes to monoterpene synthase first-tier architecture. 145
Table 7-1: Summary of terpene synthase crystal structures. 154
Table 7-2: Initial MdAFS1 crystal screens. 159

Table 9-1: Summary of TPS-d sesquiterpene synthase potassium responses. 196

ABBREVIATIONS

°C	degree centigrade
Å	Angstrom
BLAST	Basic Local Alignment Search Tool
bp	base pair
BTP	bis-tris propane
cDNA	complementary deoxyribonucleic acid
CDS	coding sequence
C-terminal	carboxyl terminal
CTP	chloroplast transit peptide
Da	Dalton
di-TPS	diterpene synthase
DLS	dynamic light scattering
DMADP	dimethylallyl diphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EC	enzyme classification
EDTA	ethylene diaminetetraacetic acid
EMBOSS	European Molecular Biology Open Software Suite
EMBL	European Molecular Biology Laboratory
EST	expressed sequence tag
FDP	farnesyl diphosphate
FPLC	fast performance liquid chromatography
GCMS	gas chromatography mass spectroscopy
GDP	geranyl diphosphate
GGDP	geranyl geranyl diphosphate
HIC	hydrophobic interaction chromatography
HPLC	high pressure liquid chromatography
IDP	isopentenyl diphosphate
IMAC	immobilised metal affinity chromatography
IPTG	isopropylthio-β-galactoside
kb	kilobase – 10^3 bases
k_{cat}	catalytic centre activity (first order rate constant)
kDa	kilodalton – 10^3 Daltons
K_m	Michaelis constant
LCMS	liquid chromatography mass spectroscopy
LDP	linalyl diphosphate
mAU	milli absorbance units
MS	mass spectroscopy
MQ-H_2O	milli-Q water
MW	molecular weight
m/z	mass to charge ratio
N-terminal	amino terminal
NCBI	National Centre for Biotechnology Information
NIST	National Institute of Standards and Technology
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis

PCR	polymerase chain reaction
pI	isoelectric point
RACE	rapid amplification of cDNA ends
rms	root mean square
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SEC	size exclusion chromatography
SEM	standard error of the mean
SPME	solid phase micro extraction
T_m	melting temperature
TPS	terpene synthase
tRNA	transfer ribonucleic acid
UTR	untranslated region
UV	ultraviolet
V_{max}	maximum enzyme velocity
Vrel	relative enzyme velocity
WT	wild-type