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**Characterisation of ACC Oxidase Isoforms
during Leaf Maturation and Senescence
in White Clover (*Trifolium repens* L.)**

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Abstract

One-aminocyclopropane-1-carboxylic acid (ACC) oxidase, the enzyme which catalyses the final step in the ACC-dependent pathway of ethylene biosynthesis in plants, has been studied during leaf maturation and senescence in white clover (*Trifolium repens* L.).

The coding regions from two white clover ACC oxidase genes, designated TR-ACO2 (expressed in mature green leaves) and TR-ACO3 (expressed in senescent leaves), have been expressed in *E. coli* as fusion proteins. The expression of the two proteins has been optimised in terms of induction time with isopropyl- β -D-thiogalactopyranoside (IPTG) and IPTG concentration. The solubility of the fusion proteins was low but lysis buffer containing 0.5 % (w/v) SDS or 0.5 % (v/v) Triton X-100 produced a higher protein yield. The recombinant TR-ACO2 and TR-ACO3 proteins were purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography and had an apparent molecular mass of 38 kDa. Enzyme activities of the purified TR-ACO2 and TR-ACO3 fusion proteins were 0.34 and 0.23 nmol ethylene/h/mg protein, respectively.

Activity *in vitro* of ACC oxidase, extracted from both mature green and senescent leaf tissues, was observed to be very labile at 20°C with lower temperature, ascorbate and 1,10-phenanthroline (PA) required to help stabilise the enzyme activity *in vitro* during enzyme extraction and purification.

Three isoforms of ACC oxidase, one from mature green leaves, designated MGI and two from senescent leaves, designated SEI and SEII, have been identified. Two of the three isoforms (MGI and SEII) were purified to homogeneity as judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with Coomassie Brilliant Blue staining and western analysis. The purified isoforms MGI and SEII had specific enzyme activities of 25.2 and 29.8 nmol ethylene/h/ mg protein at pH 7.5 with approximately 100- and 144-fold purification, respectively. During

purification, both isoforms were recognised by an antibody raised against the protein product of TR-ACO2 expressed in *E. coli*.

The native molecular mass of the purified isoforms MGI and SEII was determined to be 37.5 kDa by size exclusion chromatography and molecular masses of MGI and SEII were observed to be 37 kDa and 35 kDa, respectively by SDS-PAGE analysis. The data indicate that both isoforms are active as monomers. Both isoforms were found to be neutral or near neutral proteins with apparent isoelectric points (pIs) of 7.36 for isoform MGI and 7.0 for SEII determined by chromatofocusing. The optimal pHs for MGI and SEII were 7.5 and 8.5, respectively.

The two isoforms also displayed differences in apparent K_m and V_{max} values for the substrate ACC. The K_m values for MGI and SEII were determined to be 39.7 μM and 110.0 μM , respectively. SEII had a higher V_{max} value for ACC than MGI. The data indicate that MGI displays a higher affinity for ACC, SEII requires a higher ACC concentration to achieve the higher enzyme activity and can operate in an environment with higher levels of ACC. In addition, both isoforms exhibited absolute requirements for the co-substrate ascorbate and the cofactors bicarbonate and ferrous iron for maximal enzyme activity *in vitro* with different optimal concentrations for ascorbate and ferrous iron. The data suggest that the two ACC oxidase isoforms are differentially regulated by pH and ACC concentration and are activated by different levels of cofactors. The significant differences between the two isoforms (pH optimum and K_m for ACC) may reflect the distinct physiological status of the leaf tissue in which each isoform is active.

These results show that now widely observed transcriptional regulation of the ACC oxidase gene family is also expressed in terms of differential regulation of isoforms of this enzyme in higher plants.

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Table of Contents

ABSTRACT.....	II
ACKNOWLEDGEMENTS.....	IV
LIST OF FIGURES.....	XI
LIST OF TABLES.....	XV
LIST OF ABBREVIATIONS.....	XVI
AMINO ACID ABBREVIATIONS.....	XIX
1. Chapter One: Introduction.....	1
1.1 Introduction to the Ethylene Biosynthetic Pathway.....	1
1.1.1 SAM Synthetase.....	3
1.1.2 ACC Synthase.....	3
1.2 ACC Oxidase.....	5
1.2.1 Evidence for <i>in vivo</i> Conversion of ACC to Ethylene.....	5
1.2.2 Discovery of the Ethylene-Forming Enzyme.....	6
1.2.3 Purification and Characterisation of ACC Oxidase.....	8
1.2.3.1 Kinetic Properties of ACC Oxidase.....	13
1.2.3.2 Mechanism of Catalysis of ACC Oxidase.....	17
1.2.4 Subcellular Localisation of ACC Oxidase.....	20
1.3 Molecular and Biochemical Evidence for the Presence of ACC Oxidase Isoforms in Plants.....	21
1.3.1 Sequence Homology and Divergence of ACC Oxidase Multigene Families.....	22
1.3.2 Differential Expression and Regulation of ACC Oxidase Genes during Plant Development.....	24
1.3.2.1 Temporal Expression and Regulation.....	24
1.3.2.2 Spatial Expression and Regulation.....	26
1.3.3 Differential Expression and Regulation of ACC Oxidase Genes in Response to Different Cues.....	27
1.3.3.1 Plant Hormones.....	27

1.3.3.2 Wounding.....	28
1.3.3.3 High Temperature and Pathogen Infection.....	29
1.3.4 Biochemical Evidence for the Occurrence of ACC Oxidase Isoforms.....	30
1.4 Differential Expression of ACC Oxidase during Leaf Maturation and Senescence in Plants.....	30
1.5 Ethylene Biosynthesis during Leaf Maturation and Senescence in White Clover.....	31
1.6 Aims of the Thesis.....	32
2. Chapter Two: Materials and Methods.....	33
2.1 Expression and Purification of ACC Oxidases in <i>E. coli</i>	33
2.1.1 Chemicals.....	33
2.1.2 Growth and Storage Conditions of <i>E. coli</i>	33
2.1.3 Expression of ACC Oxidases in <i>E. coli</i>	34
2.1.4 Purification of His-Tagged Fusion Protein by Affinity Chromatography.....	36
2.1.5 Activity Assay of TR-ACO2 and TR-ACO3 Recombinant Proteins.....	38
2.2 Purification and Characterisation of ACC Oxidase from Leaves of White Clover	39
2.2.1 Chemicals	39
2.2.2 Growth of White Clover Plants and Sampling of Leaves.....	39
2.2.2.1 Plant Materials and Growth Conditions.....	39
2.2.2.2 Propagation of Stock Plant and Initiation of the Plant Growth Model System.....	41
2.2.2.3 Sampling of Leaves.....	42
2.2.2.4 Wounding Treatment for Mature Green Leaves.....	42
2.2.3 Extraction of ACC Oxidase	42
2.2.4 Ammonium Sulphate Salt Precipitation.....	44
2.2.5 Sephadex G-25 Gel Filtration Chromatography.....	45
2.2.5.1 Cleaning of Sephadex G-25 Resin.....	46
2.2.6 Fast Protein Liquid Chromatography (FPLC).....	46
2.2.6.1 Hydrophobic Interaction Chromatography.....	47
2.2.6.1.1 Column Cleaning.....	49
2.2.6.2 Anion Exchange Chromatography.....	50

2.2.6.2.1 Column Cleaning.....	51
2.2.6.3 Chromatofocusing.....	51
2.2.6.3.1 Column Equilibration.....	52
2.2.6.3.2 Desalting and Buffer Exchange.....	53
2.2.6.3.3 Column Cleaning.....	53
2.2.6.4 Gel Filtration Chromatography.....	53
2.2.6.4.1 Column Cleaning.....	56
2.2.7 Affinity Chromatography.....	56
2.2.7.1 Isolation of Immunoglobulin (IgG) from Serum.....	56
2.2.7.2 Immunoaffinity Chromatography.....	59
2.2.8 Assay of ACC Oxidase Enzyme Activity <i>in vitro</i>	61
2.2.8.1 Determination of pH Optimum.....	63
2.2.8.2 Determination of K_m and V_{max}	64
2.2.8.3 Optimal Requirements for Co-substrate and Cofactors.....	64
2.2.8.4 Determination of Relative Abundance of Enzyme Isoforms.....	64
2.2.9 Ethylene Analysis.....	65
2.2.9.1 Measurement of Ethylene by Gas Chromatography using a PhotoVac 10S 50.....	65
2.2.9.2 Measurement of Ethylene by Gas Chromatography using a Varian 3400 Gas Chromatograph.....	65
2.2.10 Quantification of Protein Concentration.....	66
2.2.10.1 Bradford Method.....	66
2.2.10.2 UV Method.....	67
2.2.11 Electrophoresis of Protein.....	69
2.2.11.1 Linear Slab Gel SDS-PAGE	69
2.2.11.2 Detection of Protein in Gels and Drying of Gels.....	72
2.2.11.3 Determination of Apparent Molecular Mass of Isoforms by SDS-PAGE.....	72
2.2.12 Western Analysis.....	73
2.2.12.1 Electrophoretical Transfer of Proteins from Gel to PVDF Membrane.....	73
2.2.12.2 Immunodetection of Proteins on PVDF Membrane.....	74
2.3 Statistical Analysis.....	75

3. Chapter Three: Results.....	76
3.1 Introduction.....	76
3.2 Expression of ACC Oxidases in <i>E. coli</i> and Purification of Recombinant Proteins by Nickel Affinity Chromatography.....	76
3.2.1 Expression of ACC Oxidases in <i>E. coli</i>	76
3.2.1.1 Optimisation of Expression of ACC Oxidases in <i>E. coli</i>	80
3.2.1.2 Effect of Concentrations of SDS and Triton X-100 and the Yield of Extracted ACC Oxidase Protein from <i>E. coli</i>	80
3.2.1.3 Sephadex G-25 Gel Filtration Chromatography of Extracted Fusion Protein Preparation	84
3.2.2 Purification of Recombinant TR-ACO2 and TR-ACO3 Proteins.....	84
3.2.3 Enzyme Activity of Purified Recombinant ACC Oxidases.....	86
3.3 Extraction and Purification of ACC Oxidase Isoforms from Leaves of White Clover.....	87
3.3.1 Extraction of ACC oxidase from Mature Green and Senescent Leaves.....	87
3.3.2 Ammonium Sulphate Precipitation and Sephadex G-25 Gel Filtration Chromatography of Crude Extract from Mature Green and Senescent Leaves.....	88
3.3.3 Stability and Stabilisation of ACC Oxidase Activity <i>in vitro</i>	90
3.3.3.1 Stability of ACC Oxidase Activity <i>in vitro</i>	90
3.3.3.2 Stabilisation of ACC Oxidase Activity <i>in vitro</i>	90
3.3.4 Column Chromatography Purification of ACC Oxidase from Mature Green Leaves.....	94
3.3.4.1 Comparison of UV Absorbance of Different Components in FPLC Buffers.....	94
3.3.4.2 Selection of Leaf Tissue for ACC Oxidase Purification.....	95
3.3.4.3 Hydrophobic Interaction Chromatography.....	97
3.3.4.3.1 Hydrophobic Interaction Chromatography on a Phenyl Superose Column.....	97
3.3.4.3.2 Hydrophobic Interaction Chromatography on a Phenyl Sepharose Column.....	99
3.3.5 Anion Exchange Chromatography on a Mono Q Column.....	103
3.3.6 Chromatofocusing on a Mono P Column.....	105
3.3.7 Gel Filtration Chromatography on a Superose 12 Column.....	105
3.3.8 SDS-PAGE Analysis of Purified Isoform MGI.....	108

3.4 Column Chromatography Purification of ACC Oxidase from Senescent Leaf Tissue.....	110
3.4.1 Hydrophobic Interaction Chromatography on a Phenyl Superose Column....	110
3.4.2 Anion Exchange Chromatography on a Mono Q Column.....	114
3.4.3 Chromatofocusing on a Mono P Column.....	114
3.4.4 Gel Filtration Chromatography on a Superose 12 Column.....	118
3.4.5 SDS-PAGE Analysis of Purified Isoform SEII.....	118
3.4.6 Comparison of Purified Isoforms of ACC Oxidase from Leaf Tissue of White Clover.....	118
3.5 Partial Purification of ACC Oxidase from Wounded Detached Mature Green Leaves by Hydrophobic Interaction Chromatography.....	121
3.6 Immunoaffinity Chromatography of ACC Oxidase Protein from Mature Green Leaves.....	123
3.6.1 Affinity Purification of Native ACC Oxidase Protein.....	123
3.6.2 Affinity Purification of Denatured ACC Oxidase Protein.....	126
3.7 Characterisation of Two Isoforms of ACC Oxidase from Leaves of White Clover	128
3.7.1 Physicochemical Properties of Two Purified Isoforms.....	128
3.7.1.1 Molecular Mass.....	128
3.7.1.1.1 Apparent Molecular Mass Determined by SDS-PAGE.....	128
3.7.1.1.2 Native Molecular Weight Determined by Gel Filtration Chromatography.....	128
3.7.1.2 pH Optimum.....	132
3.7.2 Kinetic Properties of Partially Purified Isoforms MGI and SEII.....	132
3.7.2.1 Apparent K_m for ACC.....	132
3.7.2.2 Optimal Requirements of ACC Oxidase Activity <i>in vitro</i> of Two Isoforms for Co-substrate and Cofactors.....	140
3.7.2.3 Relative Abundance of Partially Purified Isoforms of ACC Oxidase Identified from Mature Green and Senescent Leaves.....	145
4. Chapter Four: Discussion.....	146
4.1 Expression and Purification of ACC Oxidases in <i>E. coli</i>	146
4.2 Extraction and Purification of ACC Oxidase Isoforms from Mature Green and Senescent Leaves.....	149

4.3 Physicochemical Characteristics of Two Isoforms of ACC Oxidase from Mature Green and Senescent Leaves.....	158
4.4 Kinetic Properties of Two Isoforms of ACC Oxidase.....	161
4.5 Isoforms MGI and SEII Might Be Encoded by TR-ACO2 and TR-ACO3.....	165
4.6 Conclusions.....	167
4.7 Suggestions for Future Work.....	169
4.7.1 Investigation on Rhythmicity of ACC Oxidase Activity and Protein in Leaf Tissue.....	169
4.7.2 Investigation on How to Improve ACC Oxidase Stability during Extraction and Purification.....	170
4.7.3 Further Characterisation of the Two Isoforms.....	170
5. Bibliography.....	171

List of Figures

Figure 1.1 Ethylene Biosynthetic Pathway in Higher Plants.....	2
Figure 1.2 Stoichiometry of ACC Oxidation to Ethylene Catalysed by the ACC Oxidase.....	7
Figure 2.1 Map and Schematic Diagram of the pPROEX TM -1 Vector and the Vector Multiple Cloning Site.....	35
Figure 2.2 A Typical Mature Stolon of White Clover Plant.....	40
Figure 2.3 Diagram Summarising the Purification Procedure of ACC Oxidase from Mature Green and Senescent Leaves	43
Figure 2.4 Elution of IgG from a DEAE Sephacel Column using 70 mM Sodium Phosphate Buffer.....	58
Figure 2.5 A Typical Protein Standard Curve used to Estimate Protein Concentration of Samples for BIO-RAD Protein Microassay Procedure.....	68
Figure 2.6 A Diagrammatic Setup for Electrophoretic Transfer of Proteins to PVDF Membrane	73
Figure 3.1 Deduced Amino Acid Sequences of TR-ACO2 (A) and 3 (B) Genes.....	77
Figure 3.2 SDS-PAGE Analysis of the Expression of TR-ACO2 Recombinant Protein in <i>E. coli</i> Strain TB-1.....	79
Figure 3.3 The Effect of Different Induction Times after the Induction of IPTG on the Expression of TR-ACO2 Fusion Protein in <i>E. coli</i> Strain TB-1 at 27°C.....	81
Figure 3.4 SDS-PAGE Analysis of the Expression of TR-ACO3 Fusion Protein in <i>E. coli</i> Strain TB-1.....	82
Figure 3.5 SDS-PAGE Analysis of the Effect of SDS Concentrations on the Yield of Extracted TR-ACO2 Fusion Protein from <i>E. coli</i> strain TB-1.....	83
Figure 3.6 SDS-PAGE Analysis of Induced TR-ACO2 (A) and TR-ACO3(B) Proteins in pPROEX TM -1 prior to and after Purification with Ni-NTA Affinity Column.....	85
Figure 3.7 ACC Oxidase Activity <i>in vitro</i> as a Function of Added Enzyme Extract from Mature Green Leaves	89
Figure 3.8 ACC Oxidase Activity <i>in vitro</i> from Mature Green Leaf Enzyme Extracts Assayed after Different Incubation Times at 20°C.....	91
Figure 3.9 ACC Oxidase Activity <i>in vitro</i> from Mature Green Leaf Enzyme Extracts Assayed after Different Incubation Times at 4°C.....	92
Figure 3.10 Stabilisation of ACC oxidase Activity <i>in vitro</i> in Senescent	

Leaf Enzyme Extracts.....	93
Figure 3.11 Western Analysis using the TR-ACO2 Antibody of Active Fractions Eluted from the Mono Q (A) and Superose 12 (B) Columns Of Enzyme Extracts of Pooled Leaves from Nodes 4 to 9.....	96
Figure 3.12 Protein Elution Profile and ACC Oxidase Activity from a Mature Green Leaf Protein Extract after Chromatography through a Phenyl Superose Hydrophobic Interaction Column.....	98
Figure 3.13 Immunodetection of ACC Oxidase Protein using the TR-ACO2 (A), TR-ACO3 (B) and TR-ACO1 (C) Antibodies in MGI Fractions from Mature Green Leaf Protein Extract Eluted from the Phenyl Superose Column (Figure 3.12).....	100
Figure 3.14 Protein Elution Profile and ACC Oxidase Activity from a Mature Green Leaf Protein Extract after Chromatography through a Phenyl Sepharose Hydrophobic Interaction Column.....	102
Figure 3.15 Protein Elution Profile and ACC Oxidase Activity of the MGI Preparation (fractions 30 to 34 from the Phenyl Superose column (Figure 3.12)) after Chromatography through a Mono Q AnionExchange Column.....	104
Figure 3.16 Protein Elution Profile and ACC Oxidase Activity of the MGI Preparation (fractions 5 to 7 from the Mono Q column (Figure 3.15)) after Chromatography through a Mono P Column.....	106
Figure 3.17 Protein Elution Profile and ACC Oxidase Activity of the MGI Preparation fractions 3 from the Mono P Column (Figure 3.16)) after Chromatography through a Superose 12 Gel Filtration Column.....	107
Figure 3.18 SDS-PAGE Analysis of the Purified Isoform MGI from Mature Green Leaf Tissue of White Clover. (a) Coomassie Brilliant Blue Staining and (b) Western Analysis using the TR-ACO2 Antibody of the Separation Shown in (a).....	109
Figure 3.19 Protein Elution Profile and ACC Oxidase Activity from a Senescent Leaf Protein Extract after Chromatography through a Phenyl Superose Hydrophobic Interaction Column.....	111
Figure 3.20 Immunodetection of ACC Oxidase Protein in the Two ACC Oxidase Activity Peaks, SEI and SEII, from the Senescent Leaf Protein Extract Eluted from the Phenyl Superose Hydrophobic Interaction Column (Figure 3.19).....	112
Figure 3.21 Protein Elution Profile and ACC Oxidase Activity of the SEII Preparation (fractions 28 to 29 from the Phenyl Superose column (Figure 3.19)) after Chromatography through a Mono Q Anion Exchange Column.....	115

Figure 3.22 Protein Elution Profile and ACC Oxidase Activity of the SEI Preparation (fractions 33 to 35 from the Phenyl Superose column (Figure 3. 19)) after Chromatography through a Mono Q Anion Exchange Column.....	116
Figure 3.23 Protein Elution Profile and ACC Oxidase Activity of the SEII Preparation (fractions 7 to 8 from the Mono Q anion Exchange column (Figure 3.22) after Chromatofocusing through a Mono P Column.....	117
Figure 3.24 Protein Elution Profile and ACC Oxidase Activity of the SEII Preparation (fraction 4 from the Mono P Chromatofocusing column (Figure 3.23)) after Separation through a Superose 12 Gel filtration Chromatography.....	119
Figure 3.25 SDS-PAGE Analysis of the Purified Isoform SEII from a Senescent Leaf Extract. (a) Coomassie Brilliant Blue Staining and (b) Western Analysis using the TR-ACO2 Antibody of the Separation in (a).....	120
Figure 3.26 Protein Elution Profile and ACC Oxidase Activity from a Non-Wounded Mature Green Leaf Protein Extract Subjected to Phenyl Sepharose Hydrophobic Interaction Chromatography.....	122
Figure 3.27 Protein Elution Profile and ACC Oxidase Activity from Extracts of Wounded (for 6 h) Mature Green Leaf Tissue Subjected to Phenyl Sepharose Hydrophobic Interaction Chromatography.....	124
Figure 3.28 Protein Elution Profile and ACC Oxidase Specific Activity from Extracts of Wounded (for 6 h) Mature Green Leaf Tissue Subjected to Phenyl Sepharose Hydrophobic Interaction Chromatography.....	125
Figure 3.29 (A) SDS-PAGE Analysis of the Eluate from an Immunoaffinity Column with TR-ACO2 Antibody after Chromatography and Elution of Bound Protein from Mature Green Leaf Extracts Denatured with 1.0 % (w/v) SDS.....	127
Figure 3.30 Determination of the Molecular Mass of Isoform MGI using SDS-PAGE.....	129
Figure 3.31 Determination of the Molecular Mass of Isoform SEII using SDS-PAGE.....	130
Figure 3.32 Determination of the Molecular Masses of MGI and SEII using Gel Filtration Chromatography.....	131
Figure 3.33 ACC Oxidase Activity of Isoforms MGI and SEII over a pH Range from 4.0 to 9.0.....	133
Figure 3.34 ACC Oxidase Activity of Isoform SEII over a pH Range from 7.0 to 10.0 using a 100 mM Phosphate Buffer.....	134

Figure 3.35 Dependence of ACC Oxidase Activity <i>in vitro</i> of Isoform MGI on ACC Concentration at pH 7.5 in the Presence of 30 mM Sodium Bicarbonate.....	135
Figure 3.36 Dependence of ACC Oxidase Activity <i>in vitro</i> of Isoform SEII on ACC Concentration at pH 8.5 in the Presence of 30 mM Sodium Bicarbonate.....	136
Figure 3.37 Eadie-Hofstee Plot for ACC Oxidase Activity of Isoform MGI with ACC as Substrate.....	137
Figure 3.38 Eadie-Hofstee Plot for ACC Oxidase Activity of Isoform SEII with ACC as Substrate.....	138
Figure 3.39 Dependence of ACC Oxidase Activity of Isoforms MGI and SEII on Ascorbate Concentration.....	141
Figure 3.40 Dependence of ACC Oxidase Activity of Isoforms MGI and SEII on Bicarbonate Concentration.....	142
Figure 3.41 Dependence of ACC Oxidase Activity of Isoforms MGI and SEII on Fe ²⁺ Concentration.....	143

List of Tables

Table 1.1	Summary of Some Properties of ACC Oxidases Purified from Different Plant Species.....	9
Table 2.1	Manufacturers' Addresses of Commonly Used Chemicals.....	33
Table 2.2	Formulation of Buffers A, B and C used in the Affinity Chromatography.....	37
Table 2.3	Nutrients Added to Long Term Horticultural Grade Bark Base.....	41
Table 2.4	Supports used in FPLC.....	47
Table 2.5	Molecular Masses of Protein Standards.....	54
Table 2.6	Formulation of Reaction Mixture for Assay of ACC Oxidase Activity <i>in vitro</i>	63
Table 2.7	Summary of the Range of Molecular Masses of Low Range Prestained SDS-PAGE Standards used in this Thesis.....	70
Table 2.8	Formulation of Mini-Gel Buffer Solutions.....	70
Table 2.9	Separating and Staking Gel Solutions for using in SDS-PAGE.....	71
Table 3.1	Comparison of ACC Oxidase Activity in Mature Green and Senescent Leaves in Crude Extracts and after Ammonium Sulphate Precipitation and Sephadex G-25 Gel Filtration Chromatography.....	87
Table 3.2	Summary of Purification of ACC Oxidase Isoform MGI from Mature Green Leaf Tissue of White Clover.....	101
Table 3.3	Summary of Purification of ACC Oxidase Isoform SEII from Senescent Leaf Tissue of White Clover.....	113
Table 3.4	Summary of Partial Purification of ACC Oxidase Isoform SEI from Senescent Leaf Tissue of White Clover.....	113
Table 3.5	Summary of Purification Properties of Three Isoforms of ACC Oxidase Identified from Mature Green and Senescent Leaves of White Clover.....	121
Table 3.6	Summary of Kinetic Parameters Determined for Isoforms MGI and SEII from White Clover Leaves.....	139
Table 3.7	Summary of Biochemical Properties of Isoforms MGI and SEII from Mature Green and Senescent Leaves of white clover.....	144
Table 3.8	Comparison of Relative Abundance of Isoforms MGI and SEII from White Clover Leaves.....	145

List of Abbreviations

$A_{280/595 \text{ nm}}$	Absorbance at 280/595 nm
ACC	l-aminocyclopropane-1-carboxylic acid
AdoMet	S-adenosyl-L-methionine
AEC	1-amino-2-ethyl-cyclopropane-1-carboxylic acid
Amp ¹⁰⁰	Ampicillin (100 mg/ml)
APS	Ammonium persulphate
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BSA	Bovine serum albumin
⁰ C	Degrees celsius
<i>ca.</i>	Approximately
CNBr	Cyanogen bromide
DEA	Diethanolamine
DEAE	Diethylaminoethyl
DMF	N, N-dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EFE	Ethylene forming enzyme
ELISA	Enzyme-linked immunosorbent assay
EMS	Electrospray mass spectrometry
FPLC	Fast protein liquid chromatography
FW	Fresh weight
g	Gram or acceleration due to gravity (9.8 m/s ²)
h	Hour
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid

HIC	Hydrophobic interaction chromatography
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
IEF	Isoelectric focusing
IPTG	Isopropyl- β -D-thiogalactopyranoside
kDa	Kilodaltons
K_m	Substrate concentration at half maximum reaction rate
L	Litre
Log	Logarithm
LB	Luria-Bertani (media or broth)
M	Molar, moles per litre
MCP	1-methylcyclopropene
Mes	2-(N-morpholino)ethanesulphonic acid
mg	milligram
μ g	Microgram
μ l	Microlitre
Milli-Q water	Water purified by a Milli-Q ion exchange column
min	Minute
ml	Millilitre
MOPS	3-[N-Morpholino]propanesulphonate
Mr	Relative molecular mass (g/mol)
n	number of replicates
Ni-NTA	Nickel-nitrilotriacetic acid
nmol	Nanomole
nl	Nanolitre
PA	1,10-phenanthroline
PAGE	Polyacrylamide gel electrophoresis
PBSalt	Phosphate buffered saline (50 mM sodium phosphate, pH 7.4 containing 250 mM NaCl)
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
<i>pers. comm.</i>	Personal communication

pH	- Log [H ⁺]
pI	Isoelectric point
PMSF	Phenylmethanesulphonyl fluoride
ppm	Part per million
PVDF	Polyvinylidene difluoride
PVPP	Polyvinyl polypyrrolidone
RACE-PCR	Rapid amplification of cDNA ends-polymerase chain reaction
RO	Reverse osmosis
RPA	Ribonuclease protection assay
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAM	S-adenosyl-L-methionine
SDS	Sodium dodecyl sulphate
SE	Standard error of the mean
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethylenediamine
TFA	Trifluoroacetic acid
TNBS	2,4,6-trinitrobenzenesulfonic acid
Tricine	N-Tris(hydroxymethyl) methylycine
Tris	Tris(hydroxymethyl)aminomethane
Triton X-100	Octylphenoxy polyethoxyethanol
Tween-20	Polyoxyethylenesorbitan monolaurate
UTR	Untranslated region
UV	Ultra violet light
V	Volt (m ² kg/s ³ /A)
V _e	Elution volume
v/v	Volume per volume
V _{max}	Maximum rate of reaction
V _o	Initial (steady-state) reaction velocity or void volume
w/v	Weight per volume
w/w	Weight per weight

Amino Acid Abbreviations

Amino Acid	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
