EXPRESSION OF ACC OXIDASE GENES IN WHITE CLOVER (*Trifolium repens* L.) ROOTS IN RESPONSE TO PHOSPHATE SUPPLY

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

Doctor of Philosophy
in Plant Molecular Biology

at

Massey University
Palmerston North, New Zealand

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2008

ABSTRACT

The differential expression of members of the *Trifolium repens* ACC oxidase (*TR-ACO*) gene family and accumulation of TR-ACO proteins in white clover roots, and the temporal *TR-ACO* gene expression and TR-ACO protein accumulation in response to phosphate (Pi) stress has been investigated. Four-node stolon cuttings of wild type and transgenic white clover (designated *TR-ACOp::GUS* and *TR-ACO1p::mGFP5-ER*) plants were rooted and acclimatised in Hoagland's solution, and then subjected to either a Pi sufficiency (1 mM Pi) treatment or a Pi depletion (10 μM Pi) treatment over a designated time course.

Using semi quantitative Reverse Transcriptase-Polymerase Chain Reaction (sqRT-PCR) and gene-specific primers it has been determined that the *TR-ACO* genes are differentially expressed in the roots of white clover. The *TR-ACO1* transcript abundance was greater in the lateral roots when compared to the main roots. By immunodetection analysis using antibodies raised against TR-ACO1, recognition of a protein of expected size (*ca.* 36 kDa) was also greater in the lateral roots. The tissue-specific localisation of *TR-ACO1* promoter activity was investigated first by light microscopy using a single genetic line of white clover transformed with a *TR-ACO1p::GUS* gene construct, and results then confirmed by confocal microscopy using several genetically independent lines of transgenic plants transformed with a *TR-ACO1p::mGFP5 ER* gene construct. In these lines, the *TR-ACO1* promoter activity was primarily located in the meristem of the main and lateral roots, lateral root primordia as well as in the pericycle of the root with nodes of expression in the emerging lateral roots, suggesting a role for ethylene in the development of young tissues where cells are actively dividing.

In terms of *TR-ACO2*, greater transcript abundance and protein accumulation of TR-ACO2 were also observed in the lateral roots when compared to the main roots. Histochemical GUS staining of roots of a single genetically-independent line transformed with a *TR-ACO2p::GUS* construct showed predominant promoter activity in the mature tissues of both the main and lateral roots but not in the meristematic tissues. In contrast, *TR-ACO3* showed greater transcript abundance in the main roots relative to the lateral roots, and the promoter activity, as determined using a single genetically- independent line of *TR-ACO3p::GUS* transformed plants was predominantly in the mature tissues of the main roots

In response to Pi depletion, the members of *TR-ACO* gene family were temporally expressed in the white clover roots. Using sqRT-PCR, the *TR-ACO1* transcript abundance was greater in Pi depleted roots at 12 h and 24 h after Pi depletion in both wild type plants and in the one genetically-independent line of white clover transformed with the *TR-ACO1p::mGFP5-ER* construct examined. Similarly, by western analysis using both α-TR-ACO1 and commercially available α-GFP antibodies (for the transformed line), a greater accumulation of proteins was consistently observed in Pi depleted roots from the first up to the seventh day after Pi depletion. By confocal microscopy, it was determined for several genetically-independent line of white clover transformed with *TR-ACO1p::mGFP5-ER* that under Pi depletion more intense GFP fluorescence over a time course of 1 d, 4 d, and 7 d was observed, when compared to plants grown under Pi sufficiency.

For *TR-ACO2*, there was no significant difference in transcript accumulation and protein accumulation in response to short term Pi depletion of up to seven days. However, at 15 d and 21 d after Pi depletion there was a greater protein accumulation in

the roots of Pi depleted plants relative to the Pi sufficient roots. Further, when main and lateral roots were compared, a greater protein accumulation occurred in the lateral roots. For *TR-ACO3*, there was no consistent trend of transcript accumulation in response to Pi depletion over a 24 h period. While a marked reduction in transcript accumulation was noted in Pi depleted roots at 1h, 12 h, 24 h, there was an increase in transcript accumulation at 6 h and 18 h after Pi depletion, indicating that factors other than Pi supply may be affecting gene regulation.

Root morphological studies revealed an increase in the main root length and lateral root production in white clover in response to Pi depletion with a greatest growth rate noted between the sixth and ninth day after Pi depletion, and this period overlapped with accumulation of TR-ACO1 protein suggesting a role for ethylene in the Pi stress induced lateral root production in white clover. The differential regulation of the three *TR-ACO* genes in white clover roots in response to Pi depletion further suggests the divergence in terms of regulation of the ethylene biosynthetic pathway, which may play an important role in fine tuning the responses of plants to particular environmental cues.

ACKNOWLEDGEMENTS

In the completion of my thesis I am truly indebted to several individuals who in one way or another have contributed valuable inputs in making this project a worthwhile achievement.

My deepest expression of gratitude goes to my supervisors, Professors Michael T. McManus and Paula E. Jameson for all the support extended. Michael, you are indeed an excellent supervisor. Thank you for your kindness and untiring effort in mentoring, for guiding me in beating the challenges along the way, and for your patience in editing my writings. The deep concern and support you have accorded in the course of my studies have given me lots of inspiration to pursue this degree. Paula, you have shared a lot of inputs in a variety of ways. The constant moral support and concern you have shown have motivated me to carry on. Your patience in editing my thesis is highly appreciated. For all sorts of assistance you have extended, I am earnestly grateful.

Special thanks to Dr. Balance Chen for generously providing the *TR-ACOp::GUS* white clover plants used in my initial experiments, and for allowing me to use his *TR-ACO1* promoter. I also thank you Balance for sharing your valuable expertise in DNA manipulation and techniques in white clover transformation. I am equally grateful to Miss Susanna Leung for the technical assistance extended, and for providing the *TR-ACO1p::mGFP5-ER* construct used in this thesis. I would never forget when I was incapable of doing my lab work for couple of weeks due to mishap, and you had been so kind to look after my plants both in the growth chamber and in the glasshouse. Susanna, your kindness and friendliness are sincerely appreciated.

I also extend my thanks to the following: Dr Peter Farley for his kind assistance in the use of the Fluorostar plate reader for my phosphate assay; Associate Professor Al

Rowland, Chad Johnson and Suzanne Lambie for their valuable help in the confocal microscopy; Dr. Don Hunter for his kind assistance in the MUG assay; Dr Sarah Dorling for sharing her expertise in protein works, and for some constructive comments towards the improvement of my oral presentation skills; and Trish McLenahan for sharing some valuable techniques in molecular biology.

I am also thankful to all members of the MTM lab, namely, Sarah, Susanna, Matt, Fiona, Rachael, Jan, Elizabeth, Aluh, Ludivine, Phuong, Jaruwan, and,Sam, for the friendship, and for maintaining a lab atmosphere conducive for scientific pursuit for the time being.

My gratefulness also goes to some kindhearted people of the IMBS, namely: Dr. Kathryn Stowell, Pat Munro, Cynthia Cresswell and Ann Truter, for efficiently sharing bits of relevant information from time to time, and for the ready help with smiles each time their assistance is sought for.

This acknowledgement wouldn't be complete without mentioning the funding bodies particularly the Massey Doctoral Scholarship, and the Foundation for Research Science and Technology, which provided the research/study grant. Special thanks also to the New Zealand Society of Plant Biologists (NZSPB), New Zealand Society of Biochemistry and Molecular Biology NZSBMB), and IMBS, Massey University for providing the travel grants for attending conferences either local or international.

Finally, I am sincerely thankful to Lando, my husband, and my three lovely sons RJ, Joshua and Johnuel for their love and encouragements that serve as my life inspiration to strive further.

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1^{o}Ab	Primary antibody
2^{o}Ab	Secondary antibody
A_{260}	Absorbance at 260 nm
A_{595}	Absorbance at 595 nm
ACC	1- aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
Adomet	*S*-adenosyl-$_{L}$-methionine
AHK	*Arabidopsis* histidine kinase
AM	Apical meristem
Amp^{100}	Ampicillin (100 $mg.ml^{-1}$)
APS	Ammonium persulfate
APase	Acid phosphatase
ARR	Amplex red reagent
Au	Arbitrary unit
AVG	Aminoethoxyvinylglycine
6-BAP	6-Benzylamino purine
BCIP	5 bromo-4-chloro-3-indolyl phosphate
bp	Base pair
BME	2-β-Mercaptoethanol
BSA	Bovine serum albumin
^{O}C	Degrees Celsius
ca	Approximately
CaMV	Cauliflower mosaic virus
Cef^{300}	Cefotaxime ($300mg.ml^{-1}$)
cm	Centimetre
CTR	Constitutive triple response
dATP	2′ deoxyadenosine 5′-triphosphate
dCTP	2′deoxycytidine 5′-triphosphate
dGTP	2′deoxyguanosine 5′-triphosphate
DEPC	Diethyl pyrocarbonate
DMF	Dimethylformamide

DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DPX	Dibutyl phthalate xylene
DTT	Dithiothreitol
DW	Dry weight
E. coli	*Echerichia coli*
EDTA	Ethylenediaminetetraacetic acid
EFE	Ethylene forming enzyme
EIN	Ethylene insensitive
ELISA	Enzyme-linked immunosorbent assay
ETOH	Ethanol
ETR	Ethylene triple response
EtBr	Ethidium bromide
FAA	Formalin acetic acid
FU	Fluorescence unit
FW	Fresh weight
g	g force
GFP	Green fluorescent protein
GMO	Genetically modified organism
GUS	*E. coli* β-Glucuronidase
h	Hour
HCl	Hydrochloric acid
HRP	Horse radish peroxidase
IAA	Indole-3-acetic acid
IPTG	Isopropyl-β-$_D$ – thiogalactopyranoside
Kan^{200}	Kanamycin (200 $mg.ml^{-1}$)
Kb	Kilo basepair
kDa	Kilo daltons
K_M	Substrate concentration at half maximum reaction rate
kPa	kilo Pascal
L	Litre
LB	Luria-Bertani (media or broth)
LR	Lateral root

LRP	Lateral root primordium
M	Molar, moles per litre
MACC	1-(malonylamino) cyclopropane-1-carboxylate
MADS	The conserved domain of MCMI, AGAMOUS, DEFICIENS and SRF
MAPK	Mitogen activated protein kinase
1-MCP	1-methylcyclopropene
MDH	malate dehydrogenase
mg	milligram
Milli-Q water	water purified by a Milli-purification system
min	Minute
mL	Millilitre
Mr	Relative molecular mass (g/mol)
MR	Main root
MS	Murashige and Skoog base media
MUG	4-methyl umbelliferyl glucuronide
n	Number of replicates
NAA	1-naphthaleneacetic acid
NAD	Nicotinamide adenine dinucleotide
NaOAc	Sodium acetate
NBT	Nitrotetrazolium blue chloride
NCBI	National Center for Biotechnology Information
nL	Nanolitre
ng	Nanogram
nmol	Nanomole
NOS	Nopaline synthase
npt II	Neomycin phosphotransferase II
OD_{600}	Optical density 600 nm
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-Tween 20
PCR	Polymerase chain reaction
PEPC	Phosphoenolpyruvate carboxylase

PGU	Plant Growth Unit
pH	-Log [H^+]
Pi	Inorganic phosphate, orthophosphate
PI	Propidium iodide
ppm	Parts per million
PVDF	Polyvinylidine difluoride
RACE	3'- rapid amplification of cDNA ends
RH	Relative humidity
Rnase	Ribonuclease
RO	Reverse osmosis
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase- polymerase chain reaction
SAM	Shoot apical meristem
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecyl sulphate
s.e.	Standard error of the mean
SSC	Saline sodium citrate
SSPE	Saline sodium phosphate EDTA buffer
sqRT-PCR	Semi-quantitative RT-PCR
TAE	Tris-Acetate-EDTA
TBA	Tertiary butyl alcohol
TEMED	N, N, N', N'-tetramethylethylenediamine
TR-ACO	*Trifolium repens* ACC oxidase
TR-ACS	*Trifolium repens* ACC synthase
Tris	Tris(hydroxymethyl)aminomethane
µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometer
UTR	Untranslated region
UV	Ultraviolet light
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
YM	Yeast Mannitol

Chapter 1
Introduction

1.1 Overview

Ethylene is a gaseous plant hormone produced in different parts of the plant and is involved in the regulation of various plant developmental processes including seed germination, root elongation, leaf senescence and abscission, as well as fruit ripening. Ethylene is also produced in response to various forms of stress Abeles *et al.*, (1992). These multi-faceted roles indicate the complexity of the mechanisms involved in the synthesis and action of ethylene.

Briefly, ethylene biosynthesis starts from the amino acid methionine which is converted to *S*-adenosyl methionine (SAM) then to 1-aminocyclopropane-1-carboxylic acid (ACC) and finally to ethylene (Adams and Yang, 1979). Within each step, intricate mechanisms are involved which regulate biosynthesis particularly the last two steps involving the two key enzymes, the ACC synthase (ACS) (E.C.4.4.1.14) which is involved in the conversion of SAM to ACC, and the ACC oxidase (ACO) (E.C.1.4.3) which mediates the conversion of ACC to ethylene. Evidence has accumulated that, in many plants, expression of members of the *ACS* and *ACO* gene families are spatially and temporally regulated. For instance, in white clover, both the *ACS* and *ACO* gene families are differentially regulated during leaf developmental stages and in response to environmental cues (Hunter *et al.*, 1999; Gong and McManus, 2000; Murray and McManus, 2005; Chen and McManus, 2006).

The involvement of ethylene in plant responses to changes in phosphorus availability, specifically in the plant roots, has also been investigated in a number of plants (Lynch and Brown, 1997; Borch *et al.*, 1999; Ma *et al.*, 2003; Zhang *et al.*, 2003; Franco-