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EXPRESSION STUDIES OF THE ACC OXIDASE GENE FAMILY OF WHITE CLOVER

(Trifolium repens L.)

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Chih-Ming (Balance) Chen

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Abstract

Four ACO promoters and four ACO genomic sequences have been isolated and cloned from Trifolium repens L. The promoter sequences were cloned using Gene WalkerTM technology, and are defined as the 5' flanking sequences upstream of the ATG translation start codon, and designated pTR-ACO1 (1006 bp), pTR-ACO2 (1510 bp), pTR-ACO3 (1350 bp), and pTR-ACO4 (1250 bp). To confirm that each 5' flanking sequences represents distinct genes, Southern analysis was undertaken with each of the 5' flanking sequences used as probes. For TR-ACO1 and TR-ACO2, Southern analysis indicated that the genome of white clover contains two copies of each gene, while single copies of TR-ACO3 and TR-ACO4 are evident. However, the pattern of recognition of pTR-ACO3 differs from pTR-ACO4 confirming TR-ACO4 as a newly identified member of the ACO gene family of white clover. The four genomic sequences isolated cover sequences downstream of the ATG codon to the stop codon, and each comprises 4 exons interspersed by 3 introns. In terms of sequence identity, for exon 1, identities over the four genes ranges from 69% to 94%, with 94% identity between exon 1 of TR-ACO3 and TR-ACO4, while for exon 2, identities range from 60% to 99%, with 99% identity between TR-ACO3 and TR-ACO4. For exon 3, sequence identities ranged from 71% to 89%, with 89% identity between TR-ACO3 and TR-ACO4, while for exon 4, identities range from 62% to 100%, with 100% sequence identity between TR-ACO3 and TR-ACO4. For the intron sequences, significantly lower identities are observed, with again, highest identities were observed for TR-ACO3 and TR-ACO4. For intron 1, identities ranged from 40% to 81% with the highest identity of 81% observed between TR-ACO3 and TR-ACO4. For intron 2, an identity range of 32% to 72% was observed with 72% identity between TR-ACO3 and TR-ACO4, while identity values of 13% to 79%, with 79% between TR-ACO3 and TR-ACO4. Analysis, in silico, of the 5' flanking sequences was undertaken to identify putative transcriptional binding domains using the PLACE and Mat-Inspector programmes. The

TR-ACO1 5' flanking sequence contains a higher proportion of domains that are associated with young developing tissues, while the TR-ACO2 5' flanking sequence contains domains that are associated with environmental/hormonal cues. In contrast, the TR-ACO3 and TR-ACO4 5' flanking sequences contain a higher proportion of ethylene-response and wound associated domains. The expression pattern, in vivo, directed by all four 5' flanking sequences during leaf development has been examined using GUS fusions and transformation into both tobacco and white clover. In tobacco, the pTR-ACO1 directed expression in the terminal bud and in axillary buds of younger leaves, with expression declining in the older tissues. The pTR-ACO2 directed expression in the petioles and mature-green and senescent leaves, while the TR-ACO3 and TR-ACO4 promoters directed expression in the axillary buds, petioles and leaves of mature-green tissues and those in the early stages of senescence. In white clover, the TR-ACO1 5' flanking sequence directed highest expression in the apical tissues, axillary buds, and leaf petiolules in younger tissues and then declines in the ageing tissues, while the pTR-ACO2 directed expression in the axillary buds and leaf petiolules in mature-green tissues. The TR-ACO3 and TR-ACO4 5' flanking sequences direct more expression in the ontological older tissues, including the axillary buds and leaf petiolules. However, in association with this ontological pattern, all of the 5' flanking sequences directed expression in most cell types examined during leaf ontogeny. In younger tissues, the TR-ACO1 5' flanking sequence directed expression in the ground meristem and newly emerged leaf tissue at the apical bud of the stolon, the ground meristem tissue of axillary buds, vascular tissue, pith and cortex of the internode and node, and the cortex and vascular tissue of the leaf petiolule. In ontological older tissue, the TR-ACO3 and TR-ACO4 5' flanking sequences directed expression in the ground meristem of the axillary buds, the vascular tissue of the stolon and petiolule. However, staining could be observed in the pith and cortex of the stolon, and the cortex of the leaf petiolule, but at a reduced intensity. These expression studies suggest that in leaf development of white clover, the primary cues for the transcriptional regulation of the ACO gene family are ontological in nature.

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List of Abbreviations

A _{280 nm}	Absorbance at 280 nm
ABA	Abscisic acid
ACC	l-aminocyclopropane-l-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AdoMet	S-adenosyl- _L -methionine
AEC	l-amino-2-ethyl-cyclopropane-l-carboxylate
AM	Apical meristem
Amp ¹⁰⁰	Ampicillin (100 mg/ml)
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
AVG	Aminoethoxyvinylglycine
BCIP	5-bromo-4-chloro-3-indoyl phosphate
bp	Base pair
BSA	Bovine serum albumin
°C	Degrees celsius
са	Approximately
CaMV	Cauliflower mosaic virus
Cef ¹⁰⁰	Cefotaxime (100 mg/ml)
CTR	Constitutive triple response
, CNBr	Cyanogen bromide
DEA	Diethanolamide
DEAE	Diethylaminoethyl
DEPC	Diethyl pyrocarbonate
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPX	Dibutyl phthalate xylene
DTT	Dithiothreitol

E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EFE	Ethylene forming enzyme
EIN	Ethylene insensitive
ELISA	Enzyme-linked immunosorbent assay
EMS	Ethylmethane sulfonate
ETR	Ethylene triple response
FPLC	Fast protein liquid chromatography
EtBr	Ethidium bromide
FW	Fresh weight
g	Gram
g	Acceleration due to gravity (9.8 m/s^2)
GACC	l-(gamma-L-glutamylamino) cyclopropane-1-caboxylate
GC	Gas chromatography
GUS	<i>E.coli</i> β-glucuronidase
h	Hour
HCL	Hydrochloric acid
HIC	Hydrophobic interaction chromatography
IAA	Indole-3-acetic acid
IPTG	Isopropyl-β- _D -thiogalactopyranoside
Kan ¹⁰⁰	Kanamycin (100 mg/ml)
Kb	Kilo basepair
kDa	Kilo daltons
K _M	Substrate concentration at half maximum reaction rate
L	Litre
Log	Logarithm
LB	Luria-Bertani (media or broth)
Μ	Molar, moles per litre
MACC	l-(malonylamino) cyclopropane-1-carboxylate
MADS	The conserved domain of MCM1, AGAMOUS, DEFICIENS and SRF
1-MCP	l-methylcyclopropene

mg	Milligram
Milli-Q water	Water purified by a Milli-purification system
min	Minute
ml	Millilitre
Mr	Relative molecular mass (g/mol)
MS	Murashige and Skoog base media
n	Number of replicates
NAA	1-naphthaleneacetic acid
NaOAc	Sodium acetate
NBT	Nitrotetrazolium blue chloride
NCBI	National center for biotechnology information
nl	Nanolitre
nmol	Nanomole
NPT II	Neomycin phosphotransferase II
PAGE	Polyacrylamide gel electrophoresis
PBSalt	Phosphate buffered saline
PCR	Polymerase chain reaction
Pers.comm.	Personal communication
pН	-Log [H ⁺]
pI	Isoelectric point
ppm	Parts per million
PVDF	Polyvinylidine difluoride
RO	Reverse osmosis
RT-PCR	Reverse transcriptase-dependent PCR
SAG	Senescence associated gene
SAM	S-adenosyl- _L -methionine
SAM	Shoot apical meristem
SDS	Sodium dodecyl sulphate
S.E.	Standard error of the mean
TBA	Tertiary butyl alcohol
TCA	Trichloroacetic acid

TEMED	N, N, N', N'-tetramethylethylenediamine
TFBD	Transcription factors binding domain
T _m	PCR anneal temperature
TR-ACO	Trifolium repens-ACC oxidase
Tris	Tris(hydroxymethyl)aminomethylycine
μg	Microgram
μl	Microlitre
μΜ	Micromolar
μm	Micrometer
UTR	Untranslated region
UV	Ultra violet light
V	Volts
V/V	Volume per volume
WT	Wild type
W/V	Weight per volume
W/W	Weight per weight
X-Gal	5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside
X-Gluc	5-Bromo-4-chloro-3-indolyl β -D-glucuronide cyclohexylamine salt