Regulation of Ethylene Biosynthesis in Vegetative Tissues of White Clover (*Trifolium repens* L.) During Water Deficit

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

In

Plant Biology

Institute of Molecular BioSciences

Massey University

Palmerston North

New Zealand

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2009

ABSTRACT

The investigation in this thesis is divided into two parts. In the first part, the expression and accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO), the enzyme which catalyses the final step of ethylene biosynthesis in higher plants, is examined during exposure of white clover (*Trifolium repens* L.*)* to a water deficit. The second part of this thesis is focused on the identification and characterisation of a water-deficit-associated ACC synthase (ACS), the enzyme which catalyses the production of ACC.

In the first part, two white clover varieties with differing sensitivity to water deficit, a drought-tolerant Tienshan ecotype and a drought-sensitive Grasslands Challenge cv. Kopu II cultivar were exposed to two water deficit treatments: one cycle of water deficit (designated non-prestressed; NPS) and a water deficit, a rehydration period and then a second water deficit treatment (designated pre-stressed; PS) in the New Zealand Climate Environment Laboratory (NZCEL). Treatments were terminated when the petiole elongation rate (PER) in the first fully-expanded leaf reached zero. Water relations, growth responses, the expression of the white clover ACO genes, *TR-ACO1 TR-ACO2* and *TR-ACO3* and the accumulation of two of the corresponding proteins, TR-ACO1 and TR-ACO2, were then examined.

The soil water content (SWC) and leaf water potential (LWP) measured in both varieties and in both water deficit treatments declined progressively. The rate of decline in SWC and LWP was slower in the Tienshan ecotype with no difference between the NPS and PS treatments. However, the LWP in the Tienshan ecotype at the point at which the PER ceased was less negative (*ca.* -1.4 MPa) compared to Kopu (*ca.* -1.7 MPa). In addition, the decline in the PER differed between NPS- and PS-treated Kopu. In the NPS-treated Kopu, the PER was maintained at a high rate when plants were exposed to SWC above 18%, but declined sharply as the SWC declined further. However, in the PS-treated Kopu, the PER declined more progressively in a similar pattern to that determined for NPS- and PS-treated Tienshan.

Expression of *TR-ACO1* and accumulation of TR-ACO1 was observed in the apical structure of the stolon. As the water deficit progressed, no significant alteration in *TR-ACO1* expression and TR-ACO1 protein accumulation was observed in the apical structures of both the NPS- and PS-treated Tienshan ecotype suggesting some degree of protection of the meristem tissues in this more drought-tolerant variety. However, a discernable decline in expression of *TR-ACO1* and accumulation of TR-ACO1 protein was observed in the NPS-treated Kopu suggesting some degree of tissue injury in this more drought-susceptible variety. However, after the pre-stress (PS) treatment, no real changes in *TR-ACO1* expression and TR-ACO1 protein accumulation were observed, in common with the observations for the NPS- and PS-treated Tienshan ecotype suggesting that meristem protection may now be occurring. The results suggest further that the pre-stress treatment of the more drought-susceptible Kopu may result in a degree of acclimation to the water deficit.

For the first-fully expanded leaves, expression of two transcripts, *TR-ACO2* and *TR-ACO3* and accumulation of TR-ACO2 protein was monitored as the SWC decreased. The expression of *TR-ACO2* and accumulation of TR-ACO2 decreased as the water deficit progressed in both the NPS- and PS-treated Tienshan ecotype and correlated with the decrease in PER. By contrast, in the NPS-treated Kopu, *TR-ACO2* expression and TR-ACO2 protein accumulation increased, but again, after a period of pre-stress, *TR-ACO2* expression and TR-ACO2 accumulation decreased, in common with the Tienshan ecotype. Again, the pre-stress treatment of the drought-susceptible Kopu may result in a degree of acclimation to the water deficit such that the responses become similar to those observed in the more drought-tolerant Tienshan ecotype. However, in both NPS- and PS-treated Tienshan and Kopu there was no significant alteration in the expression of *TR-ACO3* in the first fully-expanded leaf.

The expression of *TR-ACO2* and *TR-ACO3* and accumulation of TR-ACO2 protein were also observed in the second fully-expanded leaves (an older tissue). Again similar patterns in the expression of *TR-ACO2 and TR-ACO3* and accumulation of TR-ACO2 protein were observed in both NPS- and PS-treated Tienshan and Kopu. In these leaves, expression of *TR-ACO2* and accumulation of TR-ACO2 protein

decreased as the water deficit progressed, but expression of *TR-ACO3* increased as the water deficit decreased to less than 10%. These results suggest that responses of younger tissues (apical structure; first-fully expanded leaf) maybe the critical determinant for the tolerant (or otherwise) of white clover plants to water deficit.

In the second part of this thesis, four *ACS* genes were identified from the Tienshan ecotype exposed to water deficit and designated *TR-ACS-T*. Three of these were similar to previously identified *TR-ACS* genes from Grasslands Challenge genotype 10F while the fourth was a novel gene designated *TR-ACS4-T*. *TR-ACS4-T* is 64%, 64% and 63% homologous to *TR-ACS1-T, TR-ACS2-T* and *TR-ACS3-T,* respectively in terms of nucleotide sequence. In the GeneBank database, *TR-ACS4-T* shares highly homology to ACC synthase sequences from a wide range of tissues including seedlings and fruit tissues, in addition to a high homology to *ACS* genes induced in auxin-, wounding- and ethylene-treated tissues.

The pattern of *TR-ACS4-T* expression observed during leaf development suggests that the gene is expressed initially in the apical structures and in the newly initiated leaves, and then again in the later mature leaves and those at the onset of senescence. Expression decreases again during senescence. *TR-ACS4-T* expression is not altered by water deficit, but is induced by both ethylene and NAA treatment, but the auxin-induced *TR-ACS4-T* is mediated by ethylene treatment.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisor Professor Michael T. McManus, Institute of Molecular BioSciences at Massey University, for the continuous support of my Ph.D study - research and writing this thesis - with patience, motivation, enthusiasm, encouragement and immense knowledge. I would have been lost without him.

I wish to also acknowledge my second supervisor Assoc. Prof M. Hossein Behboudian of Institute of Natural Resources, Massey University for his encouragement, support and valuable guidance during my study and writing.

Thanks also go to Chris Rawlingson of Institute of Natural Resources for help with TDR and pressure bomb, Steve and Lesley for their helps in Plant Growth Unit, Massey University as well as Cara Norling of New Zealand Climate Environment Laboratory (NZCEL) for helps during my experiments in the Climate Rooms. Thanks Ann Truter and Cynthia Cresswell, the IMBS secretary, and Pat Munro (IMBS HOI PA) for supports during my study.

I gratefully acknowledge the assistance of Michael's post-doc Dr. Sarah Dorling for the western analysis and for her courage and support. Thanks to Dr. Paul Dijkwel of IMBS, to whom we share the lab with, for his encouragement and support. Thanks also go to Michael's research assistance and technicians, Susanna Leung, for heaps of helps in molecular works. Thanks Susanna for yours supports, kindness, friendship and for making my life in IMBS more enjoyable. Previous Michael's technicians: Cait and Lena. Also, the others members of the SC5.19's Lab: Jan, Balance, Marissa, Jolla, Ludivine, Sam the celebrity, Diantha, Caleb, Nick, Mathew and Matthew, Fiona, Elizabeth, Tilman, Jaruwan, Paula, Anali, Afsana, Alvina, Jibran and Ian. Thanks to Phuong and Nena for friendships and discussions.

I would like to thank NZAID for granting me an NZDS Scholarship for my study, Sue Flynn and Olive Pimentel (the NZAID scholarship officers Massey University) for supports, Institute of Molecular BioSciences, Massey University and New

Zealand Society for Plant Biologist for their generous travel assistance granted allowing me to attend conferences and seminars.

My very special thanks must go to my husband, Zainal Abidin, for his love, support and encouragement. Thanks for sending your spirits continuously during the four years I had been away from home. Special thanks also to Lana and Lani, my two daughters, for the company, helps and throughout supports in New Zealand. I wish to thank my entire extended family for the supports, particularly for my mother, father and mother in a low.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µgram	Microgram
µL	Microlitre
µmol	Micromol
1-MCP	1-methylcyclopropene
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
A_{520}	Absorbance at 520 nm
A_{595}	Absorbance at 595 nm
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC Oxidase
ACS	ACC Synthase
AdoMet	$_S$-adenosyl-$_L$-methionie
Amp^{100}	Ampicillin (100 mg mL^{-1})
APS	Ammonium persufate
BCIP	5-bromo-4-chloro-3-indoyl-phosphate
BLAST	Basic Logical Alignment Search Tool
bp	Base-pair
BSA	Bovine serum albumin
ca.	*circa* (approximately)
CBB	Coomassie Brilliant Blue
cDNA	DNA complementary to a RNA transcript, synthesised from RNA by reverse transcription *in vitro*
dATP	Deoxyadenosine Triphosphate
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DMF	*N,N*-dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2´-deoxynucleotide 5´triphosphate
DTT	Dithiothreitol
dUTP	2´-Deoxyuridine 5´-Triphosphate
E.coli	*Eschercia coli*
EDTA	Ethylenediaminetetraacetic Acid
EFE	Ethylene forming enzyme
EIN	Ethylene insensitive
FW	Fresh weight
g	Acceleration due to gravity (9.8m s^{-2})
g	Gram
GAA	Glacial Acetic Acid
GACC	1-(gamma-L-glutamylamino)cyclopropane-1-carboxylic acid
GUS	*E. coli* β-glucuronidase
h	hour
IgG	Immunoglobulin G

IPTG	Isopropyl-β-$_D$-thiogalactopyranoside ($C_9H_{18}O_5S$)
Kb	Kilo base-pairs
kDa	Kilo Daltons
kW	Kilowatt
L	Litre
LB	Lauria-Bertani (media or broth)
LWP	Leaf water potential
M	Molar (moles L^{-1})
MACC	1-(malonylamino)cyclopropane-1-carboxylic acid
mg	Milligram
MGBG	Methylglyoxal bis(guanylhydrazone)
Milli-Q water	Water purified by a Milli-Q ion exchange column
min	Minute
mL	Millilitre
mol	mole
MPa	Mega Pascal
NAA	Naphtalene Acetic Acid
NBT	*p*-nitro blue tetrazolium chloride
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nmol	Nanomole
NPS	Non pre-stressed
NZCEL	New Zealand Climate Environment Laboratory
OD_{520}	Optical Density at 520 nm
OD_{595}	Optical Density at 595 nm
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline (50 mM sodium phosphate, pH 7.4 containing 250 mM NaCl)
PCR	Polymerase chain reaction
PER	Petiole elongation rate
pH	-Log (H^+)
pmol	Picomole
ppm	Parts per million
PS	Pre-stressed
PSB-T	Phosphate buffered saline containing 0.5% (v/v) Tween)-20
PVDF	Polyvinylidine difluoride
PVP-40	Polyvinyl pyrrolidone
PVPP	Polyvinyl polypyrrolidone
RH	Relative Humidity
RNA	Ribunucleic acid
RNase	Ribonuclease
RO	reverse osmosis
RT-PCR	Reverse Transcriptase-polymerase chain reaction
s	second
SAM	S-adenosyl-$_L$-methionine
SDS	Sodium dodecyl sulphate
SE	Standard error

sqRT-PCR	Semi quantitative RT-PCR
SSC	Sodium Chloride and Sodium Citrate
SWC	Soil water content (volumetric soil water content measured by TDR)
TDR	Time Domain Refractometer
TEMED	*N,N,N',N'*-tetramethylethylenediamine
T_m	Melting temperature of double-stranded DNA
Tris	Tris (hydroxymethyl) aminomethane
Tween-20	Polyoxyethylenesorbitan monolaurate
U	Unit (commercial enzymes are in U μL^{-1}, where unit is based on enzyme activity)
UTR	Untranslated Region of mRNA transcript
UV	Ultra violet
V	Volt
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
X-Gal	5-Bromo-4-chloro-3-indolyl-β-$_D$-galactopyranoside

AMINO ACID ABBREVIATIONS

Amino Acid (AA)	Three-letter abbreviation	One-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Aspargine	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
Trypthophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V