MOLECULAR CHARACTERISATION OF ETHYLENE BIOSYNTHESIS DURING LEAF ONTOGENY IN WHITE CLOVER (*Trifolium repens* L.)

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Dedication

This thesis is dedicated to my mother

Dong-Sook Kim

and to my wife

Young hee Cho

Abstract

Ethylene (C_2H_4) biosynthesis has been investigated during leaf ontogeny in white clover (*Triforium repens* L. cv. Glassland challenge, genotype 10F) with a particular emphasis on the production of the hormone in the apex and newly initiated leaves (designated as leaves 1 and 2). In these developing tissues, a relatively higher rate (5 to 6-fold) of ethylene production (0.5 to 0.9 nL C_2H_4 $gFwt^{-1}$ hr^{-1}) was associated with a higher accumulation (1.5-fold) of 1-aminocyclopropane-1-carboxylate (ACC), when compared with mature green leaves.

Genes encoding the ethylene biosynthetic enzymes, ACC synthase (ACS) and ACC oxidase (ACO) have been cloned to characterise ethylene biosynthesis at the molecular level. The partial protein-coding regions of ACS genes have been cloned using reverse transcriptase-dependent polymerase chain reaction (RT-PCR) with degenerate nested primers using cDNA templates from RNA isolated either the apex or leaf 2. These ACS genes were identified and designated as TRACS1, TRACS2 and TRACS3. TRACS1 (680 bp) is 72% and 64% homologous to TRACS2 (674 bp) and TRACS3 (704 bp), respectively, and TRACS2 and TRACS3 are 63% homologous, in terms of nucleotide sequence. TRACS1 shows highest homology with PS-ACS2, an ACS cloned from etiolated pea (*Pisum sativum*) seedlings which is induced by IAA and wounding. TRACS2 shows highest homology with MI-ACS1, an ACS cloned from mature mango (*Mangifera indica*) fruit. TRACO3 shows highest homology with VR-ACS7, an ACS cloned from etiolated hypocotyls of mung bean (*Vigna radiata*) and also PS-ACS1, an ACS cloned from etiolated pea seedlings which is induced by IAA, but not by wounding.

The TRACS3 gene was expressed as a 1.95 kb transcript mainly in the apex and newly initiated leaves as determined by northern analysis. TRACS1 has not been detected in the tissues examined. The expression of TRACS2 has not been determined.

Three ACO genes, comprising *ca.* 1100 bp of the protein-coding region and 3'-untranslated region (3'-UTR) have been cloned using a combination of RT-PCR and 3'-rapid amplification of cDNA ends (3'-RACE), and designated as TRACO1, TRACO2 and TRACO3. Comparison

of a 813 bp protein-coding region of TRACO1, generated by RT-PCR using degenerate primers on cDNA templates from RNA isolated from the apex shows 77% and 75% homology to the protein-coding region sequences of TRACO2 (804 bp) and TRACO3 (816 bp), respectively, generated by RT-PCR using degenerate primers on cDNA templates from RNA isolated from leaf 2. The TRACO2 and TRACO3 protein-coding region sequences show 84% homology. The technique of 3'-RACE generated 3'-UTR sequences of 301 bp (TRACO1), 250 bp (TRACO2), and 92 bp (TRACO3) each amplified using mRNA extracted from the apex. The 92 bp 3'-UTR of TRACO3 has been shown to be a truncated version of a 324 bp sequence amplified from TRACO3 expressed in senescent leaf tissue of white clover (Dr. D. Hunter, IMBS, Massey University, *personal communication*), and it is this full-length version that was used in further experiments. The 3'-UTR sequences of the three ACO genes are more divergent, when compared with the protein-coding regions, showing 61%, 55%, and 59% homology between TRACO1 and TRACO2, TRACO1 and full-length TRACO3, and TRACO2 and full-length TRACO3, respectively. Using these 3'-UTR sequences as gene-specific probes, Southern analysis revealed that the three ACO genes are encoded by distinct genes in the white clover genome.

Northern analysis, using either protein-coding regions or 3'-UTRs as probes, determined that the TRACO1 gene was expressed as a 1.35 kb transcript almost exclusively in the apex and the TRACO2 gene was expressed as a 1.35 kb transcript mainly in newly initiated leaves and mature green leaves, with maximum expression in newly initiated leaves. This pattern of gene expression coincides with the high rate of ethylene production from the apex and newly initiated leaves in white clover. The apex tissue-specific TRACO1 gene was also detected in axillary buds, and the mature leaf-associated TRACO2 gene was expressed in other mature vegetative tissues, including internodes, nodes and petioles. Both TRACO1 and TRACO2 genes are highly expressed in roots.

TRACO3 gene expression was not detected in apex and mature green leaf tissues examined using the 3'-UTR gene-specific probe, but two transcripts (1.17 kb and 1.35 kb) were visualised using the protein-coding region probe and with an extended exposure time.

ACO enzyme activity, *in vitro*, was highest in just fully expanded mature green leaves (leaf 3 and leaf 4) during leaf ontogeny in white clover. Apex, axillary bud and floral bud tissues show a relatively higher enzyme activity, when compared with mature nodes and internode tissues, but lower than that measured in petiole tissue. Highest activity of ACO, *in vitro*, has been detected in root extracts.

Polyclonal antibodies, raised against the TRACO1 gene product expressed in *E. coli*, recognised a high molecular weight (*ca.* 205 kD) protein complex with highest accumulation in the apex. This complex was also detected in axillary bud, floral bud, and leaf 1 tissue. Immunoaffinity-based purification of the *ca.* 205 kD protein was carried out to obtain sufficient protein for amino acid sequencing. However, no sequence was obtained.
Polyclonal antibodies, raised against the TRACO2 gene product in *E. coil*, recognised ACO protein (*ca.* 36 kD) in newly initiated leaves and mature green leaves as well as petioles and roots. This recognition pattern coincides with ACO enzyme activity, *in vitro*, as well as TRACO2 gene expression in the tissue.

Expression of the TRACO2 and TRACO3 genes has been characterised in response to a combination of wounding, ethylene, indole-3-acetic acid (IAA), aminoethoxyvinylglycine (AVG), and 1-methylcyclopropene (1-MCP) treatments using mature green leaves. Expression of TRACO2 gene is enhanced in response to ethylene and IAA. Ethylene-induced TRACO2 gene expression was not blocked by 1-MCP. Expression of TRACO3 was induced in response to wounding in mature green leaves. Wound-induced TRACO3 gene expression was not induced by the ethylene produced in the leaf tissue, indicating ethylene-independent ACO expression in the wounded leaves. Induction of either TRACO2 by IAA treatment or TRACO3 by wounding was delayed with AVG treatment of mature green tissue, suggesting that changes in ACS activity in the tissue is associated with induction of ACO gene expression.

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Abbreviation

$A_{260\ nm}$	absorbance in a 1 cm light path at 260 nm
ACC	1-aminocyclopropane-1-carboxylate
ACO	ACC oxidase
ACS	ACC synthase
AE	after excision
Amp^{100}	ampicilin (100 mg / mL)
AOA	aminoethoxyacetic acid
APS	ammonium persulphate
ATP	adenosine-5'-triphophate
AVG	aminoethoxyvinylglycine
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
ca.	approximately
CBB	Coomassie Brilliant Blue
CER	constitutive ethylene response
CHX	cycloheximide
CNBr	cyanogen bromide
DATP	2'-deoxyadenosine-5'-triphosphate
DCTP	2'-deoxycytidine-5'-triphosphate
DMF	N',N'-dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic aicd
DTT	dithiothreitol
DTTP	2'-deoxythymidine-5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EIN	ethylene insensitive

EMS	ethylmethane sulfonate
ERE	ethylene responsive element
Fwt	fresh weight
GACC	1-(gamma-L-glutamylamino)cyclopropane-1-caboxylate
GC	gas chromatography
hr	hour
IAA	indole-3-acetic acid
kb	kilo-pairs
kD	kilo-dalton
LB	Luria-Bertani (media)
MAbs	monoclonal antibodies
MACC	1-(malonylamino)cyclopropane-1-carboxylate
1-MCP	1-methylcyclopropene
min	minute
MOPS	Na[3-(N-morpholiono)propanesulphonic aicd
MTA	5'-methylthioadenosine
NaOAc	sodium acetate
NBT	*p*-nitro blue terazolium chloride
Ni-NTA	nickel-nitrilotriacetic acid
PA	1,10-phenanthroline
PAbs	polyclonal antibodies
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pH	$-\log[H^+]$

pI	isoelectro-point
PLP	pyrodoxyl phosphate
PVDF	polyvinyliden difluoride
PVPP	polyvinyl polypyrrolidone
3'-RACE	3'-rapid amplification of cDNA ends
RNase	ribonuclease
RO	reverse osmosis
RPA	ribonuclease protection assay
RT-PCR	reverse transcriptase-dependent PCR
s.e.	standard error of the mean
SAG	senescence-associated gene
SAM	S-adenosylmethionine
SA-PMP	Streptavidine Magne Sphere® Paticles
SDS	Sodium dodesyl sulphate
TEMED	*N,N,N',N*-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
U	units
UTR	untranslated region
UV	ultra violet
V	volt (m^2 kg s^{-3} A^{-1})
W	watt (m^2 kg s^{-3}) = VA
YE-elicitor	yeast-extracted elicitor