# EXPRESSION STUDIES OF THE ACC OXIDASE GENE FAMILY OF WHITE CLOVER

(*Trifolium repens* L.)

A thesis presented in partial fulfillment of the
requirements for the degree of

**Doctor of Philosophy**

in

**Molecular Plant Biotechnology**

at Massey University, Palmerston North,
New Zealand

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**2005**

# 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 Walker™ technology, and are defined as the 5′ flanking sequences upstream of the ATG translation start codon, and designated p*TR-ACO1* (1006 bp), p*TR-ACO2* (1510 bp), p*TR-ACO3* (1350 bp), and p*TR-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 p*TR-ACO3* differs from p*TR-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-AC*O3 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 p*TR-ACO1* directed expression in the terminal bud and in axillary buds of younger leaves, with expression declining in the older tissues. The p*TR-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 p*TR-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.

# Acknowledgements

I would really like to offer a very large thank you to my supervisor, Associate Professor Michael T. McManus, Institute of Molecular Biosciences, Massey University, for his excellent guidance, understanding, patience, and encouragement throughout the course of Ph.D. project. I am especially grateful for the time he has been spent towards the end of my writing, editing my Asia's style English, and offering a valuable perspective on this thesis. I feel I cannot say enough, thank you again Michael.

I thank to Dr. Richard Scott, his enthusiastic helping me in many aspects. Especially, I thank you for your valuable discussion when I encountered problems, although I still own you some chocolate fishes. I also thank to Ms. Anya Lambert and Miss Susanna Leung for their chemical ordering help always in a fast mode and with patient to listen my requests.

I thank to Ms. Trish McLenachan and Dr. Rissa Ota. Their warmly smiling to listen my stories and enthusiastically encouragement that helps me to accelerate lab works. Greg Clack, an interest lab mate, thank you companied me for rest of lab time. From you, I recognized the delicious flavor of milkshake. I also appreciate rest crew of MTM lab, Dr. Simon, Dr. Trish, Dr. Ning, Cait, Mathew, Fiona, Rachael, Ludervin, and Marrisa who have created a real-world-like place to work.

To the many staffs and students of the Institute Molecular BioScience who I have for reason or another asked for assistance, thank you.

My appreciation also goes out to the staffs of Plant Molecular Genetics Lab at AgResearch, Gresslands. All of whom have been kindly and friendly helped me during Ph.D. period, especially thank to Dr. Derek White and Roy Meeking their patient to teach me the white clover plant transgenic techniques and Dr. Nena Alvarez and Dr. Alicia Scott their consultation in GUS activity and *in-situ* hybridization assays.

I also thank to Dr. David Lewis and Mr. Ian King from Crop and Food Research, their kindly provided the GMO glasshouse for rescuing my transgenic plants when the period of GMO glasshouse of Massey University was re-constructed.

I would like to thank to my co-supervisor Dr. Mike Hay and AgResearch, Grasslands, for lab research funding support, also thank to Bright Future Scholarship providing financial assistance throughout my Ph.D. research period.

My final and very special thank to my all family members. I cannot thank them enough for all the support and encouragement they have given to me.

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# List of Abbreviations

| | |
|---|---|
| $A_{280\ nm}$ | Absorbance at 280 nm |
| ABA | Abscisic acid |
| ACC | 1-aminocyclopropane-1-carboxylic acid |
| ACO | ACC oxidase |
| ACS | ACC synthase |
| AdoMet | S-adenosyl-$_L$-methionine |
| AEC | 1-amino-2-ethyl-cyclopropane-1-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 |
| *ca* | 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 | 1-(gamma-L-glutamylamino) cyclopropane-1-caboxylate |
| GC | Gas chromatography |
| GUS | *E.coli* β-glucuronidase |
| h | Hour |
| HCL | Hydrochloric acid |
| HIC | Hydrophobic interaction chromatography |
| IAA | Indole-3-acetic acid |
| IPTG | Isopropyl-$\beta$-$_D$-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) |
| M | Molar, moles per litre |
| MACC | 1-(malonylamino) cyclopropane-1-carboxylate |
| MADS | The conserved domain of MCM1, AGAMOUS, DEFICIENS and SRF |
| 1-MCP | 1-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 |
| pH | -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 |
| µM | 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 |