CHARACTERISATION OF ACC OXIDASE DURING LEAF MATURATION AND SENESCENCE IN WHITE CLOVER (*TRIFOLIUM REPENS* L.)

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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.

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Abbreviations

$A_{260\ nm}$	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

EGTA	1,2,di(2-aminoethoxy)ethane- N, N,N',N',-tetraacetic acid
FW	fresh weight
g	gram
g	acceleration due to gravity (9.81 m s^{-2})
GARAP	goat anti-rabbit alkaline phosphatase
GACC	1-(gamma-L-glutamylamino)cyclopropane-1-carboxylic acid
GC	gas chromatography
GSP	gene-specific primer
GUS	β-glucuronidase
h	hour
HPLC	high perfomance liquid chromatography
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
IAA	indole-3-acetic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
Kb	kilobase-pairs
kDa	kilodaltons
LB	Luria-Bertani (media or broth)
L	litre
M	molar, moles per litre
MACC	1-(malonylamino)cyclopropane-1-carboxylic acid
1-MCP	1-methylcyclopropene
mcs	multiple cloning site
MES	2-[N-morpholino]ethanesulphonic acid
mg	milligram
mL	millilitre
µg	microgram

min	minute
MilliQ water	water that has been purified by passing through a MilliQ ion exchange column
Mr	relative molecular mass (g mol^{-1})
MTA	5'-methylthioadenosine
MTR	5'-methylthioribose
MOPS	Na(3-[N-Morpholino]propanesulphonic acid)
NaOAc	sodium acetate
ng	nanogram
NBD	norbornadiene
NBT	*p*-nitro blue tetrazolium chloride
2-ODD	2-oxoacid dependent dioxygenase
OD^x	optical density at x nm in a 1 cm light path
PAGE	polyacrylamide gel electrophoresis
PAG	photosynthetic associated gene
PBSalt	50 mM sodium phosphate pH 7.4 in 250 mM NaCl
PCR	polymerase chain reaction
pH	-log [H+]
PVDF	polyvinylidene difluoride
PVPP	polyvinyl polypyrrolidone
RNase	ribonuclease
RO	reverse osmosis
RT-PCR	reverse transcriptase-polymerase chain reaction
RUBISCO	ribulose bisphosphate carboxylase
s	second
sem	standard error of the mean
SAGs	senescence associated genes
SDS	sodium dodecyl sulphate

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/ 1mM 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 (m^2 kg s^{-3} A^{-1})
v/v	volume per volume
W	watt (m^2 kg s^{-3}) or (V A)
w/v	weight per volume