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Characterization of ACC Oxidase
from the Leaves of
***Malus domestica* Borkh. (Apple)**

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

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in
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ABSTRACT

The expression, accumulation and kinetic properties of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO), the enzyme which catalyses the final step in the ACC-dependent pathway of ethylene biosynthesis in plants, is examined.

The investigation is divided into three sections: (i) identification of two ACO genes in apple leaf tissue, designated *MD-ACO2* and *MD-ACO3*, (ii) kinetic analyses of each of the three isoforms of ACO in apple (*MD-ACO1*, *MD-ACO2* and *MD-ACO3*) expressed in *E. coli*, and (iii) temporal and developmental expression *in vivo* of each of the ACO genes and accumulation of the corresponding gene products in leaf and fruit tissue.

The coding regions of putative ACO gene transcripts were generated from leaf tissue using RT-PCR. Sequence alignments and interrogation of the expressed sequence tags (ESTs) database indicated that the entire open reading frame (ORF) sequences were encoded by two distinct genes, and these are designated *MD-ACO2* and *MD-ACO3*. A third *ACO* gene had been identified in apple by other research workers previously, and designated *MD-ACO1*. Differences are obvious in the number of base-pairs (bp) constituting the entire ORF of *MD-ACO1* (942 bp), *MD-ACO2* (990 bp) and *MD-ACO3* (966 bp). *MD-ACO1* and *MD-ACO2* share a close nucleotide sequence identity of 93.9 % in the ORF but diverge in the 3' untranslated regions (3'-UTR) (69.5 %). In contrast, *MD-ACO3* shares a lower sequence identity with both *MD-ACO1* (78.5 %) and *MD-ACO2* (77.8 %) in the ORF, and in the 3'-UTR (*MD-ACO1*, 68.4 %; *MD-ACO2*, 71 %). A comparison of the gene structures show that the endonuclease restriction sites are unique to each individual *MD-ACO* sequence. Genomic Southern analysis, using probes spanning the 3'-UTR and the 3'-end of the coding region confirmed that *MD-ACO3* is encoded by a distinct gene. However, while the distinction between *MD-ACO1* and *MD-ACO2* is not as definitive, different gene expression patterns adds credence to their distinctiveness. Each of the three deduced amino acid sequences contain all of the residues hitherto reported to be necessary for maximal ACO activity.

Expression of *MD-ACO1*, *MD-ACO2* and *MD-ACO3* as fusion proteins in *E. coli* was induced using isopropyl- β -D-thiogalactopyranoside (IPTG), the recombinant proteins purified by nickel-nitrilotriacetic acid (NiNTA) affinity chromatography and the products had predicted masses determined from the nucleotide sequences, including the His-tag of 38.53 kDa (*MD-ACO1*), 40.39 kDa (*MD-ACO2*) and 39.3 kDa (*MD-ACO3*). Polyclonal antibodies were raised against the *MD-ACO3* fusion in rabbit for western blot analysis. Antibodies had been raised previously

against recombinant MD-ACO1, and while it was considered likely the MD-ACO2 would be recognized by the MD-ACO1 antibodies, MD-ACO2 does not appear to be recognized *in vivo* by the antibody.

Analyses of the kinetic properties of the three apple ACOs was undertaken. Apparent Michaelis constants (K_m) of 89.39 μM (MD-ACO1), 401.03 μM (MD-ACO2) and 244.5 μM (MD-ACO3) have been determined which suggests differences in the affinity of each enzyme for the substrate ACC. Maximum velocity ($V_{\max}$) was determined for MD-ACO1 (15.15 nmol), MD-ACO2 (12.94 nmol) and MD-ACO3 (18.94 nmol). The catalytic constant (K_{cat}) was determined for MD-ACO1 (6.6×10^{-2}), MD-ACO2 (3.44×10^{-2}) and for MD-ACO3 (9.14×10^{-2}), with K_{cat}/K_m ($\mu\text{M s}^{-1}$) values of $7.38 \times 10^{-4} \mu\text{M s}^{-1}$ (MD-ACO1), $0.86 \times 10^{-4} \text{M s}^{-1}$ (MD-ACO2) and $3.8 \times 10^{-4} \mu\text{M s}^{-1}$ (MD-ACO3). The optimal pH for MD-ACO1 was 7.0 - 7.5, MD-ACO2 7.5 - 8.0 and MD-ACO3 7.0 - 8.0. All three isoforms exhibited absolute requirements for the co-substrate ascorbate *in vitro* with optimal activity at 30 mM. Similarly, ferrous iron ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; of 15 – 25 μM) and sodium bicarbonate (NaHCO_3 ; of 30 mM) were required for optimal activity, and were the same for all isoforms. No significant difference in thermostability was found in this study between the MD-ACO isoforms at the $P = 0.05$ level. However, the activities of the enzyme differed significantly between temperatures over time.

In vivo expression of each of the ACO genes in leaf tissue determined using RT-PCR and cDNA Southern analysis reveal that both *MD-ACO2* and *MD-ACO3* are expressed in young leaf tissue and in mature leaf tissue. While *MD-ACO3* is expressed predominantly in young leaf tissue, *MD-ACO2* (in common with *MD-ACO1*) is expressed predominantly in mature fruit tissue. None of the *MD-ACO*s were observed to be senescence associated genes (SAG). MD-ACO3 protein accumulated predominantly in young leaf tissue and less intensely in both mature leaf tissue and young fruit tissue, while MD-ACO1 accumulated only in mature fruit tissue. For the developmental studies, samples were collected at approximately 11 am in this study. *MD-ACO2* and *MD-ACO3* were also expressed in leaf tissue collected over a 24 h period in the spring and also in the autumn. For both genes transcripts accumulated in the presence of fruit but tended to disappear in the absence of fruit.

These results show that *MD-ACO1*, *MD-ACO2* and *MD-ACO3* are differentially expressed, and that MD-ACO3 is encoded by a gene distinct from MD-ACO1 and MD-ACO2. MD-ACO1 and MD-ACO2 are either allelic forms of the same gene or closely clustered. Although there is some variation in kinetic properties which may reflect different physiological environments, they do not vary greatly.

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TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS.....	iv
TABLE OF CONTENTS.....	vi
LIST OF FIGURES	xii
LIST OF TABLES	xv
LIST OF ABBREVIATIONS.....	xvii
ABBREVIATIONS FOR AMINO ACIDS	xxi
 Chapter One: INTRODUCTION	 1
1.1 ETHYLENE IN PLANT DEVELOPMENT	1
1.2 ETHYLENE PERCEPTION AND SIGNAL TRANSDUCTION	2
1.2.1 Ethylene Perception	2
1.2.2 Ethylene Signal Transduction.....	4
1.3 ETHYLENE BIOSYNTHESIS IN HIGHER PLANTS.....	6
1.3.1 SAM Synthetase	8
1.3.2 ACC Synthase (ACS)	10
1.3.3 Conjugation of ACC.....	14
1.4 ACC OXIDASE (ACO).....	15
1.4.1 The Ethylene-Forming Enzyme (EFE).....	15
1.4.2 Catalytic Mechanism of ACC Oxidase	17
1.4.3 ACC Oxidase Gene Expression in Higher Plants.....	22
1.5 CIRCADIAN REGULATON AND BIOLOGICAL CLOCKS.....	26
1.6 <i>MALUS DOMESTICA</i> L. BORKH. (APPLE)	28
1.6.1 ACC Oxidase in <i>Malus domestica</i>	29
1.6.1.1 Expression of ACC Oxidase in Apple.....	29
1.6.1.2 Purification, Characterization and Kinetic Properties of ACC Oxidase in apple	30
1.6.1.3 Requirement of Apple ACC Oxidase for Co-substrate and Co-factors	32
1.6.1.4 Localization of ACC Oxidase in Apple.....	36
1.6.1.5 The Focus of Apple Studies.....	37
1.7 OBJECTIVE AND AIMS OF THE THESIS.....	39

Chapter Two: MATERIALS AND METHODS	40
2.1 PLANT MATERIAL.....	40
2.1.1 Harvesting of Plant Material	40
2.1.2 Chlorophyll Quantification	43
2.1.2.1 Statistical Analysis	43
2.1.3 Leaf Carbon Assimilation Measurements	44
2.1.4 Protein Extraction from Apple Tissue.....	44
2.2 MOLECULAR METHODS.....	44
2.2.1 Growth and Storage of <i>E. coli</i>	44
2.2.1.1 Luria-Bertani (LB) Media and LB Ampicillin ¹⁰⁰ Plates.....	44
2.2.1.2 Preparation of Competent Cells	45
2.2.2 Isolation of Ribonucleic Acid (RNA)	46
2.2.2.1 Extraction of Total RNA	46
2.2.2.2 Quantification of RNA in Solution	49
2.2.3 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)	49
2.2.3.1 Generation of cDNA using Reverse Transcriptase	50
2.2.3.2 Amplification of cDNA by Polymerase Chain Reaction.....	50
2.2.3.3 Primers Used for In-Frame Cloning.....	54
2.2.3.4 Relative Quantitative RT-PCR using Quantum RNA TM 18S Internal Standards.....	57
2.2.4 Cloning of PCR Products into Plasmid Vectors.....	58
2.2.4.1 Ethanol Precipitation of DNA or RNA	58
2.2.4.2 Quantification of DNA	59
2.2.4.3 Agarose Gel Electrophoresis of DNA	59
2.2.4.4 DNA Recovery from Agarose Gels.....	60
2.2.4.5 DNA Ligation	61
2.2.4.6 Transformation of <i>E. coli</i> using the Heat Shock Method.....	63
2.2.5 Characterization and Sequencing of Cloned DNA in <i>E. coli</i>	64
2.2.5.1 Isolation of Plasmids from <i>E. coli</i> by the Alkaline Lysis Method	64
2.2.5.2 Plasmid Isolation from <i>E. coli</i> by the Rapid Boiling Method	65
2.2.5.3 Digestion of DNA with Endonuclease Restriction Enzymes	65
2.2.5.4 Purification of Plasmid DNA for Sequencing	66
2.2.5.5 Purification of PCR Products.....	67

2.2.5.6	Preparation for Automated Sequencing of DNA	68
2.2.6	DNA Sequencing Analysis.....	69
2.2.6.1	Sequence Alignment.....	69
2.2.6.2	Sequence Phylogeny.....	69
2.2.7	Southern Analysis.....	70
2.2.7.1	Isolation of Genomic DNA.....	70
2.2.7.2	Digestion of Genomic DNA	71
2.2.7.3	Southern Blotting of Genomic DNA.....	72
2.2.7.4	Southern Blotting of cDNA Fragments	73
2.2.7.5	Labelling DNA for Southern Analysis with [α - 32 P] -dATP	74
2.2.7.6	Hybridisation and Washing of DNA Blots.....	74
2.2.7.7	Stripping Southern Blots.....	75
2.3	EXPRESSION, PURIFICATION, ANALYSIS OF ENZYME	
	ACTIVITY AND PROTEIN ACCUMULATION	75
2.3.1	Preliminary Induction of Recombinant Proteins	76
2.3.2	Large Scale Induction of Recombinant Proteins.....	76
2.3.3	Isolation of Glutathione-S-Transferase (GST) Fusion Proteins	77
2.3.4	Purification of His-Tagged Proteins by Metal Chelate Affinity	
	Chromatography	79
2.3.4.1	Cleaning of Nickel Nitrilotriacetic Acid (Ni-NTA) Resin	79
2.3.4.2	Cleavage of the Histidine Tag (His-Tag) from the Fusion Protein	
	Using Tobacco Etch Virus (TEV) Protease.....	80
2.3.4.3	Acetone Precipitation of Recombinant Proteins	81
2.3.5	Sephadex G-25 Gel Filtration Chromatography.....	81
2.3.6	Fast Protein Liquid Chromatography (FPLC)	82
2.3.6.1	Anion Exchange Chromatography	83
2.3.6.2	Column Cleaning.....	83
2.3.7	The Bradford Protein Assay	84
2.3.8	ACC Oxidase Assay.....	84
2.3.8.1	Determination of pH Optimum	85
2.3.8.2	Optimal Requirements for Co-Substrate and Co-Factors.....	86
2.3.8.3	Determination of K_m , V_{max} and K_{cat}	86
2.3.8.4	Determination of Thermal Stability	86
2.3.9	Ethylene Analysis	87

2.3.9.1	Measurement of Ethylene using Gas Chromatography.....	87
2.3.9.2	Measurement of Ethylene Production in Plant Tissues.....	87
2.3.9.3	Ethylene Calculations.....	87
2.3.10	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis	
	(SDS-PAGE)	88
2.3.10.1	Preparing and Running Linear (12 %) SDS-PAGE	88
2.3.10.2	Preparing and Running Gradient (10 % to 20 %) SDS-PAGE.....	90
2.3.10.3	Coomassie Brilliant Blue (CBB) Staining	90
2.3.11	Western Analysis.....	91
2.3.11.1	Production of Primary Polyclonal Antibodies	91
2.3.11.2	Electrophoretical Transfer of Proteins from Gel to PVDF Membrane	91
2.3.11.3	Immunodetection of Proteins on PVDF Membrane.....	93
2.3.11.4	Amino Acid Sequencing of N-Terminal Peptide Sequences	94
Chapter Three:	RESULTS	95
3.1	ACC OXIDASE GENES IN <i>MALUS DOMESTICA</i> BORKH. (APPLE) ...	95
3.1.1	Molecular Cloning of Protein-Coding Regions of ACC Oxidase	
	Genes in <i>Malus domestica</i> (cultivar: Gala)	95
3.1.2	Confirmation of Putative ACC Oxidase cDNAs	97
3.1.3	Expressed Sequence Tag (EST) Searches at HortResearch.....	104
3.1.4	Summary of Gene Nomenclature.....	107
3.1.5	Genomic Structure of the <i>Malus domestica</i> ACC Oxidase Genes.....	108
3.1.6	Sequence Analysis of ACC Oxidase from <i>Malus domestica</i>	111
3.1.6.1	Nucleotide Sequence Identities Amongst the ORF of MD-ACO1, MD-ACO2 and MD-ACO3	111
3.1.6.2	NCBI Nucleotide Database BLAST Analysis of the Entire ORF for Each of the ACC Oxidase Sequences	111
3.1.6.3	Analysis of the Deduced Translational Sequences of Each of the ACC Oxidase Proteins.....	115
3.1.7	Confirmation by Southern Analysis that <i>MD-ACO3</i> is Encoded by a Gene Distinct from <i>MD-ACO1</i> and <i>MD-ACO2</i>.....	117
3.1.8	Evidence that Three Members of the <i>MD-ACO</i> Multi-gene Family are Differentially Expressed <i>In Vivo</i>.....	122

3.2	EXPRESSION AND CHARACTERIZATION OF MD-ACO1, MD-ACO2 AND MD-ACO3 AS RECOMBINANT PROTEINS.....	130
3.2.1	Protein Expression of MD-ACO1, MD-ACO2 and MD-ACO3.....	130
3.2.1.1	Expression of Recombinant Protein in <i>E. coli</i>	130
3.2.1.2	Purification of His-Tagged Fusion Proteins	133
3.2.2	Characterization of Polyclonal Antibodies Raised Against MD-ACO Fusion Proteins.....	139
3.2.2.1	Protein Accumulation of MD-ACO1/MD-ACO2 and MD-ACO3 in Apple Tissue	143
3.2.2.2	Protein Analysis: Removal of the His-Tag.....	143
3.2.3	Kinetic Properties of MD-ACO1, MD-ACO2 and MD-ACO3.....	146
3.2.3.1	Optimal Substrate and Co-factor Requirements for MD-ACO Activity.....	146
3.2.3.2	The Michaelis-Constant (K_m) and Maximum Velocity (V_{max}) of the MD-ACO Isoforms for ACC	148
3.2.3.3	Assessment of some Kinetic Parameters using the Same ACC Oxidase Isoform Generated in Two Different Expression Vector Systems.....	152
3.2.3.3	Thermostability of MD-ACO1, MD-ACO2 and MD-ACO3	154
3.3	ACC OXIDASE GENE EXPRESSION IN THE BOURSE SHOOT LEAVES OF APPLE	157
3.3.1	Physiological Measurements, MD-ACO Protein Accumulation and Gene Expression During Leaf Development.....	157
3.3.1.1	Change in Chlorophyll Concentration.....	157
3.3.1.2	Photosynthesis Measurements	159
3.3.1.3	Protein Accumulation and Gene Expression	163
3.3.2	ACC Oxidase Gene Expression and Protein Accumulation in the Leaves of Apple Over a 24 h Period	170
Chapter Four:	DISCUSSION	176
4.1	OVERVIEW.....	176
4.2	ACC OXIDASE GENES IN <i>MALUS DOMESTICA</i>	176
4.2.1	Evidence of a Multi-Gene Family in Apple by Genomic Southern Analysis.....	176

4.2.2	Gene Structure of <i>MD-ACO1</i> , <i>MD-ACO2</i> and <i>MD-ACO3</i> ; Comparison of Exons and Introns.....	178
4.2.3	Translated Products of MD-ACO Genes.....	180
4.2.4	Confirmation of <i>MD-ACO</i> Differential Gene Expression <i>In Vivo</i>	181
4.3	EXPRESSION AND CHARACTERIZATION OF MD-ACO1, MD-ACO2 AND MD-ACO3 AS RECOMBINANT PROTEINS.....	187
4.3.1	Kinetic Properties of the MD-ACO Isoforms.....	187
4.3.2	Thermostability of MD-ACO1, MD-ACO2 and MD-ACO3.....	195
4.3.3	Physiological Significance of the Kinetic Properties of MD-ACOs	197
4.3.4	Some Possible Limitations of Expressing MD-ACOs in <i>E. coli</i>	200
4.4	ACC OXIDASE ACCUMULATION AND GENE EXPRESSION IN THE BOURSE SHOOT LEAVES OF <i>MALUS DOMESTICA</i>	202
4.4.1	MD-ACO Accumulation and Gene Expression During Leaf Development	203
4.4.2	MD-ACO Accumulation and Gene Expression in Leaves Over a 24 h Period.....	206
4.5	Summary	210
4.6	Future Directions.....	211
	APPENDIX 1A.....	212
	APPENDIX IB	213
	BIBLIOGRAPHY	215

LIST OF FIGURES

Figure 1.1	Ethylene biosynthesis and the Yang (Methionine) Cycle.....	7
Figure 1.2	The stoichiometry of the reaction catalyzed by ACC oxidase.....	17
Figure 2.1	The bourse shoot leaves of Apple	42
Figure 2.1	Sequence of the primers used in RT-PCR to amplify putative ACC oxidase sequences.....	51
Figure 2.3	The map and schematic diagram of the pGEX-GP-3 Vector and the vector multiple cloning site	55
Figure 2.4	The map and schematic diagram of the pProEX-1 Vector and the vector multiple cloning site	56
Figure 2.5	pGEM [®] -T Easy Vector circle map and sequence reference points.....	62
Figure 2.6	Sequence of the promoter and multiple cloning site of the pGEM [®] -T Easy Vector	63
Figure 2.7	Arrangement of the apparatus used to transfer DNA samples to Hybond [™] -N ⁺ Membranes	73
Figure 2.8	Western blotting apparatus: assembly of the electrophoretic transfer of proteins from an acrylamide gel to PVDF membrane	92
Figure 2.9	BioRad semi-dry trans-blot cell	92
Figure 3.1	PCR amplification of a putative ACC oxidase cDNA using RT-generated cDNA templates from total RNA isolated from newly initiated green leaves, mature green leaves and senescing leaves.....	96
Figure 3.2	Cloning of putative cDNAs encoding ACC oxidase in newly initiated green leaves, mature green leaves and senescing leaves of <i>Malus domestica</i> generated using RT-PCR	98
Figure 3.3	Comparison between the translated sequences of the partial cDNAs JEB:α and JEB:β with corresponding ACC oxidase sequences as indicated	104
Figure 3.4	Comparison of the deduced amino acid sequences of JEB:α (<i>MD-ACO2</i>) with Genbank accession number AF015787 and EST 120223 and EST 153710.....	106
Figure 3.5	Comparisons of the deduced amino acid sequences of JEB:β (<i>MD-ACO3</i>) with EST 179706 and EST 192136.....	108
Figure 3.6	Gene structure of <i>MD-ACO1</i> and <i>MD-ACO2</i> with a putative structure determined for <i>MD-ACO3</i>	109
Figure 3.7	Comparison of the deduced amino acid sequences of the open reading frames of MD-ACO1, MD-ACO2 and MD-ACO3	116

Figure 3.8	Phylogenetic analysis of the three ACC oxidase amino acid sequences from <i>Malus domestica</i> with other ACC oxidase genes in the Genbank database	118
Figure 3.9	Specificity of <i>MD-ACO1</i> , <i>MD-ACO2</i> and <i>MD-ACO3</i> as probes in blotting analysis	119
Figure 3.10	Southern analysis of <i>Malus domestica</i> genomic DNA.....	121
Figure 3.11	Virtual northern.....	122
Figure 3.12	Evidence that <i>MD-ACO1</i> , <i>MD-ACO2</i> and <i>MD-ACO3</i> are expressed differentially <i>in vivo</i>	124
Figure 3.13	The position of the forward intron spanning primers of <i>MD-ACO1</i> , <i>MD-ACO2</i> and <i>MD-ACO3</i> , the forward position of the probe primers, and the reverse 3'-UTR primers used for both fragments and probes	126
Figure 3.14	Relative quantitative RT-PCR analysis of <i>MD-ACO1</i> , <i>MD-ACO2</i> and <i>MD-ACO3</i> expression <i>in vivo</i>	127
Figure 3.15	Confirmation that <i>MD-ACO1</i> , <i>MD-ACO2</i> and <i>MD-ACO3</i> are differentially expressed <i>in vivo</i> using cDNA Southern analysis	129
Figure 3.16	<i>MD-ACO1</i> , (putative) <i>MD-ACO2</i> and (putative) <i>MD-ACO3</i> expressed in <i>E. coli</i> (strain BL-21) using the pGEX expression vector.....	131
Figure 3.17	Specific activities of recombinant <i>MD-ACO1</i> , <i>MD-ACO2</i> and <i>MD-ACO3</i> expressed in the pGEX Vector.....	134
Figure 3.18	Comparison of the molecular mass of recombinant <i>MD-ACO3</i> expressed using the pGEX Vector with <i>MD-ACO3</i> expressed using the pProEX-1 vector, after separation on a 10 to 20 % SDS-PAGE gradient gel.....	135
Figure 3.19	SDS-PAGE of <i>MD-ACO1</i> , <i>MD-ACO2</i> and <i>MD-ACO3</i> following Metal Chelate Chromatography.....	136
Figure 3.20	Separation of active recombinant <i>MD-ACO</i> using a Mono Q Anion Exchange column	138
Figure 3.21	Recombinant His-tagged proteins after purification by metal Chelate Affinity Chromatography, Sephadex G-25 and Anion Exchange Chromatography	140
Figure 3.22	Detection of ACC oxidase in apple fruit and apple leaves using antibody raised against <i>MD-ACO1</i> on western blot.....	141
Figure 3.23	Detection, using western analysis, of ACC oxidase in apple fruit and apple leaves by an antibody raised against <i>MD-ACO3</i>	142
Figure 3.24	Accumulation of <i>MD-ACO1</i> / <i>MD-ACO2</i> and <i>MD-ACO3</i> in various tissues of apple	144
Figure 3.25	Cleavage of the histidine tag from recombinant <i>MD-ACO2</i> and <i>MD-ACO3</i> using tobacco etch virus (TEV) protease, and subsequent evaluation of enzymatic activity.....	145

Figure 3.26	Effect of MD-ACO concentration on the $V_{\max}$ of MD-ACO1, MD-ACO2 and MD-ACO3.....	147
Figure 3.27	Activity assays of MD-ACO1, MD-ACO2 and MD-ACO3 without the presence of sodium bicarbonate (NaHCO_3) in the reaction mixture	150
Figure 3.28	Recombinant MD-ACO1, MD-ACO2 and MD-ACO3 activity as a function of ACC concentration.....	151
Figure 3.29	Recombinant MD-ACO2 activity as a function of ACC concentration, expressed in the pGEX vector	153
Figure 3.30	Thermal stability of <i>Malus domestica</i> ACC oxidase at incubation temperatures of 25 °C, 35 °C and 45 °C	155
Figure 3.31	Changes in chlorophyll concentration as an indicator of leaf maturation in <i>Malus domestica</i>	158
Figure 3.32	Changes in chlorophyll concentration as an indicator of leaf senescence in <i>Malus domestica</i>	160
Figure 3.33	Chlorophyll concentration as an indicator of leaf senescence in <i>Malus domestica</i>	161
Figure 3.34	Leaf carbon assimilation in <i>Malus domestica</i> as an indicator of leaf senescence	162
Figure 3.35	ACC oxidase accumulation in the leaves of <i>Malus domestica</i> , from early to mid-season, using antibodies raised against MD-ACO1	164
Figure 3.36	ACC oxidase accumulation in the leaves of <i>Malus domestica</i> , from early to mid-season, using antibodies raised against MD-ACO3	165
Figure 3.37	Changes in ACC oxidase protein accumulation in leaf tissue from mid-season through to senescence (2003) in <i>Malus domestica</i>	166
Figure 3.38	RT-PCR analysis and cDNA Southern blot analysis of ACC oxidase gene expression (2003)	168
Figure 3.39	Gene expression and protein accumulation of ACC oxidase from mid-season through to senescence	169
Figure 3.40	Circadian rhythm of ACC oxidase gene expression over 24 h in the leaves of <i>Malus domestica</i> (November, 2003)	171
Figure 3.41	Circadian rhythm of ACC oxidase gene expression over 24 h in the leaves of <i>Malus domestica</i> (April, 2004)	172
Figure 3.42	Circadian rhythm of ACC oxidase gene expression over 24 h in the leaves of <i>Malus domestica</i> (fruit removed from the tree; April, 2005)	174
Figure 3.43	Circadian rhythm of ACC oxidase gene expression over 24 h in the leaves of <i>Malus domestica</i> (with fruit; April, 2005)	175

LIST OF TABLES

Table 1.1	Summary of some properties of ACC oxidase purified from apple.....	33
Table 1.2	Summary of the requirements of apple ACC oxidase for co-substrate and co- factors.....	35
Table 2.1	Names and addresses of the manufacturers of reagents and specialized equipment.....	41
Table 2.2	Primers used in this study	52
Table 3.1	Distribution of colonies and clones containing ACC oxidase inserts from apple leaf tissue	97
Table 3.2A	Results of a nucleotide BLAST search against JEB:α, 819 bp cDNA sequence	99
Table 3.2B	Results of a nucleotide BLAST search against JEB:β, 816 bp cDNA sequence	100
Table 3.3A	Partial cDNAs of JEB:α and JEB:β aligned with ACC oxidase sequences from the NCBI Genebank database.....	102
Table 3.3B	Nucleotide identity (%) between JEB:α and JEB:β and ACC oxidase sequences from three cultivars of <i>Malus domestica</i>	103
Table 3.4	Identity (%) between EST 153710 and the cDNA X61390 (pAP4), JEB:α:AF015787 and JEB:β	105
Table 3.5	Identity (%) between ESTs (192136 and 179706) and the cDNA pAP4, JEB:α and JEB:β sequences.....	107
Table 3.6	Identity amongst the genomic DNA sequences of <i>MD-ACO1</i> , <i>MD-ACO2</i> and <i>MD-ACO3</i>	110
Table 3.7A	Opening reading frame nucleotide sequence identities of <i>MD-ACO1</i> , <i>MD-ACO2</i> and <i>MD-ACO3</i>	112
Table 3.7B	Nucleotide sequence identities of the 3'-UTR of <i>MD-ACO1</i> , <i>MD-ACO2</i> and <i>MD-ACO3</i>	112
Table 3.7C	Translated amino acid sequence identities of <i>MD-ACO1</i> , <i>MD-ACO2</i> and <i>MD-ACO3</i>	112
Table 3.8A	Results of a nucleotide BLAST search against the <i>Malus domestica</i> ACC oxidase (<i>MD-ACO1</i>) 942 bp cDNA entire open reading frame sequence.....	113
Table 3.8B	Results of a nucleotide BLAST search against the <i>Malus domestica</i> ACC oxidase (<i>MD-ACO2</i>) 990 bp cDNA entire open reading frame sequence.....	114
Table 3.8C	Results of a nucleotide BLAST search against the <i>Malus domestica</i> ACC oxidase (<i>MD-ACO3</i>) 966 bp cDNA entire open reading frame sequence	114

Table 3.9	A comparison of MD-ACO1, (putative) MD-ACO2 and (putative) MD-ACO3	132
Table 3.10	NaCl concentration at which MD-ACO1, MD-ACO2 and MD-ACO3 elute from the Mono Q column at pH 7.5, compared with the estimated pI of each of the proteins	139
Table 3.11	Summary of substrate and co-factor requirements for MD-ACO1, MD-ACO2 and MD-ACO3 for optimal activity	149
Table 3.12	Summary of kinetic properties of MD-ACO1, MD-ACO2 and MD-ACO3.	152
Table 3.13	Some kinetic properties using the pGEX and pProEX-1 expression vectors	153
Table 3.14	Summary of thermal stability of <i>Malus domestica</i> ACC oxidases at incubation temperatures of 25 °C, 35 °C and 45 °C	156
Table 4.1	Comparison of the kinetic properties of recombinant and purified ACOs from apple and other species	189
Table 4.2	Summary of the specific activities of ACOs.....	191

LIST OF ABBREVIATIONS

A ₅₉₅	Absorbance at 595 λ nm
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC Oxidase
ACS	ACC Synthase
AEC	1-amino-2-ethyl-cyclopropane-1-carboxylic acid
AdoMet	S-adenosyl-L-methionine
AEC	1-amino-2-ethyl-cyclopropane-1-carboxylic acid
Amp ¹⁰⁰	Ampicillin (100 mg mL ⁻¹)
amu	Atomic mass unit
ANS	Anthocyanidin synthase
APS	Ammonium persulphate
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BLAST	Basic Logical Alignment Search Tool
Borax	di-Sodium tetraborate decahydrate
bp	Base-pair
BSA	Bovine serum albumin
°C	Degrees Celsius
ca.	<i>circa</i> (approximately)
CAPS	3-(Cyclohexylamino)propanesulfonic acid
CBB	Coomassie Brilliant Blue
cv.	Cultivar
Ci	1 Curie = 3.7 x 10 ¹⁰ disintegrations per second
CTAB	Hexadecyltrimethylammonium bromide
DAFB	Days after full bloom
cDNA	DNA complimentary to a RNA, synthesized from RNA by reverse transcription <i>in vitro</i>
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DAOCS	Deacetoxycephalosporin C synthase
DEAE	Diethylaminoethyl
DEPC	Diethyl pyrocarbonate
DMF	N,N-dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease
DSBH	Double stranded beta helix (jellyroll)
dNTP	2'-deoxynucleotide 5'-triphosphate
DTT	Dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
DW	Dry weight
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetra acetic acid
EFE	Ethylene forming enzyme
EIN	Ethylene insensitive
EST	Expressed Sequenced Tag
ExPASy	Expert protein analysis system: proteomics server of the Swiss Institute of Bioinformatics (SIB)
FID	Flame ionization detector
FPLC	Fast protein liquid chromatography
FW	Fresh weight
GACC	1-(gamma-L-glutamylamino)cyclopropane-1-carboxylic acid
<i>g</i>	Acceleration due to gravity (9.8 m/s ²)
<i>g</i>	Gram
GC	Gas Chromatography
GCG	Gene Computer Group
GST	Glutathione S-Transferase
GUS	<i>E. coli</i> β -glucuronidase
<i>h</i>	Hour
His-tag	Histidine tagged
IgG	Immunoglobulin G
IEC	Internal ethylene concentration
IPTG	Isopropyl- β -D-thiogalactopyranoside (C ₉ H ₁₈ O ₅ S)
IPNS	Isopenicillin N synthase
Kb	Kilo base-pairs
kDa	Kilo Daltons
K _i	Inhibition constant
K _m	The Michaelis constant (substrate needed to occupy 50 % of the active sites)
L	Litre
LB	Luria-Bertani (media or broth)
Log	Logarithm
M	Molar (moles per litre)

MACC	1-(malonylamino) cyclopropane-1-carboxylic acid
MCS	Multiple cloning site
MD-ACO	<i>Malus domestica</i> ACC Oxidase
mol	mole (amount of a substance, Avogadro's number)
μmol	Micromole
mg	Milligram
μg	Microgram
mL	Millilitre
μL	Microlitre
Milli-Q water	Water purified by a Milli-Q ion exchange column
min	Minute
MOPS	Sodium [3-(<i>N</i> -Morpholino)]propanesulphonate
Mr	Relative molecular mass (g mol^{-1})
n	Number of replicate
NBT	<i>p</i> -nitro blue tetrazolium chloride
NCBI	National Center for Biotechnology Information
ng	Nanograms
Ni-NTA	Nickel-nitrilotriacetic acid
nL	Nanolitres
nmol	Nanomole
2-ODD	2-oxoglutarate dependent dioxygenase
OD ₅₉₅	Optical Density at 595 λ nm
ORF	Open reading frame
PA	1,10-phenanthroline
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline (50 mM sodium phosphate, pH 7.4 containing 250 mM NaCl)
PBS-T	Phosphate buffered saline containing 0.5 % (v/v) Tween-20
PCR	Polymerase chain reaction
<i>Pers. Comm.</i>	Personal communication
pH	$-\text{Log}(\text{H}^+)$
pI	Isoelectric point
pmol	Picomole
pn	Net photosynthesis (carbon assimilation measurements)
ppm	Parts per million
PVDF	Polyvinylidene difluoride
PVP-40	Polyvinyl pyrrolidone
PVPP	Polyvinyl polypyrrolidone

3' RACE	Three prime-rapid amplification of cDNA ends
RNA	Ribonucleic acid
RNase	Ribonuclease
RO	Reverse osmosis
RT-PCR	Reverse Transcriptase-polymerase chain reaction
s	Second
SAM	S-adenosyl-L-methionine
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SSPE	Saline, sodium phosphate, and EDTA buffer
TEMED	<i>N, N, N', N'</i> -tetramethylethylenediamine
T_m	Melting temperature (in molecular biology: the temperature at which DNA strands separate in preparation for annealing)
TEV	Tobacco etch virus
Tris	Tris (hydroxymethyl) aminomethane
Triton X-100	Octylphenoxy polyethoxyethanol
Tween-20	Polyoxyethylenesorbitan monolaurate
U	Unit (commercial enzymes are in $U\ \mu L^{-1}$, where unit is based on enzyme activity)
UTR	Untranslated region
UV	Ultra violet light
V	Volt ($m^2\ kg\ s^{-3}A^{-1}$)
V_{max}	Maximum velocity
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
X-Gal	5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside

AMINO ACID ABBREVIATIONS

Amino Acid (AA)	Three-letter abbreviation	One-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V