Characterization of ACC oxidase during leaf ontogeny in white clover (*Trifolium repens* L.) and *Trifolium occidentale*

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2004

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This is to certify that the research carried out in the Doctoral Thesis entitled "Characterization of ACC oxidase during leaf ontogeny in white clover (*Trifolium repens* L.) and *Trifolium occidentale*" in the Institute of Molecular BioSciences at Massey University, New Zealand:

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CANDIDATE'S DECLARATION

This is to certify that the research carried out for my PhD thesis entitled "Characterization of ACC oxidase during leaf ontogeny in white clover (*Trifolium repens* L.) and *Trifolium occidentale* " in the Institute of Molecular BioSciences at Massey University, Palmerston North, New Zealand is all my own work and that the thesis material has not been used in part or in whole for any other qualification.

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18/08/04

Date

Characterization of ACC oxidase during leaf ontogeny in white clover (*Trifolium repens* L.) and *Trifolium occidentale*

A thesis presented in partial fulfillment of the requirements
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2004

ABSTRACT

To produce plant material for this thesis, *Trifolium repens* (white clover) (genotype 10F) and *Trifolium occidentale* (genotype 18Z) were propagated to produce individual stolons trained over a plastic matrix to inhibit nodal root formation. These stolons comprised leaf tissue representative of all developmental stages, from leaf initiation, maturation through to senescence. The developmental pattern for both species in terms of leaf ontogeny was generally reproducible between vegetatively propagated clones.

Three distinct 1-aminocyclopropane-1-carboxylate (ACC) oxidase genes expressed during leaf ontogeny in white clover (*Trifolium repens* L.) have been identified (Hunter *et al.,* 1999). Of the three ACC oxidase genes identified, one designated *TR-ACO2* is expressed in newly initiated and mature green leaves while *TR-ACO3* is expressed predominantly in the senescent leaf tissue. In order to further characterize the protein products of these genes, a series of FPLC columns was used to partially purify isoforms of ACC oxidase from leaf tissue of white clover at different developmental stages, followed by 2D gel electrophoresis to obtain further purification. Two distinct isoforms of ACC oxidase were identified and partially purified from newly initiated green (designated the NIGI isoform) and senescent (designated the SEI isoform) leaf tissue. Both purified NIGI and SEI proteins were recognized by western analysis using an anti-(*Trifolium repens*) TR-ACO2 antibody after SDS-PAGE or 2D gel electrophoresis. To determine whether NIGI is coded for by *TR-ACO2* and SEI is coded for by gene *TR-ACO3*, protein spots (after 2D gel electrophoresis) were digested with trypsin and the masses of the peptide determined by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometric analysis. For NIGI, the coverage of the putative protein sequence (TR-ACO2) by tryptic digestion ranged from 24.5% to 37.6%, while the observed pI (5.1) and molecular mass (37 kDa) were close to the theoretical pI (5.3) and computed mass (35.7 kDa). For SEI, the percentage coverage of the putative protein sequence (TR-ACO3) from the peptides identified ranged from 13.4% to 18.0%, while the observed pI (5.2) and molecular masses (35.0-35.5 kDa) were also close to the theoretical pI (5.5) and computed mass (35.2 kDa). These data suggest that the NIGI isoform is encoded by *TR-ACO2*, while the SEI isoform is encoded by *TR-ACO3*.

ACC oxidase activity *in vitro* and ACC oxidase protein accumulation over 24 h in mature green leaf tissue extracts during both short and long days has been shown to be under circadian control. There are two ACC oxidase activity peaks observed, in which the pattern of fluctuation in ACC oxidase activity resulted in a high level of enzyme activity at 12:00 am (0.18-0.27 nmol ethylene/h/mg), and maximum activity at 12:00 pm (0.24-0.31 nmol ethylene/h/mg). Lowest activity was observed in both long and short days at 9:00 pm (0.09-0.10 nmol ethylene/h/mg). In addition, northern analysis indicated that the *TR-ACO2* mRNA level also displayed a circadian pattern of expression.

Investigation of the effect of protein phosphorylation and dephosphorylation on ACC oxidase activity indicated that ACC oxidase activity *in vitro* during the periods of maximum activity increased 36% (at 12:00 am) and 56% (at 12:00 pm) after dephosphorylation, respectively. However, there was only 21% increase in enzyme activity at the time point with lowest activity (9:00 pm) in the dephosphorylated extracts. SDS-PAGE using a mini-protein gel system or a gradient gel system showed that the molecular mass of ACC oxidase decreased after dephosphorylation when compared with phosphorylation of the enzyme. These results suggest that the phosphorylation and dephosphorylation of the ACC oxidase proteins occurs *in vitro* and the state does affect enzyme activity.

In the second part of this thesis, the coding regions of putative ACC oxidase gene transcripts were generated from leaf tissue of genotype 18Z of *T. occidentale* using RT-PCR. Sequence alignments indicated that the sequences could be grouped into two distinct classes, and these coding regions were designated *TO-ACO2* (*Trifolium occidentale* ACC oxidase 2) and *TO-ACO3* (*Trifolium occidentale* ACC oxidase 3). *TO-ACO2* and *TO-ACO3* share 82% similarity in nucleotide sequence and 84% similarity in amino acid sequence. The *TO-ACO2* and *TO-ACO3* sequences were validated as encoding ACC oxidases by comparison with other ACC oxidases in the GenBank database and both TO-ACO2 and TO-ACO3 deduced amino acid sequences contain all the residues hitherto shown to be important for maximal activity of the enzyme. Further, *TO-ACO2* had 97% identity with *TR-ACO2* at the nucleotide level, and 98% identity at the amino acid level. *TO-ACO3* had 97% identity with *TR-ACO3* at the nucleotide level, and 96% identity at the amino acid level. Genomic Southern

analysis, using 3'-UTRs of *TR-ACO2* and *TR-ACO3* as probes, could not confirm that *TO-ACO2* and *TO-ACO3* are encoded for by distinct genes.

Expression studies of *TO-ACO2* and *TO-ACO3* genes during leaf maturation and senescence of *T. occidentale* were examined using northern analysis. *TO-ACO2* is expressed predominantly in newly initiated and at the onset of the mature-green leaf stage, while *TO-ACO3* shows maximal expression in senescent leaf tissue. The changes of ACC oxidase activity during leaf ontogeny of *T. occidentale* coincided with the pattern observed for ACC oxidase protein accumulation using western analysis and image analysis.

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LIST OF ABBREVIATIONS

$A_{595\ nm}$	absorbance at 595 nm
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AdoMet	*S*-adenosyl-$_L$-methionine
Amp^{100}	ampicillin (100 mg/ml)
AOA	aminoethoxyacetic acid
APS	ammonium persulphate
AU	absorbance unit
AVG	aminoethoxyvinylglycine
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
°C	degree celsius
ca.	*circa* (approximately)
CBB	coomassie brilliant blue
DMF	*N, N*-dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EIN	ethylene insensitive
EDTA	ethylenediamine tetra-acetic acid
EFE	ethylene forming enzyme
FPLC	fast protein liquid chromatography
FW	fresh weight
GACC	1-(gamma-$_L$-glutamylamino) cyclopropane-1-carboxylic acid
g	gram
g	acceleration due to gravity (9.8 m/s^2)
GC	gas chromatography
GUS	β-glucuronidase
h	hour
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid

HIC	hydrophobic interaction chromatography
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
IPG-IEF	immobilized pH gradient- isoelectric focusing
IPTG	isopropyl-β-$_D$-thiogalactopyranoside
kb	kilobase-pairs
kDa	kilodaltons
K_m	substrate concentration at half maximum reaction rate
L	litre
LB	luria-Bertani (media or broth)
Log	logarithm
MACC	1-(malonylamino) cyclopropane-1-carboxylic acid
M	Molar, moles per litre
mg	milligram
µg	microgram
µl	microlitre
MilliQ water	water purified by a MilliQ ion exchange column
min	minute
ml	millilitre
MOPS	sodium [3-(*N*-morpholiono)] propanesulphonic acid
Mr	relative molecular mass (g mol^{-1})
NBT	*p*-nitro blue tetrazolium chloride
ng	nanogram
2-ODD	2-oxoacid dependent dioxygenase
OKA	okadaic acid
PA	1, 10-phenanthroline
PAGE	polyacrylamide gel electrophoresis
PBSalt	50 mM sodium phosphate, pH 7.4, containing 250 mM NaCl
PCR	polymerase chain reaction
Pers. comm.	personal communication
pH	- log [H^+]
pI	isoelectric point
PLP	pyridoxal-5'-phosphate

ppm	part per million
PVDF	polyvinylidene difluoride
PVPP	polyvinyl polypyrrolidone
3'-RACE	3'-rapid amplification of cDNA ends
RNase	ribonuclease
RO	reverse osmosis
RT-PCR	reverse transcriptase-dependent polymerase chain reaction
s	second
SAM	*S*-adenosyl-$_L$-methionine
SDS	sodium dodecyl sulphate
SE	standard error of mean
TEMED	*N,N,N',N'*-tetramethylethylenediamine
Tris	tris (hydroxymethy) aminomethane
Triton X-100	octylphenoxy polyethoxyethanol
Tween-20	polyoxyethylenesorbitan monolaurate
UTR	untranslated region
UV	ultra violet light
V_{max}	maximum rate of reaction
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

ABBREVIATIONS FOR AMINO ACIDS

Amino acid	Three-letter abbreviation	One-letter symbol
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