Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.
CHARACTERISATION OF ACC OXIDASE DURING LEAF MATURATION AND SENESCENCE IN WHITE CLOVER
(TRIFOLIUM REPENS L.)

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

Doctor of Philosophy

at

Massey University

DONALD ALEXANDER HUNTER

1998
Dedication

This thesis is dedicated to my parents

John & Marie

and to

Stephanie
Abstract

ACC oxidase, the enzyme which converts 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, has been studied in leaves of a single genotype of white clover (*Trifolium repens* L., Genotype 10F) during leaf maturation and senescence.

Leaf senescence in genotype 10F is associated with an increase in both ACC content and ethylene evolution of the leaves. The increase in levels of ACC slightly precedes that of ethylene production, but occurs concomitantly with the onset of senescence (as judged by chlorophyll loss).

The coding regions of two distinct ACC oxidases were generated from leaf tissue of genotype 10F using RT-PCR with degenerate oligonucleotide primers. The coding regions were designated TR-ACO1 and TR-ACO2. TR-ACO1 and 2 are 84% and 87% similar in nucleotide and amino acid sequence respectively. Genomic Southern analysis using these regions as probes confirmed that both sequences are encoded by distinct genes, but also suggested that there may be additional genes closely related to both TR-ACO1 and TR-ACO2.

Gene expression studies during leaf maturation and senescence were undertaken using these regions as probes. TR-ACO1 hybridised to a single RNA transcript of ca. 1.35 Kb on the northern blot. Expression of this transcript was high in mature green leaves but declined as the leaves senesced. By contrast, TR-ACO2 hybridised equally to two RNA transcripts of ca. 1.17 Kb and 1.35 Kb on the northern blot, and unlike expression of TR-ACO1, the levels of these transcripts were low in mature green leaves and increased as the leaves senesced.

The 3'-UTRs of TR-ACO1 and TR-ACO2 were generated using 3'-RACE (Randomly Amplified cDNA Ends). Repeating the genomic Southern analysis with these regions as probes indicated that the gene for TR-ACO1 may be polymorphic, and that there may be an additional ACC oxidase gene in the genome that encodes a transcript with a similar coding region, but divergent 3'-UTR to TR-ACO2.

The 3'-UTRs of TR-ACO1 and 2 confirmed the expression patterns of their cognate coding regions in northern analysis, except that the 3'-UTR of TR-ACO2 hybridised only to the 1.35 Kb and not the 1.17 Kb transcript.
ACC oxidase activity assayed in vitro correlated with the levels of gene expression of TR-ACO1 but not the senescence-associated TR-ACO2, with the activity being highest in mature green leaves and declining as the leaves senesced. The decrease in activity was greatest when expressed on a per unit fresh weight basis (ca. 8-fold) than per unit protein basis (ca. 3-fold). The extraction and assay conditions altered in this study were not able to prevent the decline in ACC oxidase activity in vitro that occurred during leaf senescence.

Polyclonal antibodies raised against the translation products of TR-ACO1 and 2 expressed in E. coli recognised a protein of the expected size (ca. 36.4 kDa) for ACC oxidase using western analysis. The pattern of protein accumulation recognised by the antibodies raised against TR-ACO1 (in rabbits) broadly matched gene expression of TR-ACO1 and activity of ACC oxidase. That is, antibody recognition was highest in mature green leaves and declined as the leaves senesced. By comparison, the antibodies raised against TR-ACO2 (in rats) recognised a protein of the same size with weak avidity. The pattern of accumulation was similar to that observed with the TR-ACO1-raised-antibodies, and therefore not consistent with the gene expression pattern of TR-ACO2. It was concluded that these antibodies were cross-reacting with the same protein as that recognised by the TR-ACO1-raised-antibodies and that the lower enzyme activity was due to lower TR-ACO1 ACC oxidase protein.

Wounding mature green leaves increased TR-ACO2 gene expression, but as observed for the senescing leaves, increased gene expression of TR-ACO2 was not correlated with increased protein accumulation or ACC oxidase enzyme activity.

Potential explanations as to why the increased gene expression of TR-ACO2 during leaf senescence (or wounding) is not accompanied by increased protein accumulation and ACC oxidase activity are discussed.
Acknowledgments

I wish to thank my supervisors, Dr Michael T. McManus and Professor Paula E. Jameson for their encouragement and guidance throughout the course of this project. As my chief supervisor, Michael has been everything people said he would be, extremely competent, enthusiastic, supportive, and a perfect role model. I feel I cannot say enough, thank you again Michael. Paula has been brilliant in a supportive role throughout. I am especially grateful for the time she has spent towards the end of my writing, offering a fresh and valuable perspective on organisation of the thesis, as well as assisting in proofing. Thank you again Paula.

I can’t go much further without mentioning Lyn, Michael’s research assistant, and the person responsible for keeping the lab running smoothly. “Lyn - look I’ve finished!!!!!” Always willing to help, I don’t know how many thousands of chocolate fish I must owe you, thanks again. To Sang Dong (Sangie !), my partner in oxidase, thank you for your very generous help throughout.

Thanks to rest of the crew of lab C 5:19, Trish, Simone, Celia, Deming, Marian, Michelle, Huaibi, and Nigel who have made it such a great place to work.

There are various people who have helped with specific aspects of the project. I would like to thank Steve Butcher for initial help in getting this project underway, Jocelyn Tilbrook, Sang Dong and Trish for helping with harvesting tissue, Marty Hunt for helping me get up to speed with the GC and for loan of a chart recorder, Dr Peter Lockhart and Richard Winkworth for phylogenetic analysis, and Steven Northover for help with the bibliography.

To the many staff and students of the Department of Plant Biology and Biotechnology who I have for one reason or another asked for assistance, thank you.

My appreciation also goes out to the staff of the Plant Molecular Genetics Lab at AgResearch, Grasslands. All of whom have been friendly, and generous to the (what do you want this time!) guy who kept popping up at their doorstep. In particular, I would like to thank, Andrew, Tina, Ferenc, Briggida, Nick, Margaret, Mush and Anne (CRI Campus Library).

I’m also grateful to the New Zealand Pastoral and Agricultural Research Institute Limited for providing financial assistance in the form of a three year stipend, and to the New
Zealand Society of Plant Physiologists for providing generous travel assistance grants. In addition, I would like to thank the Department of Plant Biology and Biotechnology for providing me with a J. P. Skipworth Scholarship in 1998.

My final and very special thanks must go to both my parents, John and Marie, and to my partner, Stephanie. I cannot thank them enough for all the support and encouragement they have given to me.
Table of Contents

ABSTRACT ........................................................................................................................................... III

ACKNOWLEDGEMENTS ....................................................................................................................... V

LIST OF FIGURES ............................................................................................................................... XVI

LIST OF TABLES ................................................................................................................................... XX

ABBREVIATIONS ................................................................................................................................. XXI

1. INTRODUCTION ................................................................................................................................. 1

1.1 Ethylene in plant development ........................................................................................................ 1

1.2 Mode of ethylene action .................................................................................................................... 4

1.3 Ethylene biosynthesis ........................................................................................................................ 6

1.3.1 ACC-mediated ethylene production .............................................................................................. 7

1.3.2 Enzymes of the ACC-mediated biosynthetic pathway ................................................................. 10

1.3.2.1 AdoMet synthetase .................................................................................................................... 10

1.3.2.2 ACC synthase ............................................................................................................................ 11

1.3.2.3 ACC conjugation ........................................................................................................................ 14

1.4 ACC oxidase ....................................................................................................................................... 16

1.4.1 Identification of EFE as ACC oxidase .......................................................................................... 16

1.4.2 Biochemical characterisation of ACC oxidase ............................................................................. 19

1.4.3 Localisation of ACC oxidase ......................................................................................................... 21

1.4.4 ACC oxidase activity during plant development ........................................................................... 22

1.4.5 Molecular characterisation of ACC oxidase ............................................................................... 23

1.4.6 ACC oxidase: A rate limiting enzyme ......................................................................................... 26

1.4.7 Role of ethylene in leaf maturation and senescence .................................................................... 28

1.5 ACC oxidase during leaf maturation and senescence ...................................................................... 29

1.5.1 White clover and leaf senescence .............................................................................................. 31
1.5.2 Thesis Aims..................................................................................... 32

2. MATERIALS AND METHODS................................................................. 33

2.1 Propagation and Harvesting Methods .............................................. 33
  2.1.1 Plant material ............................................................................. 33
  2.1.2 Plant growth conditions ........................................................... 33
  2.1.3 Plant propagation ................................................................. 33
  2.1.3.1 Stock Plants ........................................................................ 33
  2.1.3.2 Model System ..................................................................... 34
  2.1.4 Leaf harvesting ................................................................. 36
  2.1.5 Leaf wounding ................................................................. 36
  2.1.6 Experimental conditions used for wounding experiments .......... 36
     2.1.6.1 Conditions used for detached leaves .................................. 36
     2.1.6.2 Conditions used for attached leaves .................................... 37

2.2 Biochemical and physiological methods ......................................... 37
  2.2.1 Chemicals ................................................................................. 37
  2.2.2 Chlorophyll quantitation.......................................................... 37
  2.2.3 Ethylene analysis ................................................................. 38
     2.2.3.1 Measurement of ethylene by Gas Chromatography ......... 38
     2.2.3.2 Measurement of ethylene by PhotoVac ......................... 38
  2.2.4 Ethylene Calculations ............................................................. 39
  2.2.5 Measurement of ethylene evolution from attached leaves of white clover .... 39
  2.2.6 ACC quantitation ................................................................. 40
     2.2.6.1 ACC extraction for leaf tissue down the stolon ............... 40
     2.2.6.2 Paper chromatography ................................................. 40
     2.2.6.3 ACC assay ..................................................................... 41
  2.2.7 Measurement of ACC Oxidase activity .................................. 42
2.2.7.1 Extraction procedure ................................................................. 42
  2.2.7.1.1 Extraction procedure 1 ......................................................... 42
  2.2.7.1.2 Extraction procedure 2 ......................................................... 42
2.2.7.2 Preparation of Sephadex G-25 spin columns ......................... 43
2.2.7.3 Assay procedure ..................................................................... 44

2.2.8 Protein quantitation .................................................................... 44

2.2.9 SDS-PAGE of Protein ................................................................. 46
  2.2.9.1 Linear slab gel SDS-PAGE using the BioRad Mini-Protean apparatus .... 46
  2.2.9.2 Gradient (8-15 %) slab gel SDS-PAGE ...................................... 47
  2.2.9.3 Staining of gels after SDS-PAGE ............................................. 48
  2.2.9.4 Drying of gels after SDS-PAGE ................................................ 48

2.2.10 Western analysis of SDS-PAGE gels .......................................... 49
  2.2.10.1 Production of primary antibodies .......................................... 49
    2.2.10.1.1 Preliminary induction of His-tagged fusion proteins in E. coli .......... 49
    2.2.10.1.2 Large scale induction of His-tagged fusion proteins in E. coli ...... 51
  2.2.10.2 Purification of His-tagged fusion protein by metal chelate affinity chromatography ............................................................. 52
  2.2.10.3 Protein sequencing ................................................................. 53
  2.2.10.4 Immunisation of animals with affinity purified His-tagged fusion proteins and collection of antisera .................................................. 54
  2.2.10.5 Western blotting .................................................................... 55
    2.2.10.5.1 Transfer of proteins from SDS-PAGE gel to membrane support .... 55
    2.2.10.5.2 Immunodevelopment of membrane ........................................ 56
    2.2.10.5.3 Estimation of protein molecular mass ...................................... 56
  2.2.10.5.4 Quantification of protein accumulation in western blots by image analysis ................................................................. 57
2.3 Molecular methods ........................................................................................................ 57
  2.3.1 Chemicals .................................................................................................................. 57
  2.3.2 DNA cloning procedures ......................................................................................... 57
    2.3.2.1 Growth conditions for bacteria ........................................................................... 57
    2.3.2.2 Plasmid isolation from E. coli by the alkaline lysis method .............................. 58
    2.3.2.3 Precipitation of DNA and RNA with ethanol or isopropanol ......................... 59
    2.3.2.4 DNA quantitation ............................................................................................. 59
    2.3.2.5 Restriction digestion of DNA ............................................................................. 60
    2.3.2.6 Agarose gel electrophoresis of DNA ............................................................... 62
    2.3.2.7 Size determination of nucleic acid ..................................................................... 63
    2.3.2.8 DNA recovery from agarose gels .................................................................... 63
      2.3.2.8.1 Spin column method .................................................................................. 63
      2.3.2.8.2 Freeze-squeeze method ............................................................................. 64
    2.3.2.9 DNA Ligation ..................................................................................................... 64
      2.3.2.9.1 DNA ligation using linearised T-Vector ..................................................... 64
      2.3.2.9.2 DNA ligation using restriction digested vector ........................................... 66
    2.3.2.10 Transformation of E. coli ................................................................................ 66
      2.3.2.10.1 Preparation of competent cells .................................................................. 66
      2.3.2.10.2 Transformation by standard heat shock protocol ..................................... 67
      2.3.2.10.3 Transformation by 5-minute protocol ....................................................... 67
      2.3.2.10.4 Transformation by TA cloning kit protocol .............................................. 68
  2.3.3 DNA sequencing procedures .................................................................................... 68
    2.3.3.1 Purification of DNA for sequencing ............................................................... 68
    2.3.3.2 Manual sequencing ........................................................................................... 69
    2.3.3.3 Sequencing gels ............................................................................................... 70
    2.3.3.4 Automated DNA sequencing ............................................................................ 71
2.3.3.5 Sequence alignment .......................................................... 71
2.3.3.6 Sequence phylogeny .......................................................... 72
2.3.4 Southern analysis procedures .................................................. 72
  2.3.4.1 Genomic DNA isolation method 1 ....................................... 72
  2.3.4.2 Restriction Digestion of Genomic DNA .................................. 74
  2.3.4.4 Southern (capillary) blotting of genomic DNA ....................... 75
  2.3.4.5 Southern (capillary) blotting of cDNA fragments .................... 76
  2.3.4.6 Labelling Probe DNA for use in Southern and northern analysis .... 77
    2.3.4.6.1 Labelling DNA with the Ready-To-Go\textsuperscript{TM} \(\alpha\textsuperscript{32P}\)-dCTP DNA Labelling Kit ........................................... 77
    2.3.4.6.2 Labelling DNA with the Megaprime\textsuperscript{TM} \(\alpha\textsuperscript{32P}\)-dATP DNA Labelling Kit ........................................... 77
  2.3.4.7 Hybridisation and washing of DNA and RNA blots .................. 78
  2.3.4.8 Stripping Southern and northern blots .................................. 79
2.3.5 RNA Isolation .......................................................................... 79
  2.3.5.1 Isolation of total RNA ....................................................... 80
  2.3.5.2 Isolation of poly (A)+ RNA ................................................. 81
  2.3.5.3 RNA Quantitation ............................................................. 82
2.3.6 Amplification of DNA by RT-PCR ........................................... 82
  2.3.6.1 Reverse Transcriptase Synthesis of cDNA .............................. 82
  2.3.6.2 PCR amplification of DNA .................................................. 83
    2.3.6.2.1 Primers ...................................................................... 83
    2.3.6.2.2 Degenerate primers used in RT-PCR ............................... 84
    2.3.6.2.3 Primers used for in-frame cloning into the pPROEX\textsuperscript{TM}-1 vector ....... 85
    2.3.6.2.4 Primers used for 3'-RACE ........................................... 86
    2.3.6.2.5 PCR conditions .......................................................... 87
2.3.7 Northern Analysis Procedures ................................................ 87
2.3.7.1 Electrophoresis of RNA..........................87
2.3.7.2 Northern (capillary) blotting by downward alkaline transfer........88
2.3.7.3 Northern (capillary) blotting by downward SSPE transfer ..........89

3. RESULTS .................................................................90

3.1 Stolon growth of white clover.................................................90
3.2 Chlorophyll content of leaf tissue during leaf initiation, maturation and
senescence of white clover.......................................................90
3.3 Changes in evolved ethylene during leaf initiation, maturation and
senescence of white clover..........................................................93
3.4 Changes in ACC content during leaf initiation, maturation and
senescence of white clover..........................................................93
3.5 Changes in activity of ACC oxidase during leaf maturation and
senescence of white clover..........................................................101
3.5.1 Optimisation of ACC oxidase extraction and assay in vitro ..........101
3.6 Activity of ACC oxidase during leaf maturation and senescence
of white clover.............................................................................111
3.7 Gene expression of ACC oxidase during leaf maturation and
senescence of white clover.............................................................114
3.7.1 Confirmation of putative ACC oxidase sequences by manual sequencing.114
3.7.2 RT-PCR amplification of putative ACC-oxidase gene transcripts ....116
3.7.3 Confirmation of putative ACC oxidase gene transcripts by sequence
analysis.........................................................................................116
3.8 Confirmation by genomic Southern analysis that TR-AC01 and
TR-AC02 are encoded by distinct genes .........................................124
3.9 Changes in gene transcript expression of ACC-oxidase during
leaf maturation and senescence of white clover by northern analysis ....127
3.9.1 Gene expression of TR-AC01 and TR-AC02 during leaf maturation
and senescence............................................................................127
3.10 Confirmation of Southern and Northern analysis using 3'-RACE ..........135
3.10.1 Identification of the 3'-UTRs of TR-AC01 and TR-AC02 .............136
3.10.1.1 Development of 3'-RACE: degenerate primer approach .............136
3.10.1.2 Development of 3'-RACE:Gene-specific primer approach .................140
3.10.1.3 Confirmation of putative ACC oxidase 3'-UTR gene transcripts by sequence analysis.................................................................145
3.10.1.4 Isolation of TR-AC01 3'-UTR by the GSP approach.........................147
3.10.1.5 3'-UTR probe production for blot analysis ........................................149
3.10.2 Specificity of TR-AC01 and TR-AC02 3'-UTR probes in blotting analysis .........................................................................................152
3.10.3 Confirmation that the 3'-UTRs of TR-AC01 and TR-AC02 identify distinct genes ..............................................................................152
3.10.4 Changes in gene expression of ACC oxidase during leaf maturation and senescence using the 3'-UTRs of TR-AC01 and TR-AC02 as probes ....157
3.11 Extraction and assay requirements for ACC oxidase activity in vitro of mature green and senescent leaf material ........................................159
3.12 Protein accumulation of ACC oxidase during leaf maturation and senescence .................................................................166
3.12.1 Directional cloning of TR-AC01 and TR-AC02 into the pPROEX vector....167
3.12.1.1 Preparation of cDNA inserts of TR-AC01 and TR-AC02 by attachment of restriction sites to their 5' and 3' ends ......................167
3.12.1.2 Preparation of pPROEX vector for ligation by digestion with EcoR1 and Hind III restriction enzymes ............................................167
3.12.2 Purification of TR-AC01 and TR-AC02 His-tagged fusion proteins in preparation for animal inoculations .................................................168
3.12.3 Confirmation that the fusion protein is the translation product of TR-AC01171
3.12.4 Polyclonal antibody production to TR-AC01 and TR-AC02 His-tagged fusion proteins .................................................................................171
3.12.5 Changes in chlorophyll concentration, protein concentration and ACC oxidase activity during leaf maturation and senescence .............171
3.12.6 ACC oxidase protein accumulation during leaf maturation and senescence ......................................................................................172
3.13 Exogenous control of ACC oxidase expression in leaf tissue of white clover ......................................................................................176
3.13.1 Ethylene evolution of detached wounded leaves ................................176
3.13.2 Changes in ACC oxidase gene expression in detached wounded leaves. 176
3.13.3 Changes in ACC oxidase activity in detached wounded leaves. 177
3.13.4 Changes in ACC oxidase protein accumulation in detached wounded leaves. 177
3.13.5 Changes in ethylene evolution in non-wounded detached leaves. 181
3.13.6 Changes in ACC oxidase gene expression in non-wounded detached leaves. 181
3.13.7 Changes in ACC oxidase activity in non-wounded detached leaves. 181
3.13.8 Changes in ACC oxidase protein accumulation in non-wounded detached leaves. 181
3.13.9 The effect of an ethylene adsorbing compound on TR-AC02 gene expression and ACC oxidase activity in detached mature green leaves. 184
3.13.10 The effectiveness of Purafil in removing ethylene from around leaves. 184
3.13.11 The effect inclusion of Purafil had on TR-AC02 gene expression in leaves. 185
3.13.12 The effect of Purafil on ACC oxidase activity in leaves. 185
3.13.13 Changes in TR-AC01 and TR-AC02 gene expression in attached wounded and non-wounded mature green leaves. 188
3.13.14 Changes in ACC oxidase activity in vitro in mature and senescing leaves over a 24 h time period. 193

4. DISCUSSION.................................................................195

4.1 Biochemical characterisation of ACC oxidase. 198
4.2 Molecular characterisation of ACC oxidase. 200
4.3 Gene expression of TR-AC01 and TR-AC02 during leaf maturation and senescence. 205
4.4 Changes in ACC oxidase activity during leaf maturation and senescence. 205
4.5 ACC oxidase protein accumulation during leaf maturation and senescence. 210
4.6 Gene expression, protein accumulation, and activity of ACC oxidase in wounded mature green leaves. 211
5. SUMMARY ........................................................................................................... 214

5.1 Future Work ....................................................................................................... 219

6. BIBLIOGRAPHY .................................................................................................. 221
List of Figures

Figure 1. The Ethylene Biosynthetic Pathway .............................................................. 9
Figure 2. Stoichiometry of reaction catalysed by ACC oxidase .................................. 19
Figure 3. Stolons of white clover with leaves ready for harvesting ............................. 35
Figure 4. A typical standard curve for the Bio-Rad protein assay microassay procedure ........................................................................................................ 45
Figure 5. The map and schematic diagram of the pProEX vector and the vector multiple cloning site ................................................................. 50
Figure 6. Setting up the cassette for transfer of protein from gel to membrane .......... 55
Figure 7. Diagram of the pCR 2.1 plasmid used for TA-cloning PCR generated sequences ...................................................................................................... 65
Figure 8 Arrangement of the apparatus used to transfer DNA and RNA samples to Hybond-N’ membranes ............................................................... 76
Figure 9. Sequence of the ADAPdT primer ................................................................ 83
Figure 10. Sequence of the primers used in RT-PCR to amplify putative ACC oxidase sequences ...................................................................................... 84
Figure 11. Primers used for amplification of sequences for directional in-frame cloning into the pProEX vector ..................................................... 85
Figure 12. Primers used in 3’-RACE ........................................................................... 86
Figure 13. Chlorophyll concentration as an indicator of senescence ............................ 91
Figure 14. Fresh weights of leaves of white clover during leaf ontogeny ...................... 92
Figure 15. Ethylene evolution and chlorophyll content in leaves at nodes 1 to 16 ...... 94
Figure 16. ACC content, and ethylene evolution during leaf ontogeny ........................ 95
Figure 17. Assessment of paper chromatography as a quantitative tool for measuring ACC content ................................................................. 97
Figure 18. Specificity of Lizada and Yang method for determination of tissue ACC content ........................................................................... 99
Figure 19. Time course of ACC oxidase activity and effect of extract concentration on ACC oxidase activity measured *in vitro*..........................103

Figure 20. Optimisation of ACC oxidase activity measured *in vitro*..........................104

Figure 21. Effect of extraction time on ACC oxidase activity *in vitro*..........................107

Figure 22. Effect of assay buffer pH on ACC oxidase activity *in vitro*..........................108

Figure 23. Effect of freeze/thaw on ACC oxidase activity *in vitro*. ..............................109

Figure 24. Extraction requirements for ACC oxidase *in vitro*.................................110

Figure 25. Activity of ACC oxidase in leaves at mature green, onset and chlorotic stages, and after a 3 h storage on ice..............................112

Figure 26. Activity of ACC oxidase at individual nodes in mature and senescing leaves.................................113

Figure 27. First round PCR amplification of putative ACC oxidase cDNA sequences and restriction digests of plasmids cloned with these sequences.................118

Figure 28. Nucleotide and deduced amino acid sequence of TR-ACO1 .......................119

Figure 29. Nucleotide and deduced amino acid sequence of TR-ACO2 .......................120

Figure 30. Alignment of coding frame regions of TR-ACO1 and TR-ACO2 gene transcripts........................................................................121

Figure 31. Alignment comparison of the deduced polypeptide sequences of TR-ACO1 and TR-ACO2 with a consensus sequence derived from 23 ACC oxidases........................................................................122

Figure 32. Phylogenetic analysis of amino acid sequences of 2-ODD members........123

Figure 33. Specificity of TR-ACO1 and TR-ACO2 (coding-frame probes) in blotting analysis.................................125

Figure 34. Southern analysis of white clover genomic DNA.................................126

Figure 35. Concentration of total RNA extracted from mature and senescing leaves of white clover ...............................................................129

Figure 36. Chlorophyll analysis and northern analysis of ACC oxidase gene expression expressed on a per unit fresh weight basis in mature and senescing leaf tissue of white clover using the coding frame regions of TR-ACO1 and TR-ACO2 as probes.................................130
Figure 37. Chlorophyll analysis and northern analysis of ACC oxidase gene expression expressed on a per unit total RNA basis in mature and senescing leaf tissue of white clover using the coding frame regions of TR-AC01 and TR-AC02 as probes.

Figure 38. Chlorophyll analysis, and northern analysis of ACC oxidase gene expression expressed on a per unit poly A⁺ RNA basis in mature and senescing leaf tissue of white clover using the coding frame regions of TR-AC01 and TR-AC02 as probes.

Figure 39. Development of 3'-RACE: degenerative primer approach.

Figure 40. Development of 3'-RACE: gene-specific primer approach.

Figure 41. Confirmation of transformed colonies after TA-cloning of putative 3'-UTRs.

Figure 42. Nucleotide and deduced amino acid sequence of TR-AC02 3'-translated and untranslated regions generated by 3'-RACE.

Figure 43. Nucleotide and deduced amino acid sequence of the TR-AC01 3'-translated and untranslated regions.

Figure 44. Nucleotide and deduced amino acid sequence of the 3'-UTR TR-AC01 and TR-AC02 sequences generated with the primers ACOF4b (TR-AC01), ACOF2b (TR-AC02) and ADAP.

Figure 45. Nucleotide alignment of 3'-UTRs of TR-AC01 and TR-AC02 sequences.

Figure 46. Specificity of TR-AC01 and TR-AC02 3'-UTRs in blotting analysis.

Figure 47. Genomic Southern blot analysis of ACC oxidase genes. A comparison of hybridisation patterns obtained with coding frame regions and 3'-UTRs of TR-AC01 and TR-AC02.

Figure 48. Chlorophyll analysis and northern analysis of ACC oxidase gene expression during leaf maturation and senescence using the 3'-UTRs of TR-AC01 and TR-AC02 as probes.

Figure 49. A comparison of extraction requirements of mature green and senescent leaves for recovery of ACC oxidase activity in vitro.

Figure 50. Effect of assay buffer pH on ACC oxidase activity measured in vitro.

Figure 51. Cofactor and cosubstrate requirements for ACC oxidase extracted from mature green and chlorotic leaves.

Figure 52. Activity of ACC oxidase after incubation on ice.
Figure 53. Effect of extraction time on recovery of ACC oxidase. ................................165

Figure 54. Induction of TR-AC01 and 2 fusion proteins within the genetic background of *E. coli* strain TB-1. .................................................................169

Figure 55. Identification of column fractions that contained the induced TR-AC02 fusion protein. .............................................................................170

Figure 56. Chlorophyll concentration, protein concentration and ACC oxidase activity of mature and senescent leaves........................................173

Figure 57. Changes in activity and protein accumulation of ACC oxidase during leaf maturation and senescence..............................................174

Figure 58. Calculation of the molecular weight of ACC oxidase protein. ..............175

Figure 59. Effect of wounding detached mature green leaves on ethylene evolution, ACC oxidase activity *in vitro*, TR-AC01 and TR-AC02 gene transcripts and ACC oxidase protein accumulation .......................179

Figure 60. Effect of detachment of mature green leaves on ethylene evolution, ACC oxidase activity *in vitro*, TR-AC01 and TR-AC02 gene transcripts and ACC oxidase protein accumulation .............................................182

Figure 61. Ethylene evolution of detached wounded and non-wounded mature green leaves in the presence or absence of Purafil. .........................186

Figure 62. TR-AC02 gene transcript accumulation and ACC oxidase activity *in vitro* in detached wounded and non-wounded mature green leaves incubated with or without Purafil. ........................................187

Figure 63. The effect of wounding attached leaves either with or without a 30 min pretreatment with 1-MCP on transcript expression of TR-AC01 ....191

Figure 64. The effect of wounding attached leaves either with or without a 30 min pretreatment with 1-MCP on transcript expression of TR-AC02. ..........192

Figure 65. ACC oxidase activity measured *in vitro* in mature green, onset of senescence and senescent leaf tissue over a 24 h time period. .............194
List of Tables

Table 1. Nutrient addition to horticultural grade bark base. ...........................................33

Table 2. Composition of resolving and stacking gels used for SDS-PAGE
with the Mini-Protean apparatus. ..................................................................................46

Table 3. Composition of resolving and stacking gel solutions used in the
SDS-PAGE gradient gels. ..............................................................................................47

Table 4. Antibody production: timing of inoculation and collection of antisera. ..........54

Table 5. Percentage homology comparison of sequences amplified from mature
green leaves of white clover by RT-PCR and designated TR-AC01. ........115
Abbreviations

$A_{280\,\text{nm}}$ absorbance $[\log(I_0/I)]$ in a 1 cm light path at 260nm
ACC 1-aminocyclopropane-1-carboxylic acid
AdoMet S-adenosyl-L-methionine
Amp$^{100}$ ampicillin (100 mg / mL)
AOA aminoethoxyacetic acid
APS ammonium persulphate
ATP adenosine 5'- triphosphate
AVG aminoethoxyvinylglycine

BCIP 5-bromo-4-chloro-3-indoyl phosphate
BSA bovine serum albumin

°C degrees celsius
ca. approximately
CAMV cauliflower mosaic virus
CBB coomassie brilliant blue

dATP 2'-deoxyadenosine 5'-triphosphate
dCTP 2'-deoxycytidine 5'-triphosphate
dGTP 2'-deoxyguanosine 5'-triphosphate
DMF N, N-dimethyl formamide
DMSO dimethyl sulphoxide
DNA deoxyribonucleic acid
DNase deoxyribonuclease
DTT dithiothreitol
dTTP 2'-deoxythymidine 5'-triphosphate

EDTA ethylenediaminetetraacetic acid
EDTA(Na$_2$) ethylenediaminetetraacetic acid, disodium salt
EFE ethylene forming enzyme
EFS ethylene forming system
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA</td>
<td>1,2,di(2-aminoethoxy)ethane-N,N',N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>FW</td>
<td>fresh weight</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>acceleration due to gravity (9.81 m s(^{-2}))</td>
</tr>
<tr>
<td>GARAP</td>
<td>goat anti-rabbit alkaline phosphatase</td>
</tr>
<tr>
<td>GACC</td>
<td>1-(gamma-L-glutamylamino)cyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GSP</td>
<td>gene-specific primer</td>
</tr>
<tr>
<td>GUS</td>
<td>(\beta)-glucuronidase</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid</td>
</tr>
<tr>
<td>IAA</td>
<td>indole-3-acetic acid</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-(\beta)-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase-pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani (media or broth)</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>M</td>
<td>molar, moles per litre</td>
</tr>
<tr>
<td>MACC</td>
<td>1-(malonylamino)cyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>1-MCP</td>
<td>1-methylcyclopropene</td>
</tr>
<tr>
<td>mcs</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-morpholino]ethanesulphonic acid</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre</td>
</tr>
<tr>
<td>(\mu)g</td>
<td>microgram</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>nit.</td>
<td>minute</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>water that has been purified by passing through a MilliQ ion exchange column</td>
</tr>
<tr>
<td>Mr</td>
<td>relative molecular mass (g mol(^{-1}))</td>
</tr>
<tr>
<td>MTA</td>
<td>5'-methylthioadenosine</td>
</tr>
<tr>
<td>MTR</td>
<td>5'-methylthioribose</td>
</tr>
<tr>
<td>MOPS</td>
<td>Na(3-[N-Morpholino]propanesulphonic acid)</td>
</tr>
<tr>
<td>NaOAc</td>
<td>sodium acetate</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NBD</td>
<td>norbornadiene</td>
</tr>
<tr>
<td>NBT</td>
<td>(p)-nitro blue tetrazolium chloride</td>
</tr>
<tr>
<td>2-ODD</td>
<td>2-oxoacid dependent dioxygenase</td>
</tr>
<tr>
<td>OD(^x)</td>
<td>optical density at x nm in a 1 cm light path</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAG</td>
<td>photosynthetic associated gene</td>
</tr>
<tr>
<td>PBSalt</td>
<td>50 mM sodium phosphate pH 7.4 in 250 mM NaCl</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pH</td>
<td>-log [H(^+)]</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>PVPP</td>
<td>polyvinyl polypyrrolidone</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RO</td>
<td>reverse osmosis</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>RUBISCO</td>
<td>ribulose bisphosphate carboxylase</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>sem</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SAGs</td>
<td>senescence associated genes</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
</tbody>
</table>
SSPE  
saline, sodium phosphate, and EDTA buffer
SA-PMPs  
Streptavidin MagneSphere® Particles

TE  
10 mM Tris/1 mM EDTA, pH 8.0
TEMED  
N,N,N',N'-tetramethylethylenediamine
Temp.  
temperature
TES  
10 mM Tris/100 mM sodium chloride/1 mM EDTA, pH 8.0
Tris  
tris (hydroxymethyl)aminomethane
Triton X-100  
octylphenoxy polyethoxyethanol
Tween 20  
polyoxyethylenesorbitan monolaurate

U  
units
UTR  
untranslated region
UV  
ultra violet light

V  
volt (m² kg⁻³ A⁻¹)
v/v  
volume per volume

W  
watt (m² kg⁻³) or (V A)
w/v  
weight per volume